Stewart Sell *Editor*

Stem Cells Handbook

Second Edition

Humana Press

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Preface

Our understanding of embryonic cells has increased exponentially over the last 3 decades. It was only 30 years ago when embryonic stem cells were first cultured from mouse embryos. Fifteen years later, human embryonic stem cells were derived from human embryos that were donated from early blastocysts not needed for in vitro fertilization. In the 9 years since the publication of the first edition of *Stem Cells Handbook*, much has changed, yet much remains the same. Obviously, this second edition of *Stem Cells Handbook* concentrates on what has changed and provides a source for experts' critical reviews of their results in various aspects of stem cell research during the last 10 years. The chapters cover what stem cells are, how they contribute to diseases, such as cancer, how bad stem cells can be converted to good stem cells, and how good stem cells can be manipulated and used for therapy. What has not changed is the limited ability to use embryonic cells to treat disease. We hope that this book will help in reaching the goal of many FDA-approved uses of stem cells, both embryonic and adult.

This edition starts with an overview of stem cells in general and ethical problems that need to be addressed in any clinical use. Part I covers the properties of embryonic and fetal stem totipotential cells and how they may be manipulated. This includes how to get them, what signals maintain them as stem cells, how to differentiate them to selected tissue stem cells, and what immunological questions need to be answered if they are to be used for transplantation.

The area of greatest advance since the first edition is the development of methods to produce and apply iPSCs to generate cells that could be used to replace essentially any lost or diseased tissue in the body. The contribution of pluripotent stem cells in adult tissues to repair injury and replace amputated limbs in an experimental model opens Part II. Then we move on to a thorough look of the four critical steps in the use of iPSCs: obtaining them, expanding them, getting them to differentiate into functional tissue stem cells, and then successfully transplanting them. Finally, the vast commercial opportunities of iPSCs are presented.

Part III covers tissue-specific stem cells which are the cells in adult organs responsible for maintaining normal tissue renewal. Understanding how to manipulate normal tissue stem cells could lead to many approaches to preventing or curing various human diseases. The properties and characteristics of tissue stem cells is presented for individual organs or types of tissue and includes a discussion of the role of stem cells in aging.

Part IV deals with transplantation and translating therapeutic approaches, a critical stage of application of stem cell therapy. This includes transplantation of mesenchymal stem cells, use of stem cells in treatment of burns and wounds, as well as treatment of diseases of the eye and diabetes.

Part V examines the stem cell origin of cancer and cancer stem cells. The role of tissue stem cells as the cells of origin of cancer and how to target the signals that maintain cancer stem cells are discussed in general. Then approaches for targeting the stem cells of leukemia, liver and breast cancer, as well as a particular type of kidney cancer, nephroblastoma, for which cancer stem cells are readily identified, are adumbrated.

In closing, we have put together representative, timely, and substantive chapters covering critical aspects of current stem cell research, both basic and clinical. This is done with the full understanding that, given the rate of data accumulation, it is impossible to be all inclusive. Thus, there are many exciting and important aspects of stem cell research that are not covered in this book. What is in this book is a sampling of some of the most critical ongoing studies in stem cell research.

I would like to thank the numerous authors of the chapters in the book for their critical contributions. I owe a particular thanks to my coworkers in the laboratory: Zoran Ilic and Ian Guest, who keep things going productively. Then there are my mentors, who are too numerous to mention, but include Frank J. Dixon, William Weigle, Richard Farr, and Hank Fennel from the University of Pittsburgh; Benjamin Castleman, Robert Scully, and Byron Waksman (Massachusetts General Hospital), John Fahey (NIH), and Phillip Gell (U. Birmingham, England); as well as my long-time collaborators: Hyam Leffert (UCSD), Fred Becker (M.D. Anderson), Ed Smuckler (UCSF), and Gennadi Glinsky (Sanford-Burnham Inst.). Finally, I owe a special thanks to Barry Pierce, who taught me what stem cells are.

Albany, NY Stewart Sell, M.D.

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Introduction to Stem Cells

Rob Burgess

Omnis cellula e cellula. (All cells come from cells.)

Rudolph Virchow (1858)

Stem cells are present within most if not all multicellular organisms and are the ultimate drivers of growth and regeneration. They and are defined as biological cells capable of self-renewal and the capacity to differentiate into a variety of cell types. They are considered to be the most critical biological components necessary for proper growth and development during embryogenesis. Yet they have also been demonstrated to play indispensable roles in adult species, providing a much needed source of cellular replenishment for virtually every mature, differentiated cell type. All stem cells originate from what one might consider the ultimate stem cell, the fertilized egg. As a *totipotent* entity, the fertilized egg has the capacity to drive the formation of all intra- and extraembryonic tissues during growth and development. It is during the process of embryonic maturation that *determination* occurs wherein a variety of more specialized stem cell types are generated with differing properties that allow for the development of specific tissues and organs. For example, embryonic stem cells have the *pluripotent* capacity to drive the formation of all tissues of the embryo proper, but not extraembryonic tissues such as the placenta or amniotic membrane. As the embryo matures, determined *multipotent* stem cells are produced which provide a limited, albeit extremely powerful ability to produce more differentiated cell types. An example of multipotency is the stem cell population of the hematopoietic system, which drives blood formation from a common precursor stem cell both during embryonic development and for a lifetime after birth. While hematopoietic stem cells (HSCs) are capable of differentiation into a variety of blood cell types, they do not contribute to other organ systems; this restricted differentiation capacity defines them as adult in origin. Eventually in the mature adult, *unipotent* stem cells reside in a few select systems such as the heart and central nervous system (CNS) which have the capacity to differentiate into only one mature lineage (Fig. 1).

Gleaning a firm understanding of the genetic and biochemical hierarchies involved in embryonic and adult stem cell differentiation will no doubt lead to new cutting-edge cell-based and non-cell-based therapeutic strategies. In addition, whereas stem cells play crucial roles in embryonic development and adult tissue maintenance, their powerful mitotic properties may potentially mediate cancer development. The ability of stem cells to rapidly propagate can be deregulated and derailed resulting in oncogenic and ultimately tumorigenic properties. The theory of the existence of cancer stem cells is rapidly emerging and may open the door to new avenues for cancer treatment. Gaining a firm understanding of how cancer stem cells contribute to tumorigenic and metastatic phenotypes is key to developing new technologies and methods for cancer diagnosis and treatment. For example, targeting therapeutic entities to cancer stem cells present within a large population of tumor cells may be a powerful technique to rid the body of certain forms of cancer. Part V of this book takes an intimate look at this controversial field, outlining data gleaned on the existence and properties of cancer stem cells present in breast cancer, melanoma, and Wilms' tumor.

A firm understanding of stem cell origins and biology is critical to the development of new modes of therapy. This chapter introduces the origins and basic concepts of stem cells, from embryonic to adult to cancer, and emphasizes key areas of stem cell research and focus that are described and highlighted by leaders in the field in following chapters. Particular attention is paid to the signaling cascades and genetic regulatory mechanisms underlying embryonic and adult stem cell development as well as the differentiation of stem cells into mature, cell type-specific lineages. Unless otherwise noted, the focus of this chapter and the majority of the book sections is on mammalian stem cells.

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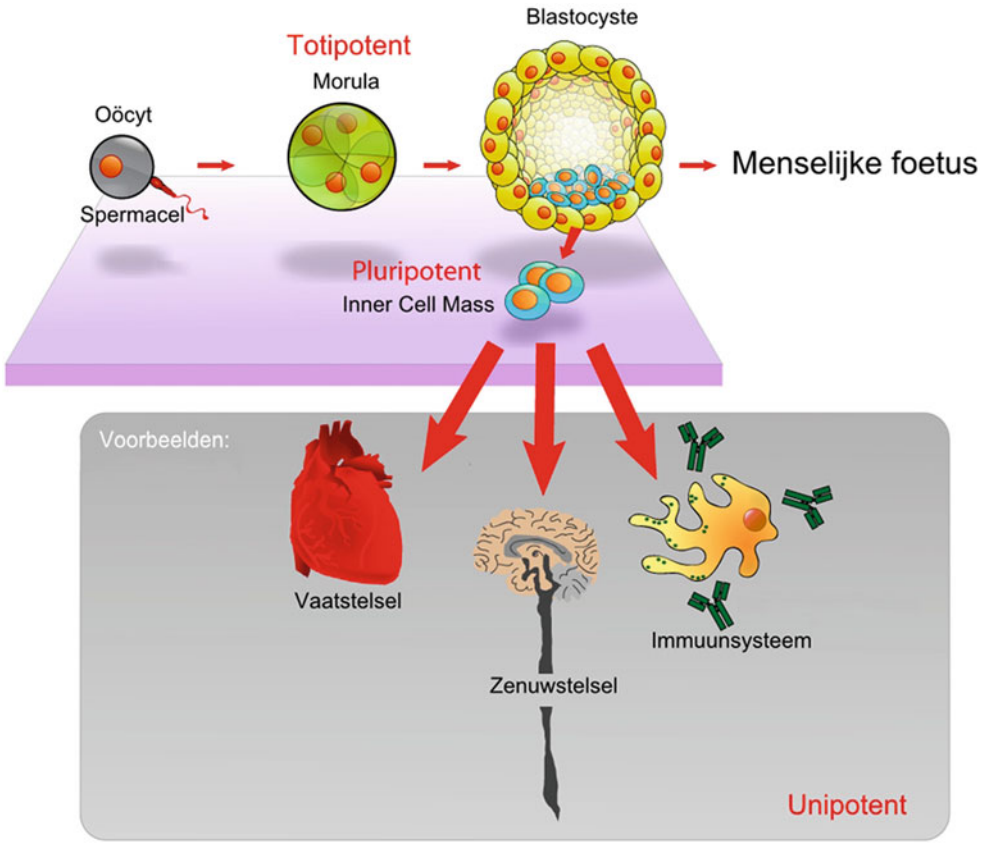

Fig. 1 The origin and specialization of stem cells. See text for details (Courtesy Wikimedia Commons; reprinted with permission)

A Brief History of Stem Cell Research

The first records of man contemplating the origin of life and human development can be traced back to when the ancient Greeks believed that living entities could arise spontaneously (*Generatio spontanea*). Aristotle (384–322 bc) did not agree with the theory of spontaneous generation, but he did believe that order could arise from disorder. This led him to hypothesize that the embryo originates from the mother's menstrual blood. Aristotle's hypotheses are well documented in Leslie Brainerd Arey's comprehensive textbook *Developmental Anatomy: A Textbook and Laboratory Manual of Embryology* [1]. The concept of spontaneous generation was widely accepted and prevailed for the better part of 2,000 years until the mid-1600s when the Italian physician Francesco Redi demonstrated that not all forms of life arise spontaneously in his infamous "six jar experiment" [2] (Fig. 2).

It was in the mid-1800s when Franz Leydig proposed that spontaneous generation in fact did not occur in any context and that all life comes from preexisting life (*omne vivum ex vivo*). Leydig was a German zoologist and comparative anatomist who specialized in the study of neural tissue at the University of Tubingen in Germany. In his seminal publication *Lehrbuch der Histologie des Menschen und der Tiere* he not only outlined crucial developments in the study of histology (including the groundbreaking research of Jan Evangelista Purkyne in 1837), he also emphasized his theory on the origin of life [3]. Purkyne was a Czech anatomist and physiologist who specialized in the study of the brain. His analysis of the histological properties of the cerebellum (he was the first in the world to use a microtome to study tissue slices) resulted in the discovery of Purkinje cells, large neurons possessing a high degree of branched dendrites. Although Robert Hooke is widely credited with the discovery of the cell, this observation is generally accepted as the first definitive documentation of cells. Leydig's theory that all life comes from preexisting life was expanded upon by Rudolph Virchow, a leading Prussian scientist who vehemently disagreed with the theory of spontaneous generation. Virchow carried out a number of experiments in nematodes to demonstrate the prerequisite that all cells come from preexisting cells (*Omnis cellula e cellula*) in 1858 and was a major advocate of this "cell theory." The research and theories of both Leydig and Virchow have withstood the test of time and laid the groundwork for the considerable advancement in cell biology research and stem cell research in

Fig. 2 Portrait of Francesco Redi, the Italian physician first to disprove the theory of spontaneous generation

particular. It was through studies on the role of microorganisms in fermentation in 1864 that Louis Pasteur finally and formally disproved the theory of spontaneous generation [4]. Almost 100 years later, in 1961, researchers James Till, a biophysicist, and Ernest McCulloch, a hematologist, inadvertently discovered the existence of adult stem cells in a suspension of murine bone marrow cells capable of indefinite proliferation. These cells were found to be transplantable and the first colony counting methodology to characterize stem cell numbers was established [5]. These early findings by Till and McCulloch have had perhaps the most significant impact on stem cell research and therapeutic advancements to date. Other key findings are temporally outlined in specific sections below and in Fig. 3. The discovery and characterization of particular types of embryonic and adult stem cells and their potential uses in regenerative medicine are described therein.

Embryonic Development and the Origin of Stem Cells

Over the last 100 years, a massive effort by developmental biologists has been directed at understanding the biochemical, molecular, and morphological mechanisms behind embryonic development, from fertilization through birth. It is only in the past 30 years, however, that significant advancements in understanding cellular potential and lineage commitment as a function of internal cues, environmental influences, and time have revealed unique mechanisms underlying embryogenesis. Research by countless developmental biologists has resulted in the amassing of a wealth of data and information delineating the unique morphological, signaling, and molecular events that drive embryogenesis in a variety of species. In order to understand the capacity of stem cells, it is necessary to review their origins from the perspective of early embryonic development.

Initial Events in Embryogenesis

During the process of embryonic development, the pivotal early event following fertilization is *cleavage*, a stage at which the single cell fertilized egg divides, setting the stage for multiple *symmetric* cell divisions primarily directed at increasing the size of the embryo by amassing large populations of undifferentiated cells in preparation for later cell type specialization. Cells resulting from early cleavage-stage symmetric divisions are known as *blastomeres*, and retain the genetic potential to divide and produce daughter cells that will eventually become specialized. The embryo proper becomes known as the blastocyst and consists of three unique groups of cells: the primitive ectoderm, epiblasts, and the trophectoderm. It is only the epiblast lineage which gives rise to the embryo proper and is a component of the *inner cell mass* (ICM), from which the embryo proper is formed. Transcriptional regulation of the development and anatomical organization of these three lineages is critical to setting up the morphological domains that will later give rise to endoderm, mesoderm, and ectoderm of the embryo itself. In fact, it has been well established that the identity of at least one of these three groups of cells is regulated by the homeodomain transcription factor Cdx2. This notion is illustrated by the fact that overexpression of Cdx2 in murine embryonic stem cells drives them to differentiate into trophoblasts and to exhibit characteristics related to trophoblast stem cells [6]. Interestingly, Cdx2 is expressed in an asymmetric manner at the morula stage on outside cells, thus setting the stage for trophectoderm formation even at this early time point in embryonic development [7, 8]. *Eomes*, a T-box transcription factor, has been placed downstream of Cdx2, yet gene targeting experiments in mice have revealed that neither gene is required for the formation of trophectoderm, which suggests that other factors are involved [8, 9]. Indeed, TEAD4 of the TEA domain transcription enhancer factor family has been placed upstream of Cdx2 with mutants exhibiting a more severe

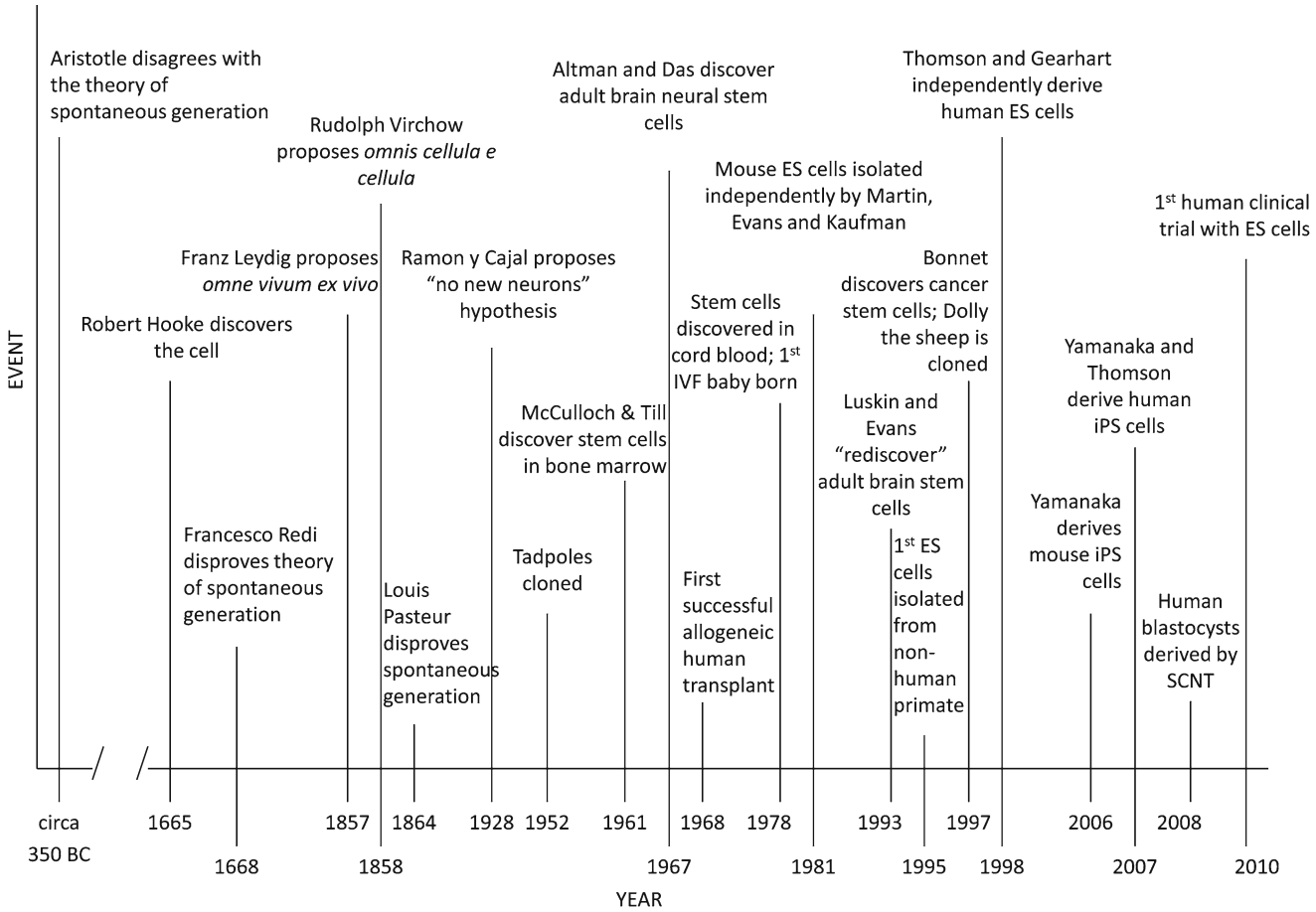

Fig. 3 Timeline of historical events related to stem cell research

phenotype that than of the latter transcription factor. Finally, Cdx2 has been shown to be a potent negative regulator of the pluripotent transcription factors Sox2, Nanog, and Oct4 in trophectoderm cells after blastocyst formation [8]. This is a classical example of transcriptional repression driving extraembryonic lineage commitment. Thus an important transcriptional cascade has been outlined involving multiple positive and negative factors which sets the stage for trophectoderm development. More descriptions of the powerful pluripotent transcription factors Sox2, Nanog, and Oct4 are outlined immediately below and throughout this book.

Inner Cell Mass Regulatory Identity

The ICM of mammalian blastocysts consists of pluripotent stem cells and gives rise to all cells of the embryo proper. The regulatory mechanisms underlying ICM formation have thus been well studied. As mentioned above, it is actually the epiblast subcompartment of the ICM from which the embryo is derived. Interestingly, perhaps the most high profile transcription factors that denote the pluripotency of ICM cells, specifically Nanog and Oct4, are expressed even at the earliest stages of cleavage in all cells (stochastically expressed), yet the expression of these factors becomes restricted to the ICM post-blastocyst formation. As mentioned above, this restricted expression pattern is dependent upon the activity of Cdx2. Thus it has been postulated that early lineage specification throughout the blastocyst begins with the upregulation of key trophectoderm targets and the repression of ICM-specific loci in outside cells. Later during embryonic maturation, the factors Oct4, Sox2, and Nanog actively repress trophectoderm specification and promote pluripotency in the ICM. Positive autoregulatory feedback of these factors subsequently allows for the maintenance of ICM lineage specification [10]. Finally, the growth factor receptor bound protein, Grb2, acts to simultaneously inhibit Cdx2 expression and activate Gata6 expression in a population of ICM cells that will later give rise to the primitive endoderm [11]. These are the first and perhaps most crucial positive and negative transcriptional events that set up both extra- and

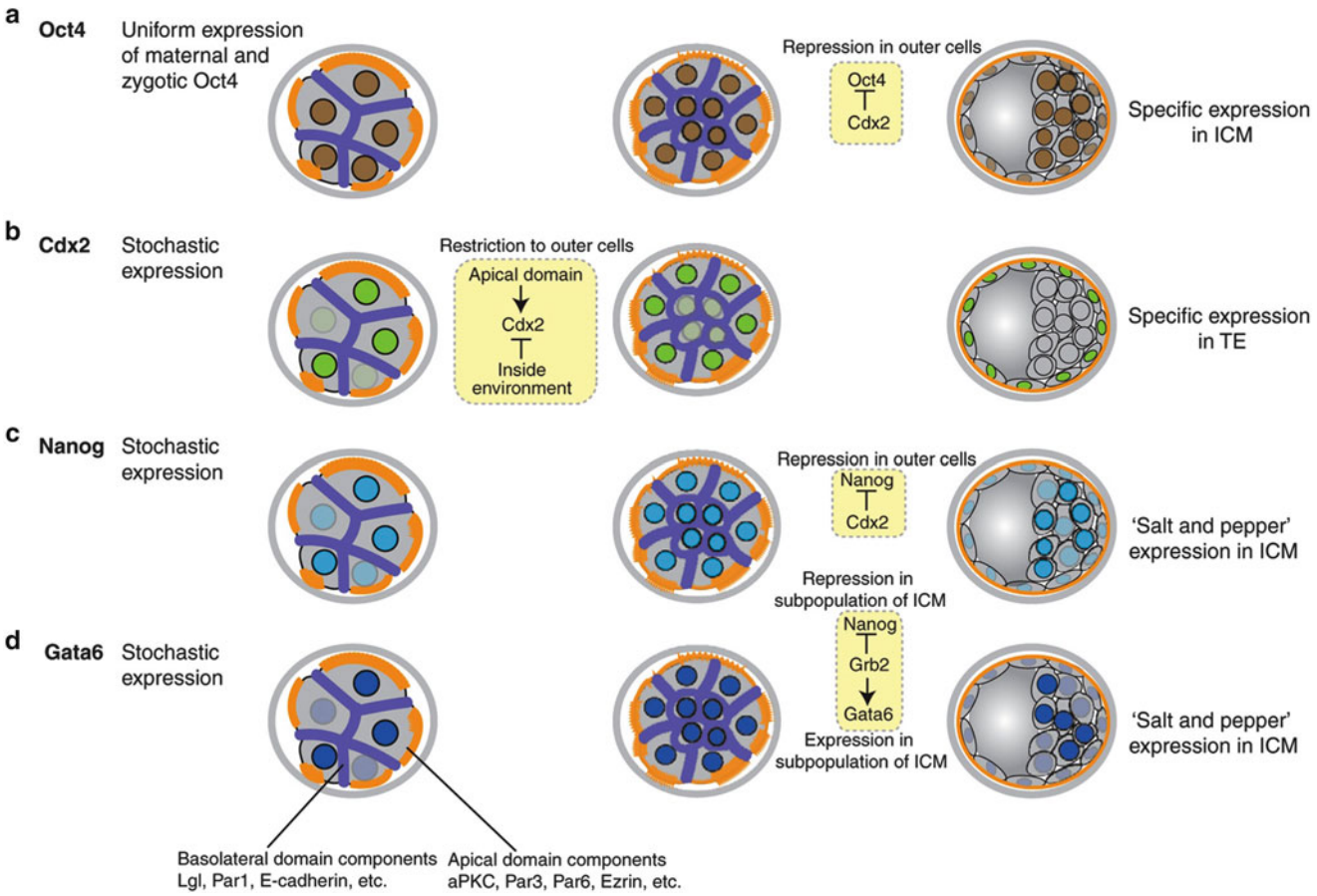

Fig. 4 Molecular players in the formation of early embryonic lineages. Four lineage-specific transcription factors, Oct4, Cdx2, Nanog, and Gata6, are important for the generation of the first three lineages in the blastocyst. The initial expression of these transcription factors is not restricted to specific cell populations. Lineage-specific expression is gradually established in association with the maturation of cellular structures (such as apical-basolateral cell membrane domains, intercellular junctions) and of positive and negative interactions among the transcription factors themselves. (**a**) Oct4: Oct4 protein is observed in all blastomeres throughout early cleavage stages. At the eight-cell stage, all blastomeres contain Oct4. At the blastocyst stage, Oct4 is gradually downregulated in the outer trophectoderm (TE) cells by Cdx2 through direct physical interaction and transcriptional regulation. (**b**) Cdx2: Cdx2 protein is detected beginning at the 8- to 16-cell stage, its initial expression appears to be stochastic. By the early morula to early blastocyst stages, Cdx2 expression is ubiquitous but higher in outer, apically polarized cells. Restricted expression in outer TE cells is established by the blastocyst stage. (**c**) Nanog and (**d**) Gata6: Nanog and Gata6 are detected from the eight-cell stage. Both proteins are expressed uniformly in all cells until the early blastocyst stage. Nanog expression is downregulated in outer cells by Cdx2 and in a subpopulation of the ICM by Grb2-dependent signaling. By contrast, Gata6 expression is maintained by Grb2-dependent signaling. By the late blastocyst stage, ICM cells express either Nanog or Gata6 exclusively (Courtesy Janet Rossant, Patrick P.L. Tam and *Development* (Rossant and Tam [12]); reproduced with permission)

intra-embryonic lineage specification, moving from a totipotent to pluripotent phenotype (Fig. 4 and for a comprehensive review see [12]).

Upon blastocyst formation, ICM cells have already lost totipotency as all cells within the ICM are demarcated as either epiblast or primitive endoderm in phenotype. This has been well documented by comparing Nanog (epiblast) to Gata4 and Gata6 (primitive endoderm) expression and is now known as the "salt and pepper" mosaic pattern in the ICM [11, 165]. The molecular and morphological transition from fertilization through *gastrulation*, defined as the process by which three primary germlayers are acquired, is perhaps the most crucial early-stage developmental process, defining the future of every cell type derived from the one-cell stage fertilized embryo. Gastrulation is the specific stage at which a morphological transition occurs whereby invagination of specific cells of the ICM sets up the formation of the three primary germ layers: endoderm, ectoderm, and mesoderm. The *endoderm* will give rise to the organs, the *ectoderm* to brain and other neural tissue and the *mesoderm* to muscle, bone, and vasculature. It is thus during and after gastrulation that stem cells begin to lose some of their capacity to differentiate into all embryonic and extraembryonic cell types whereby the majority of cells have transitioned from a

totipotent to pluripotent or multipotent capacity for differentiation. As mentioned above, this loss of potency and initiation of specialization is known as *determination*.

What transcriptional mechanisms are crucial to determination of the three primary germ layers? As outlined above, a percentage of ICM cells express the homeodomain transcription factors Gata4 and Gata6 as well as Lrp2. These factors drive the expression of genes crucial for and specific to the endodermal phenotype. The mesodermal and ectodermal layers also express unique transcription factors that drive the eventual maturation of cell types specific to these layers. For example, the basic helix-loop-helix (bHLH) transcription factor twist is expressed at the earliest stages of mesoderm formation [13]. At later stages, the bHLH protein paraxis is expressed in the paraxial mesoderm and is thought to be either a direct or indirect target of twist. Mouse mutants of paraxis have severe defects in somitogenesis and musculoskeletal patterning, most likely the result of aberrant transcriptional signaling in the mesodermal compartment which also affects patterning of neighboring ectoderm along the entire embryonic axis [14, 15]. The ectodermal layer expresses transcription factors in a more restricted fashion. For example, Pax3 is active in a narrow band of ectoderm contiguous with future neural folds yet is not present throughout the entire ectodermal layer at early stages [16]. Ultimately, it is the transcriptional regulatory mechanisms underlying determination events which drive gastrulation, ICM compartmentalization, and stem cell development, with stem cells becoming restricted and specialized to differentiate into mature lineages during and even post-embryonic development throughout adulthood.

The Establishment of Germ Cell Identity

No discussion of stem cell potentiality would be complete without a mention of germinal (germ) cell specification and maturation. *Germinal cells* exist in both the developing embryo and the adult and are totipotent in nature. During embryogenesis, cells committed to the germinal lineage populate the *genital ridge*, a component of the embryo that will develop into the future gonads. It is here that these cells will commit to a germinal lineage of either male or female germ cells; this commitment is defined by an XX or XY genomic makeup. Thus they give rise to sperm as well as egg cells through a process known as *meiosis* in which each daughter cell derived from a common progenitor contains only half of the requisite chromosomal complement needed for a viable developing embryo. The full complement is therefore provided upon fertilization with the transfer of either an X or Y chromosome driving sex determination. As early as 1970, researchers realized the capacity of these cells to differentiate into all cell types and were thus classified as totipotent. The tumorigenic potential of germ cells was demonstrated in these early studies through murine transplant experiments which produced *teratocarcinomas*, malignant teratomas containing a wide range of cell types representing both embryonic and extraembryonic tissues [17]. Recent studies in murine models have suggested three unique molecular events which are crucial to establishing germ cell specification: a full repression of the somatic cell phenotype; pluripotency reacquisition; and epigenetic reprogramming. What transcriptional mechanisms drive germ cell development? Not surprisingly it is some of the same factors that drive ES cell development and pluripotentiality. For example, after gastrulation, Oct4 expression becomes restricted to the primordial germ cells (PGCs) [18, 19]. Interestingly, it is much later that PGC specification is thought to occur, suggesting the existence of other factors upstream of Oct4 which drive PGC commitment. Yet Oct4 has been shown to be indispensable for PGC survival in mouse conditional knockout experiments [20]. Oct4 also specifically marks cells with pluripotential properties in human germ cell tumors [21]. Upon closer look, BLIMP1/PRDM1, a PR (RIZ) domain-containing transcription factor, has been shown to be expressed very early, specifically in epiblast cells that later commit to the PGC lineage [22]. These same epiblast cells express a multitude of homeobox-containing (Hox) genes known to specify cell type and even axial structure in other systems. There is a transient repression of the expression of pluripotency transcription factors Sox2, Zic3, and Nanog, yet these are upregulated later in development [23, 24]. This suggests that PGCs transiently take on a mesodermal fate, and pluripotentiality reemerges as development proceeds. A second PR domain-containing protein, PRDM14 is exclusively expressed in PGC precursors and mature PGCs. Mouse knockouts of either PR domain-containing transcription factor have severe developmental defects in germ cell development [24, 25]. PRDM1 has since been demonstrated to function upstream of PRDM14 and is indirectly negatively regulated by the RNA binding protein Lin28 [26]. These factors working in concert via their activation by bone morphogenetic proteins such as BMP4 act to drive the expression of a variety of transcription factors to drive and secure germ cell identity (Fig. 5).

For a thorough and excellent review of the transcriptional regulatory mechanisms underlying mammalian germ cell specification see Saitou and Yamaji [135]. As they are beyond the scope of this text oogenesis and spermatogenesis will not be covered here but in a related context, Marco Seandel, Assistant Professor of Cell and Development Biology in the Department of Surgery at Weill Cornell Medical College in New York, will discuss the development and function of adult spermatogonial stem cells in Part III, Chap. 14 of this book.

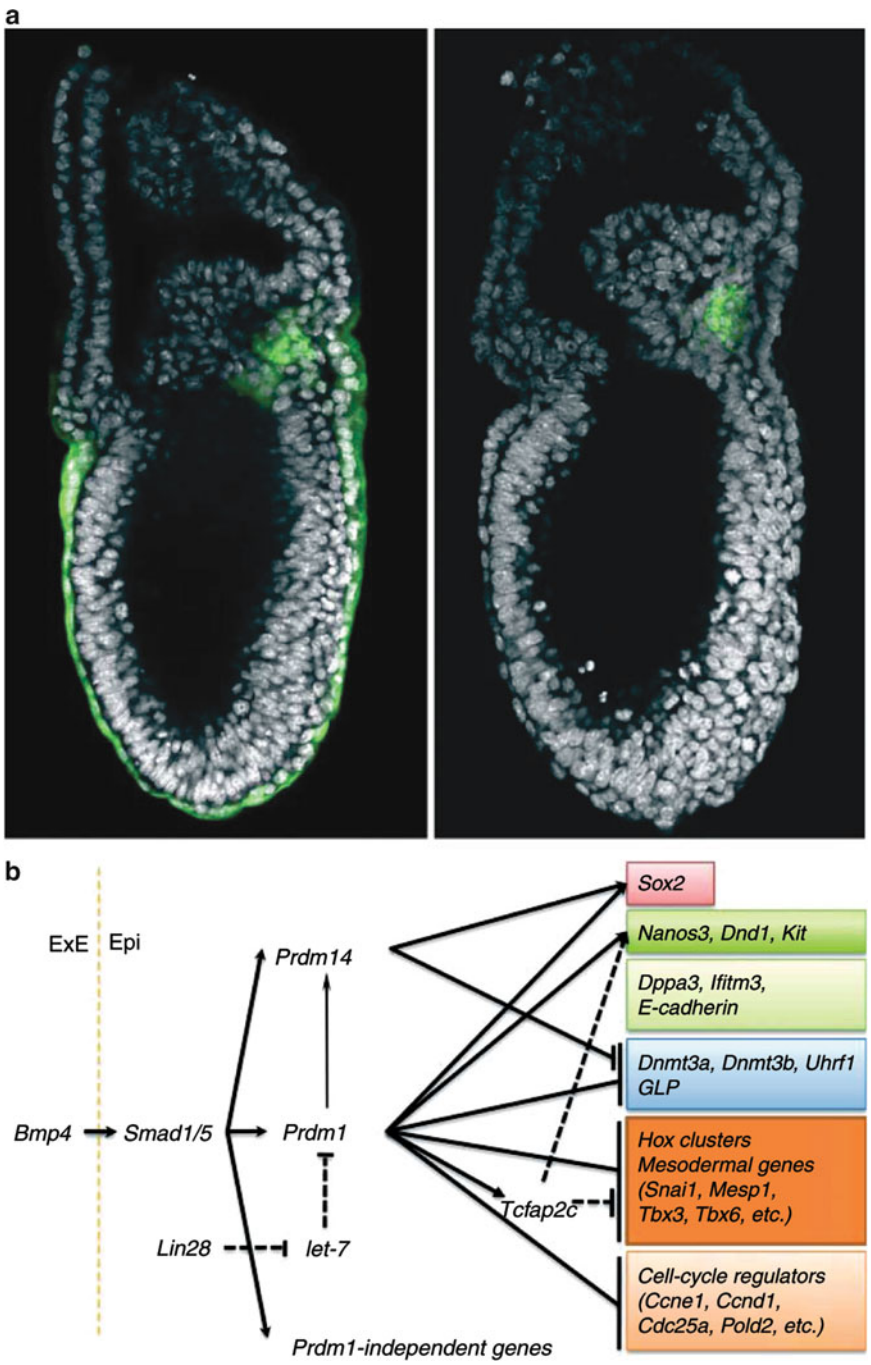

Fig. 5 (**a**) Expression of Prdm1 (left) and Prdm14 (right) in the LS stage embryo visualized by the Prdm1-mVenus and Prdm14-mVenus reporters respectively. Prdm1 is expressed in the nascent PGC precursors emerging from the most proximal part of the posterior epiblast as well as in the visceral endoderm. Prdm14 is exclusively expressed in the germ cell lineage and pluripotent cell lines. (**b**) A summary of genetic pathways for PGC specification

Embryonic Stem Cells

Perhaps the stem cell type that has received the most attention during the past 20 years is that of the *embryonic stem* (ES) *cell*. Embryonic stem cells are pluripotent in nature and derived from the ICM of blastocyst stage embryos. Mammalian ES cells were first isolated in 1967 in a seminal study by Robert Edwards and colleagues, who cultured rabbit blastocysts on feeder layers and demonstrated their capacity to differentiate into a variety of adult cell types representing hematopoietic, neural muscular, and connective tissue [27]. In 1981, murine embryonic stem cells were successfully isolated and cultured by two independent research teams, that of Gail Martin in the Department of Anatomy at the University of California—San Francisco and a team lead by Martin Evans and Andrew Kaufman in the Department of Genetics at the University of Cambridge [28, 29]. Martin's research showed that embryos could be successfully cultured

in vitro and embryonic stem cells derived there from which could be directed to differentiate into a variety of terminal, mature adult cell types. Her team accomplished this via whole embryo culture in serum followed by microdissection of the ICM and further expansion of isolated ICM cells in the presence of a fibroblast feeder layer. Individual colonies resulting from ICM cell plating and propagation were analyzed for pluripotential properties by assessing their ability to differentiate *in vitro* and to form embryoid bodies and teratomas in nude mice. Evans and Kaufman instead focused on the relatively low number of ICM cells present in a mammalian blastocyst and in particular how to increase this number to improve the chances of ES cell isolation and culture. Their group outlined a unique intra-uterine culture technique that allowed for increased ICM cell number, thus enabling successful ES cell isolation. Their technique was devised to delay embryonic implantation and involved hormonal manipulation of the pregnant mother through progesterone administration and ovary removal. This technique allowed for an increase in ICM cell number in utero. Embryos were subsequently isolated and cultured on arrested feeder cells in a manner similar to that employed by the Martin group and pluripotentiality confirmed. In 1987 and 1989, researchers Mario Capecchi, Martin Evans, and Oliver Smithies independently utilized mouse embryonic stem cells and genetic manipulation technologies to disrupt and thereby inactivate the hypoxanthine phosphoribosyl transferase (HPRT) locus [30, 31]. These techniques were later refined and the use of murine embryonic stem cells to inactivate specific loci through gene targeting experiments in mice has become one of the most widely used *in vivo* techniques for defining gene function in mammals. Capecchi, Evans, and Smithies went on to win the 2007 Nobel Prize in Medicine for this work.

What marks a truly pluripotential embryonic stem cell? During the 1990s a defined set of molecular and biochemical markers was established and accepted as minimum criteria for pluripotentiality. These include the stage-specific embryonic antigen cell surface markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Of course, the presence of the key transcription factors Oct4 and Nanog also have been accepted as classical markers as well as drivers of pluripotentiality. In fact, these transcription factors not only define but are required for ES cell pluripotentiality. Oct4 knockout embryos fail to develop pluripotent stem cells, with the ICM instead skewing towards the extraembryonic trophoblast lineage [32]. Similarly, Nanog deficient ICMs failed to form epiblast cells, instead producing parietal endoderm-like cells [33]. These markers have enabled researchers to define minimum requirements for specific cell types to be considered -pluripotent, however, the ultimate proof is confirming the ability of a cell to contribute to lineages representing the three primary germ layers. In 1995, a research team led by James A. Thomson of the University of Wisconsin—Madison modified the classical embryonic and cell culture conditions employed for murine ES cell research over the last 17 years to successfully isolate primate embryonic stem cells. These cells were demonstrated to maintain a normal XY karyotype after 1 year of propagation and to have the capacity to differentiate into trophoblasts as well as derivatives of embryonic endoderm, mesoderm, and ectoderm. Two key differences were noted between the derivation of primate and murine ES cells. First, the cells were demonstrated to contribute to derivatives of both the ICM and extraembryonic trophectoderm. This suggests the possibility that the primate lines were isolated from an earlier developmental time point. Second, in the absence of feeders, primate ES cells were shown to undergo significant differentiation, even in the presence of leukemia inhibitory factor (LIF), a key supplement in the inhibition of murine ES cell differentiation in tissue culture. These findings implicated additional or other as yet unknown factors secreted by feeder cells required for maintaining a pluripotent phenotype [34]. In a seminal study 3 years later, Thomson's group employed similar methodologies to isolate human embryonic stem cells from embryos produced by *in vitro* fertilization methods and donated after informed consent. These cells exhibited morphological and marker presence features similar to ES cells derived from other species and were confirmed for pluripotential capacity (teratoma formation) and self-renewal. They also demonstrated high levels of telomerase activity indicating a replicative lifespan that will exceed that of somatic cells ([35] and for an example see Fig. 6).

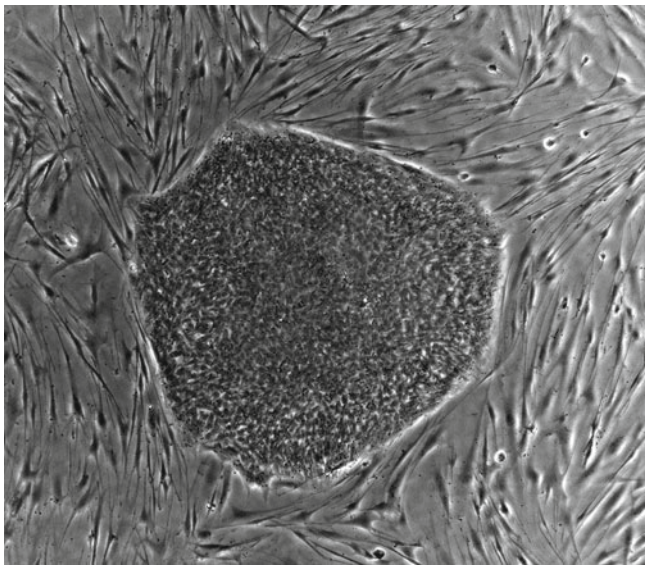

Fig. 6 Brightfield microscopy of a human embryonic stem (hES) cell colony grown from cell line SA02 on a mouse embryonic fibroblast (MEF) feeder layer (Courtesy Wikimedia Commons)

Thus, in a developing human embryo, ES cells can be successfully isolated from the ICM at the blastocyst stage in a similar manner to that of other species. The two classical hallmarks of ES cells are pluripotentiality and indefinite replication capacity. Their pluripotential nature means that these cells can give rise to differentiated derivatives of the three primary germ layers endoderm, mesoderm, and ectoderm including over 220 adult cell types. In Part I of this book, researchers Virginia Papaioannou, Professor in the Department of Genetics & Development at Columbia University Medical Center in New York, Ihor Lemischka, Director of the Black Family Stem Cell Institute and Mount Sinai Hospital in New York and Evan Snyder, Associate Physician of Pediatrics at the University of California—San Diego and Professor at Sanford Burnham Medical Research Institute outline three critical areas of research pertaining to embryonic stem cell origin and identity respectively: (1) The existence and derivation of embryonic stem cells from early mammalian embryos (Chap. 3); (2) A discussion of the key signals driving "stemness" (Chap. 4); and (3) The growth and differentiation dynamics of human fetal neural stem cells (Chap. 5).

It is thus the indefinite replication capacity of ES cells, if cultured properly such as in the presence of feeder cells and/or various growth factors, that allows for a powerful research and potential cell transplant therapeutic reagent source. Albeit controversial due to the use of human embryos, the use of ES cells in cell replacement therapies has been a main goal of ES cell research over the last 20 years. Some anomalies which could be addressed include immune system and hematopoietic diseases, neurological disorders such as Parkinson's Disease, spinal cord injuries, and juvenile diabetes. And existing human ES cell lines may provide a valuable unlimited resource for the development and implementation of cell-based drug screening platforms.

Adult Stem Cells

Adult stem cells can be defined as undifferentiated cells, often found among mature organs or tissues, which undergo self-renewal and have the capacity to differentiate into some or all of the specialized cell types of that organ or tissue system. Adult stem cells are considerably restricted in differentiation capacity, having already become both determined and committed to ultimately become or drive the production of specified mature lineages. They are hence defined as either multipotent or unipotent. Adult stem cells are sometimes referred to as *somatic stem cells* ("soma" means body) thus distinguishing them from stem cells of embryonic or germinal origin. Beginning with the early studies in bone marrow by McCulloch and Till, the past 50 years have seen the discovery of a multitude of somatic stem cells and the characterization of their potential to populate tissues and organs with much needed specialized cells. Adult stem cells have now been discovered in a variety of tissues and organ systems including brain, bone marrow, the vasculature and peripheral blood, skeletal muscle, cardiac tissue, hepatic tissue, ovarian epithelium, gut teeth, and testis. These cells may provide a valuable resource for the treatment of numerous medical disorders. Table 1 lists some of the more high profile adult stem cells discovered and characterized to date.

In each of the tissues mentioned above there exists a *stem cell niche*, an ideal microenvironment, within which adult stem cells reside. This niche is conducive to both stem cell propagation, and in some instances, differentiation. It should also be noted that adult stem cells have a finite capacity to undergo cellular division, and differentiation capacity is considerably limited to a few or one lineage(s). These two properties are the most notable differences between adult and embryonic stem cells. The following sections provide brief introductory synopses for some of the more high profile adult stem cell types studied. Greater detail on these and other categories of adult stem cells is found in later chapters of this book.

Hematopoietic Stem Cells

HSCs can be defined as a heterogeneous population of multipotent stem cells that can differentiate into the myeloid or lymphoid cell types of the adult blood system. During early vertebrate embryonic development HSCs exist as extraembryonic hemangioblasts which differentiate into both endothelial cells and erythrocytes to drive the development of the yolk sac vasculature. What are referred to as "adult" HSCs arise later in development and are unrelated to hemangioblasts, yet similar signaling and transcriptional control mechanisms that drive early HSC formation during embryogenesis are thought to play a role later in fetal development and even in the adult. In fact, as early as 1978 Schofield contemplated the existence of a HSC residing within a specific "niche" of the bone marrow postnatally where complex signaling crosstalk provides the cues needed for both stemness and proper differentiation [36]. The crucial "stemness" nature of the hematopoietic system is now known to be required throughout life for the constant generation of the different blood cell types. Over the last 40 years this niche concept has been expanded upon, with a delineation of concise crosstalk between bone marrow endosteal and vascular niches driving the development of HSCs and their function in the developing and mature blood system. The *endosteum*, the region interfacing bone marrow with bone, is infiltrated with *osteoblastic* cells that secrete numerous cytokines thought to drive the development, maintenance, and behavior of HSCs through the "endosteal niche." For example, thrombopoietin and angiopoietin are thought to enhance

Table 1 Sources of adult stem cells and their differentiation capacity (Adapted from the National Institutes of Health resource for stem cell research)

Tissue of origin	Adult stem cell type	Mature lineage produced	Reference
Blood	Circulatory	Adipocyte	Kuznetsov et al. [174]
	Skeletal	Osteocyte	
Bone marrow	Angioblast (endothelial precursor)	Mature endothelia and newly formed blood vessel	Kocher et al. [173]
	Hematopoietic stem cell (HSC)	Hepatocyte	Alison et al. [155]
		Cholangiocyte	Theise et al. [140]
	Human marrow stromal	Stromal-derived cell engrafted in rat brain	Azizi et al. [156]
	Mesenchymal stem cell (MSC)	Adipocyte	Pittenger et al. [136]
		Chondrocyte	
		Osteocyte	
	MSC	Neuron	Woodbury et al. [149]
	MSC	Neuron	Sanchez-Ramos et al. [138]
	MSC	Adipocyte	Liechty et al. [177]
		Bone marrow stromal cell	
		Cardiomyocyte	
		Chondrocyte	
		Myocyte	
		Thymic stromal cell	
Bone marrow (fetal)	HSC	HSC	Baum et al. [157]
		Red blood cell lineages	
		White blood cell lineages	
Brain	Neural stem cell (NSC)	Muscle cell	Galli et al. [163]
Brain (adult and neonatal)	Neural progenitor cell (NPC)	Astrocyte	Palmer et al. [135]
		Neuron	
		Oligodendrocyte	
Brain (fetal)	Human central nervous system stem cell (hCNS-SC)	Astrocyte	Uchida et al. [144]
		Neuron	
		Oligodendrocyte	
Fat	Stromal vascular cell fraction of processed lipoaspirate	Adipocyte precursor	Zuk et al. [152]
		Osteocyte precursor	
		Chondrocyte precursor	
		Myocyte precursor	
Liver (fetal)	HSC	Hematopoietic progenitor cell (HPC)	McCune et al. [154]
		Red blood cell lineages	Namikawa et al. [134]
		White blood cell lineages	
Pancreas	Nestin-positive islet-derived progenitor cell (NIP)	Pancreatic	Zulewski et al. [153]
		Hepatic	
Umbilical cord	HPC	Most red and white blood cell lineages	Broxmeyer et al. [158]
	HSC	Most red and white blood cell lineages	Erices et al. [162]
	Mesenchymal progenitor cell (MPC)	Osteoblasts	
		Adipocytes	

HSC quiescence [37, 38]. These osteoblasts also express membrane-bound ligands such as Jagged and N-cadherin, and chemokines including stromal derived factor, SDF-1. These signaling molecules have been demonstrated to drive stem cell self-renewal and myelopoiesis [39–41]. The homing and migration properties of HSCs within the bone marrow can be largely attributed to regulation by the chemokine stromal derived factor (SDF-1), although this factor is not restricted to the endosteal niche but rather is secreted by a variety of cell types, including endothelial cells of the vascular niche, stromal cells, and osteoblasts. The TGFB superfamily of bone morphogenetic proteins TGFB1, BMP2, and BMP7A are released as a result of osteoclast bone breakdown and have been suggested in *in vitro* studies to cause HSCs to quiesce [42, 43]. In addition, even high endosteal ionic concentrations can regulate HSC behavior, with elevated levels of endosteal calcium promoting surface migration [44]. A component of the endosteal niche, the sympathetic

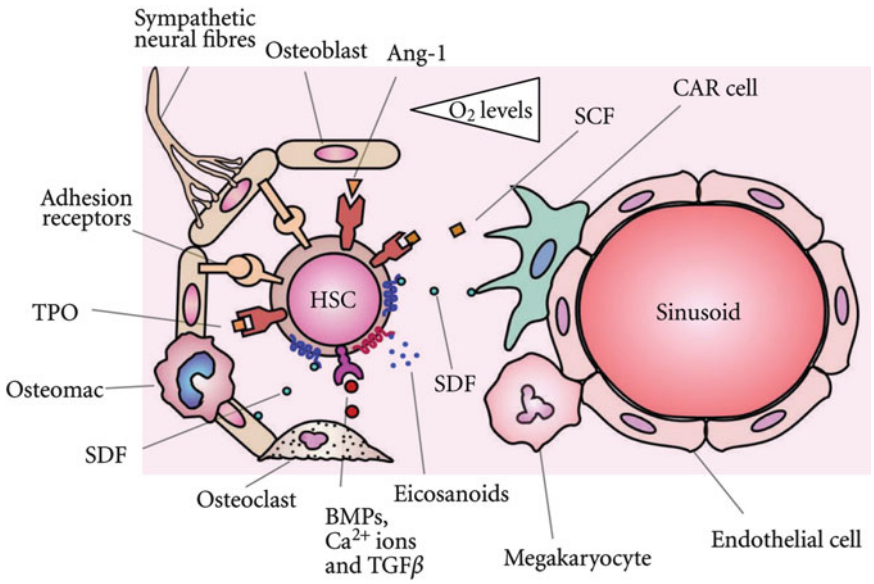

Fig. 7 The interplay between endosteal and vascular niches in the control of hematopoietic stem cells. Cells of both the endosteal and vascular niches communicate and the balance of signaling molecules between the two niches along with signaling from oxygen levels and small molecules regulates HSC behavior (Courtesy Andrew J. Lilly and *Stem Cells International* (Lilly et al. [178]); reprinted with permission)

nervous system (SNS) has also been demonstrated to provide signaling cues for HSC mobilization from the bone marrow [45]. A second key component of the regulatory environment is known as the "vascular niche," as it has been well documented that the bone marrow vasculature also plays a role in HSC maintenance. As mentioned above, extraembryonic vasculature is formed from hemangioblasts, yet the endothelial components of this vasculature have been shown to give rise to HSCs demonstrating close developmental regulation. In adults it has been shown that the vasculature of the liver and spleen can drive hematopoiesis in these organs [46, 47]. The signaling components of the vascular niche that regulate HSC proliferation, maintenance, and differentiation capacity are less well defined than for the endosteal niche, but it is clear that endothelial cells are crucial components of the vasculature that drive hematopoiesis *in vivo*. The cytokine receptor gp130, aka IL6-ST, ILbeta, and CD130, has been shown to be a crucial factor expressed by endothelial cells in the vascular niche to promote hematopoiesis. This has been definitively confirmed in conditional mouse knockouts deleting gp130 from both HSCs and endothelial cells [48]. These mice exhibited hypocellular bone marrow and died within a year after birth. In addition, bone marrow transplants from gp130 deficient mice to normal irradiated mice restored normal hematopoiesis, but the converse transplant failed to yield the same result [49]. Thus the glycoprotein gp130 acts as a key regulator of hematopoiesis, exerting its effect from the vascular niche. The localization of HSCs within the bone marrow has also been shown to be driven by the vascular niche. CAR reticular cells proximal to the sinusoidal endothelium have been demonstrated to play a role in the migration and localization of HSCs, specifically the attraction of HSCs via the secretion of SDF-1 [50]. HSC proliferation is also directly influenced by factors secreted from the CAR cell lineage residing in the sinusoidal endothelium, aptly named for CXCL12 abundant reticular cells. Strikingly, it has been demonstrated that the vast majority of HSCs, upwards of 97%, are proximal to CAR cells within both the bone marrow and endosteum, suggesting an intimate relationship between the two cells types from a signaling and perhaps migratory perspective. Stem cell factor, SCF, a cytokine aptly named for its pro-proliferative effects, has been shown to be secreted by CAR cells within this region [51]. Finally, irrespective of the endosteal or vascular niches, it should be noted that both small bioactive signaling molecules such as Eicosanoids and a hypoxic environment directly affect HSC behavior. Prostaglandins, for example, the most widely studied subgroup of the Eicosanoids, have been shown to drive increased expression of CXCR4 on the surface of HSCs, thereby enhancing migratory capacities [52]. Under hypoxic conditions, when oxygen levels drop below a certain threshold in the bone marrow, hematopoiesis has been shown to increase [53, 54]. Figure 7 illustrates the crosstalk between endosteal and vascular niches as well as other factors to drive HSC behavior.

Many other signaling factors emanating from both the endosteal and vascular niches have been shown to affect hematopoiesis and are beyond the scope of this introductory chapter. For a comprehensive review of the HSC niche and the interplay between the HSC, endosteal, and vascular niches, see Lilly et al. 178]. Part III of this book focuses exclusively on tissue stem cells. Specifically, Pierre Charbord of the Institut National de la Recherche et Santé Médicale (INSERM) in Tours, France will discuss the HSC niches further and expand on this brief introduction through a detailed analysis of the molecular and developmental pathways that drive hematopoiesis.

Liver Stem Cells

The hepatic system has one of the most unique and widely studied adult stem cell niches, and actually consists of numerous niches, both extra- and intrahepatic, which depend upon a considerable amount of proximal and distal signaling to drive the different stages of stem cell maturation (Fig. 8). Much of the paracrine signaling that occurs mimics the defined molecular crosstalk that drives the classical epithelial-to-mesenchymal transition during early embryonic development. The reciprocal signaling that occurs between parenchymal and mesenchymal cells in the developing and adult liver is based upon gradients of paracrine signals that regulate cellular identity. Feedback loop signaling also plays a role in later lineage specification, with both positive and negative signaling cues emanating from dying cells and actively proliferating hepatoblasts to promote not only proliferation, but also to set up terminal lineage commitment [55]. Both paracrine and feedback loop signaling ultimately allow for the generation of hepatic stem cells, hepatoblasts, angioblasts, and committed progenitors, each of which are precursors for the various cell types needed for proper liver function. Below is a brief summary and description of the cell types arising chronologically during the differentiation cascade (Fig. 8).

1. *Hepatic stem cells*: Hepatic stem cells are multipotent in nature and have the capacity to give rise to hepatoblasts, committed progenitors and ultimately mature adult cells. The location of hepatic stem cells varies depending upon age and can be found in the ductal plates of fetal and neonatal livers. Later in life, these cells restrict to the canals of Hering and remain there throughout adulthood [56–62]. Morphologically, hepatic stem cells have a high nuclear-to-cytoplasmic ratio and are about 8 μm in diameter. They represent up to 2% of the parenchymal (non-connective tissue) cell population of postnatal human livers. Some classic markers for hepatic stem cells include EpCAM, NCAM, sonic, and Indian hedgehog and the transcription factors Sox9, Sox17, and FoxA2. The Wnt-beta catenin signaling pathway has been shown to be crucial for activation of EpCAM and driving of the hepatic stem cell phenotype and Wnt1 specifically has been demonstrated

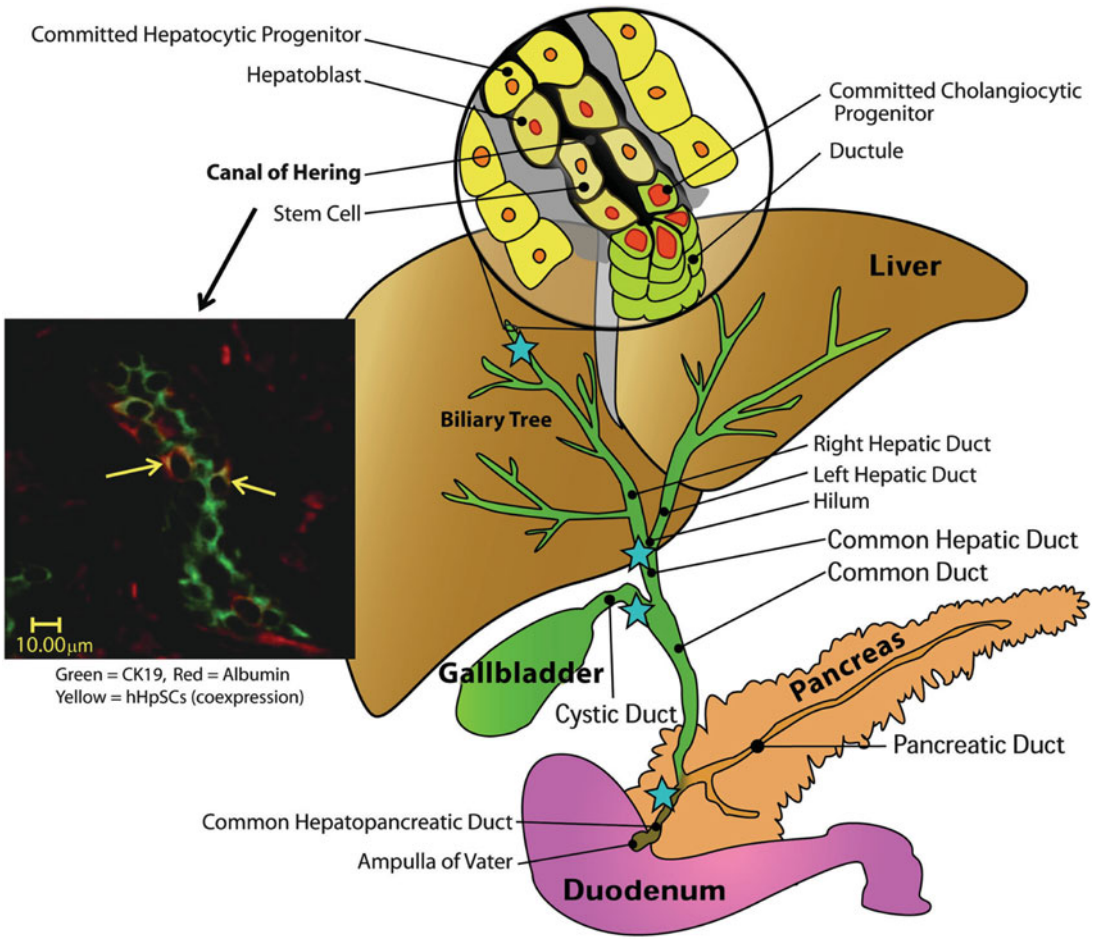

Fig. 8 Schematic image of liver, the biliary tree, and pancreas and their connections with the duodenum. The blue stars indicate sites at which there are high numbers of peribiliary glands, the stem cell niches of the biliary tree (Courtesy Rachael Turner and *Hepatology* (Turner et al. 2011) [73]; reprinted with permission)

to be required for the efficient regeneration of liver by oval cells after hepatic injury [63, 64].

2. *Hepatoblasts*: Hepatoblasts are undifferentiated bipotent stem cells which arise from the foregut endoderm during embryonic development, specifically form hepatic stem cells, and can give rise to mature hepatocytes and biliary epithelial cells [65]. In a manner similar to that for hepatic stem cells, their localized presence in the liver is dependent upon age, with widespread presence in the parenchymal region of fetal and neonatal livers and later clumped in the canals of Hering in adults [58]. Terminal differentiation of hepatoblasts requires a gradient of activin A and TGFb [66, 67]. They are identified morphologically as oval cells and express the markers OV-6, albumin, and cytokeratins CK-19 and CK-7 [68]. In contrast to hepatic stem cells, hepatoblasts do not express markers for mesenchymal cells or hematopoietic endothelial cells and exhibit up to fivefold more telomerase activity than hepatic stem cells. These are two distinguishing factors for the highly related cell types.
3. *Progenitor cells*: Two types of committed progenitor cells, *intermediate hepatocytes* and *small cholangiocytes,* arise from hepatic stem cells throughout the liver and within the bile ducts, lose stem cell marker expression such as NCAM and begin to express either biliary or hepatocytic terminal markers. Both cell types are typically unipotent, giving rise to either mature hepatocytes or cholangiocytes, respectively. Intermediate hepatocytes range in size from 12 to 15 μm in diameter, are polygonal in shape and tend to be present throughout fetal and neonatal liver tissue, expressing albumin and other terminal markers. Small cholangiocytes are smaller at 6–8 μm in diameter, cuboidal in shape, and tend to co-localize with hepatic stem cells in the ductal regions and canals of Hering. Marker expression is widespread for these cells and includes cystic fibrosis transmembrane conductance regulator (CFTR, humans only), anti-apoptotic proteins annexin V and bcl2 as well as endothelin receptors A and B [69]. Interestingly, small cholangiocytes have been shown to proliferate in response to H1 histamine receptor stimulation by activation of the IP3/CaMK I/CREB pathway [70].
4. *Proliferative adult cells*: Following commitment to a specific terminal lineage, adult hepatic cells, known as *small hepatocytes*, express terminal differentiation markers yet retain their ability to divide for on average 6–7 generations as confirmed by *in vitro* studies [71]. In fact, when cocultured with differentiated hepatocytes, these cells have been demonstrated to express terminal markers such as α-fetoprotein, albumin, and Mrp1 and form organoids exhibiting a fully differentiated transporter expression phenotype [72]. *Large cholangiocytes* also retain some proliferative capacity, and are present primarily in the ductal regions. They express the terminal markers CFTR, aquaporin 4, aquaporin 8, and others and play a primary role in regulating ductal bile secretion and absorption. Thus while small cholangiocytes tend to play a role in generating sufficient cell numbers via potent proliferative capacity large cholangiocytes are more focused on secretory and absorption functions. For a more thorough comprehensive review of the various stages involved in the development and differentiation of hepatic terminal lineages, please see the excellent review by Rachel Turner and colleagues at the University of North Carolina—Chapel Hill [73].

What are the clinical implications of adult liver stem cells? Cell and tissue replacement therapies could become routine and a reality for a variety of liver degeneration disorders utilizing the various stages and types of liver-specific stem cells as cell replacement candidates. It is widely known that the liver by itself is indeed capable of undergoing varying degrees of regeneration after partial hepatectomy or due to toxic injury-driven loss of pericentral cells. A more thorough analysis of the liver stem cell phenotype, pathways involved in its generation and the use of liver stem cells in clinical scenarios will be covered in detail in Chap. 22 by Malcolm Alison, Professor of Stem Cell Biology and Lead, Centre for Diabetes at the Blizard Institute of Cell and Molecular Science.

Neural Stem Cells

One of the primary characteristics of the CNS is that, unlike the liver its tissues do not regenerate (discussed above). Damage occurring as a result of disease or injury is usually permanent and its deleterious effects chronic. The foundation of this hypothesis was Santiago Ramon y Cajal's concept of "no new neurons" in the adult [74]. Conceptualized over 80 years ago, this idea was widely accepted until 1967 (initial findings occurred in 1961) when Joseph Altman and Gopal Das of Massachusetts Institute of Technology in Boston observed mitotic activity in the brains of adult guinea pigs. Altman further observed, using tritium radiolabeled thymidine, that these mitotic neural cells differentiated into a mature neuronal phenotype they termed "microneurons" [75]. As fate would have it the Altman/Das findings went largely ignored until 30 years later when adult neurogenesis was "rediscovered." In the 1990s, neurogenesis in the adult human brain was again confirmed [76–78]. These findings have led to a rebirth in the study of neurogenesis utilizing neural stem cells as key tools for deciphering the biochemical and molecular signaling events that drive neural lineage determination and commitment postnatally. They have also driven an intense effort at applying neural stem cell plasticity for therapeutic gain, i.e., CNS repair.

The beginning of CNS maturation during embryonic development, termed *neural induction,* is a temporal point at which *neural stem cells* (NSCs) or NSC-like precursor cells

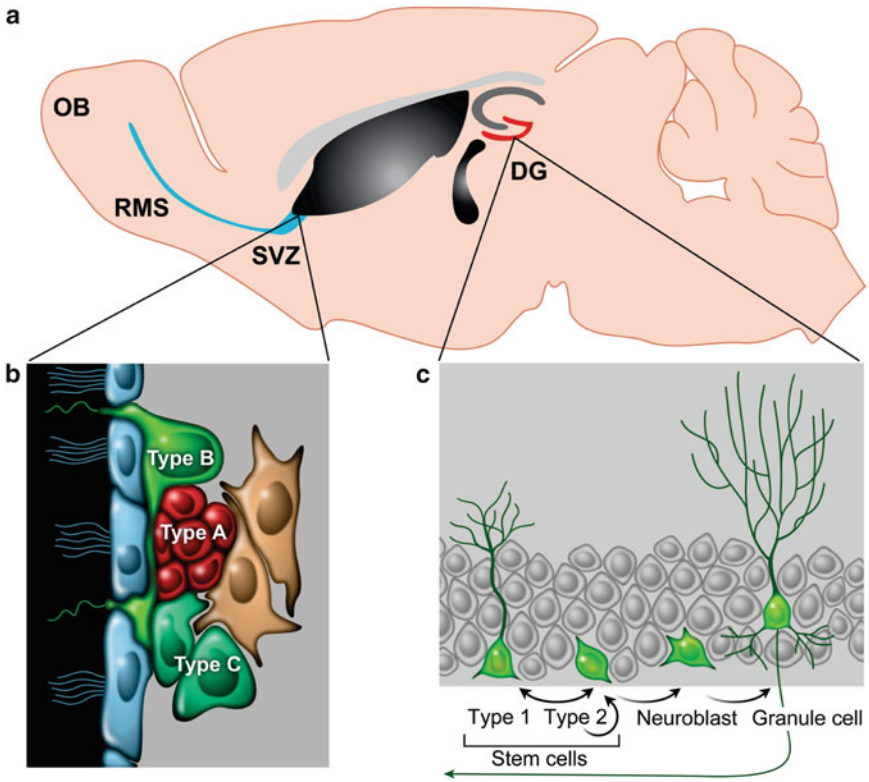

Fig. 9 Neurogenesis in the adult brain. (**a**) Adult NSCs are primarily located in two germinal zones of the brain: the SVZ of the lateral ventricles and the SGZ of hippocampal DG. (**b**) A subset of relatively quiescent GFAP+ radial cells (type B cells) in the SVZ has the potential to serve as adult NSCs and generate rapidly dividing, transit-amplifying non-radial NSCs (type C cells), which in turn give rise to neuroblasts (type A cells) that migrate through the RMS towards the OB. (**c**) In the adult SGZ, a population of GFAP+ Sox2+ radial cells corresponds to quiescent NSCs (type 1 cells). They coexist with actively proliferating, GFAP− Sox2+ non-radial NSCs (type 2 cells) that generate both astrocytes and neuroblasts. Neuroblasts then migrate into the granule cell layer and mature into neurons (Figure courtesy Fred H. Gage and Elsevier, Ltd (Mu et al. 2010) [90], reprinted with permission)

become determined to give rise to particular neural and neuronal phenotypes. NSCs can therefore be defined as *multipotent precursor cells* that have both the capacity and the restriction to give rise to neurons, astrocytes, or oligodendrocytes. In order to understand the inherent capacity of neural stem cells to drive the development of not only individual neural and neuronal lineages but also the complex development of an entire organ system crucial for such processes as sensory and locomotor activities, it is necessary to grasp the developmental maturation of NSCs during embryonic development. Many of these principles may apply in adult neurogenesis. Reflected under the guise of embryonic development as a model system, NSC behavior can be broken down into three primary phases:

1. *Expansion*: During the early stages of embryonic development, columnar neural stem cells, often called *neuroepithelial cells*, undergo symmetric division to drive population growth in preparation for later determination and commitment events [79, 80]. In the vertebrate embryo, this occurs proximal to the early embryonic ventricle and pial surfaces. In addition to increasing neuroepithelial cell numbers, this division also begins to populate these surfaces with mature neurons. Collectively these events will drive CNS organization and function after birth.
2. *Neurogenesis*: During the neurogenic phase of embryonic development neuroepithelial cells, now most commonly defined as *neural stem cells*, divide in an asymmetric fashion, with one daughter cell maintaining the mitotic state while a second daughter cell exits the cell cycle and differentiates into a neuronal lineage. This cell division rapidly populates the germinal ventricular zone (VZ) with NSCs needed to ultimately produce many of the terminally differentiated cell types crucial for neural function. The vast majority of these neurogenic events occur in the VZ. As the neural tube thickens, NSCs receive multiple signaling cues that drive a morphological transition from a columnar to a radial appearance, resulting in the development of *radial glia*. These cells ultimately give rise to cortical astrocytes and, perhaps through continued asymmetric division, cortical neurons [81, 82]. In the adult brain, neurogenesis continues to occur throughout life, primarily in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus (DG) (Fig. 9). What are the extrinsic signaling and intrinsic

molecular events which drive both embryonic and adult CNS neurogenesis? The Wnt signaling pathway has been implicated in driving both embryonic and adult neurogenesis, specifically in the developing and mature hippocampal region of the brain. NSCs have been demonstrated to receive inductive cues not only from Wnt-producing astrocytes but also from their own Wnt signaling [83, 84]. It is the Wnt/b-catenin pathway which ultimately drives the expression of NeuroD1, a bHLH transcription factor involved in late-stage terminal differentiation of neural stem cells into neurons [59]. In fact, the bHLH class of transcription factors has broad influence on neurogenesis during embryonic development and into adulthood. Neurogenin1 and 2 are well characterized as neuronal determination factors, controlling waves of neurogenesis in the developing dorsal root ganglia (DRG); Mash1 (Ascl1) drives GABAergic interneuron development in the olfactory bulb; Neurogenin2 and Tbr2 promote glutamatergic neuronal development in the juxtaglomerular region; and the Hes family of inhibitor bHLHs act to antagonize the neurogenic effects of these and other bHLH proteins to maintain neural stem cell numbers required for later neurogenic diversity [85–87]. No mention of neuronal induction signaling would be complete without a reference to the sonic hedgehog/smoothened pathway, which acts as a potent mitogenic cascade to drive NSC proliferation in the developing embryo and adult brain [88]. Finally, the transcription factor Sox2 has been shown to play a crucial role in maintaining appropriate NSC numbers in the adult brain. As an inhibitory transcription factor, Sox2 is speculated to inhibit glial fibrillary acidic protein (GFAP) expression [89]. GFAP is a marker of the switch to gliogenesis (described below) and is an intermediate filament protein expression in glial cells such as astrocytes and ependymal cells. A complete outline of the key transcription factors and signaling events involved in embryonic and adult neurogenesis is beyond the scope of this introductory chapter. For more detail on adult neurogenesis please see the excellent review by Fred Gage and colleagues at the Salk Institute for Biological Studies [90] (Fig. 9).

3. *Gliogenesis*: Upon completion of the vast majority of neurogenesis, in the VZ a subset of neural stem cells will become competent to generate various glial cell types. This restriction occurs mostly in a proximal region of the brain referred to as the SVZ. During this gliogenic phase, NSCs lose some multipotent competency, committing to a glial phenotype. These glial progenitor cells will ultimately give rise to terminally differentiated glial cells, including astrocytes and radial glia. What transcription factors drive the loss of multipotency at this stage? Hideyuki Okano and colleagues have performed elegant *in vitro* knockdown studies on mouse embryoid bodies in combination with known neural inducers such as noggin and retinoic acid. These studies demonstrated a crucial role for the transcription factor COUP-TFII, which is suggested to play a role in alleviating the epigenetic silencing of such terminal differentiation markers as GFAP [91]. Other transcription factors positively regulating gliogenesis include neurogenin2, olig2, and Sox10 for oligodendrocyte maturation [92, 93]. Negative transcriptional regulators of gliogenesis, thus promoting the "default" neurogenesic pathway, include the homeobox transcription factors Emx2, Sox5, Sox6 and the winged helix transcription factor Foxg1, albeit through entirely distinct mechanisms [94]. Also, Notch, BMP, sonic hedgehog and FGF signaling in this system is crucial both for the early inhibition of neurogenesis and the later promotion of gliogenesis. These growth and differentiation factors have been demonstrated to act directly through many of the transcription factors mentioned above.

Thus it is clear that the capacity for CNS-derived NSCs to act as a timely source of terminally differentiated neurons and glial cells is of key importance, both during embryonic development and postnatally. The therapeutic potential for NSCs and the signaling pathways regulating them is considerable. The potential to supply NSCs as a source for the generation of new neurons or glial cells could positively impact a multitude of medical disorders, from spinal cord injury to Parkinson's Disease. For a more comprehensive review of neural stem cells as they pertain to regeneration of the CNS see Okano [91]. As described above, this review illustrates some elegant *in vitro* studies that mimic the temporal changes in NSC multipotency and provides insight into the timing of lineage commitment at multiple levels. In Part III, Chap. 24 of this book Magdalena Gotz, Professor in the Department of Physiology, Development and Neuroscience at the Wellcome Trust/Cancer Research UK Gurdon Institute in Cambridge, UK and Swetlana Sirko, Scientist in the Department of Physiological Genomics at the Ludwig Maximilian University in Munchen, Germany will outline the differentiation potential of glial cells.

It should be noted that adult stem cells as they relate to other organ systems will be covered in detail in later chapters of this book and will include the stem cells of the hematopoietic system (Chap. 15), gastrointestinal system (Chap. 19), pancreas (Chaps. 20 and 21), glandular system (Chap. 16) and mammary epithelia (Chap. 17). In addition, Heidi Scrable, Associate Professor of Biochemistry/Molecular Biology in the Division of Experimental Pathology and Laboratory Medicine at the Mayo Clinic in Rochester, Minnesota will give a comprehensive overview of adult stem cells and their role in as well as their impact on aging (Part III, Chap. 25). For more information on stem cells and aging describing human adipose-derived stromal/stem cells (hADSCs) as a model system see the review by Tollervey and Lunyak [142].

Nuclear Reprogramming and Induced Pluripotency

The issue of immunorejection as it applies to tissue and cell replacement regenerative therapies has long been a driving force in the search for patient-specific stem cell platforms that might be scalable for clinical use. Therapeutic and clinical strategies that involve non-genetically matched cells run the considerable risk of rejection by the patient's immune system. For example, the derivation of an individual's stem cells and their scalable differentiation into desired terminal lineages has therapeutic potential for a wide variety of disease states; this type of treatment represents one of the holy grails of stem cell research. This autologous replacement regenerative therapy eliminates the possibility of cell, tissue, or organ rejection, thus making it possible to effectively treat a disorder with the patient's own cells. Disorders such as Parkinson's disease, Alzheimer's disease, diabetes, etc. are all candidates for stem cell-related regenerative medicine therapy should the barrier of immunorejection become removed. Furthermore, deriving stem cells from an individual's own adult cell types through induced pluripotency (iPS) could eliminate the need for using embryos in the development of pluripotent stem cell-based regenerative medicine therapies (Fig. 10).

Three independent but related methods have been developed which may ultimately allow generation of patient-specific stem cells through nuclear reprogramming. Below is a brief synopsis of each technique. Note that iPS through nuclear reprogramming, iPS cell induction, and directed differentiation in particular will be discussed in much more detail throughout this book. For a more thorough outline of reprogramming strategies and technology developed to date see the review by Yamanaka and Blau [95].

1. *Cell fusion*: In cell fusion two or more cells are brought together to form a single entity (*hybrid* if dividing or *heterokaryon* if nondividing) and the transcriptional regulatory mechanisms of all combined cells tend to influence the resulting fusion's phenotype. Cell fusion is not a new con-

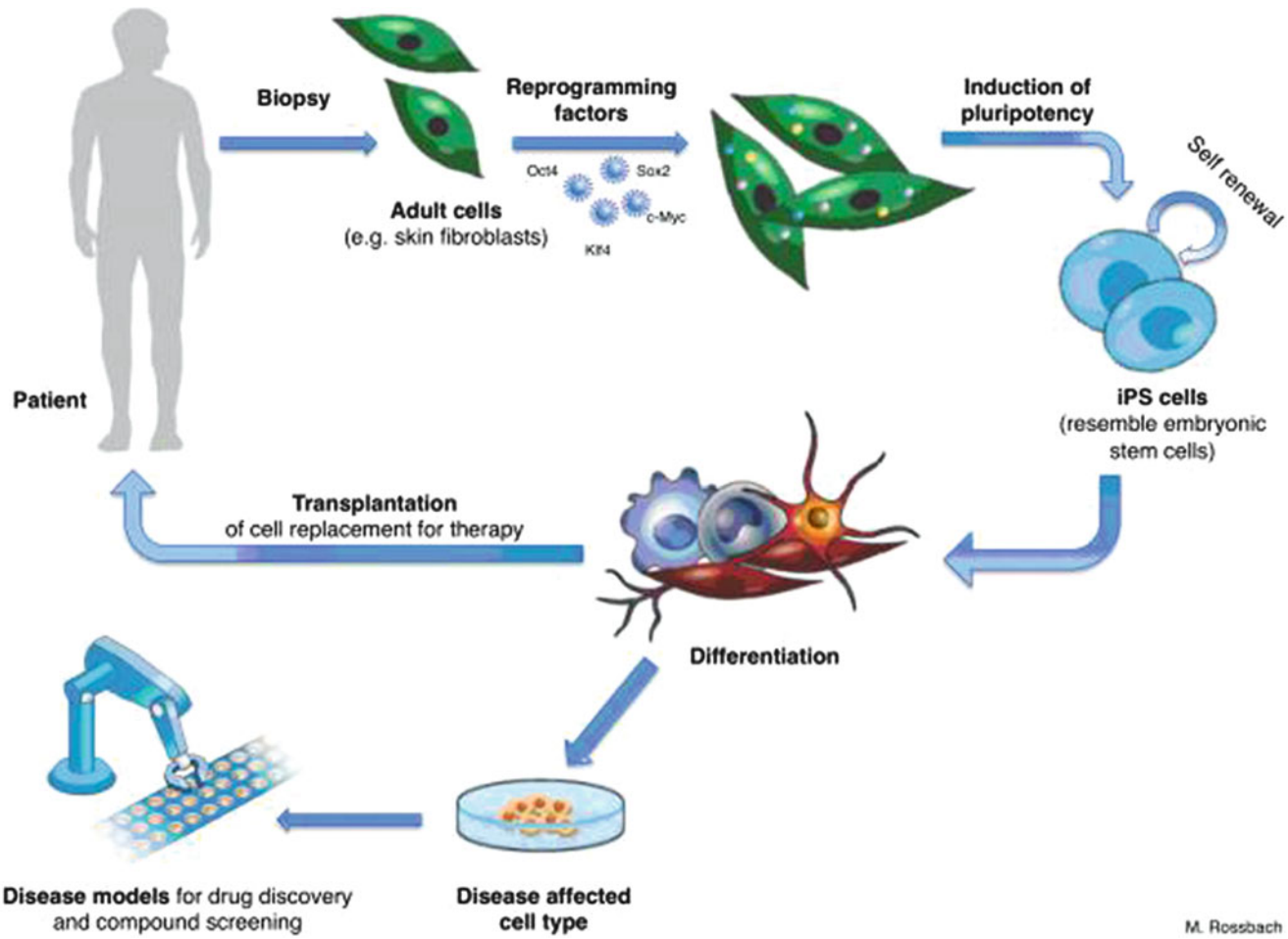

Fig. 10 Illustrative overview of the procedure for generating patient-specific iPS cells (Courtesy Michael Rossbach and *EuroStemCell*; reprinted with permission)

cept or technique and was actually first successfully accomplished in the 1960s. These initial studies along with later studies by pioneering researcher Helen Blau, currently Director of the Baxter Laboratory for Stem Cell Biology at Stanford University School of Medicine, revealed that cell fate is not fixed and can be transient if not actively regulated by appropriate gene transcription [96–98]. Blau's team focused on interspecies cellular fusion to allow gene products from each donor cell to be easily characterized. Collectively, these cell fusion studies have culminated in the realization that not only transcriptional activation but also active repression provides crucial regulatory signals to maintain a specific cellular identity. Loss of transacting repression mechanisms resulted in the activation of silenced genes and in some cases, a reprogramming of cellular identity. Early examples include the activation of blood- and liver-specific genes in fibroblast mixed species heterokaryons [99, 100]. These studies confirmed that mature lineage cellular phenotypes are not reversible but are rather plastic and actively regulated by both activation and repression-based transcriptional mechanisms. Takashi Tada and colleagues in the Department of Development and Differentiation, Institute for Frontier Medical Sciences at Kyoto University have taken these findings a step further, revealing that adult somatic cells can acquire a pluripotent phenotype upon fusion with embryonic stem cells. In studies of fusions between murine thymocytes and ES cells, Tada's group demonstrate the reactivation of genes silenced on the X-chromosome through both hyperacetylation and demethylation, indicating that an alteration of both the transcriptional and epigenetic identities of these loci resulted in fusions acquiring pluripotency [101, 102]. Loci reactivated in interspecies mixed heterokaryons have now been shown to include the key pluripotency factors Nanog and Oct4 [103, 104].

2. *Nuclear transfer*: Nuclear transfer, often referred to as *somatic cell nuclear transfer* (SCNT) when used in the context to reprogram adult or somatic cells, involves the direct transplantation of a donor nucleus into an enucleated oocyte. The oocyte environment signals and induces changes in gene expression, driving an ultimate total reversal of the differentiated phenotype. The technique, when implemented to generate either daughter cells or an entire organism originating from the donor somatic cells, is also referred to as *cloning*. Cloning of entire organisms was successfully demonstrated in the early 1950s in amphibians, but it was not until 1997 that mammals were successfully cloned by SCNT with the generation of "Dolly the Sheep" by Sir Ian Wilmut and colleagues at the Roslin Institute and PPL Therapeutics in Scotland [105, 106]. In these studies, Wilmut and colleagues utilized mammary nuclear donor cells starved of serum to eliminate any effect of cell cycle on chromatin organization. Nuclear transfer was accomplished by electrical pulse-mediated cell fusion with unfertilized enucleated oocytes acting as recipients. The cloning of other species such as pigs, dogs, and wolves soon followed, including perhaps the most significant milestone which was the mouse a year after Dolly was first produced. This work has now been extended to the use of nuclear donor cells derived from frozen tissue sources, even after long-term (a decade or more) storage [107, 108]. A cautionary note: Cloned organisms derived via the SCNT process often exhibit a variety of abnormalities including increased risk of cancer, immunological disorders, and often premature death [109]. These phenotypes are speculated to be due to epigenetic consequences whereby progeny cells retain some genomic imprinting characteristics of the original donor somatic cell, thus affecting appropriate gene expression. It is clear that, should SCNT ever be utilized to generate clinically relevant cells or tissues for regenerative medicine strategies, a firm knowledge and control of chromatin remodeling mechanisms such as methylation and histone modification will be required (Table 2).
3. *Transcription factor-based induction*: It was 25 years ago that Walter Gehring and colleagues at the University of Basel in Switzerland demonstrated that an entire tissue's fate can be altered via the introduction and expression of a single gene. The discovery of the transcription factor Antennapedia, which drove the replacement of antennae with legs in *Drosophila,* was a seminal finding in the field of gene regulation, demonstrating that individual genes have the power to guide cellular fate and whole body plans [110]. Since then, a multitude of transcription factors have been identified as potent regulators of cell fate, driving muscle (MyoD), blood (C/EBP), and neuronal (neurogenin) lineage development, to name but a few [85, 111, 112].

What factors might perform the opposite function, resulting in a reversal of the terminally differentiated phenotype? Shinya Yamanaka and colleagues at the Institute for Frontier Medical Sciences, Kyoto University in Japan addressed this question through a systematic characterization of 24 individual loci known to be expressed at high levels in undifferentiated ES cells. Yamanaka's group introduced each gene into both embryonic and adult murine fibroblasts via retroviral transduction and characterized the expression of a reporter gene, Fbx15, known to be expressed uniquely in ES cells. Through a process of elimination, four key transcription factors, Oct4, Sox2, Klf4, and c-Myc, were identified that could not only activate the reporter, but in combination were sufficient to drive a morphological and molecular conversion and resulting resemblance of the fibroblasts to ES cells. Teratoma formation in mice confirmed the *in vivo* pluripotentiality of the resulting cells and solidified these four factors as the major players in driving pluripotency [113]. Other groups later showed that c-Myc was dispensable for pluripotency

Table 2 Species cloned to date

Year cloning achieved	Species cloned	References
1952	Frog (tadpole)	Briggs and King [105]
1963	Carp	Liao et al. [176]
1986	Sheep (from early embryonic stem cells)	Willadsen et al. [148]
1987	Mouse (from an embryonic donor cell),	Tsunoda et al. [143]
1995	Sheep (from early ES cells)	Highfield [170]
1997	Mouse, Cattle, Sheep (from somatic cells)	Wakayama et al. [107]; Wells et al. [147]; Campbell et al. [159]
2000	Pig, Rhesus Monkey (by embryo splitting)	Walker et al. [145]; Chan et al. [161]
2001	Cat, Guar (wild cattle), Mouflon	Shin et al. [139]; Loi et al. [175]
2003	Rat, Rabbit, Horse, Mule, Deer	Zhou et al. [151]; Tian et al. [141]; Galli et al. [164]; Woods et al. [150]; Carrolton [160]
2005	Dog, Water Buffalo, Drosophila	Jordan [172]; Haigh et al. [168]
2006	Ferret	Li et al. [179]
2007	Rhesus Monkey, Wolf	Highfield [171]; Grant [166]
2009	Camel, Pyrenean ibex	Wani et al. [146]; Gray and Dobson [167]
2012	Pashmina Goat	Hassan [169]

induction [114, 115]. Since these initial groundbreaking studies, iPS cells of various origins have, in a manner similar to that of embryonic stem cells, been shown to colonize the germline of chimeras. iPS cells have now been derived from a variety of cell types and species including mice, nonhuman primates, humans and highly endangered species including the drill and the northern white rhinoceros [113, 116, 117]. The future of iPS induction will no doubt involve the use of small molecules to induce aforementioned endogenous factors or perhaps an as-yet-undefined master regulator of pluripotency. This approach would eliminate the need to introduce exogenous genes thus removing genome alterations as safety concerns. For a more thorough review on the three primary methodologies currently employed to drive nuclear reprogramming and pluripotentiality see Yamanaka and Blau [95].

Part II of this book is dedicated to defining and describing the extent of cellular pluripotency as it pertains to a variety of systems, from limb regeneration to immune system reconstitution. For example, David Stocum, Professor and Director, Center for Regenerative Biology and Medicine at Indiana University—Purdue University in Indianapolis deciphers the cellular and molecular mechanisms underlying dedifferentiation of cells for regeneration of the amphibian blastema (Chap. 7). Hans Snoeck at Columbia University's Center for Translational Immunology has made much progress in generating pluripotent cells from thymic epithelial cells to generate "personal immune (PI)" mice, whereby the human immune system is represented in a mouse model. He will discuss the utilization of human pluripotent stem cells for reconstitution of the immune system (Chap. 12). Steve Duncan, Director of the Medical College of Wisconsin Program in Regenerative Medicine and Stem Cell Biology in Milwaukee will review progress made in the generation of hepatic cells from iPS precursors (Chap. 10). Derrick Rancourt, Professor in the Departments of Oncology and Biochemistry & Molecular Biology at the University of Calgary in Canada will round out the discussion by focusing on bioreactor scaling of iPS cells for translational medicine initiatives (Chap. 9).

Tumor Cell Plasticity and Cancer Stem Cells

Cancer originates when control of the cell cycle, senescence or apoptosis goes awry. This may occur due to the compound effect of accumulated inherited (genetically predisposed) or somatic (environmental) gene-specific mutations that result in an alteration of transcriptional expression levels or the structure of the corresponding protein product. These changes in genetic makeup may thus represent coding sequence alterations, aberrant activation or inhibition of upstream regulatory regions, or changes in proximal promoter elements. Any of these polymorphisms may drive inappropriate functions of proteins that play roles in maintaining normal mitotic activity and inhibiting the transformed cellular phenotype. *Oncogenes* are defined as genes that promote cellular transformation. *Tumor suppressor genes* typically provide a checks-and-balances effect on cell division. Therefore, much attention has been paid to the molecular switches which either activate or repress the tumorigenic phenotype. It has been previously demonstrated that genetic alterations driving the initiation of cancer could occur randomly in virtually any cell type, leading to the emergence of the *stochastic hypothesis* in which any cell could become tumorigenic if the right combination of tumor suppressor down-regulation and oncogene upregulation were to occur. Yet recently a new, albeit controversial, hypothesis has been proposed whereby tumor development and growth are driven by a small subset of cells within the tumor known as *cancer stem cells*. Cancer stem cells were first hypothesized to be present in hematological malignancies, but the idea of their

Table 3 Cancer stem cell markers for various tumor types (Adapted from the book *Cancer Stem Cells*, William L. Farrar, Editor)

Tumor Type	Phenotype	Cell fraction (%)	Reference
Breast	CD44+ CD24−	11–35	Goodell et al. [192]
Brain	CD133+	5–30	Singh et al. [180]; O'Brien et al. [123]
Prostate	CD44+ CD133+ a2b1hi or CD44+ CD24−	0.1–3	Du et al. [181]; Dalerba et al. [182]
Pancreatic	CD44+ CD24− ESA+	0.2–0.8	Prince et al. [183]
Hepatocellular	CD133+	1–3	Collins et al. [124]
Colon	CD133+ or ESAhi; CD44+	1.8–24.5	Ricci-Vitiani et al. [184]; Eramo et al. [185]; Li et al. [186]
Head and neck	CD44+	<10	Matsui et al. [187]
Lung	CD133+	0.3–22	Ma et al. [188]
AML	CD34+ CD38+	0.2–1	Lapidot et al. [189]; Masters et al. [190]
Multiple myeloma	CD138+	2–5	Bonnet et al. [121]
Melanoma	CD20+	~20	Ponti et al. [191]

existence was later also proposed for that of solid tumors. Cancer stem cells are thought to have unique stem cell-like morphological, biochemical, and molecular (genetic) characteristics that enhance self-renewal capacities and thereby the ability to drive tumor growth and development. The hypothesis of the existence of cancer stem cells is not new, and was first proposed by the researchers Julius Cohnheim and Rudolph Virchow (see above) in the nineteenth century. It was Virchow's *embryonal rest hypothesis* that illustrated the striking similarity between early-stage developing embryonic and fetal tissue and that of cancer cells (for review see [118, 119]). This idea was later expanded upon by Virchow who suggested that mature organs may in fact have embryonic remnants that could drive tumor development. It was in 1961 when the first definitive evidence emerged that the heterogeneous population of cells in a tumor may have differing tumorigenic potential. In the same year, Southam and Brunschwig performed a series of elegant autologous tumor cell transplantation studies which demonstrated a minimum threshold of cell number required for tumor development, suggesting differences in cellular potency [120]. The first definitive isolation and characterization of cancer stem cells occurred when John E. Dick's group in the Department of Genetics, Research Institute, Hospital for Sick Children, University of Toronto, Canada isolated a CD34+, CD38− subpopulation of cells and classified it as a primitive hematopoietic cancer stem cell after confirming its tumorigenic potential [121]. *Tumorigenic potential* is defined as the ability of cells to drive tumor formation at low cell densities. This is accomplished by injecting a population of CSC-containing cells into nude or non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and observing tumor formation. A second key identification of a stem cell-like population of tumor cells was accomplished by Margaret A. Goodell and colleagues when they observed via flow cytometry that a small subset of cells in bone marrow did not accumulate Hoechst 33342 dye. Extrusion of this dye from cells is typically thought to be carried out by the ATP-binding cassette (ABC) transporter, thus Goodell's group suggested that expression of this transporter might define it as a cancer stem cell marker and this was later confirmed [122]. Other cancer stem cell markers that have been widely studied include various cell surface antigens. These allow for not only CSC identification but isolation via flow cytometric cell sorting. Over the years researchers have focused on cell surface markers known to be highly expressed in hematopoietic or embryonic stem cells. While the two main markers identifying a CSC population have now been defined as CD133 and CD44, a variety of cell surface antigens have been characterized and categorized as marking CSCs present in various types of tumors as listed in Table 3.

Following the identification, isolation, and initial characterization of marker phenotypes for CSCs present in a multitude of tumors (as outlined in Table 3), much effort has been directed at defining the inherent properties of these cells that enable them to drive the tumorigenic phenotype. As one would expect, CSCs isolated from different tumor types have widely varying tumorigenic potential, which can range from as little as 100 to as high as 50,000 cells required to induce the tumorigenic phenotype [123, 124]. *Self-renewal* properties of CSCs have also been defined via either *in vivo* serial tumor transplantation or *in vitro* via soft agar or sphere formation. While *in vivo* serial propagation studies are a more rigorous test of self-renewal capacity, they are also more costly and thus many researchers turn to *in vitro* methods to address CSC self-renewal capacities. *Establishment of tumor heterogeneity* is the third primary criteria for defining a CSC. CSCs must not only be able to form tumors and self-replicate, but also demonstrate an ability to comprehensively reproduce the tumorigenic phenotype from which they were originally isolated. This means that CSCs drive the growth of the heterogeneous population of cells normally found in the original tumor. Muhammad Al-Hajj, Michael F. Clarke, and colleagues at Stanford's Institute for Stem Cell Biology and Regenerative Medicine were the first to demonstrate the ability of a defined CSC population, specifically that of a breast

cancer stem cell line, to give rise to a phenotypically diverse mixture of tumorigenic and non-tumorigenic cell populations resembling the initial tumor from which the line was isolated. They accomplished this through the isolation and serial passaging of a CD44+ CD24– low population of breast cancer CSCs and subsequent characterization of tumor formation potential in NOD/SCID mice [125, 126]. As outlined in Table 3, in recent years many cancer researchers have utilized the above criteria and experimental methodologies to identify and classify cancer stem cells from numerous tumor types. Part V of this book will outline the developmental origins of CSCs and outline examples from breast cancer, melanoma, and nephroblastoma/Wilms' tumor. Specifically, Max Wicha, Director of the University of Michigan Comprehensive Cancer Center in Ann Arbor and Sarah Conley will outline the phenotypic and morphological makeup of breast cancer stem cells and the cytokine signaling as well as the tumor microenvironment regulating their metastatic potential and resistance to treatment (Chap. 34). Mary Hendrix, President and Scientific Director of the Children's Memorial Research Center at Northwestern University Feinberg School of Medicine in Chicago has made significant advances in demonstrating that the presence of ES cells may reprogram cancer cells to a less aggressive state. She will review these and other findings that allow for the targeting of tumor stem cell plasticity (Chap. 35). Rounding out the section on cancer stem cells, Alan Perantoni, Head, Differentiation and Neoplasia Section and Laboratory Chief at the National Cancer Institute in Frederick, Maryland will discuss the correlation and relationship between stem cells and nephroblastoma/Wilms' tumor (Chap. 36).

The Emerging Stem Cell Industry

The emergence of stem cell biology as a basic research field has been met with cautious speculation that therapeutic potential may indeed exist which could significantly impact certain areas of medicine. As mentioned in numerous instances above, a variety of disorders are potentially treatable, if not curable, utilizing stem cells as a base platform for cell or tissue replacement strategies. This last introductory section outlines the emergence of the growing stem cell industry, from a tissue replacement origin to regenerative medicine and finally stem cell medicine.

Tissue Replacement and Regeneration: A Historical Perspective

The replacement of diseased tissues and cells with other healthy living material for therapeutic purposes or the utilization of living tissues in generation or regeneration of new tissue has long been contemplated by scientists and doctors as a methodology to treat a variety of disorders. The science behind it has evolved considerably over the past 30 years. Cellular seeding in the context of therapy was initially studied from a 2D culture and preparation perspective, without much success. After many struggles without 3D matrices, scaffolds were realized to be a key component to cellular seeding. In the 1970s, pioneering researcher and medical doctor W.T. Green of Harvard Children's Hospital pursued the spurring of new cartilage growth in nude mice via implantation of bone spicules seeded with chondrocytes [127]. These studies were unsuccessful, but the concept of utilizing a novel 3D matrix to provide a cellular source for tissue replacement or regeneration had been born. Throughout the 1980s researchers expanded upon this concept and developed a variety of platforms for *in vivo* cell seeding and the term *tissue engineering* was first coined and proposed at a National Science Foundation meeting in 1987 (NSF Bioengineering and Research to Aid the Handicapped (BRAH) Program within the Engineering Directorate, 1987). Perhaps the most cited manuscript in this arena, at least at the time, was that published by Robert Langer of MIT and Joseph Vacanti of Harvard Children's Hospital, which confirmed the utility and functional capacity of a biocompatible and bio-absorbable synthetic matrix (yet another 3D platform) to seed a variety of cell types into host animals [128]. Since this time the field of regenerative medicine has evolved to encompass a broad range of therapeutic applications. From an industrial perspective, the 1990s saw an explosion of tissue engineering companies, peaking at over 3,000 full-time employees and $610 million in private sector investment capital in 2000 ([129] and see also the section on stem cells in regenerative medicine below). The vast majority of the companies were classified as those focused on tissue engineering for the replacement of living skin. Companies such as Advanced Tissue Sciences, Integra Life Sciences and Organogenesis developed products that were derived from highly adaptive human foreskin or manufactured with collagen, each produced with allogeneic cells but immunorejection turned out not to be a factor. Examples include ATS's Transcyte® and Dermagraft®. Transcyte is a human fibroblast-derived temporary skin substitute consisting of a polymer membrane and neonatal human fibroblast cells cultured under aseptic conditions *in vitro* on a nylon mesh. Dermagraft is a polymer coated with fibroblasts derived from human foreskin. It is composed of fibroblasts, extracellular matrix, and a bioresorbable scaffold. Genzyme also entered the skin replacement arena with Epicel®, an autograft of epidermal fibroblasts utilized primarily for burn victims with deep dermal damage over 30% of the body's surface area. Interestingly, Epicel is manufactured in the presence of murine fibroblasts and is thus considered a xenotransplantation product, carrying the minimal risk of viral transmission from mice to humans. As such it is only utilized under the guise of a humanitarian use device (HUD) per FDA restrictions. In addition, around the same time Genzyme developed Carticel®, a product based on

autologous cultured chondrocytes used to repair articular cartilage injuries of the knee in adults who have not responded to a prior arthroscopic or other surgical repair procedure. It was based on methodology developed by researchers in Department of Orthopedic Surgery, University of Göteborg, Sahlgrenska University Hospital, Sweden whereby autologous chondrocytes were cultured for a period of 2–3 weeks in the patient's own serum, eliminating any possibility of immune rejection ([130] and Fig. 11).

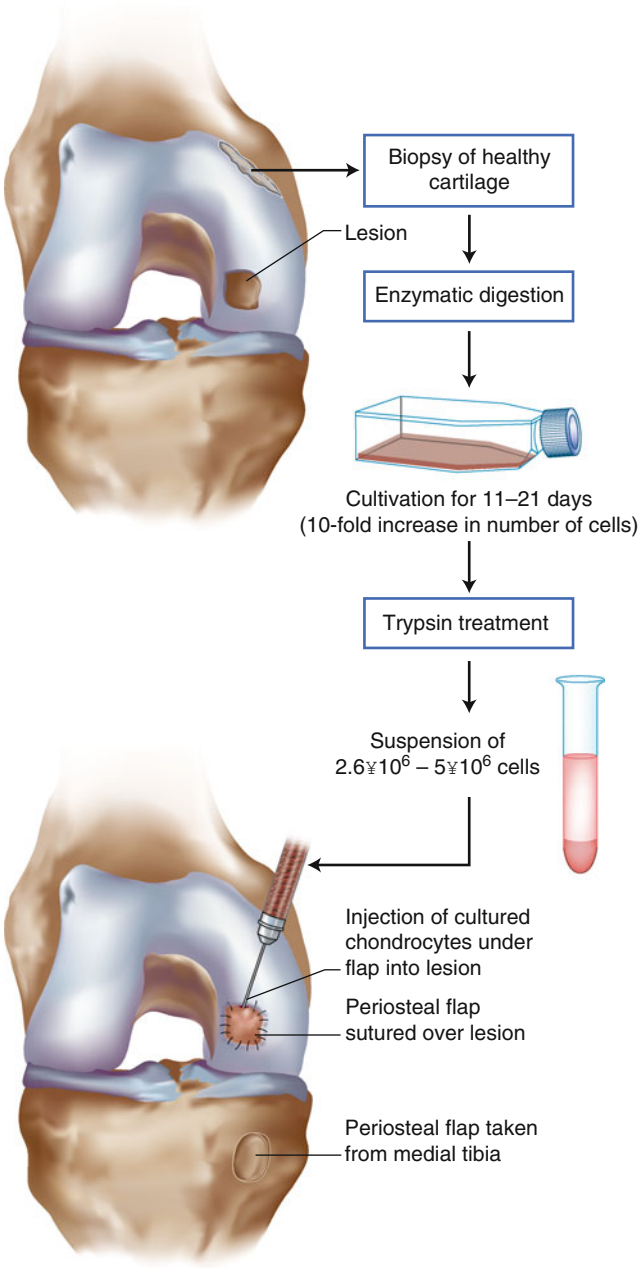

Fig. 11 Diagrammatic illustration of the methodology on which Carticel is based (Courtesy Mats Brittberg [130] and the *New England Journal of Medicine*; reprinted with permission)

In 1997 it was the only FDA approved autologous chondrocyte cell replacement therapeutic on the market, yet it was an example of an effective treatment for disorders and anomalies other than those of the skin. Thus towards the end of the 1990s it became abundantly clear that tissue and cell replacement strategies were viable modes of effective, and profitable, therapeutics for numerous disorders.

Stem Cells as a Regenerative Medicine Platform

By the late 1990s not only had tissue engineering and replacement taken a firm foothold in industry it had become apparent that stem cells could indeed act as a platform for the development of cell-replacement therapeutics, and the field of regenerative medicine quickly emerged. Key scientific advancements such as the identification of neural stem cells in the adult brain [75, 76] and derivation of the first human embryonic stem cell line [35] set the stage for an explosion of scientific advancements in the first decade of the new millennium. These included the identification of dermal stem cells in adult skin tissue [131], the discovery of cancer stem cells [132], the first derivation of adult, fully mature dopaminergic neurons from human embryonic stem cells [133] successful iPS [113] and initiation of the first clinical trial of human embryonic-derived stem cells for treatment of spinal cord injury (Geron Corporation 2010) (see also Fig. 3 for a more comprehensive historical breakdown of scientific advancements in stem cell research). Yet it was not until 2007 that the stem cell and regenerative medicine industry was considered legitimate. A clear breakthrough in industry interest from Wall Street occurred this year, and in the middle of 2007 there were over 170 regenerative medicine companies fielding over 6,100 full-time employees. Market capitalization of publically traded tissue engineering or regenerative medicine companies jumped to $4.7 billion in 2007 from $300 million just 48 months prior in 2003. This was in contrast to an industry downturn where the total number of all regenerative medicine business units was a mere 89 [129]. Thus the tissue engineering and regenerative medicine industries, for which stem cells have played a major role in driving their maturation, have survived both scientific advancement lulls and market fluctuations to be poised for a considerable impact on therapeutics. Figure 12 illustrates a fraction of the diseases and anomalies that are being pursued for treatment via the use and application of stem cell platforms. For a more comprehensive review of the broader tissue engineering and regenerative medicine disciplines converging and emerging as an industry see Nerem [129].

The industrial aspect of stem cells and stem cell biology would not exist if there were not a demand, or at least the prospect of a demand, at the medical and therapeutics levels.

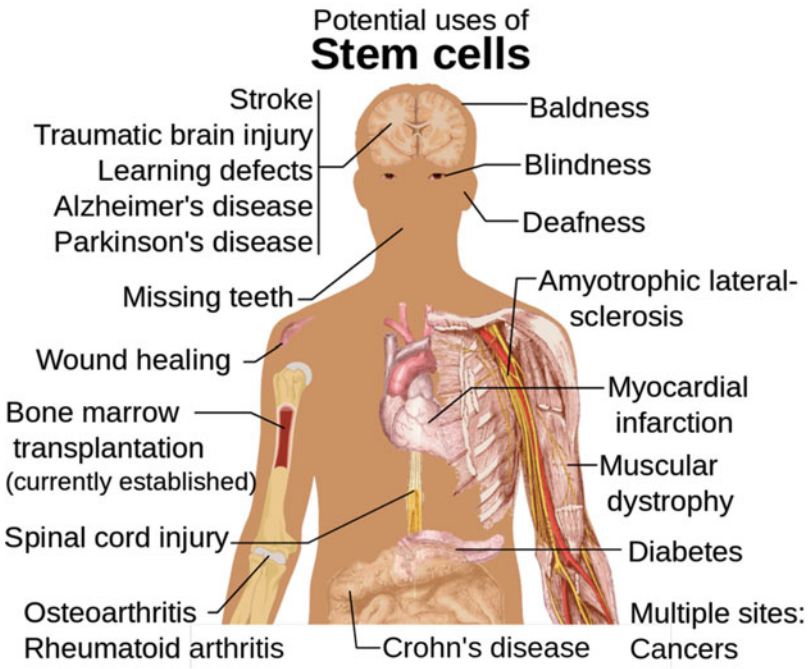

Fig. 12 Diagrammatic illustration of stem cell applications in medicine (Courtesy Wikimedia Commons)

Part IV of this book focuses on stem cell transplantation as a cell replacement therapeutic strategy for the treatment of a variety of medical disorders. In it numerous stem cell-based strategies are described for the treatment of disorders such as liver degeneration, burns, diabetes, heart and ocular disorders. For example, Shuibing Chen, Assistant Professor in Weill Cornell Medical College's Department of Surgery in New York will discuss the application of human pluripotent stem cells for the treatment of diabetes via the directed differentiation of these cells into pancreatic and endocrine progenitors. Dennis Clegg, Professor in the Department of Molecular, Cellular and Developmental Biology at the University of California—Santa Barbara will discuss the application of stem cells for the treatment of age-related macular degeneration. Alan Trounson, President of the California Institute of Regenerative Medicine and Professor Emeritus at Australia's Monash University will provide a comprehensive review of stem cell discoveries as they relate to translational medicine and real-world therapeutics applications. It is clear that recent advancements in stem cell research have given rise to a strong foundation of scientific knowledge setting the stage for almost limitless possibilities in tissue and cell replacement therapeutics. The regenerative medicine industry, albeit somewhat fledgling and immature at the moment, is here to stay and poised to revolutionize medicine as we know it in the coming years.

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Contemporary Ethical Issues in Stem Cell Research

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Introduction

Recent developments in stem cell technologies hold out the promise of finding treatments and cures to a wide range of conditions and diseases. With these advancements, however, come ethical concerns. Some ethical issues have been the focus of extensive commentary and analysis since the derivation of human embryonic stem cells (hESCs) in 1998 [1]. In particular, literature and policy debate have focused on the ethical appropriateness of using human embryos for stem cell research [2, 3]. As research in the field of hESCs has advanced, the debate evolved from focusing solely on the propriety of destroying embryos for research purposes to a broader discussion of appropriate methods of creating embryos for research [4–6]. Much attention has been generated in response to the technique of somatic cell nuclear transfer (SCNT)—in which the nucleus of a somatic, or differentiated, cell is placed into an oocyte, usually after the oocyte's nucleus has been removed, thereby creating a type of cloned cell [7]—including whether SCNT will encourage human cloning for reproductive purposes [8, 9]. Additionally, ethicists and policy-makers have endeavored to address the development of human-animal chimeras [10], by focusing on the normative concerns related to animal welfare and questions about whether the creation of chimeras implicates notions of human dignity and the moral acceptability of crossing the "species barrier."

In light of the substantial literature on these topics, this chapter will focus on more contemporary bioethical issues central to individuals' participation in stem cell research as "human subjects"—both in the initial stages of research (as donors of gametes, somatic cells, or embryos) and, as the research progresses from bench to bedside, where humans may be participants in stem cell clinical trials or patients receiving innovative therapies. Both the acts of donation to stem cell research and participation in stem cell clinical trials are considered human subjects research, and protocols must adhere to the core principles of research: beneficence, respect for persons, and justice [11, 12]. Beneficence and its corollary, non-malfeasance, place responsibility on researchers and research institutions to maximize the benefits and minimize the harm to subjects [11]. Respect for persons requires recognition of the importance of individual autonomy and personal decision-making [11]. Finally, justice dictates the fair distribution of the risks and benefits of research, as well as availability of the products of research, across individuals and populations [11].

This chapter explores how these core ethical principles may be implicated in research requiring donations of gametes, embryos, and somatic cells and in clinical trials of stem cell therapies. The first section of this chapter addresses certain rights of individuals who donate biological materials for the purposes of hESC research, including the necessity of providing voluntary informed consent and how compensation for participation may affect the voluntariness of such consent. The second section will evaluate ethical issues relating to the study of stem cell-based therapies in humans.

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Issues Arising from Donations of Biological Materials to Human Embryonic Stem Cell Research

Autonomous decision-making about donating biological materials for stem cell research requires the provision of fully informed, voluntary authorization or agreement [11]. It obligates institutions and researchers to disclose the nature and purpose of the research, as well as the potential risks and benefits of participation, in a manner that promotes true comprehension of the information provided.

While ensuring that fully informed, voluntary consent can be challenging to all forms of stem cell research and human subjects research generally, it may be more complicated in the hESC research context due to the complexity of the research, the potentially wide distribution of stem cell lines derived from donated biological materials, the downstream potential of this research, and the provision of compensation for participating in research through acts of donating. Donations to stem cell research also challenge the central notion of what it means to "participate" in research, particularly when examining various stakeholders' rights to control, direct, or financially benefit from stem cell lines and other derivative products that have been substantially altered from the original donated material.

Consent to Donation of Clinically Excess Gametes and Embryos for Research Purposes

Gametes and embryos typically are donated to stem cell research after they have been harvested in the context of reproductive treatment (i.e., in vitro fertilization (IVF)) but are no longer considered clinically necessary. Analyzing the adequacy of informed consent for donation of clinically excess gametes and embryos requires an examination of (1) whether the donor was apprised of all alternatives to participating in the research by donating, (2) the timing of the consent in relation to the transfer of the materials to the research protocol, (3) the specificity of the consent to research, and (4) from whom consent was obtained.

First, in order for an individual to make a fully informed decision to participate in research, he or she must understand the alternatives to participation. In the case of donating clinically excess gametes and embryos to stem cell research, donors should be apprised, at a minimum, that these materials may be (1) provided to others for procreative purposes, (2) donated for research, or (3) destroyed [5, 13].

Second, at the initiation of the reproductive treatment, those undergoing IVF are often offered the option of providing clinically excess gametes and embryos to research after the treatment is either successful or halted for other reasons. The timing and nature of this initial consent raise ethical issues when it is relied on as the sole consent to donation to stem cell research. It is unlikely that a general consent to research at the beginning of a reproductive process would include specific information regarding the nature and type of research that would occur if the excess biological materials were donated to research. Individuals also may not be fully aware of the consequences of their decision to participate in a specific research project or the implications of allowing derivation of stem cell lines from excess materials. Individuals also likely would not be apprised of any new information generated after the time of the initial consent, including possible advances in research techniques or additional alternatives to donation. Obtaining re-consent—particularly where such re-consent is more specific to the research study and the potential risks and benefits of participating in the research—closer to the time of transfer to the research facility may help obviate concerns that the individual did not make a fully informed choice about participating in research. However, because of the difficulties in tracking down individuals for re-consent after significant time has elapsed, many commentators have asserted that it may also be ethically acceptable to use gametes and embryos in stem cell research if general consent to research was provided at the time gametes or embryos were harvested for IVF purposes [6] and re-consent is prohibitively difficult [4].

Third, when considering the donation of clinically excess embryos to research, determining which parties should provide consent research is a contentious issue. In other research contexts, informed consent need only be provided by a singular person who may become a research participant. In contrast, to procure an embryonic stem cell line, multiple parties' autonomy rights may be implicated—including those who have control or custody of embryos (who may or may not be biologically linked to the embryos) and third-party donors of gametes (who may have intended those gametes to be used only for reproductive purposes) [4, 6, 13]. Because the parties who have dispositional authority over the embryos may not be the same individuals whose biological materials comprise the embryo, significant privacy and autonomy concerns arise [14, 15].

Ethical Issues Arising from Stem Cell Line Banking and Wide Distribution of Lines

Once stem cell lines are derived, they are commonly deposited in stem cell banks for storage and distribution. These repositories maintain records of donor information, such as ethnic background, infectious disease screening results, and medical history, so that proper lines will be disseminated to researchers seeking to study certain traits or diseases [16]. These repositories facilitate information sharing among

researchers regarding stem cell lines available to the scientific community and promote efficient domestic and international transfer of lines [16]. But while banking and registries help facilitate research, they also raise a host of autonomy issues for donors.

Consent to Future Research

As a basic aspect of informed consent, a participant should comprehend the nature and type of research in which they may participate. However, while researchers may be able to disclose and describe to gamete, embryo, or tissue donors the types of research they intend to conduct with any stem cell lines derived from their biological materials, the eventual banking and wide distribution of the lines makes it a near impossibility either for the researcher to disclose or the participant to consent to all types of future research. This particularly applies once stem cell lines are transferred internationally, where research rules may differ substantially from those in the donors' home countries.

At a minimum, an effective informed consent process should make donors aware of whether they will be able to place restrictions on immediate or future uses of their donated tissues. In order to allow for future research using donated biological materials, it is necessary for potential donors to understand that derived stem cell lines may be kept and stored for many years and used in future studies and that future research protocols may use the lines in ways that are currently not known or unforeseeable. Although the option of allowing donors to consent to certain types of research but not others has been considered [6], doing so would arguably impede research and may be practically unenforceable. Some jurisdictions have dealt with this issue by ensuring that donor consent forms clearly state that if the individual is uncomfortable with the idea that stem cell lines derived from their biological materials may be used in ways that are unknown at the current time, they should abstain from donating [13].

Withdrawal of Donated Materials from Research

Stem cell research challenges traditional notions of research "participation" and its attendant rights—including the right to withdraw from the research. According to generally accepted principles of human subjects research, any participant who enrolls in a research protocol has the right to withdraw from the study at any time without prejudice [17].

The issue of withdrawal arises on two levels in stem cell research: the right to withdraw the originally donated biological materials and the right to withdraw the stem cell lines derived from these materials. It is generally accepted that a donor has the right to withdraw his or her biological sample until the time the sample has itself been used in the research [5]. A donor arguably has the ethical right to withdraw consent to the usage or storage of any tissue that has not been used in the research. Currently, most ethical guidelines include the opportunity to withdraw consent up until an individual's gametes or embryos are used in cell line derivation and/or when identifying information has been stripped from the donated sample [4, 15].

However, the issue of withdrawal becomes more challenging once stem cell lines have been derived, manipulated, and combined with other biological materials, implicating a key concern: at what point—if any—is the original donor no longer considered a participant in research?

For those who believe that these downstream products are tied inextricably to the original donor (regardless of the degree of derivation and manipulation), the donor arguably should have the right to withdraw not only his or her actual biological sample but also the stem cell lines derived therefrom. Others counter that the protections contained in human subjects research laws and regulations were originally conceived in order to prevent involuntary participation in research [18], particularly protocols involving physical harm or bodily violations. Depending on how one defines "research participant," it may follow that once derivations and manipulations of stem cell lines have proceeded, the original donor does not have the same rights and is not entitled to the same degree of protection as other research participants. Notably, however, existing policy and current academic literature appear to acknowledge that the US federal rules and regulations governing informed consent for research with human subjects also apply to most research with biospecimens, except in some limited conditions [19–21].

Drawing a line after which withdrawal is prohibited—regardless of where that line is drawn—calls into question donors' continued status as a research participant and implicates their autonomy interests and privacy rights [22, 23]. It also gives rise to concerns related to bodily integrity and property rights in the tissue [24].

Importantly, even if the right to withdraw downstream stem cell products is ethically appropriate, its practical unenforceability could render the right moot. More specifically, worldwide distribution of stem cell lines—which are almost universally coded or otherwise de-identified—may severely limit the ability to trace or identify the lines a donor wishes to withdraw [23]. Accordingly, it is essential to inform a potential participant of the extent to which he or she may be able to withdraw consent to usage of the donated biological materials as well as the stem cell lines and other products derived therefrom.

Return of Research Results

Another fundamental question relating to the donation of biological materials is whether there is an ethical duty to return research findings—whether the results are directly

related to the research ("individual research results") or not directly related to the central research question ("incidental" findings)—to research participants [25]. Stem cell research raises particular challenges to result verification. For example, induced pluripotent stem cells (iPSCs) may have been genetically modified, or embryos derived from donor gametes may not be identical to donor genomes. Thus, it may be uncertain whether a presumed finding is valid unless it can be replicated with a fresh specimen from the donor.

The ethical implications of requiring return of research results may be intensified as the discovery of clinically relevant and scientifically valid information becomes more frequent and donors increasingly express a desire to receive these findings. Supporters of a duty to return results rely upon the principles of respect for persons and autonomy, as well as the notion that individuals have an interest in obtaining personally relevant results and information about the research in which they participated. Among other factors, the nature and duration of the relationship between the research participant and the researcher may be the most important in determining whether a duty to return results exists [26]. A duty to return may also hinge on considerations related to ownership, property rights, autonomy, and an individual's status as a "research participant."

There are also practical considerations in considering the return of results to donors. First, the obligation to return results may strain already limited resources and delay research—particularly in jurisdictions that require that any discussion of genetic information be conducted by a clinician with particular expertise. Second, where samples are de-identified, locating the individual who donated the sample may be practically impossible. Third, if, during the consent process, researchers tell potential participants that research results may be returned, some donors may not seek medical treatment or undergo testing that they otherwise would have because of a misunderstanding that researchers would notify them of any and all negative findings.

Compensation of Oocyte Donors for hESC Research

Encouraging involvement in scientifically valuable research by providing compensation to research subjects is the subject of much ethical discussion [27]. In the context of stem cell research, it is well settled that no compensation should be provided to those who donate clinically excess gametes and embryos because these donors have not expended any additional time or been subject to additional risk, and compensation could be construed as purchasing the bodily materials themselves. However, the issue of whether or not and to what extent women who donate their oocytes directly and solely for stem cell research should be compensated has been the subject of much debate [9]. While direct reimbursements for the costs associated with the donation process, such as travel costs or time taken out of work, are relatively commonplace [6], some commentators and policy-makers assert that women should be compensated beyond direct expenses for the substantial time, burden, and discomfort associated with the donation process [15].

The process of undergoing hormonal stimulation for egg harvesting can be a lengthy and potentially risky process. Although it appears that serious complications rarely accompany the egg harvesting process, some uncertainty remains regarding the frequency and severity of such risks, due to the dearth of long-term studies of risk conducted on donors [28–30]. Some scholars assert that compensation for donation fairly promotes a mutual benefit for both the researcher and the donor [31] and appropriately acknowledges the woman's contribution and effort.

Others have argued that justice demands that compensation beyond direct expenses should be provided to women who donate their oocytes directly to research, as women in the USA have historically been allowed such compensation for the parallel act of donating oocytes for reproductive purposes [9]. Indeed, the medical procedures and risks associated with oocyte donation are the same, regardless of the purpose for which the eggs are intended.

However, some commentators have asserted that compensation of oocyte donors may compromise a potential donor's ability to provide free and voluntary informed consent. Compensation may become coercive if it blinds a person to the risks involved in the research or if it leads a person to conceal or misrepresent information that would disqualify his or her from being eligible to participate [32]. Few studies have been conducted that support the argument that reasonable compensation affects a person's perception of risk presented by a protocol [33, 34]. Exactly what may constitute an undue inducement to participate in hESC research is unclear, as it must be determined on a case-by-case basis. In determining whether compensation qualifies as undue inducement, ethicists and policy-makers may consider whether the financial incentive serves as the primary or sole reason for an individual to donate oocytes for research [35, 36].

Further, it has been asserted that compensation of oocyte donors may not protect donors from exploitation or appropriately respect the integrity of their biological materials. Compensating oocyte donors may exploit underprivileged populations who would not otherwise choose to engage in research. Scholars also worry that compensation of oocyte donors may lead to the "commodification" of human biological materials, undermining the dignity or meaning of human life. These concerns may lead to prohibitions on compensation for the number or quality of eggs donated [15, 32, 37]. Whether or not compensation for oocyte donation is analogous to purchasing biological materials or providing an incentive commensurate with the risk undertaken by the provider is still a topic of great debate [38–40].

While donors of oocytes for hESC research in the USA have historically not been compensated beyond reimbursement for out-of-pocket costs and medical expenses related to the donation [6, 7], New York State has adopted policies that permit capped compensation of women for the time, burden, and discomfort associated with the donation process, so long as the donor also has been fully apprised of—and comprehends—the risks associated with donation [41]. This policy reflects how compensation for donation of oocytes for hESC research may promote a balance between the individual and societal benefits of the research and the risks posed to research participants.

Ethical Issues Involving Translational Stem Cell Research

Clinical stem cell research has the potential to expand the range of therapies available for neurological or other kinds of disease and may be progressing more rapidly than the normative considerations applying to these research practices [42, 43]. While some cell-based therapies are already the standard of care for certain medical conditions, many other types of stem cell products may soon have clinical value. The increased focus on moving stem cell science from bench to bedside makes the need for ethical guidance for testing and using novel stem cell therapies in humans particularly acute [44]. The following section will focus on the ethical considerations associated with clinical application of stem cell products.

Challenges to the Already Fluid Boundary Between Medical Practice and Scientific Research

Translational stem cell research involves the close—and often overlapping—connection between research and medical practice. Indeed, stem cell-based clinical research involves the shared interests of the two enterprises, as researchers and medical practitioners alike employ novel therapies and scientific theories. However, prevailing ethical principles distinguish scientific research from medical treatment. It is commonly accepted that scientific research promotes "generalizable knowledge" for the entire population, whereas medical treatment focuses on the well-being of the individual patient [11].

Clinical Research Paradigm and Medical Innovation Model

Clinical application of cell-based therapies may fall within the traditional research paradigm. Typically, research proceeds in three sequential stages, in which researchers conduct experiments in vitro, then in vivo, and finally in human subjects. Clinical research, defined as any trial to evaluate the effectiveness and safety of medications or medical devices by monitoring their effects on large groups of individuals, represents the commonly recognized model for human subjects research [12]. These trials aim to produce generalizable knowledge that will yield benefits to a wider population or society at large. A number of clinical trials involving stem cell-based therapies for a variety of different diseases are currently underway [45].

Where trials of stem cell-based therapies are presented as an alternative to existing treatment options, researchers and oversight committees should consider the principle of clinical equipoise—i.e., the genuine uncertainty among expert clinicians about the relative merits of an investigational intervention and the available alternatives—when deciding which model to pursue [46]. In doing so, researchers should address whether a protocol or intervention is designed solely to deal with the research question or if it may have potential therapeutic benefit [47]. Clinical equipoise assessments are based on the expected benefits and burdens of the interventions for the overall patient population rather than on particular individuals' unique characteristics. When an intervention is known to be particularly risky, or individuals are randomly assigned to an inferior therapy, clinical equipoise does not exist [48, 49].

The medical innovation model may offer clinicians the opportunity to provide potentially beneficial stem cell-based therapies outside the context of a formal clinical trial. Innovative therapies should (1) have an appropriate scientific rationale, (2) provide an explicit explanation for why the researchers need to pursue a more experimental option, and (3) include a well-articulated characterization of the treatment regimen as well as a sufficient data reporting plan, particularly for adverse events [50]. This model is distinguishable from the clinical research model in that the former concentrates on the welfare of the individual patient, while the latter focuses on scientific results that can be applied to a broader patient population [51, 52]. Although a sufficient evidentiary base is necessary for using medical innovations, medical innovation is not standardized to a point where a trial is appropriate.

Notably, although the clinical research model and the medical innovation model differ, the two paradigms are not incompatible. In some cases, medical innovation (with adequate preclinical evidence to support the intervention and appropriate ethical oversight) [51] may be an important alternative to the slower-moving clinical research process for individuals with serious illnesses and limited treatment options [53].

Moreover, the distinctions between the protections offered by the clinical research model and the medical innovation model may be mitigated by encouraging physician-scientists who provide innovative therapies to share any relevant information with other researchers and to move therapies that are

successful for critically ill individuals into large-scale clinical trials when possible [54]. In addition, by applying the criteria of medical innovation in surgical procedures to stem cell interventions, the medical innovation model could further approach the level of ethical oversight and protection ensured under the clinical research paradigm [51, 55]. In the case of studies of cell-based therapies, particularly those that deviate from the standard of care, the two models at issue—clinical research and medical innovation—should emphasize the scientific validity of the intervention. Both models also require intensive follow-up and evaluation.

Clinical Use of Unproven Medical Interventions

In contrast to the clinical research and medical innovation models, an ethically unacceptable approach to translational stem cell research is the clinical use of unproven medical interventions that do not have sufficient previous research or demonstrable evidence that the procedures are safe and effective in the target population. This model, often referred to as the unproven intervention model, is distinguishable from the medical innovation model by its failure to support its interventions with adequate preclinical evidence [56]. As a result, although the medical innovation model and the clinical research paradigm may be able to coexist within a single ethical and research framework, the use of unproven stem cell interventions may not meet the ethical standards established and expected by the research community and beyond.

As noted above, a sufficient evidentiary base is one of the core ethical requirements for using medical innovations [53]. Unlike medical innovations, unproven interventions generally do not include objective follow-up of patients and independent evaluation. The ability to differentiate objectionable unproven treatments from valid medical innovations is one of the key challenges facing those concerned with the integrity of stem cell treatments.

Some commentators have maintained that people should be permitted to select medical treatment based on their personal level of risk tolerance, but respect for patient autonomy may not always justify softening basic ethical protections. On the contrary, it may be imperative to ensure that appropriate safeguards apply to procedures offered to the desperately ill, as they may be particularly vulnerable to abuse or coercion.

In the absence of regulatory oversight, patients and clinicians are often left to weigh the risks and benefits of a particular stem cell therapy, despite a lack of equipment to successfully perform this task. However, physicians can still provide invaluable protection against ethically unsupportable unproven interventions. The professional obligations of physicians, as exemplified by the duty to minimize harm, may prohibit them from providing these therapies to patients. Empirical evidence suggests that many novel stem cell-based therapies are not effective or safe enough at this time to warrant distribution in the clinic [54, 57]. Recent deaths or severe physiological responses of patients who received unproven medical treatments have raised concerns about the stem cell clinics that have delivered these treatments [58].

Patients seeking stem cell interventions frequently travel—sometimes abroad to foreign countries—giving rise to what is commonly referred to as “stem cell tourism” [59]. Although not all travel for innovative therapies is ethically problematic, many in the scientific community have expressed concern that lucrative stem cell treatments are being aggressively marketed to desperate patients without appropriate safeguards to ensure the safety and potential effectiveness of the advertised interventions and without a mechanism to prevent misleading or fraudulent claims [60–62]. Approved stem cell therapies remain rare, and clinics offering unapproved stem cell treatments are frequently, though not exclusively, located in countries that are not known for their biomedical research [60]. In such cases, the jurisdiction in which an advertised procedure is offered does not indicate the extent to which such treatment “adheres to the widely accepted translational pathway from basic science to clinical application” [61].

In response to the explosion of stem cell-related marketing and tourism, efforts should be made to promote safe and effective treatments and prevent misleading or fraudulent advertising through demands for improved transparency of the methods, results, expertise, and oversight of clinics offering stem cell interventions [61].

Challenges to the Ethical Principles Central to Human Subjects Research

As with other first-in-human clinical trials, translational stem cell research may require adopting and altering existing research oversight procedures in order to uphold the core ethical principles of human subjects research [54, 55, 63]. The clinical application of novel stem cell therapies may present unforeseeable risks and benefits to subjects and therefore require additional or different forms of oversight [64]. Research oversight committees, including institutional review boards (IRBs) and, in some instances, Embryonic Stem Cell Research Oversight Committees (ESCROs) or Stem Cell Research Oversight Committees (SCROs), must carefully weigh the risks and benefits to participants and monitor them over the course of the trial and consider study design issues (e.g., the potential harms, benefits, and safety of different clinical trials) [6, 54].

While IRBs and ESCROs or SCROs are traditionally based in a single institution, effectively overseeing complex translational stem cell research may require expertise from more than one type of institution [65]. Relying on a more centralized strategy could encourage a “higher-level” review

process [51, 65, 66]. By promulgating ethical standards pertaining to risks and benefits, a central committee could also facilitate information sharing among different research institutions that are using the same stem cell lines to produce and test their potential therapies [2].

In addition to differing oversight procedures, the informed consent process must also account for the complex risks and the potential for unknown complications necessitating periodic health assessments or withdrawal from a study [63, 67]. Ensuring that participants provide fully informed consent to participate in stem cell clinical trials is particularly challenging in light of the unique level of publicity surrounding this area of research. Stem cell research also may exacerbate the therapeutic misconception, which arises when research participants do not understand the distinction between treatment and research [68, 69]. Individuals participating in novel stem cell trials may believe that their participation will necessarily include a therapeutic benefit. When the therapeutic misconception is coupled with the promises of commercial stem cell therapies that have not been substantiated by scientific evidence, participants may become further confused or misled as to the benefits of participation. Thus, a particularly rigorous informed consent process is essential to ensure that potential participants are fully informed of the risks and benefits of participating in stem cell research and understand the distinction between research and clinical care.

Additionally, where stem cell therapies seek to treat or cure conditions that impair cognitive function, such as Alzheimer's disease, clinical trials will, by necessity, need to enroll individuals suffering from those conditions [43]. Because these individuals may lack the capacity to provide first-person consent, special procedures should be in place to ensure that these clinical trials are conducted according to stringent ethical standards. Institutional oversight committees should consider additional protections for vulnerable populations, methods of communicating the risks and benefits to individuals that will maximize their comprehension and foster individual's assent or dissent to participation, and rules for appointing surrogate decision-makers in the event that the individual is incapable of providing fully informed, valid consent.

Finally, once therapies become widely available, the principle of distributive justice may mandate affordable access to resulting therapies and treatments for those who participate in publicly funded translational stem cell research. It may be advisable that relevant stakeholders—including researchers in academic and private companies, government funding organizations, patients, and providers—consider, in advance, how to ensure the fair and transparent distribution of stem cell therapies and products. Providing access to the benefits of translational stem cell research for the broader community may ameliorate increasing societal concern about health disparities.

Conclusion

Applying well-established ethical frameworks to the design and conduct of stem cell research involves recognition of the varied ways individuals can contribute to or participate in stem cell research—as donors of gametes, cells, or embryos for hESC research, as participants in clinical trials, or as recipients of innovative stem cell-based therapies. Acquiring the biological materials to perform stem cell-based research should abide by norms that recognize donors' rights. Additionally, the biological nature of stem cells may make it difficult for research institutions to abide by the three main principles governing human subjects research in testing novel stem cell-based therapies: beneficence, respect for persons, and justice. Finally, experiments studying the clinical application of stem cells continue to blur the distinction between research and medical treatment. The three approaches to introducing stem cell-based therapies in humans—the clinical research paradigm, the medical innovation model, and the clinical use of unproven interventions—are not always adequately protective of participants nor are they necessarily compatible.

Despite the continuing need to address ethical questions central to stem cell research involving human subjects, it is clear that stem cell research will continue to advance. As it does, policy-makers, ethicists, researchers, and oversight bodies should consider how best to ensure that donors' rights are respected and research participants are adequately protected.

Disclaimer The views expressed in this chapter are solely those of the authors and do not necessarily represent those of the Empire State Stem Cell Board, the New York State Task Force on Life and the Law, the New York State Department of Health, Health Research, Inc., or the New York State Government.

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Part I

Embryonic/Fetal Stem Cells

Stem Cells from Early Mammalian Embryos

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Abbreviations

2i	Two inhibitors
AVE	Anterior visceral endoderm
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
E	Embryonic day
EC	Embryonal carcinoma (stem cell)
EG	Embryonic germ (stem cell)
emVE	Embryonic visceral endoderm
EPI	Epiblast
EpiSC	Epiblast-derived stem cell or epiblast stem cell
ES	Embryonic stem (cell)
exVE	Extraembryonic visceral endoderm
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
gp 130	Glycoprotein 130
hES	Human embryonic stem (cell)
HSC	Hematopoietic stem cell
ICM	Inner cell mass
iPS	Induced pluripotent stem (cell)
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MEF	Murine embryonic fibroblasts
NT ES	Nuclear transplant embryonic stem (cell)
PE	Parietal endoderm
PGC	Primordial germ cell
PrE	Primitive endoderm
SF	Steel factor
TE	Trophectoderm
TS	Trophoblast stem (cell)
VE	Visceral endoderm
XEN	Extraembryonic endoderm (cell)

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Multipotential Cells in Early Mammalian Development

Throughout mammalian development, there exist only a few proven totipotent cells. Following fertilization, the zygote has the potential to differentiate into all cell types of the fetal membranes and placenta, as well as all cell types of the body, a capacity that defines totipotency. As the cleavage divisions take place, the earliest blastomeres also share this capacity, but by the time the blastocyst forms, cells have become limited or fixed in their potential such that pluripotent but not totipotent cells are present. In the course of development, the progeny cells of the zygote continue to divide and differentiate into cell types with different characteristics. As a part of this progression, specific populations of cells are imbued with the dual capacity of producing more cells exactly like themselves and of producing cells different from themselves through cellular differentiation. These self-renewing cells with the potential to differentiate are termed stem cells and are an essential part of normal growth and development. Their existence and persistence for different periods of time during embryogenesis has been the subject of curiosity and investigation since the earliest days of embryology. Questions regarding the potency of stem cells, their determination, and the control of their differentiation have been posed in order to better understand the great mystery of embryogenesis. Recently, interest in pluripotent stem cells has taken a more practical turn as their potential clinical application for the repair or replacement of failing or damaged organs enter the realm of possibility. Whether stem cells from the embryo can be harnessed and utilized in this way has yet to be demonstrated, but the ease of their isolation, purification, and propagation, as well as control over their ultimate differentiation,

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will determine the eventual usefulness of embryo-derived stem cells in regenerative medicine.

Role of Embryonic Stem Cells in Development

Lineage Specification and Pluripotent Cells Within the Early Embryo

The totipotent cells of the cleaving mammalian embryo compact to form a morula and become radially polarized. This ball of cells gives rise to a cystic structure called the blastocyst by a process of cavitation at around embryonic day (E) 3.5 (Fig. 1). During this process, the developmental potential of the blastomeres gradually becomes restricted, giving rise to distinct cell lineages. Prior to its implantation in the uterus, the late blastocyst-stage embryo comprises three molecularly and spatially distinct cell lineages: the epiblast (EPI), the primitive endoderm (PrE), and the trophectoderm (TE) (Fig. 2). The pluripotent EPI lies in the interior of the inner cell mass (ICM) of the blastocyst and is encapsulated by the predominantly extraembryonic PrE, located on the surface of the ICM, and the TE, which comprises the epithelial surface of the blastocyst and is in contact with the outer environment [1–3]. The TE forms the fetal portion of the placenta, while the PrE gives rise to the yolk sac. By contrast the EPI gives rise to most of the fetus and adult organism.

Two cell fate decisions take place during preimplantation development that ensure the proper specification and spatial segregation of the extraembryonic lineages from the pluripotent EPI. The first involves the specification of the TE from the ICM; the second occurs within the ICM and involves the segregation of the PrE from the EPI. It was originally thought that position alone influences lineage allocation, such that outer blastomeres give rise to TE, segregating it from the ICM, and thereafter outer ICM cells in contact with the blastocoel cavity form the PrE. However, this strictly position-

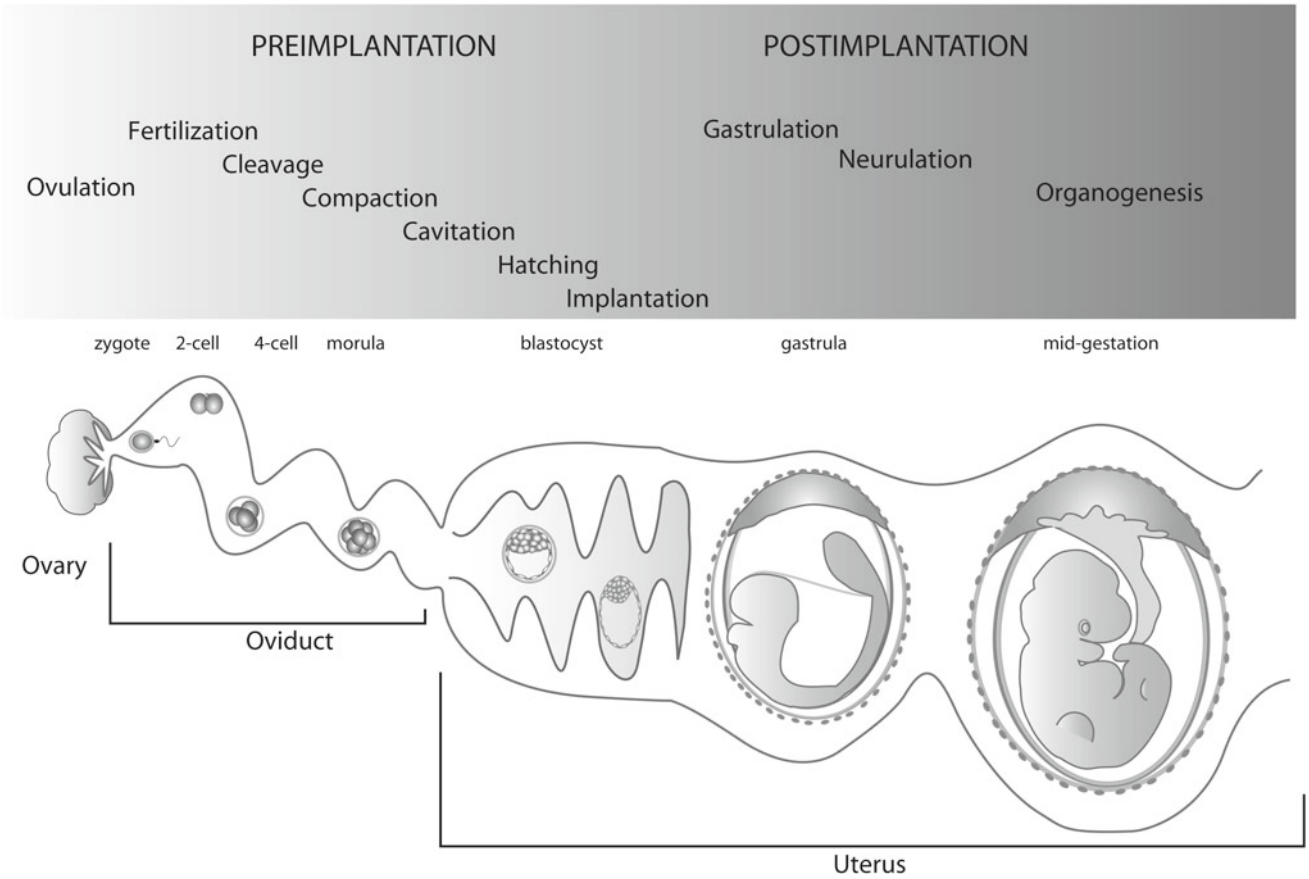

Fig. 1 Overview of mouse embryonic development: from fertilization to midgestation. After fertilization has taken place within the oviduct, the preimplantation stage embryo makes its way to the uterus. The first cell division, or cleavage, occurs approximately a day after fertilization. Thereafter cleavages take place approximately every 12 h. Transcription from the zygotic genome begins at the 2-cell stage (E1.5). Totipotency is lost by the time the embryos have entered the uterus (~E2.5). After hatching out of the zona pellucida, the mature blastocyst induces a decidual response and implants into the receptive uterus (E4.5). The latter half of embryonic development takes place within the uterus, with a maternal-fetal interface established between the trophoblast of the embryo and the maternal deciduum, eventually forming the mature placenta

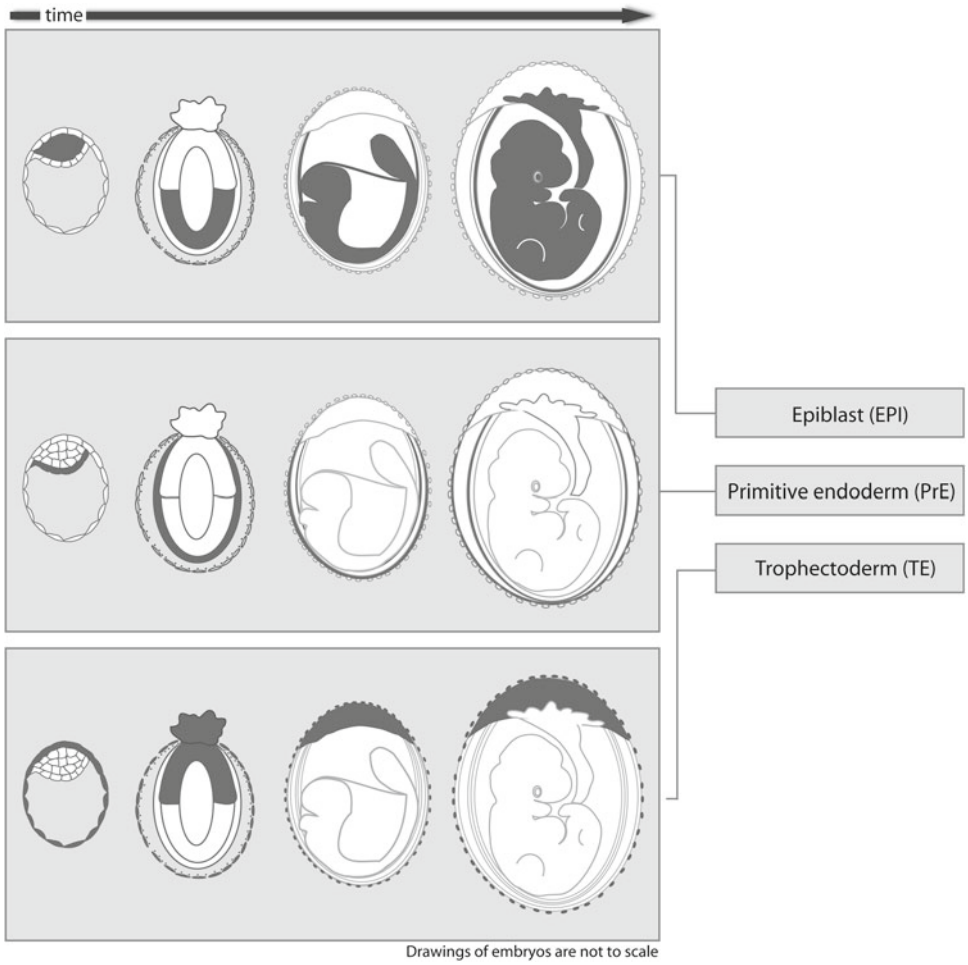

Fig. 2 Lineages of the first three cell types to differentiate in the mouse embryo. The fetus and the extraembryonic mesoderm develop exclusively from the EPI. The PrE, which differentiates from the ICM at the late blastocyst stage, gives rise to the extraembryonic endoderm. The TE of the blastocyst gives rise to the trophoblast giant cells and a large part of the chorioallantoic placenta

based model has been challenged by studies showing that cell lineage allocation is strongly influenced by the expression of certain lineage-specific transcription factors [4, 5]. Initially, markers of derivative lineages, such as NANOG for EPI and GATA6 for PrE, are co-expressed in individual cells at variable levels, but expression subsequently becomes mutually exclusive as cells are biased to a particular lineage. It is debatable, however, whether these precursor cells, although biased to a specific lineage, exhibit plasticity depending on context. A cell's potential, i.e., what it is capable of doing under experimental conditions, may not necessarily reflect the lineage for which it exhibits marker-specific expression. Recent studies have addressed this question by revealing that PrE precursor cells exhibit greater developmental plasticity than EPI precursor cells and that plasticity is lost once the cells have sorted to their respective tissue layers and the PrE begins to epithelialize [6]. Moreover, the developmental potential of early ICM cells is greatly influenced by signaling pathways such as the FGF/MAPK signaling pathway [7]. In the mouse, inhibition of FGF/MAPK signaling shifts all ICM cells to a NANOG-positive EPI fate, whereas removal of inhibition results in the restoration of the PrE lineage, indicating that the ICM cells retain a highly plastic state [8]. Rather surprisingly, however, this lineage segregation event in early bovine and human embryos, in contrast to rodents, does not appear dependent on FGF signaling [9, 10].

After implantation, the process of gastrulation takes place whereby cells of the EPI rearrange by morphogenetic movements through the primitive streak to form the three germ layers: ectoderm, mesoderm, and definitive endoderm. As tissues are progressively formed in development, embryonic cell potential gradually becomes narrower until almost all of the cells of the embryo have acquired a highly restricted developmental potential. The property of pluripotency then resides only in a relatively small population of cells, the primordial germ cells (PGCs), which are the progenitors of the male and female germ cells and eventually the gametes (Fig. 3).

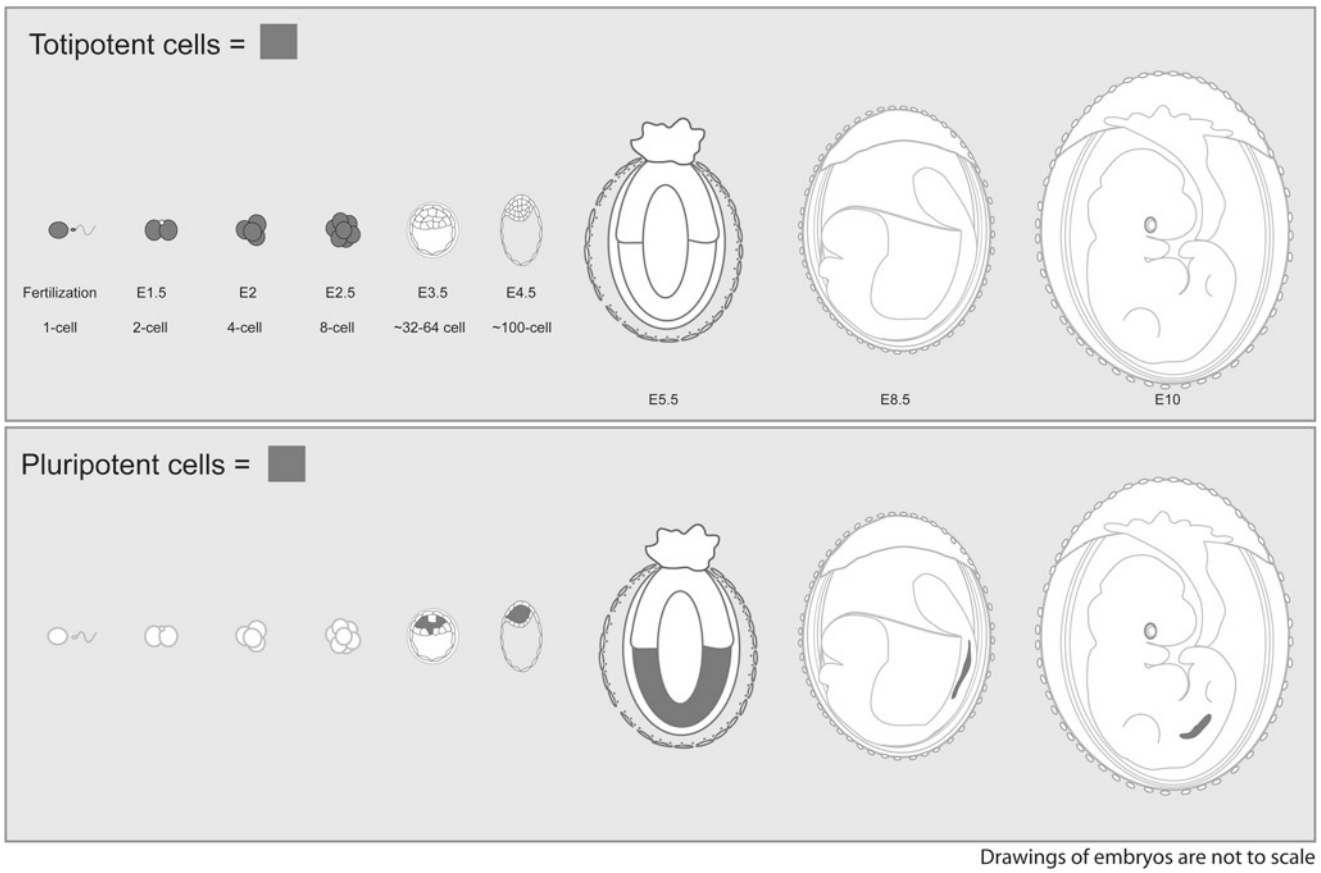

Fig. 3 Totipotent and pluripotent cells in the mouse embryo. Only the zygote and the blastomeres of cleavage stage embryos are truly totipotent. After the differentiation of the first lineage, the TE, totipotency is lost. The TE makes up the outer layer of cells in the cavitated embryo and is limited to making placental cell types. The epiblast lineage of the ICM is pluripotent; it can give rise to all cell types of the fetus, but not to trophoblast. At later stages of gestation, pluripotency resides only in the PGCs of the gonads

Harnessing the Potential of Embryonic Cells

Embryos increase in size at a phenomenal rate while at the same time differentiating a variety of cell types. One means to accomplish this feat is the rapid expansion of multipotential cell populations in specific lineages at different stages of development, prior to their further differentiation. This results in what can be thought of as transitory populations of stem cells with the capacity to reproduce similar cells, often referred to as transit amplifying cells, with similar developmental potential [11]. These stem cell populations exist only briefly in the rapidly changing embryo, but their stem cell capacity can be revealed and exploited by experimental intervention, which essentially "captures" the cells in a proliferative, stem cell state. Study of these cells constitutes the field of embryonic stem cell research, which began with the study of spontaneous gonadal tumors of germ cell origin, the teratocarcinomas. In the 1970s, it was discovered that teratocarcinomas could also be derived following the grafting of early embryos into ectopic sites in adults. These tumors consist of a chaotic array of differentiated embryonic cell types, as well as undifferentiated stem cells that can be propagated and/or differentiated in culture and can take part in embryonic development in chimeras in vivo [12–15]. The stem cells of teratocarcinomas were termed embryonal carcinoma (EC) cells and were thought to be the counterpart of pluripotent cells within the early embryo. Work with EC cells spurred the search for a means of deriving stem cells directly from embryos without the intervening step of tumor formation. In the following decades, stem cell lines were successfully isolated from the pluripotent EPI lineage of blastocyst-stage embryos and also from PGCs. The resulting cell lines are called embryonic stem (ES) and embryonic germ (EG) cells, respectively (reviewed by [16, 17]). In addition, pluripotent stem cells have been isolated from the epiblast lineage of postimplantation stage mouse embryos, namely the epiblast stem cells (EpiSC) [18, 19]. Finally, stem cells with a more restricted potential were isolated from the TE and PrE cell lineages of the mouse blastocyst, namely the trophoblast stem (TS) and extraembryonic endoderm stem (XEN) cells, respectively [20, 21]. Each of these embryo-derived stem cell types will be considered in detail in the following sections.

Stem Cells Derived from Early Mouse Embryos

Embryonal Carcinoma (EC) Cells

EC cells were first derived by culturing cells from germ cell-derived teratocarcinomas that occur spontaneously in the testes or ovaries of certain strains of mice. These tumors may contain differentiated tissues such as epithelia, bone, cartilage, muscle, fat, and hair, in addition to areas of rapidly dividing, undifferentiated stem cells [14, 15]. The tumors are not metastatic but are transplantable from one animal to another, and the EC cells derived from them can be propagated indefinitely in vitro.

In the 1970s, it was discovered that normal embryos from the blastocyst through early postimplantation stages, as well as isolated genital ridges, the primordia of the gonads, give rise to identical tumors when transplanted to ectopic sites in histocompatible hosts. In other words, teratocarcinomas can be derived from all stages of embryogenesis in which a high proportion of pluripotent cells can be identified (Fig. 3). Stem cells can be isolated and maintained in vitro from these embryo-derived tumors and are identical to EC cells derived from spontaneous teratocarcinomas (Fig. 4) [13–15]. EC cells can proliferate indefinitely in the undifferentiated state and still retain the ability to differentiate under specific conditions, although there is considerable variability among different cell lines in the range of developmental potential, with some lines being very limited. One explanation for this variable restriction of potential is aneuploidy, a condition that might arise during the transition from embryonic growth to tumor growth. Aneuploid cells or cells that have lost differentiation and growth checkpoints may be at a proliferative advantage during the formation of tumors.

EC cells have close parallels to embryonic cells. The protein synthesis profile resembles that of the EPI of the egg-cylinder-stage embryo. When placed into the embryonic environment by injection into a blastocyst, a stringent test for normal developmental potential, some EC cells have the ability to participate in development to form chimeras [22–24], although compared with normal embryonic cells, the contribution of EC cells to chimeras is less extensive and less uniform, and EC cells seldom, if ever, differentiate into germ cells (Fig. 5) [25]. Furthermore, EC cells frequently continue to proliferate in an undifferentiated state, resulting in tumors in the chimeras. This incomplete regulation of EC cell proliferative potential by the normal embryonic environment may reflect genetic changes that occurred during teratocarcinoma formation or during the derivation or culture of the EC cells [12]. Nonetheless, the existence of stem cells in these embryo-derived tumors and the tantalizing similarity between EC cells and the putative stem cells of the embryo fueled hopes of isolating stem cells directly from embryos, bypassing the tumor formation step.

Embryonic Stem (ES) Cells

In the early 1980s, three groups independently succeeded in deriving stem cell lines directly from early mouse embryos using different blastocyst culture conditions [26–28]. These primary cell lines, called embryonic stem (ES) cell lines, corresponded closely to the stem cells of the ICM and EPI. ES cell lines can be routinely derived from embryos by in vitro outgrowth of blastocysts, followed by disruption of the ICM and culture of the disaggregated cells in the presence of serum and the cytokine leukemia inhibitory factor (LIF) [29], or by growth on murine embryonic fibroblasts (MEFs), which provide a source of LIF (Fig. 4). Serum can be replaced by BMP4, so that mouse ES cells can be derived and cultured in serum-free media in the presence of LIF and BMP4 [30, 31]. In contrast to human ES cells that require FGF signaling for propagation (discussed later), mouse ES cells are induced to differentiate by FGF [32]. It was recently demonstrated that pluripotency of mouse ES cells does not require activation of signaling pathways, but rather the inhibition of FGF/ERK signaling and glycogen synthase kinase 3 (GSK-3) [33]. In this double-inhibitor condition (referred to as 2i), ES cells can be derived and propagated more efficiently from recalcitrant genetic mouse strains, as well as from other mammalian species including rat. It is thought that under 2i conditions ES cells remain in a "naïve" state of self-renewal that is more refractory to differentiation [17, 34]. Interestingly, this naïve state is established in the absence of FGF signaling, which is nevertheless required for the derivation of other pluripotent cell types such as mouse EpiSCs and human ES cells (discussed later).

ES cells can be maintained as permanent, undifferentiated cell lines when propagated in the presence of serum with LIF or LIF and BMP4 or in 2i conditions. They generally retain a normal, euploid karyotype as well as the capacity to differentiate into multiple cell types in vitro, in teratomas following transplantation to ectopic sites in host mice and in chimeras following blastocyst injection (Fig. 5). The majority of established mouse ES cell lines are male (XY), since the XX karyotype appears to be less stable and one X chromosome is frequently lost. Gene and protein expression studies further define the identity of ES cells. It is now known that a core regulatory network of pluripotency-associated transcription factors expressed in the EPI lineage of the ICM, including OCT4, NANOG, and SOX2, is essential for the maintenance of the ES cell state. These transcription factors positively regulate their own expression and repress the expression of genes promoting differentiation [35–40]. For example, ICM cells lacking OCT4 differentiate to TE in vivo and similarly

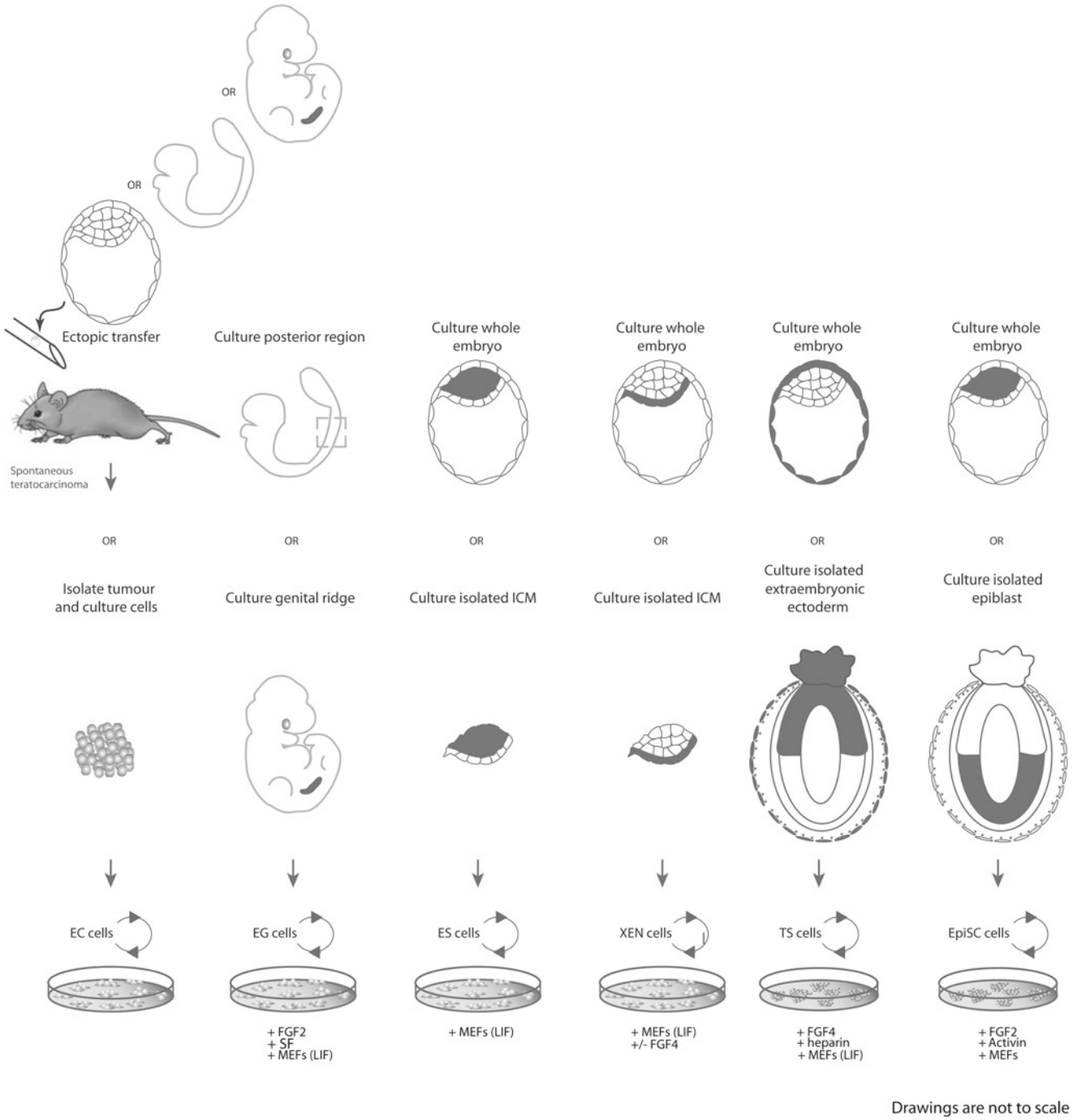

Fig. 4 Derivation of stem cell lines from early mouse embryos. Embryonal carcinoma (EC) stem cells can be derived from spontaneous teratocarcinomas or from teratocarcinomas resulting from the ectopic transfer of pluripotent embryonic tissues. Embryonic germ (EG), embryonic stem (ES), extraembryonic endoderm (XEN), trophectoderm stem (TS), and epiblast stem cells (EpiSC) can all be derived from embryos by a combination of dissection and specialized culture conditions (see text for details). The resulting cell lines have characteristics that mirror those of the early embryonic lineages from which they were isolated

downregulation of OCT4 in ES cultures in vitro induces TE differentiation [37, 41].

With the exception of the trophectoderm and extraembryonic endoderm, ES cells can contribute to all cell types in chimeras, including the germ cells [42], a potential similar but not identical to that of cells derived from the ICM of the blastocyst, which consists of pluripotent EPI as well as extraembryonic PrE precursor cells (Fig. 2). ES cells thus appear to represent the in vitro counterpart of EPI lineage of the blastocyst-stage embryo.

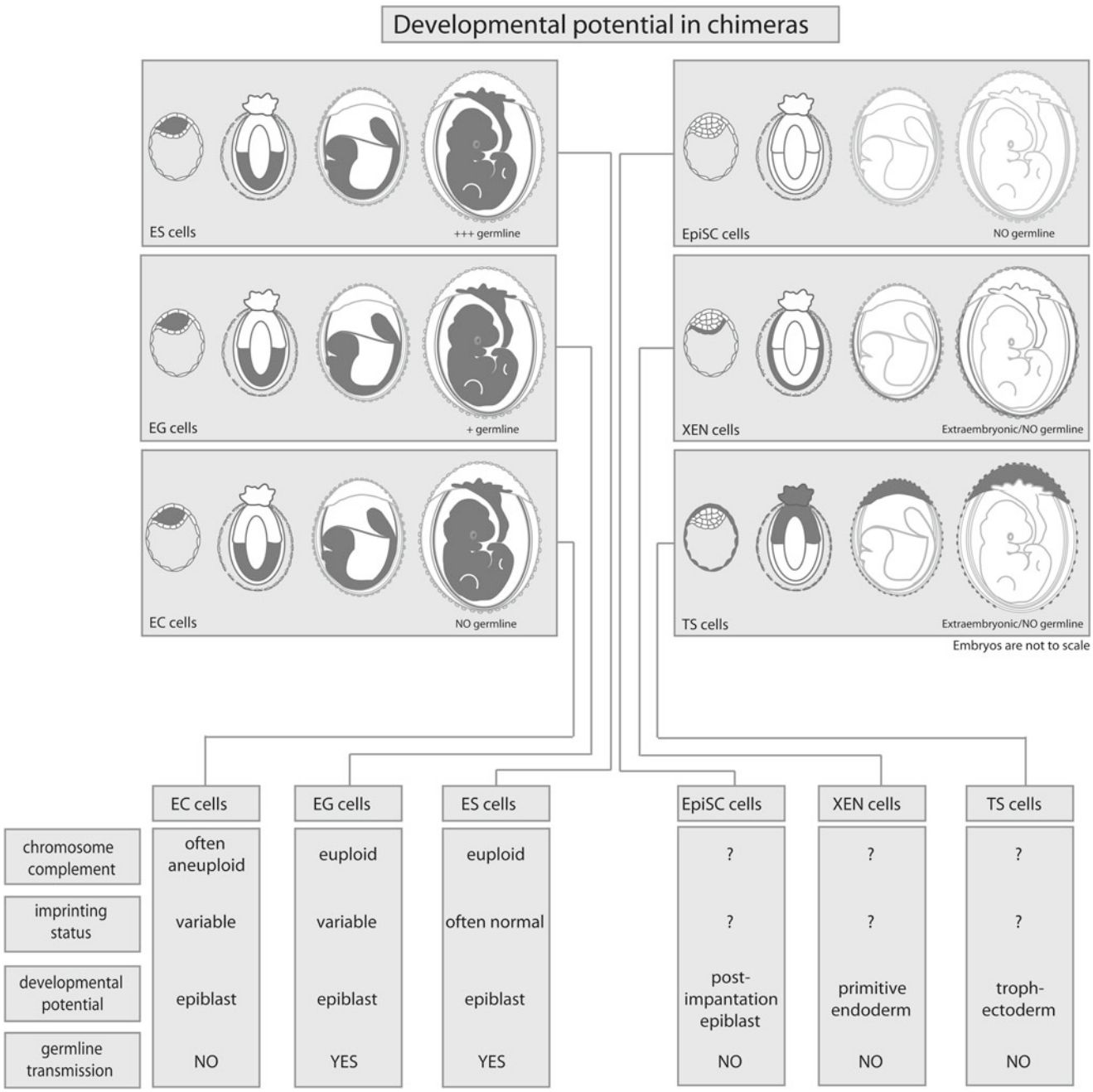

Fig. 5 Developmental potential and comparison of properties of mouse embryo-derived stem cells

When LIF is withdrawn during culture, ES cells differentiate and pluripotency is lost. LIF acts by binding a heterodimeric receptor complex comprised of the LIF receptor (LIFR) and glycoprotein 130 (GP130 or IL6ST). Receptor binding results in activation of GP130 signaling through the JAK/STAT pathway, which is essential for maintenance of pluripotency in vitro [43]. Several cytokines related to LIF, including ciliary neurotrophic factor, cardiotrophin 1, and oncostatin M, bind the LIFR/GP130 heterodimer and can substitute for LIF in vitro. Additionally, a combination of interleukin 6 and a soluble version of its receptor can substitute for LIFR action. This combination of ligand and receptor can activate GP130 homodimers and can be used to derive and maintain ES cells. In an interesting twist, the parallel between ES cells and the pluripotent cells of the EPI lineage of the embryo was challenged by the finding that GP130 signaling is not essential for early embryonic development, since mice carrying mutations in either *Lif*, *Lifr*, or *gp130*

develop beyond peri-implantation stages (see [44] for review). However, additional studies suggest that GP130 signaling is essential for maintaining pluripotency of the EPI during implantation delay or diapause [44], an explanation that would restore the parallel between ES cells and EPI cells of the blastocyst.

Embryonic Germ (EG) Cells

Spontaneous teratocarcinomas arising within gonads and teratocarcinomas developing from transplanted genital ridges were indications that stem cells were derived from PGCs prior to their differentiation into the highly specialized gametes. The discovery that steel factor (SF), the c-*kit* ligand, is essential for the survival and proliferation of migrating PGCs in the embryo (reviewed by [45]) provided a clue to the necessary in vitro culture conditions for deriving stem cells of PGC origin. In 1992, two groups were successful in deriving stem cell lines, which became known as embryonic germ (EG) cells, directly from PGCs [46, 47]. As with the derivation of ES cells, LIF was an important ingredient in establishing permanently growing, pluripotent cell lines, but in addition, FGF2 (bFGF) and SF were required (Fig. 4). Both male and female EG cell lines can be isolated directly from PGCs prior to or during their migration in gastrulating embryos or shortly after their arrival in the genital ridges. The dedifferentiation of PCGs to EG stem cells is promoted by activation of the PI3K/AKT pathway [48, 49]. These cells have many characteristics of ES cells with respect to their differentiation potential in vitro [50, 51] and their contribution in chimeras and, like ES cells, are capable of contributing to the germ line of chimeric mice [52, 53] (Fig. 5).

The status of imprinted genes is one important respect in which EG cells differ from ES cells. The expression of imprinted genes is dependent on their parental origin as reflected in the heritable, differential methylation of maternally or paternally derived alleles [54–56]. The imprint is, however, reversible, as it is erased and established anew in the germ cells at each generation and this appears to have profound consequences for the expression of genes in EG cell lines. Some chimeras developing with EG cell contributions are normal and transmit the EG cell-derived genotype to the next generation. Others, however, show fetal overgrowth and skeletal abnormalities, features characteristic of chimeras made with androgenetically derived ES cells, which have a paternal imprint [52, 57]. This observation indicates variability in the expression of imprinted genes in independently derived EG cell lines. EG cell lines derived from early PGCs are highly heterogeneous with respect to the methylation status of imprinted genes whereas EG lines derived from later PGCs more closely resemble the uniform pattern seen in ES cells [52, 57, 58]. With respect to experimental or therapeutic uses of EG cells, this important variable in the state of imprinted genes is essential to consider.

Epiblast Stem Cells (EpiSC)

Mouse ES cells derived from the EPI lineage of the blastocyst were the primary in vitro model of pluripotency for almost 25 years. Recently, however, pluripotent cell lines have been derived from early postimplantation stage mouse embryos, namely the EpiSC [18, 19]. Even though both ES cells and EpiSCs are pluripotent (Fig. 5), they differ in several respects: EpiSC cannot colonize the ICM of a blastocyst; consequently, the ability of these cells to contribute to chimeras has not been demonstrated; EpiSCs have a flattened colony morphology compared to the rounded ES cell colonies; EpiSCs express the core pluripotency-associated factors NANOG/OCT4/SOX2 but not some other factors such as KLF4, STELLA, and DAX1 expressed by ES cells; most importantly, however, EpiSCs require different extrinsic factors for their isolation and propagation, namely Activin and FGF2 (Fig. 4). These requirements are similar to those used for propagation of human ES cells and suggest major mechanistic differences in the establishment of pluripotency in mouse ES cells vs. mouse EpiSCs and human ES cells. Interestingly, mouse EpiSCs have also been derived from preimplantation embryos, the source of ES cells, raising questions as to the exact in vivo counterpart in the early embryo that is represented by EpiSCs [59].

Trophoblast Stem (TS) Cells

At first glance, the TE would appear to be a highly differentiated tissue. Certainly, within the TE, many cells rapidly become terminally differentiated, post-mitotic giant cells that undergo endoreduplication of DNA. However, cells directly in contact with the ICM, which are known as the polar trophectoderm, normally remain diploid and continue dividing in a relatively undifferentiated state, eventually forming the ectoplacental cone and extraembryonic ectoderm, which in turn form components of the placenta, including the secondary trophoblast giant cells (Fig. 2). The polar TE constitutes a limited-potential stem cell population, balancing proliferation (self-renewal) with differentiation into highly specialized, physiologically active, and post-mitotic cells.

It has long been known that the maintenance of proliferation in the polar trophectoderm is dependent on signals from the ICM and its later derivatives, and that without these signals the cells differentiate into giant cells or other terminally differentiated cells of placental lineages [60–62]. FGF signaling has been strongly implicated in mediating the

interaction between ICM and trophectoderm to maintain a proliferative TE cell population. This evidence comes both from the expression patterns of the FGF ligands and receptors (FGFRs) and from mutant and transgenic mice. In the case of the *Fgf4* and *Fgfr2* null embryos, no diploid trophoblast cells are detected either in vivo or in embryo outgrowths in vitro, as all the cells become trophoblast giant cells [63, 64]. A presumed hypomorphic allele of *Fgfr2* allows survival past the peri-implantation period, but embryos die later in gestation with multiple defects including a deficiency of trophoblast cells and a complete lack of the labyrinthine component of the placenta [65]. Expression of a transgenic, dominant-negative FGF receptor in polar trophectoderm causes the cells to cease division and differentiate into trophoblastic giant cells [66]. Another line of evidence comes from studies on a targeted mutation in *Oct4*, a gene coding for a transcription factor that synergizes with SOX2 to cooperatively bind the *Fgf4* promoter and regulate gene expression [67]. OCT4-deficient embryos develop to the blastocyst stage, but the inner cells differentiate along the trophoblast lineage and trophoblast proliferation is not maintained. When FGF4 is added to cultures of the inner cells of *Oct4* mutant embryos, ICM pluripotency is not restored but instead, dividing, undifferentiated cells that appear to be diploid trophoblast cells emerge from the differentiated trophoblast layer [41].

These observations pointed the way to the in vitro "capture" of a stem cell for the trophectoderm lineages by implicating FGF4 in the maintenance of the diploid trophectoderm precursors. FGF4 added to the culture medium of isolated extraembryonic ectoderm suppresses differentiation into giant cells and maintains the population of undifferentiated trophoblast precursors [41]. Furthermore, culture of extraembryonic ectoderm on MEFs with the addition of FGF4 and heparin facilitates the isolation of diploid epithelial cell lines that are capable of indefinite growth in vitro (Fig. 4). These cells, called trophoblast stem (TS) cells, differentiate into trophoblast giant cells upon the removal of either FGF4 or MEFs [20]. When TS cells are tested for their developmental potential by injection into blastocysts, they contribute exclusively to trophoblast subtypes in chimeras [20]. Gene expression studies confirm the molecular identity of TS cells and indicate that as they differentiate in vitro, they closely recapitulate the gene expression profile of the trophoblast lineage in vivo (Fig. 5) [68]. Indeed, similar to the pluripotency-associated network that governs pluripotency in ES cells, a network containing TE-specific transcription factors such as CDX2 and GATA3 is essential for TS cell self-renewal; each of these factors, when misexpressed in ES cells, is sufficient to force them to turn into TS cells [69, 70]. Other transcription factors important for TS cell self-renewal include EOMES, TCFAP2C, SMARCA4, ELF5, and ETS2 [71–73]. These studies have provided further support to the notion that TS cells are indeed the in vitro counterparts of the proliferating polar trophectoderm.

TS cells can be derived from blastocysts, from the extraembryonic ectoderm at E6–7, and from the chorionic ectoderm of E7.5 embryos at high efficiency regardless of the strain or sex of the embryo [20] (and our unpublished observations) (Fig. 4). FGF1 or FGF2 can substitute for FGF4 in vitro, but the factor(s) supplied by the MEFs has not yet been identified. However, since MEF-conditioned medium can substitute for MEFs, it suggests that a soluble factor is involved [20]. To this end, MEF-conditioned medium can be replaced by Activin and TGFβ (but not NODAL), highlighting a critical role for these members of the TGFβ superfamily for TS cell derivation and propagation [74, 75].

Extraembryonic Endoderm (XEN) Cells

PrE and EPI precursors are specified within the ICM of the mid- to late blastocyst. Prior to implantation, the PrE lineage is spatially segregated from the EPI and forms an epithelial layer next to the blastocyst cavity. After implantation, PrE gives rise to parietal endoderm (PE), which comes into contact with the TE, and visceral endoderm (VE), which covers the developing EPI and extraembryonic ectoderm. Extraembryonic endoderm (XEN) cells were first derived from blastocyst-stage mouse embryos [21]. XEN cells express PrE-specific factors, such as GATA4, GATA6, and SOX17, which play critical roles in their self-renewal, supporting the notion that XEN cells are the in vitro counterpart of the PrE lineage (Fig. 5) [21, 76]. XEN cell lines can be derived from intact blastocysts or isolated ICMs in TS cell conditions (on feeders or in hanging drops in the presence of FGF4 and heparin) followed by withdrawal of FGF4/heparin and culture on feeders [21, 77, 78]. They can also be isolated from blastocysts explanted in ES cell derivation conditions (on feeders in the presence of LIF) (Fig. 4) [21, 79]. The fact that these cells can be derived under a variety of conditions suggests that XEN cells do not require specific growth factor signaling pathway activation or repression to facilitate their derivation. Moreover, XEN cells have also been isolated from preimplantation rat embryos [80–82]. Interestingly, these rat-derived XEN cells have been reported to possess a more immature expression profile exhibiting characteristics of ES, TS, and XEN cells and exhibit a highly dynamic and plastic potential contributing to different lineages in donor rat and mouse embryos [81, 82].

XEN cells have been widely used as an in vitro model to understand how the extraembryonic endoderm lineage is specified and expanded. For example, PDGF signaling has been shown to be required for the expansion of the PrE lineage of the blastocyst, XEN cell isolation, and proliferation [79]. Moreover, the differentiation of XEN cells can be

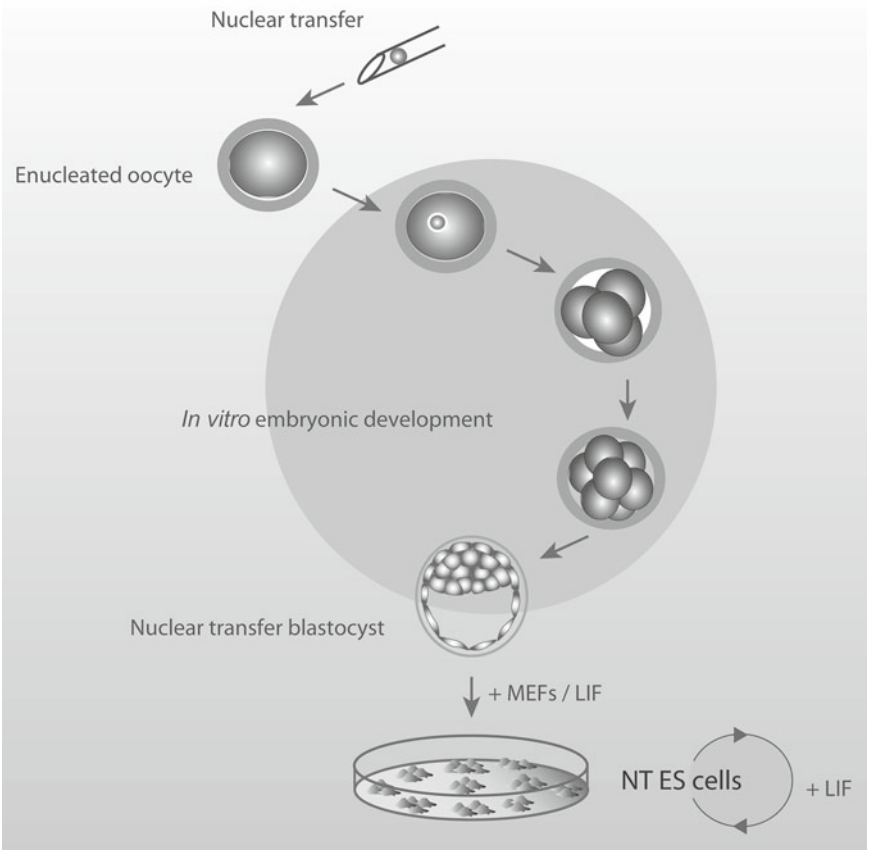

Fig. 6 NT ES cells: ES cells from cloned embryos. A somatic cell nucleus is transferred into an enucleated oocyte and allowed to develop in vitro to the blastocyst stage. The cloned embryo is then cultured on mouse embryonic fibroblasts (MEFs) in the presence of LIF for the derivation of NT ES cells

directed into specific extraembryonic endoderm derivatives such as the embryonic visceral endoderm (emVE) [77] and extraembryonic visceral endoderm (exVE) upon BMP4 stimulation [83, 84]. Finally, another study reported the functional similarity of XEN cells to the heart-inducing anterior visceral endoderm (AVE), highlighting the potential utility of these cells for developing improved protocols for cardiomyocyte differentiation [85].

Stem Cells from Cloned Embryos: NT ES Cells

Cloning is a mode of asexual reproduction resulting in offspring bearing the identical nuclear genome to their parent. In recent years, mammalian cloning has been achieved by the introduction of somatic cell nuclei into enucleated oocytes. Wakayama et al. [86] were the first to report the derivation of ES cell lines from cloned blastocysts (Fig. 6). These cells are referred to as nuclear transfer (NT) ES cells. NT ES cells are identical in their developmental potential to normal ES cells in that they can be maintained as pluripotent stem cells when propagated in the presence of LIF, and they are able to contribute widely in chimeras. Furthermore, their transcriptional and posttranscriptional profiles are indistinguishable from ES cells derived from normal embryos [87, 88].

The ability to derive ES cells from cloned embryos allows for partnering of the technology of cloning with that of genetic engineering in ES cells and thus offers unprecedented opportunities for exploitation. In light of the ethical and scientific controversies surrounding cloning, it is worthwhile making a distinction between cloning with the intent of generating individuals (reproductive cloning) and cloning with the intent of deriving genetically matched pluripotent ES cells (therapeutic cloning). NT ES cells can be used in therapeutic regimes, where ES cells generated from sick adult individuals could provide the basis for therapy by providing genetically matched stem cells. Genome manipulation could be undertaken to correct defects in the stem cells and the resulting cells could be used for the treatment of the sick individual through cell-based therapy (Fig. 7). An early study described the first successful application of therapeutic cloning, where immunodeficient mice were treated with their own genetically repaired NT ES cells [89] and subsequent studies have applied the technique to ameliorate the effects of Parkinson disease [90, 91].

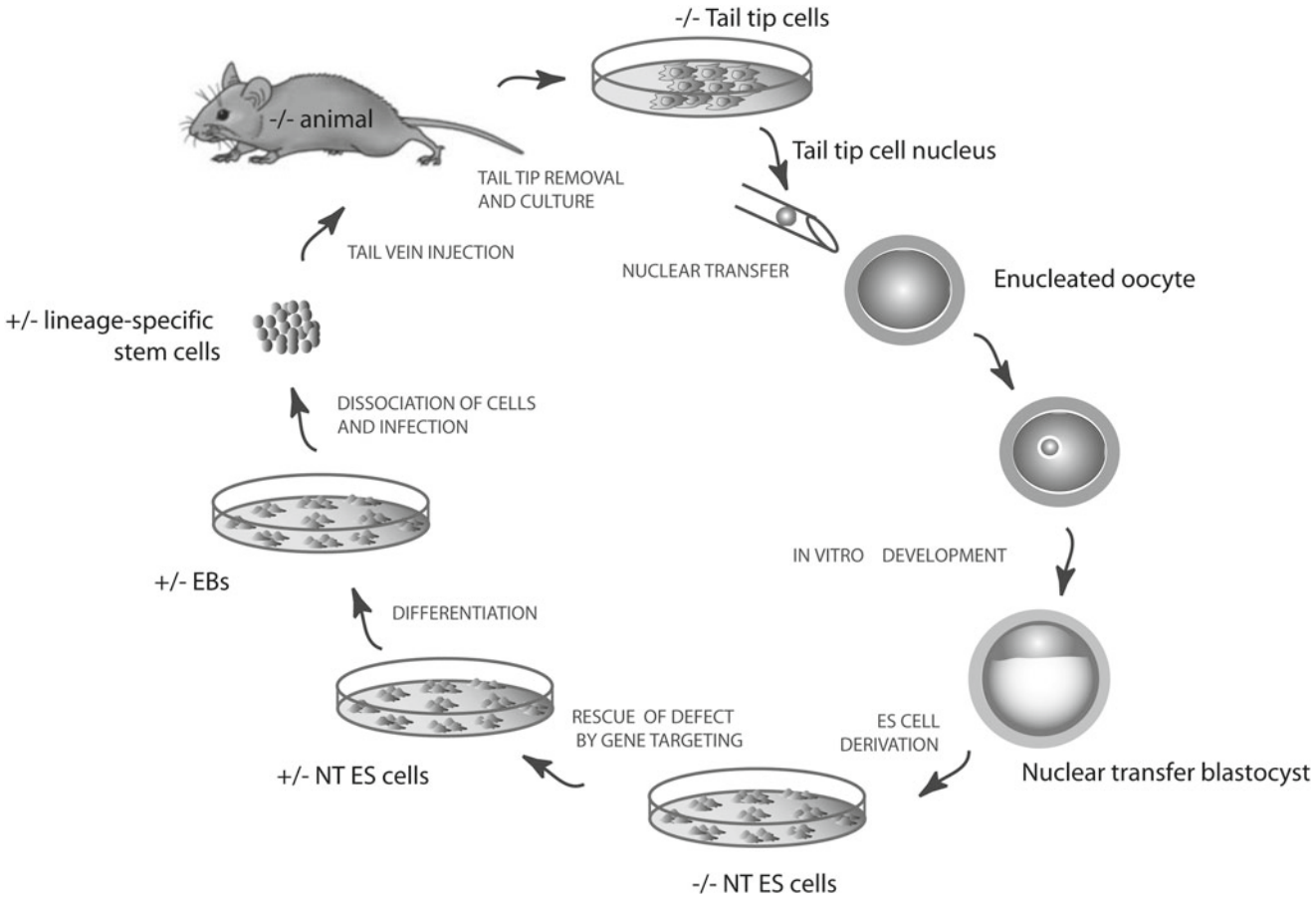

Fig. 7 A scheme for the treatment of genetic disorders, combining therapeutic cloning with gene therapy. Somatic cells from a mutant (–/–) animal are cultured and used as nuclear donors for injection into enucleated oocytes. The resulting cloned embryos are used to derive ES cells. The genetic defect is then corrected (+/–) by gene targeting in the ES cells which are then differentiated and returned to the mutant mouse

Exploiting Mouse ES Cells

Mouse ES cells have been invaluable tools in embryological studies of cell fate and cell lineage, and they have also provided a versatile tool for gene manipulation. Pluripotent ES cells are capable of differentiation into the germ cells of a chimera, even after extensive in vitro culture, electroporation of genetic material, and drug selection. This capacity of ES cells has allowed for specific genetic changes to be engineered into ES cells, selected in vitro, and subsequently introduced into mice through germ line transmission from ES cell chimeras [92]. The development of this "gene-targeting" technology and its exploitation during the past 20 years have been the single most important use for ES cells to date. Both directed genome alterations, such as "knock-outs," "knock-ins," single-base changes, and gene replacements, and random alterations, such as "gene traps," insertional mutagenesis, and chemically induced mutagenesis, have become commonplace and thousands of mutations have been produced.

Once mutations are in the germ line, ES cells can again be put to use in the study of mutant effects on the development of embryos and/or organs. Combinations of mutant ES cells and normal cells in chimeras address questions about the cell autonomy of a mutant effect and the phenotypic consequences of the mutant effect on cell-cell interactions and induction during development. Thus, in addition to being the means through which specific mutations are introduced into the mouse germ line through targeted mutagenesis, ES cells are also a tool in the analysis of those mutations.

Transdifferentiation of Embryo-Derived Stem Cells: Altering Developmental Potential

The coordinate regulation of specific signaling pathways is pivotal for the first differentiation event involving the specification of TE vs. ICM lineages. Similarly, the isolation and maintenance of stem cells representing those lineages are promoted by the regulation of intracellular signaling

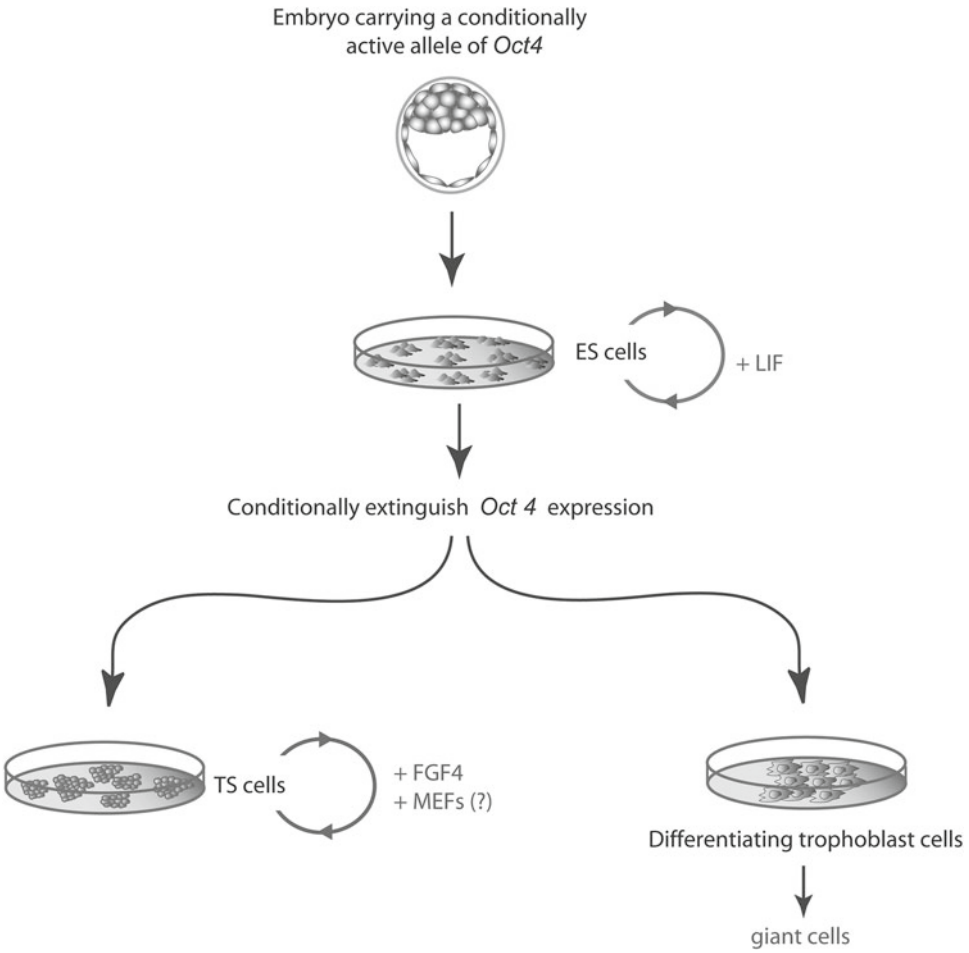

Fig. 8 Interconversion of stem cell identity. The expression of *Oct4* is pivotal for maintenance of ES cell identity. Loss of *Oct4* and stimulation of the MAP kinase pathway by exposure to FGF4 can change the developmental potential and identity of stem cells from that of ES to that of TS cells

cascades though culturing under appropriate conditions (Fig. 4). This raises the possibility that transdifferentiation between ES and TS cells could be encouraged by controlling the on/off status of key signaling pathways and transcriptional regulators. For example, FGFR/MAP kinase signaling off, GP130/JAK/STAT signaling on, and OCT4 on characterize the ICM lineage, whereas the diploid trophoblast lineage would be specified by FGFR/MAP kinase signaling on, GP130/JAK/STAT signaling off, and OCT4 off. Thus when the in vitro culture conditions of ES cells are changed to those of TS cells (i.e., withdrawal of LIF and addition of FGF4- and MEF-conditioned media), the ES cells transdifferentiate. The central premise is that *Oct4* expression is extrinsically manipulated. In ES cell lines, when *Oct4* is repressed, the ability of the cells to self-renew is lost and they differentiate into what resemble trophoblast giant cells, even in the presence of LIF [93]. However, if *Oct4* expression is repressed and the culture conditions promoting TS cells maintenance are substituted for those promoting ES cells, the cells continue to self-renew, but now resemble TS cells in morphology and adopt a TS cell-specific gene expression profile (Fig. 8). It was also demonstrated that misexpression of the two major TE-specific transcription factors CDX2 and GATA3 in mouse ES cells results in the transdifferentiation of these cells to TS cells [69, 70]. Conversely, two studies have shown that TS cells can be reprogrammed to ES-like pluripotent stem cells via overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* or by *c-Myc* or *Oct4* alone [94, 95]. These observations suggest that ES cells and TS cells have the potential to transdifferentiate to each other and that the expression of key lineage-specific markers such as *Oct4*, *Cdx2*, and *Gata3* is pivotal in dictating their status and developmental potential in these transdifferentiation events.

Similar evidence has also been shown for transdifferentiation events between ES and XEN and ES and EpiSC stem cells. Forced expression of PrE-specific transcription factors, such as GATA4, GATA6, SOX7, and SOX17, is sufficient to drive ES cells to adopt a XEN-like phenotype [76, 79, 96].

Together these studies suggest that a change in developmental potential can be induced by manipulating the expression of key intrinsic regulators. Alteration of extrinsic signals could further reinforce the transdifferentiation event. ES cells can be converted to an EpiSC identity solely by a change in culture conditions [97]. Similarly, EpiSCs derived from postimplantation epiblast can spontaneously revert to a "naïve" ES-like state when cultured for several passages in regular ES cell conditions (on feeders with LIF) [98]. Conversely, EpiSCs can be reprogrammed to ES cells via forced expression of "naïve" pluripotency-associated factors such as KLF4, OCT4, LIF/STAT3, and the NR5A nuclear receptors [97, 99–102]. Moreover, another means to convert EpiSCs to ES cells is by culturing in ES cell media supplemented with LIF and small molecules targeting epigenetic modifiers, which highlights the importance of the epigenetic signature in transdifferentiation [103].

Human Embryonic Stem Cells

Pluripotent stem cell lines have been derived from human blastocysts [104] and PGCs [105] with techniques similar to those developed for mouse embryos. These lines have been designated with the same terminology of ES and EG cells, respectively. Human ES (hES) cells differ significantly from murine ES cells in colony morphology, proliferation rate, growth factor requirements, and epigenetic status. In contrast to murine ES cells, hES cells proliferate slowly and form flattened two-dimensional colonies and are dependent on FGF2 and Activin/NODAL signaling [106]. Unlike murine ES cells, which tolerate passage as single cells following trypsin digestion, hES cells require mechanical or collagenase-mediated dissociation and passage as small clusters of cells [107]. Epigenetically, murine and human ES cells display a different pattern of X chromosome inactivation and promoter occupancy by pluripotent transcription factors [101].

hES cells can proliferate indefinitely in vitro and can maintain a stable karyotype under stringent culture conditions. However, prolonged culture can also result in chromosomal abnormalities, such as trisomy 12 or 17 [108, 109], thought to arise during growth adaption to culture [110]. hES cells can be clonally derived and still maintain pluripotency when differentiated in vitro or as teratomas in vivo in nude mice. Their differentiation potential cannot, however, be tested in vivo in chimeras for obvious ethical reasons.

Until recently the differences between mouse and human ES cells were thought to be a variation of an otherwise comparable pluripotent population. The derivation of EpiSCs from explanted murine or rat postimplantation EPI changed this view. EpiSCs show striking similarities to hES cells with regard to their molecular properties, growth factor requirements, colony morphology, X-inactivation status, and culture dynamics [18, 19, 97]. Thus in the mouse, two functionally distinct pluripotent states exist: a "naïve" LIF-dependent pluripotent stem cell state corresponding to the preimplantation ICM and a "primed" FGF2-dependent pluripotent stem cell state reminiscent of the postimplantation EPI [17, 34]. The overlap of characteristics between hES and mouse EpiSCs suggests that despite their blastocyst origin, hES cells exist in a primed pluripotent state. It remains to be determined whether it is possible to derive naïve pluripotent hES cells that more closely resemble mouse ES cells than EpiSCs [17]. In a recent study, treatment of hES cells with 2i medium supplemented with LIF and forskolin led to ectopic induction of key pluripotency factors OCT4, KLF4, and KLF2 and conversion of hES cells into a cell type that possessed the characteristics of mouse ES cells, including two active X chromosomes and responsiveness to LIF [111]. Understanding the precise nature of human ES cells as well as their relationship to pluripotent cells in the embryo is a prerequisite for their exploitation for biomedical benefit. Although there are serious ethical considerations in the derivation and use of human stem cells from embryos, their potential value for therapeutic uses as a readily available, renewable source of stem cells for a variety of organs and tissues has sparked an intensive research effort as well as an active public debate. The possibility of repairing or replacing failing organs with stem cells holds enormous appeal, especially if the stem cells can be engineered in vitro to avoid the host's immune response.

Therapeutic Use of Stem Cells: Challenges and Perspectives

The extensive background of knowledge on mouse embryo-derived stem cells provides an experimental model for human embryonic stem cell research and a means of testing ideas on the biological basis of therapeutic interventions involving stem cells. Given the extensive data base of genomic and developmental information and the long history of mutagenesis and embryonic stem cell experimentation, the mouse is the ideal model organism for this purpose. The differentiation pathways leading to many specific cell types have been elucidated for stem cells in vitro and are supported by studies on developmental potential as assayed through contribution in vivo (reviewed by [112]). Even highly organized structures such as insulin-expressing cells with the three-dimensional structure of pancreatic islets have been differentiated from ES cells, demonstrating their potential for the assembly of functional organs [113]. The feasibility of using ES cells to effect cures through tissue transplantation has been tested and validated [114]. Through the manipulation of histocompatibility genes, mouse stem cells can be engineered to escape the immune response [115].

With the advent of cloning mammals by nuclear transplantation into enucleated egg cytoplasm, another technical innovation to avoid immune rejection is being tested in mice. Embryos cloned by nuclear transfer of somatic cell nuclei have been used as a source for the derivation of new ES cell lines that retain full developmental potential [86, 116]. Using this method, unique ES cell lines could be "tailor-made" through cloning from a donor's somatic nuclei for eventual stem cell therapy of that same donor. Because the nuclear material originated with the donor, there would be no danger of immune rejection [89] (Fig. 7). Studies like these demonstrate the potential for therapeutic uses of stem cells, and it is through the use of mouse stem cells that the strategies and therapies can be developed with a view to possible application to humans.

The ethical and legal controversy surrounding hES research initiated the quest for alternative sources of human pluripotent stem cells. In a breakthrough discovery, Takahashi and Yamanaka used ectopic co-expression of four transcription factor genes (*Oct4*, *Klf4*, *c-Myc*, and *Sox2*) to reprogram adult mouse fibroblasts into induced pluripotent stem (iPS) cells [117]. This methodology was quickly applied to human fibroblasts and other cell types, and a variety of reprogramming methods to derive iPS cells have been developed (reviewed by [118]). This alternative source of pluripotent stem cells with ES properties offers hope for personalized regenerative cell therapies. However, questions remain as to how equivalent iPS cells are to ES cells and whether differences in their functionality will affect their behavior in vivo. There is an emerging consensus that iPS and ES cells are neither identical nor completely distinct but rather have overlapping characteristics [118]. Differences between human iPS and hES cells will affect their utility for clinical applications. While iPS cells have clear advantage for disease modeling, both iPS and ES cells can successfully serve as drug screening and toxicity testing platforms.

Bone marrow transplant is the oldest and most widely available stem cell therapy (reviewed by [119]). Hematopoietic stem cells (HSCs) have been successfully used for decades to treat leukemia, thalassemias, or myeloid hypoplasia. HSCs do not need to be expanded ex vivo prior to transplantation and there is no need to reconstitute a complex organ architecture [119]. However, the use of other types of pluripotent stem cells for therapeutic applications is still in its infancy and many hurdles need to be surmounted before their safe clinical use. The ideal therapeutic-quality stem cells should be pathogen-free, feeder-free, have no tumorigenic potential and have no exposure to cells or products from other species [120, 121]. Key priorities in translating stem cell research into therapies are generating stringent markers of pluripotency and assays to determine the capabilities of a given human iPS or hES cell line and improving our understanding of directed differentiation. Establishment of rigorous standardized protocols in early preclinical and clinical trials is of extreme importance for achieving safety and efficacy in stem cell therapeutic applications.

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Quantitative Approaches to Model Pluripotency and Differentiation in Stem Cells

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Problems, Paradigms, and Systems Solutions

Current Limits in Reprogramming and Differentiation Fields

Today, much biological information in the stem cell field is obtained from empirical studies. Thus, the original reprogramming "cocktail" of four factors (Oct3/4, Sox2, c-Myc, and Klf4) and its consequent modifications [1–3] were determined by testing essentially random combinations of candidate factors suggested by genome-wide screens. Currently, the efficiency of reprogramming remains low and the quality of the obtained cells is often questionable [4]. The original retroviral reprogramming method can transform only about 0.01 % of fibroblasts into induced pluripotent stem cells (iPSC). More relevant to medicine, "cleaner" adenoviral reprogramming or direct delivery of reprogramming proteins into cells is even less efficient (0.0001–0.001 %) [5]. The low efficiency and associated expense still impose barriers to broad medical application of the reprogramming methods [4, 6, 7].

Directed differentiation depends on predictive and efficient conversion of a large number of pluripotent stem cells into a nearly homogeneous population of cells representing the desired cell lineage; this requires laborious testing of numerous factor combinations and growth conditions [8–10]. Among the most successful examples of directed differentiation is a recent method of human cardiac differentiation, producing 64–89 % of contacting cells resembling cardiomyocytes; routinely, only 10–20 % of pluripotent stem cells can be differentiated into cardiomyocytes [9]. Current technologies allow even direct lineage conversion, whereby brief exposure to a reprogramming cocktail precedes incubation with differentiation factors [11]. In some cases, the lineage conversion seems to be very efficient (up to 100 % of cells converted), but it remains to be seen to what extent the conversion mimics normal differentiation and whether the qualities of the obtained cells are affected by any remaining unerased epigenetic memory [12].

Beyond identifying combinations of reprogramming and differentiation "cocktails," the molecular mechanisms underlying reprogramming, directed differentiation, or lineage conversion remain largely unknown. Obtaining and culturing of many cell types [including hematopoietic stem cells (HSCs)] in sufficient quantities is still difficult or impossible [13]. Systems analysis of gene interactions, reconstruction of gene regulatory networks (GRNs), and building quantitative predictive models will help us to explain the conversion of cell phenotypes, identify effective culturing conditions, and improve quantities and qualities of the converted cells.

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Deterministic and Stochastic View of Self-Renewal and Differentiation

One of the key features shared by both embryonic and adult stem cell types is their ability to self-renew. It is believed that self-renewal and pluripotency depends on a relatively small set of gene network circuits (kernels) connecting a few transcriptional regulators [14]. It is assumed that these core transcriptional circuits occupy top positions in the regulatory hierarchy within a stem cell of any kind and can regulate expression of thousands of genes, which define the self-renewal state [5, 15]. In the case of embryonic stem cells

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(ESCs), the most frequently suggested regulatory kernel involves the transcription factors Nanog, Oct4, and Sox2 [16, 17]. More recent studies also include Esrrb and Sall4 as yet additional *bona fide* core pluripotency factors [18–22]. In the case of adult bone marrow HSCs, the suggested potential core regulators include Fli1, Scl, Gata2 [23], Tcf7 [24], and perhaps a few other transcription factors. Pluripotency in ESC and HSC is most likely regulated by different underlying gene networks; however, some reports also suggest the importance of certain ESC factors, such as Sall4 for self-renewal of HSC [25].

The topology and molecular interactions in the pluripotency core networks ensure the presence of stable pluripotency cell states. Mathematically, the stable states are described as *attractors* or vectors of the core transcription factor concentrations, towards which the concentrations evolve over time [26–29]. In ESCs, two or even more attractors apparently determine the pluripotent state [28, 30], since pluripotent cells express higher and lower concentrations of major pluripotency regulators, such as Nanog [28, 30, 31]. Theoretical studies of network motifs, such as fully connected triads [32] with topologies similar to the suggested core pluripotency network and few published quantitative models, largely support the presence of more than one pluripotency attractor in ESCs [30, 33, 34]. The described studies allow the narrowing down of the general problem of "what is self-renewal and pluripotency?" to specific goals of (1) reconstruction of stem cell regulatory gene networks and (2) identifying and explaining attractor states for the most critical network motifs and domains.

The core regulatory networks are placed in the context of much broader array of genes controlling vital stem cell functions (see Fig. 1a). One such function is susceptibility and response to external signals promoting pluripotency or differentiation. Thus, Klf4 is essential for maintenance of iPSC [35, 36] and members of Klf family of transcription factors mediate LIF (leukemia inhibitory factor) signaling. At the same time, the core pluripotency factors Nanog, Oct4, and Sox2 positively regulate expression of Klfs [37]. The transcriptional repressor Tcf3 is another example of interaction between the extrinsic signaling pathways and the core pluripotency transcriptional network. Together with Lef1 and other Tcf-like factors, Tcf3 mediates Wnt signaling [38–42]. Activation of the Wnt signaling pathway relieves Tcf3 repression and promotes pluripotency [43]. Tcf3 occupies a high position in the regulatory hierarchy and, at the same time, may be an integral part of the core pluripotency network [44]. ESCs are able to maintain their pluripotent state even in the absence of external signals, required for culturing (LIF, BMP) [42]. External signaling becomes completely dispensable if cell proliferation suppressors Erk1/2 and Gsk are inhibited [34, 45]. Thus, the core pluripotency network may be described as self-sustainable. Self-sustainability means that the core pluripotency network operates with a substantial amount of information, which the network can independently process and make decisions related to self-renewal or differentiation.

With a certain probability, the concentrations of the pluripotency factors may cross the concentration limits defined by the stable attractor states and pluripotent cells differentiate. Variation in gene expression may be the reason why the stable attractor states are lost. This hypothesis emphasizes the role of *stochastic* gene expression in cell fate determination, so many of the current models explaining stem cell behavior include both deterministic and stochastic components [27, 28, 30, 34, 46]. Indeed, concentrations of the core factors, measured in individual pluripotent ES cells, form statistical distributions featuring local peaks (bimodality), possibly corresponding to dynamical semi-stable states or pluripotent attractors [28–30, 47]. In extreme cases, stochastic variations in gene expression may lead to a loss of one or more of the key self-renewal factors. The remaining skewed and/or incomplete combinations of the core factors may result in commitment to a specific cell lineage [47, 48]. For instance, alternative expression of Oct4 or Sox2 in mouse ESCs may result in commitment of cells to either mesendodermal (ME, Oct4) or neuroectodermal (NE, Sox2) fates [34, 49–51].

The observed variations in gene expression levels or gene product concentrations may not be explicitly explained by the stochastic behavior of large and rare biological molecules. Instead, there is a broad spectrum of molecular events, roughly divided into extrinsic and intrinsic fluctuations [52–54]. Quantitative models aimed at describing stem cell behavior should take into account both the deterministic solutions with predicted attractor states and the substantial (stochastic) deviations from the deterministic paths and attractors.

Types of Gene Networks and Models Describing Stem Cells

In general, the first step in model construction includes broad integration of data, involving genome-wide expression or epigenetic studies [55, 56]. Typically, these studies identify the most prominent candidate genes selectively expressed in self-renewing or differentiating stem cells. Linking such candidate genes into networks is based on their co-expression or the presence of similar binding patterns for transcriptional regulators in the gene control regions. However, the resulting GRNs are often too complex and require filtering and partition steps before a dynamical model can be constructed. Largely, models in the stem cell field are focused on a single kernel and involve few transcriptional regulators [28, 30, 33, 34, 46, 57, 58].

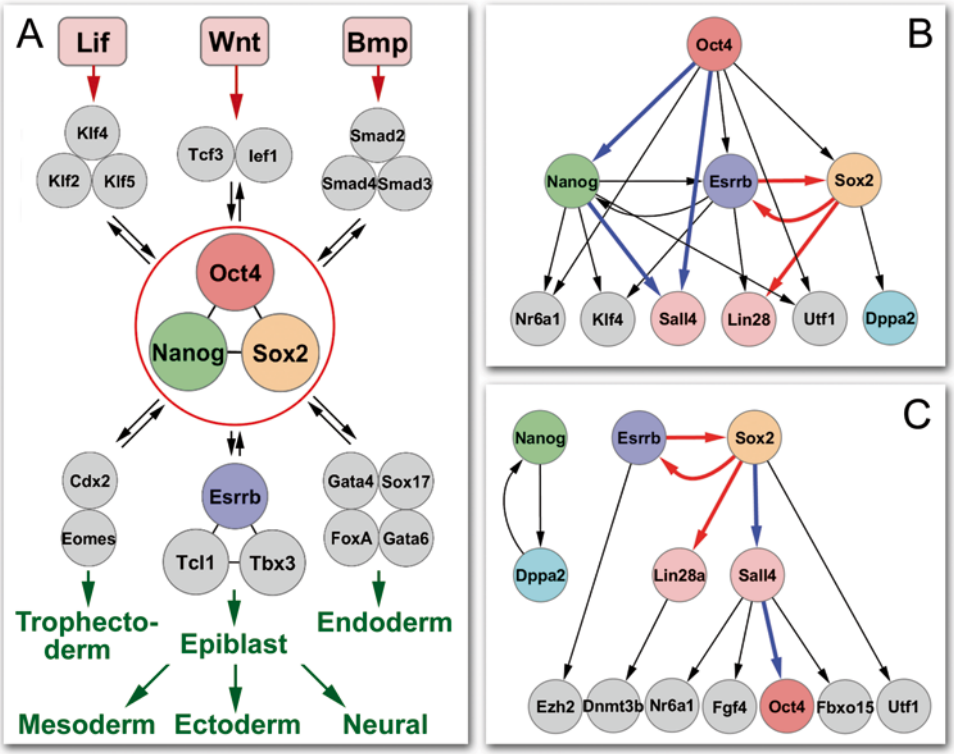

Fig. 1 Networks proposing gene interactions in mouse embryonic stem cells. (**a**) The network reflects genetic interactions (interactome) and information flow across the pluripotency network in mESC. A common hypothesis suggests that a relatively narrow core circuit (*red circle*) may be responsible for processing information received from upstream signaling pathways (shown in *pink* on the *top*) and making decisions, including those leading to differentiation (*green arrows*). Each information processing step may involve additional factors (*gray nodes*), which are more peripheral to the core circuit. (**b**) Hierarchical view of the pluripotency network for mESC; the network has been constructed based on gene expression data obtained from differentiating cells. (**c**) Hierarchical gene network based on expression data obtained during reprogramming of mouse fibroblasts to induced pluripotent stem cells (iPSC). The *red arrows* mark highly similar Esrrb-Sox2 domain; the *blue arrows* show strikingly rearranged Oct4-Sall4 domain. During reprogramming, the network is inverted with the main activator Oct4 present on the low hierarchical level (compare (**b**) and (**c**))

Quantitative dynamical models are biological hypotheses expressed in a formal way. Quantitative models provide room for capturing diversity and complexity of biological systems but require formalization and/or quantification of biological data. Current quantitative predictions still require experimental validation since the models contain many unknown or ambiguous parameters.

Roughly, all quantitative models are focused either on biochemical (BRN—biochemical reaction networks) or statistical interactions observed between genes in gene networks (SIN—statistical influence networks) [59] (see Fig. 2). Typically, BRN-based models describe gene product dynamics and rely on systems of ordinary differential equations (ODE) or stochastic differentiation equations (SDE) [30, 60], wherein the reaction constants are often represented by unknown parameters. Certain BRN-based models are limited by consideration of the reaction's steady states (or gene response potentials) and rely on systems of logistic functions describing, for instance, fractional occupancy of binding sites for transcription factors (BSTF) in the gene control regions [61–63]. Since BRN-based models attempt to capture many biological details, they are better at handling smaller gene networks, typically about a dozen genes.

A second broad class of models, based on SIN, requires no specific knowledge of biology and may rely on genome-wide readings of gene expression patterns, results of statistical analysis of gene associations in large databases, or both. SIN-based models include random and probabilistic Boolean models (RBN and PBN) [60]. In the late 1960s, Kauffman [64] first proposed the use of random Boolean networks (RBNs) to model GRNs. More recently, the probabilistic Boolean networks (PBNs) have been developed as the stochastic extension of classic Boolean network models [65, 66]. In a PBN, each node could potentially have more than one possible Boolean function to be randomly selected at each time step and the resultant output of each function carries a probability. Subsequently, the long-run steady-state behavior can be studied in the context of a Monte Carlo Markov chain. It is assumed that concentration levels in GRNs can be approximated by the Hill function, which in

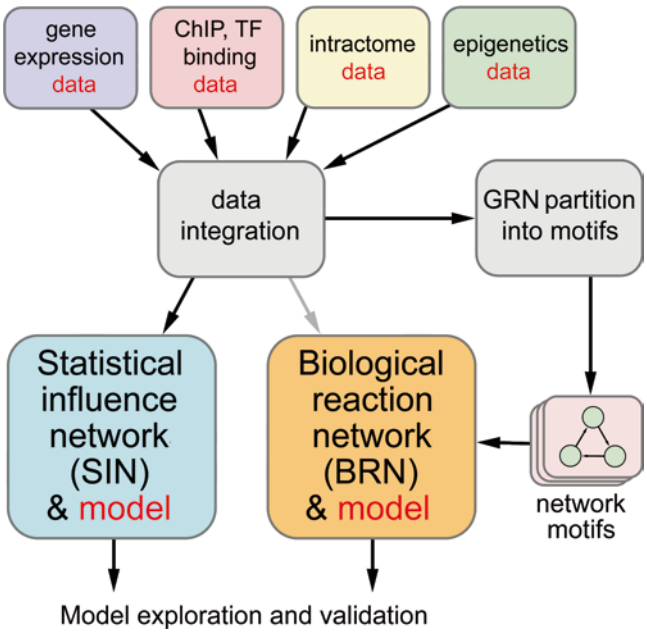

Fig. 2 Data integration and model construction strategies. Flow chart shows possible ways of data integration and construction of SIN- and BRN-based models and their relationships. While construction of SINs, such as Boolean networks and models, requires data integration, construction of BRNs may require analysis and partition of the integrated networks into smaller domains and network motifs. Simple network motif models with known input/output characteristics may be consequently integrated into larger BRN networks and models

extreme cases (high cooperativity) approaches a step function [67]. Thereby, the Boolean formalism can capture many dynamic properties of GRNs.

As it appears today, exploration of a complex system, such as the self-renewal gene network in ESCs, with largely unknown gene interactions, may be successfully modeled by starting from SINs, such as Boolean networks. Critical network domains and kernels, crystallized from these preliminary studies, may be analyzed in detail, using steady-state or dynamic BRNs, as long as the required biological knowledge is available for the interacting genes.

Gene Networks and Deterministic Quantitative Models

Data Integration and Network Construction

Functional properties of various biological systems can be uncovered by inferring the architecture of biological networks and modeling GRN dynamics using either reaction-based (BRN) or statistical-based (SIN) models (see Fig. 2). With the advent of high-throughput biotechnologies, the GRNs of stem cells have been mapped and reverse engineered. The past few years have witnessed the development of resources for stem cell-centered networks and databases for the broad stem cell community. A number of regulatory interactions are deposited in network-based repositories such as PluriNetWork [68], Plurinet [69], and iScMiD [17]. Additionally, WikiPathways provides a resource that highlights pathways contributed and maintained by the stem cell community [70]. In the context of transcriptome data, databases such as FunGenES and StemBase contain gene expression data and interactive query tools with a Web interface [71, 72]; in addition to gene expression data, genome-wide protein–gene binding ChIP-seq/chip data in stem cells are collected in the public domain at NCBI's GEO [73] database, while databases such as ChEA [74] and ESCDb [75] include a number of processed ChIP-seq/chip results and allow users to query genes of interest. Other miscellaneous databases include StemDB (http://www.stemdb.org), which holds stem cell-related information (mRNA expression profiles, antibodies, primers, and protocols). Recently, regulatory regions relevant to stem cell activity in mouse genome have been mapped using either integrative analysis of chromatin modification patterns [76] or direct experimental testing of extracted nucleosome-free DNA segments [77]. These data, along with sequence analysis based on binding motif models [78–80], may define directed transcriptional networks and biological reaction models (see below).

Currently, new provisional gene networks for mouse ESCs are generated and updated constantly [15, 17, 28, 55, 56, 68]. However, these networks are often focused on various specific aspects of mESC biology, constructed from data obtained under different conditions (e.g., different knockdowns or differentiation conditions), and have resulted in substantial disagreements. Similarly designed, genome-wide studies of the core pluripotency factors protein–protein interactions (interactomes) may produce only 20–40 % of true positives that are consistent among the studies [81–83]. Comparisons of larger number of genome-wide data sets produce even weaker agreements.

Examples of gene networks constructed for the core pluripotency factors based on various types of data [19, 48, 84] are shown in Fig. 1, the different views of how the same gene network compensate then contradict each other. One prominent but challenging goal is to integrate the enormous amount, diversity, and ambiguity of currently available genome-wide data sets across multiple informational layers in order to build more consistent GRNs and predictive quantitative models.

Statistical Interaction Networks and Boolean Models

Even very general, abstract information regarding regulatory interactions between genes can be approximated by rather simple Boolean on/off switches; Boolean networks are among the common computational approaches to model the

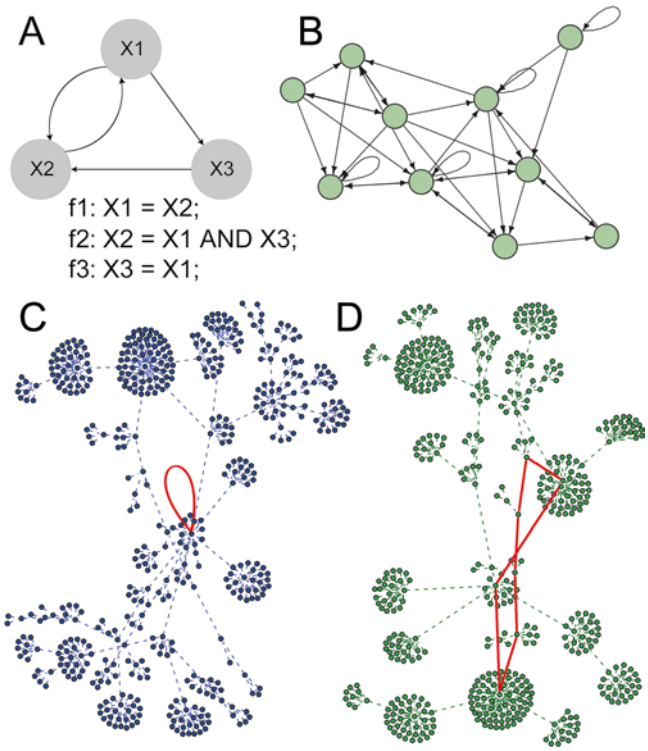

Fig. 3 Boolean networks and basins of attraction. (**a**) A formal description of a directed Boolean network; three Boolean functions (f_1–f_3) link the network nodes. (**b**) Example of Boolean network and (**c**, **d**) its dynamical solutions. (**c**) A basin for a single-point attractor. The network nodes correspond to vectors of on/off node states. *Red* edge (loop) corresponds to a transition between the attractor states (the same state). (**d**) A basin for a cycle attractor. The *red* edges show transition between the consecutive attractor states. These cyclic attractors were observed in networks describing periodic biological processes, such as the cell cycle

dynamics of the GRNs [64, 67, 85, 86]. A Boolean network can be represented by a directed graph $G(V, F)$. An example is shown in Fig. 3a. G is defined a set of vertices (nodes) $V=\{x_1, \ldots, x_n\}$ connected by a set of Boolean functions $F=\{f_1, \ldots, f_n\}$. Under the Boolean framework, each variable can take binary values of 1 representing "ON" or active state or 0 representing "OFF" or inactive state. The state of each node at time t is determined by the value of other nodes in the previous time step according to a list of Boolean functions $F=\{f_1, \ldots, f_n\}$. A Boolean function f_k is a logical function operating on the values of upstream nodes regulating the activity of node k via Boolean operators such as "and," "or," and "not." At each time step, the nodes can be updated in a synchronous or asynchronous manner. Synchronous updating schemes simply assume that the reactions in the network G have similar timescales. Therefore, a variable is updated after all rules have been applied and all downstream nodes are updated simultaneously. Alternatively, asynchronous updating schemes take temporal ordering into account and can be categorized as either (1) un-deterministic, stochastic asynchronous such as random ordering updates [87] or (2) deterministic updating [88]. The total number of states of a network is finite (2^N for a network G consisting of N nodes). For synchronous and deterministic asynchronous updating, the system relatively quickly arrives to steady states characterized by either a limit-cycle or a fixed-point attractor. In contrast, states outside the attractors are transient and unstable. For each attractor, the set of all transient states leading to that attractor constitute the basin of attraction, a common feature, found in both discrete and continuous variable dynamical systems (see Fig. 3c, d).

Boolean network modeling can capture the collective behavior of sophisticated regulatory networks and has been applied to explore several complex biological systems. A Boolean network has been developed to simulate the yeast cell cycle and to predict cell cycle events [89]. A PBN has been successfully applied to analyze the dynamical behavior of a subnetwork consisting of 15 genes in human glioma [90]. Based on the analysis of joint steady-state probabilities for Tie-2, NF-κB and TGF-β3, NF-κB, the model predicted function for Tie-2, a receptor tyrosine kinase involved in tumor development. Frequently, Boolean networks are used to infer the underlying structure of GRNs from high-throughput time series of microarray data [91–94]. The REVEAL algorithm developed by Liang was among the first formalism to infer Boolean model structure. The algorithm uses Mutual Information to determine the dependency and regulation among nodes based on state transition tables corresponding to time series of gene expression patterns. As a result, a minimal set of inputs are identified that can uniquely determine the output at the next time point for each variable in the network. In practice, a semiquantitative approach has been developed to infer the regulatory interactions in differentiating ESCs from gene expression data. The method combines the Boolean updating framework with internal continuous expression levels [95]. The resulting optimized network revealed a hierarchical structure with Oct4, Nodal, and E-cadherin on top and regulatory flow to Foxa2 through Oct4.

To facilitate the construction of BNs, there are several software packages available for (re)constructing, simulating, and visualizing Boolean networks (see Table 1). For example, general toolboxes such as the RBN toolbox, the PBN toolbox [66], and the CellNetOptimizer [96] are publicly available under the Matlab environment. Additionally, CellNetOptimizer can be downloaded as a bioconductor package in R. Other interactive graphics tools such as NetBuilder [97] and DDLab [98] allow users to create, visualize, and simulate genetic regulatory networks including discrete Boolean networks. Additionally, BooleanNet [99] is a tool used to simulate GRNs in a Boolean formalism; similarly, the R package BoolNet [100] can generate, simulate, and reconstruct Boolean networks with support for three types of BNs: synchronous, asynchronous, and probabilistic.

Table 1 Software for network construction and network modeling

Name	Source	Implementation environment
BNM	www.rustyspigot.com/software/BooleanNetwork/?url=/software/BooleanNetwork	Standalone
BooleanNet	code.google.com/p/booleannet/	Python
BoolNet	cran.r-project.org/web/packages/BoolNet/	R/bioconductor
CellNetAnalyzer	www.mpi-magdeburg.mpg.de/projects/cna/cna.html	Matlab
CellNetOptimizer	sites.google.com/site/saezrodriguez/software/cellnetoptimizer	Matlab; R/bioconductor
DDLab	www.ddlab.com/	Standalone
DVD v1	dvd.vbi.vt.edu/cgi-bin/git/dvd.pl	Web-based
NetBuilder	strc.herts.ac.uk/bio/maria/NetBuilder/	Standalone
Odefy	www.helmholtz-muenchen.de/cmb/odefy	Matlab
PBN toolbox	code.google.com/p/pbn-matlab-toolbox/	Matlab
RBN toolbox	www.teuscher.ch/rbntoolbox/	Matlab
RBNLab	sourceforge.net/projects/rbn/	Java
LearnBoo	www.maayanlab.net/ESCAPE	Matlab

It also integrates with existing visualization tools such as Pajek [101] and BioTapestry [102].

Biological Reaction Models for Transcriptional Gene Networks

Sometimes, molecular mechanisms of gene interactions are difficult to capture by the Boolean network models described above. For instance, high or low concentrations of Oct4 trigger differentiation, whereas moderate Oct4 levels promote pluripotency in mESC [103, 104]. Modeling this rich response of Oct4 target genes may require concentration-dependent thermodynamic models for transcriptional regulation [105–108]. Currently, there are several models for transcriptional regulation; steady-state models consider only responses of downstream genes to upstream regulators (in a transcriptional cascade), and more complex models take into account feedback loops and dynamics of the reaction components. Steady-state models for transcriptional gene networks are based on sigmoid (logistic) functions and include standard sets of features, such as interaction strength constants (binding affinity of transcription factors to DNA), cooperativity (energy of protein–protein interactions), and saturation of the target gene response. Below, we provide an example of a simplest quantitative framework for transcriptional regulation based on a single biochemical step binding of a transcription factor to promoter/enhancer of a target gene [109]. It is assumed that the binding reaction is the rate-limiting step in the cascade of biochemical reactions (binding, transcription, translation, etc.) leading to activation of the target gene.

Given concentration $[x]$ of a transcription factor X and a binding constant (binding affinity) of a site K in a promoter of downstream gene Y, rate of Y activation by X is found from the equation describing probability p of occupancy of the binding site by X (fractional occupancy model):

$$p_X = \frac{K[x]}{1+K[x]}; \quad K = e^{-\frac{\Delta G_{X-DNA}}{RT}} \tag{1}$$

Here denominator is a partition function describing the sum of statistical weights for all possible states of the system (binding site + transcription factor), where 1 corresponds to empty (unbound) state. Numerator in (1) is a sum of statistical weights for all successful states, promoting initiation of gene Y expression; (1) corresponds to a single, bound state. If there are N binding sites for X with equal affinity in the promoter of Y, and any combination of the occupied binding sites initiates expression of Y (successful), then (1) becomes

$$p_X = \frac{(1+K[x])^N - 1}{(1+K[x])^N} \tag{2}$$

If X binds to the target sites cooperatively, then the model needs to include the energy of protein–protein interactions C:

$$p_X = \frac{(1+CK[x])^N - 1}{C+(1+CK[x])^N - 1}; \quad C = \mathrm{e}^{-\frac{\Delta G_{X-X}}{\mathrm{RT}}} \tag{3}$$

Model (3) is exact for $N=1, 2$. For $N>2$, the model still approximates the response very well [110]. The (3) ($N=2$) may serve as an alternative to the Hill function; the main advantage is that the above equation can accommodate actual values for the binding site affinity and cooperativity. The model can be extended to arrays of binding sites with unequal affinities as well:

$$p_X = \frac{\prod_{i=1}^{N}\left(1+CK_i[x]\right)-1}{C+\prod_{i=1}^{N}\left(1+CK_i[x]\right)-1}; \quad K_i = \gamma_x \mathrm{e}^{H_i} \tag{4}$$

Typically, the binding site affinities K are unknown; therefore, they are approximated via informational scores H, found from the actual DNA sequences of the binding sites using binding motif models (such as the consensus binding site) for X [78, 111]. TF-specific binding coefficient g may serve to emphasize unequal inputs from "strong" and "weak" transcriptional regulators [62].

Complex transcription regulatory regions may contain arrays of binding sites for multiple transcription factors. Typically, all inputs from arrays of activator and repressor binding sites are considered independently and the cumulative outcome is calculated as a product of the positive and the negative inputs as follows [108, 112]:

$$P = \left(1 - \prod_{j=1}^{N}\left(1 - p_j\right)\right)\left(\prod_{k=1}^{M}\left(1 - p_k\right)\right) \qquad (5)$$

The first multiplicative term in (5) assumes that either array of occupied activating sites will add to the expression of the target gene; the second multiplicative term assumes that either array of occupied repressor sites will diminish the expression. Notice that model (5) operates on arrays of sites, where arrays may represent identical or different site types or even single binding sites, so (5) offers an opportunity to partition complex regulatory regions and then calculate the outcome of TF binding.

In the case of dynamic models, the calculated transcriptional responses P are used to approximate the synthesis rates P_i in standard ODE describing gene product dynamics:

$$\frac{d[x_i]}{dt} = \alpha_i P_i - \beta_i [x_i] \qquad (6)$$

Dynamic of ith component (node, gene product) in (6) depends on the synthesis and degradation with proportionality constants α and β, correspondingly. In the absence of any specific knowledge regarding distribution and affinity of binding sites, P may be approximated by the general response function (3) ($N=2$) and by the function (5) integrating multiple inputs [108].

Estimation of Model Parameters, Overfitting Problems

Typically, a quantitative model incorporates a network describing a sequential order of gene interactions, dependencies describing gene interactions themselves, and parameters. In the case of Boolean models (see above), the parameters may correspond to logical rules for integration of multiple inputs. In the case of biological reaction models, the parameters are the reaction rates and constants, which are usually unknown. According to (3) and (6) above, a dynamic model for a network consisting of n nodes and m edges may require at least $3n+m$ parameters (synthesis, decay and cooperativity for n, binding constants for m). Fitting methods, such as Metropolis–Hastings or simulated annealing algorithms [113], are capable of identifying the parameter values, where model fits the data; however, the solutions may not be unique. The resulting parameters may be unrealistic and, sometimes, the model can even fit any data, suggesting that there is an improper balance between the number of open parameters and the amount of input data (overfitting).

In the case of BRN models (transcriptional gene networks), the number of parameters may be reduced by learning the relative values of binding constants from promoter and enhancer DNA sequences or using global parameters for most of the reaction components. For instance, degradation and synthesis constants may be set identically for all network components regulated in a similar manner. Biological networks have sufficient robustness to tolerate significant changes in component concentrations; hereby, adequate models are likely to tolerate certain global parameters as well. Perhaps, the realistic number of open parameters, when the model is still comprehensible to a human, should not exceed 3–5 in the case of network motifs (3–5 nodes, such as the core pluripotency circuit) and 10–20 parameters in the case of larger networks incorporating 3–5 network motifs [108].

Possible benchmarking tests, which might help to detect overfitting, include randomization of input data; further computational validation of a model requires mimicking genetic mutations in silico. Usually, a model is considered valuable if it predicts the in silico outcome for 50 % or more of the known mutations. More complex predictions, such as multiple knockdowns, are tested in vitro by collecting additional experimental data.

In the case of stem cells, the major experimental challenge is to obtain a reliable protein level readout in single cells for as many network components as possible. Tools available for such measurements include transgenic cell lines expressing GFP under the control of regulatory elements for the core pluripotency factors Nanog or Oct4 [48]. These cell lines provide the power to measure gene expression in living cells and record gene expression dynamics. However, these measurements can be done in a single channel only for either Nanog or Oct4. Routine immunostaining in combination with flow cytometry will allow an increase in the number of channels to 3–4 (1 transgenic Nanog + 2–3 channels for immunostaining) [114]. This number of channels will satisfy the elementary network motif models, such as the core circuit model, but will be insufficient for larger models. Methods based on high-throughput real-time PCR assays (BioMark™ System, Fluidigm Corporation) are capable of delivering readings for up to 96 genes (mRNA) in a single cell, but the number of cells (96) in this method requires using multiple

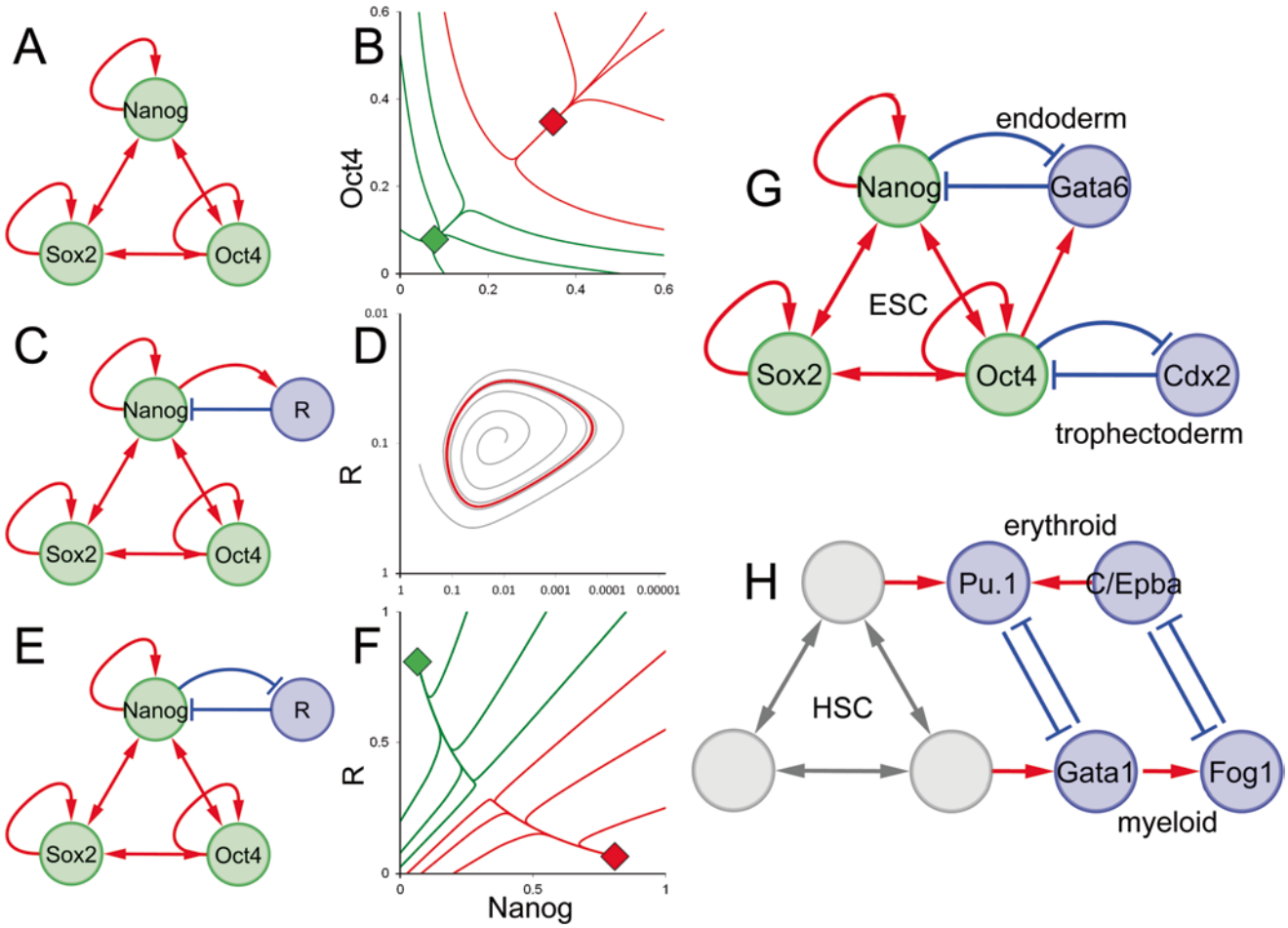

Fig. 4 Known models for embryonic and hematopoietic stem cells. (**a**–**f**) Models for pluripotency in ESC. Dynamic solutions (phase spaces) are shown for the suggested network configurations. Cooperative interactions between the core factors (**a**) may produce a bistable system (**b**) with two-point attractors; (**c**) unilateral repression, combined with self-activation, produces oscillatory behavior (limit-cycle attractor (**d**)); (**e**) mutual repression results in bistability (**f**) but with different distribution of the point attractors. (**g**) Model describing alternative cell lineage commitment in ESC. Two loosely linked bistable switches ensure choice between endoderm (Gata6 expression) and trophectoderm (Cdx2 expression) lineages. (**h**) Two parallel bistable switches reinforce each other and govern alternative commitment of hematopoietic progenitors (here HSC) to erythroid or myeloid cell fates. Possible core HSC circuit is shown by the *gray* nodes

chips to achieve statistical sound cell numbers. Recently emerging mass cytometry-based methods [115] read the stable lanthanide isotope tags attached to antibodies using metal-chelating labeling reagents and allow reading of up to 30 channels (proteins) or more in a large number of cells.

Known Quantitative Models for Stem Cells

ESCs are becoming a major subject for quantitative modeling due to the relatively high level of understanding of this particular system. Early models focused on the structure of the core pluripotency network and transitions between alternative pluripotent states, corresponding to dynamic attractors. Three potential scenarios were identified, all producing bimodal statistical distributions for Nanog concentrations, observed in ESC culture in vivo [29]. In the first scenario, highly cooperative interactions between the core components produced a classical bistable switch with two attractors, the first corresponding to the high and the second corresponding to the low concentration of Nanog (see Fig. 4a, b) [28, 30]. It has been assumed that stochastic variations in gene expression may lead to transition between the states without loss of pluripotency [46]. Addition of a transcriptional repressor to the core pluripotency network produced a second scenario—oscillation (Fig. 4c, d) [30]. Interestingly, in the context of the oscillator model, no stochastic noise is actually required to achieve transition between the suggested "high" and the "low" states. Inhibition of the core circuit may reflect known antagonistic interactions between the transcriptional regulators Cdx2 and Oct4 [116], Zfp281 and Nanog [117], or Tcf3 and Nanog [118]. Some of the core factors, such as Oct4, may be involved in both activation and repression [3]; the dual regulation properties of such pluripotency factors open

the possibility for a third possible scenario, involving mutual repression (Fig. 4e, f) [119]. While unidirectional repressive interactions may produce oscillatory behavior of the system, mutual repression often results in a scenario similar to the classical phage lambda switch [120], but rather different from the bistability of cooperativity switch considered above.

Differentiation of stem cells provides a source of models appealing to mutual repression, associated bistability, and alternative lineage commitment [33]. Mutual repression between Cdx2 and Oct4 has been suggested to promote differentiation towards the trophectoderm lineage, whereas mutual repression between Gata6 and Nanog seems to promote differentiation towards the endoderm (see Fig. 4g). One can see that the model integrates a pair of bistable switches, each combining a single pluripotency factor and a single lineage commitment regulator [58]. This model predicted the quite interesting concentration-dependent ("bell-shaped") response of Gata6 to the concentration of Oct4, resulting from direct activation and indirect repression of Gata6 by Oct4. Analogous concentration-dependent responses have been found in other well-studied systems, such as *Drosophila* embryogenesis [107]. Mutual exclusion between the alternative cell fates may be achieved, in part, via Oct4 activation of Cdx2 (trophectoderm primed cells); in its turn, Cdx2 represses Oct4, prevents Oct4-mediated activation of Gata6, thus blocking endodermal cell fate. Interestingly, the explored network topology and the model suggested that Nanog overexpression, not suppression of the lineage-specific factor Gata6, is the optimal way of reprogramming endodermic cells into iPSC.

HSCs are adult stem cells found in myeloid tissues such as red bone marrow with frequency ~10^{-4}, relative to the other cell types. Unlike ESCs, HSC culturing is not a trivial task. A large fraction of HSCs in mice is present in a nonproliferating or quiescent (dormant) state. Under native conditions, when a very low frequency HSCs may enter the cell cycle, it typically leads to irreversible differentiation of HSCs to blood lineage progenitors. In this respect, it is still not quite clear whether the quiescent HSC state is analogous to the pluripotent ESC state. An architecture for the core HSC network, responsible for the transition between the quiescent and differentiated states, still remains obscure [23]. A simple three-gene model for quiescence based on Boolean networks has been proposed [121]. In Boolean terms, the stem cell genes should be "ON" and the differentiation genes "OFF" at the quiescent state; the genes switch to the opposite states when the cells are fully committed. Analysis of the model suggested that a hematopoietic stem cell represented by three genes can end up in either "rest" or "cycling" attractor states. Interestingly this may correspond to switching between the bistable and the oscillatory scenarios, described for ESCs above (see Fig. 4a–d). More detailed models explaining quiescence are not available, with the exception of models describing HSC pool exhaustion and cellular aging due to proliferation [122, 123].

One well-studied model for hematopoiesis focuses on a switch governing differentiation of HSCs into erythroid or myeloid lineages [124]. The switch is mediated by the mutual repression between transcription factors Pu.1 and Gata1 (see Fig. 4h) [125]. In this case, however, the bistable switch responsible for the alternative cell fate commitment is placed directly between these factors, and it is also reinforced by yet another parallel switch downstream (C/Epba—Fog1). In both considered cases (see Differentiation of ESC above), differentiation events are typically associated with mutual repression; however, in the considered models the bistable switches occupy quite different positions relatively to each other and to the upstream/downstream genes. In the case of ESCs their communication is indirect and much "softer"; in the case of HSC the bistable switches appear to safeguard one another. Inferring differentiation and reprogramming conditions from future predictive quantitative models may have a great impact in the area of personal and regeneration medicine.

Variation of Gene Expression and Stochastic Models

Observed Variations in Gene Expression

Stems cells cultures are heterogeneous and they often contain pluripotent naive stem cells along with cells primed for differentiation and differentiating cells [29, 126]. For this simple reason gene expression assessment en masse may inadequately represent gene activity in either of the present cell types. On the other hand, measuring gene expression in single cells is much less reliable than in cell culture or entire embryos. Nearly any available method, including real-time PCR, flow cytometry, or imaging, will inevitably produce noisy readings and failed data points. The gene expression data recovered from the single-cell analyses will already *appear to carry* variations in gene expression. The levels of the technical noise can be high, so the single-cell gene expression data are sometimes only suitable for construction of Boolean or other statistical inference networks [19]. The means to measure and separate the technical noise from the actual variation of gene expression are limited [127]. Fluorescent reporters seem to produce less technical noise than immunodetection or real-time PCR. Currently emerging detection methods based on multiple fluorescent labels allow reliable lineage tracing and analysis of history of single cells [128].

While the technical problems associated with detection in single cells are well known, problems associated with biological interpretation of the actual variations (detected reliably) are often obscure. In fact, in the absence of any

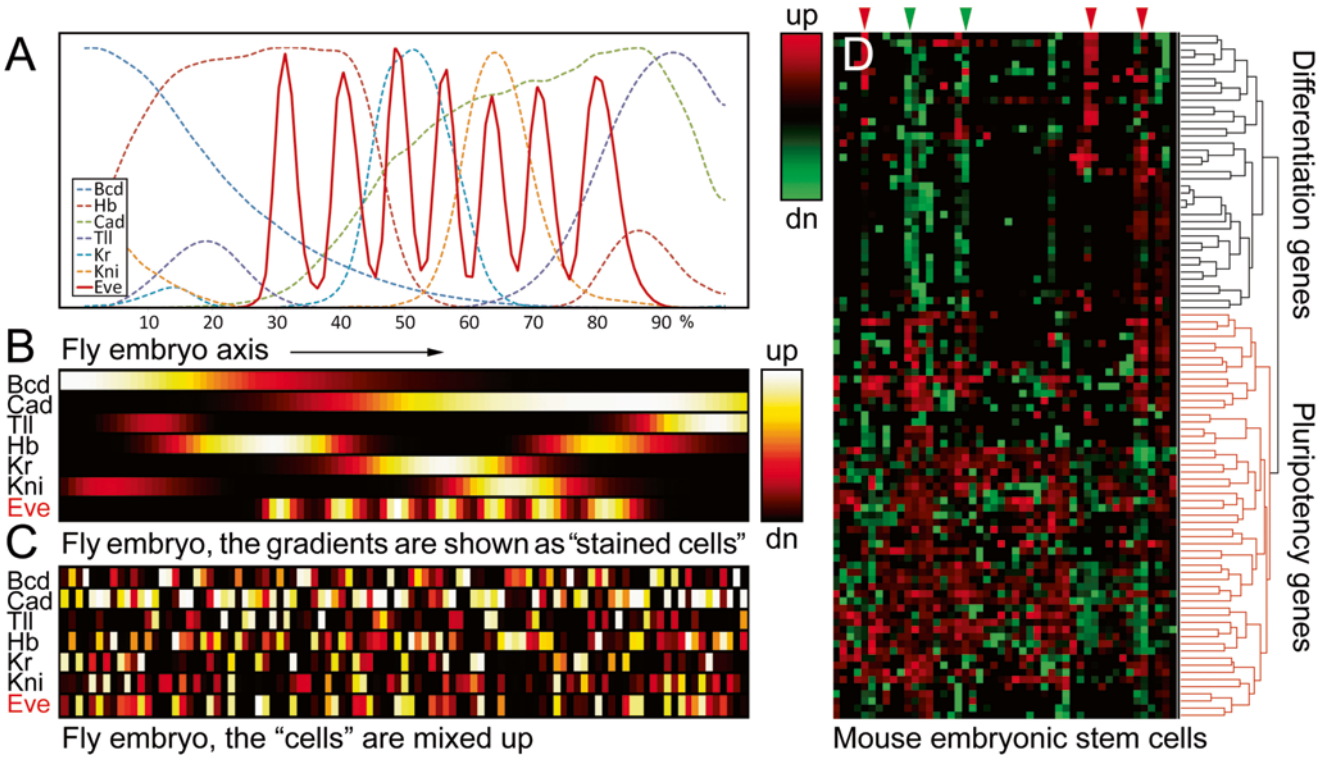

Fig. 5 A deterministic scenario for the observed variations in gene expression. (**a**) In fly embryo, formation of the eve stripes (output, in *red*) is usually explained from the gradients of upstream transcriptional regulators (Bcd, Cad, Tll, Hb, Kr, Kni, etc.). (**b**) The same gradients as in (**a**) but represented as stained cells (see the *color box* on the *right side* of the panel). (**c**) The embryo is disintegrated and the "cells" are mixed up. Panel (**c**) resembles variations of gene expression observed in stem cell culture. (**d**) Single-cell real-time PCR (Fluidigm chip) analysis of gene expression in mouse ESC. Even after two-way clustering (clusters are marked by the *arrows* on *top*), the expression appears "noisy." (**a**–**c**) demonstrates that the noisy pattern on (**d**) may be obtained from a purely deterministic network

knowledge regarding the structure and the function of underlying gene network, any gene expression pattern would appear to an observer as "random," "variable," or "stochastic." Figure 5 demonstrates this interesting paradigm. A model system, where gene expression is explained from purely deterministic models and which requires no variation in gene expression (fly embryo gradients) [129–131], is formatted to mimic gene expression patterns observed in stem cell cultures. The existing models suggest that each peak level of expression of the downstream gene *eve* (Fig. 5a–c) is explained by completely different combinations of the input gradients [132, 133]. In the absence of exact knowledge, the presence of such expression patterns in stem cells would only be explained by variations of gene expression or stochasticity. In reality this might be a purely deterministic outcome produced by the underlying network.

Detected *actual variations* in gene expression may be of different biological origins and may occur on different temporal scales as well [134–136]. Altogether, given the technical difficulties, problems with biological interpretation and high diversity of the noise, exploring variations in gene expression on the purpose of network modeling, should be approached with great care.

Models for Intrinsic and Extrinsic Noise

Deterministic models predict gene product concentrations at the time point t_1 based on the concentration of the components at the previous time point (t_0), assuming that all synthesis and decay processes proceed with absolute precision. In reality, the stochastic nature of chemical reactions and imprecision of mRNA and protein synthesis machineries add substantial deviations to the concentrations calculated using deterministic models (see (6)). The stochasticity inherent to biochemistry of gene expression is considered as *intrinsic noise* or *intrinsic fluctuations* [54, 134]. Stochastic differential equations (SDE) are commonly used to describe intrinsic fluctuations in biological systems [28, 60]:

$$\frac{d[x_i]}{dt} = \alpha_i P_i - \beta_i [x_i] + \sqrt{x_i}\,\xi_i(0,\sigma_i) \qquad (7)$$

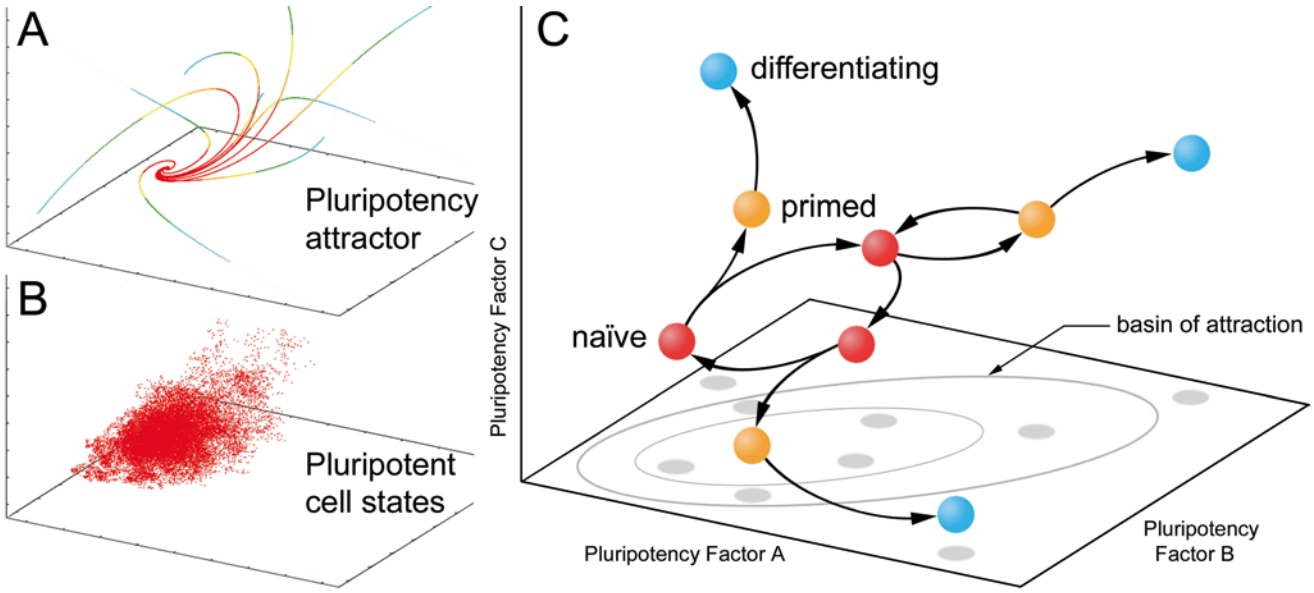

Fig. 6 Deterministic and stochastic components of a model for pluripotency. (**a**) Phase space with a dynamic attractor corresponding to a pluripotent state. Color (from cold to warm) shows the potential of the attraction basin. The attractor shown may be classified as a damping oscillator. (**b**) Behavior of cell states is simulated in the same phase space in the presence of intrinsic noise. The states (cells) are now forming a cloud "rotating" around the attractor. (**c**) Detailed consideration of hypothetical cell states present in the system. Naive pluripotent cells (in *red*) are close to the attractor; cells pushed to the edge of the attractor basin (see the *ellipse* on XY plane) become primed to differentiation (in *orange*); differentiating cells (in *blue*) are already left in the attractor basin

The last term in (7) models *intrinsic noise* in the system, where x is a zero-mean white (Gaussian) noise and s is the noise level (standard deviation). For $s>0$, the model solutions become distributions centered on dynamic attractor states. This model is convenient since it assumes no specific source of the gene expression noise and adds a single parameter (x) to the model. The square root function of the concentration is selected based on the assumption that higher gene product concentrations should provide better signal/noise ratio (SNR). Based on (7), concentration deviations caused by the intrinsic noise occur on the same timescale as the gene product synthesis cycle (minutes to hours). Figure 6 shows a possible solution of the SDE and possible trajectories of cells "traveling" around the basin of attraction (Fig. 6c).

Extrinsic noise is a more delicate matter; these fluctuations of gene expression are often attributed to changing cell environments [134] and epigenetic levels of gene regulation, such as promoter DNA methylation or chromatin modification [136], meaning that the extrinsic noise may represent unexplained deterministic events unaccounted by a given model (see also section "Observed Variations in Gene Expression"). The extrinsic noise may have a greater amplitude and may occur on a longer timescale (cell cycle, hours to days) than the intrinsic noise, thus contributing more to the observed cell-to-cell variability of gene expression [136]. A common example of extrinsic noise is switching of a gene locus between active and inactive epigenetic states. In the active state the gene promoter becomes accessible to transcriptional machinery, which initiates a series of events leading to mRNA synthesis or "bursts" of transcription. Figure 7 demonstrates the potential order of epigenetic and transcriptional levels of regulation (Fig. 7a), dynamics of the gene product synthesis (Fig. 7b–d), and the corresponding cell-to-cell variability of the gene product under alternative epigenetic modes of regulation (Fig. 7e).

Few quantitative frameworks have been proposed for modeling the bursts of transcription and the resulting cell-to-cell variability [54, 137–140]. Epigenetically active state of promoter may be described by a Boolean function F, which depends on the "concentration" (epigenetic state/grade of promoter) of the epigenetic factor:

$$F_{t=n} = \begin{cases} 1, r \le p_1 \cap F_{t=n-1} = 0; & r > p_2 \cap F_{t=n-1} = 1 \\ 0, r > p_1 \cap F_{t=n-1} = 0; & r \le p_2 \cap F_{t=n-1} = 1 \end{cases} \quad (8)$$

In (8), p_1 and p_2 are the probabilities of promoter activation or deactivation, r is a random number, and t is an integration step (time). Simulations shown in Fig. 7c assume that the probability of turning the promoter "ON" (association) p_1 linearly depends on the "concentration" of the epigenetic factor $p_1 \sim E$ (Fig. 7a). If the promoter is in "OFF" state ($F=0$), at any given moment of time (integration step), there is a probability p_1 of transition into an active "ON" state ($F=1$); if the promoter is active, there is a probability p_2 of transition into an inactive state. No transcriptional regulation is present in this particular model (but the probability p_1 may

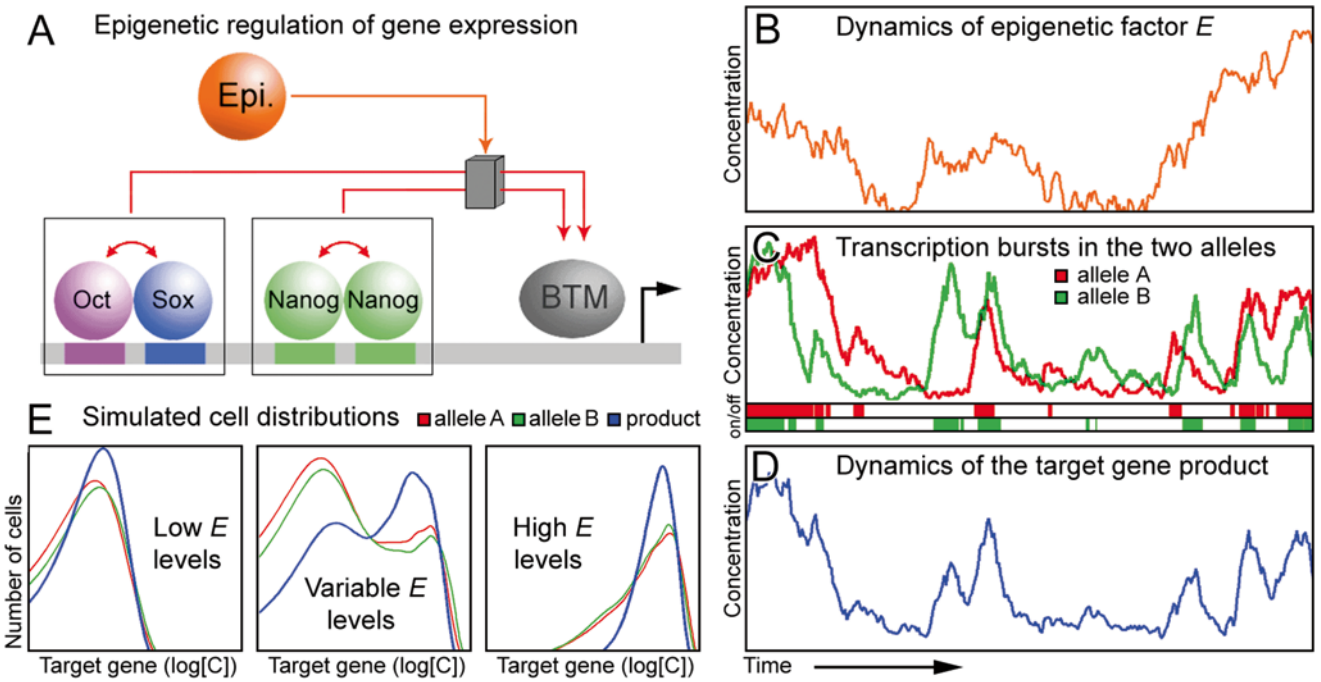

Fig. 7 Contribution of epigenetic signals to variations in gene expression. (**a**) Architecture of regulatory levels in a promoter of an abstract "pluripotency gene." Epigenetic factors, ("Epi."), such as DNA methylation or chromatin modification, serve as a "valve" locking the function of basal transcriptional machinery (BTM) at the lower level. (**b**) Dynamic changes in the activity of the epigenetic factor affect switching the alleles of the downstream gene between "ON" and "OFF" states. (**c**) Transcriptional bursts in the "ON" states result in accumulation of the gene product (shown independently here for each allele). (**d**) Variations in the gene product of the downstream gene (sum of the two alleles). (**e**) Cell distributions with respect to the target gene product concentration, simulated for different modes of action of the upstream epigenetic factor

be linked to the fractional occupancy $p_1 = P$; see (3), (4), and (5)). Synthesis of the gene product X now depends on the state of the Boolean function F (in discrete form):

$$\frac{\Delta[x_i]}{\Delta t} = \alpha_i F_i - \beta_i [x_i] + \sqrt{x_i}\,\xi_i(0,\sigma_i) \qquad (9)$$

This model now takes into account both the extrinsic (F) and the intrinsic (x) variations. The described method may be applied to study allelic variations in the gene expression, which may contribute to the formation of stem cell subpopulations [141] (see Fig. 6e).

Information Flow and Epigenetic Landscapes in Differentiation

Information Flow and Epigenetic Memory

One interesting problem emerging from quantitative analysis of stem cell gene networks is informational flow in the system during self-renewal and differentiation. Information content of any pluripotent cell state and each transient or terminally differentiated state can be characterized, for instance, by a genome-wide set of gene expression levels. This rather trivial representation is supported by the frequently observed specific "molecular signatures" characterizing pluripotent and differentiated cell types in microarray studies [55]. During transition between two (rather arbitrary defined) neighboring cell states n and $n+1$, genes specific to $n+1$ are activated, while some genes specific to n are shut down and some genes specific to n maintain or slightly change their activity levels (see Fig. 8). One can see that the new information, required to achieve the state $n+1$ from state n, has been read from the genome. Intuitively, the amount of new information may not exceed the amount of binary information contained in the DNA, encoding the newly activated genes, with the account of their transcription regulatory regions as well. In the case of differentiation, the genome plays a role similar to that of a storage device on a computer, such as CD or "read-only memory"—ROM. Interestingly, epigenetic information (genes which are still active, DNA methylation, chromatin modification states, and so on) from state n to state $n+1$ may be considered as temporary memory or, by analogy with computers, "random access memory—RAM." The epigenetic memory provides means for self-renewal, where a given number of active network components fluctuates between different semi-stable states.

Finding cell-specific markers or tissue-specific transcriptional regulators is, in fact, tracing the emergence of new information in differentiating cells. Separation of the new,

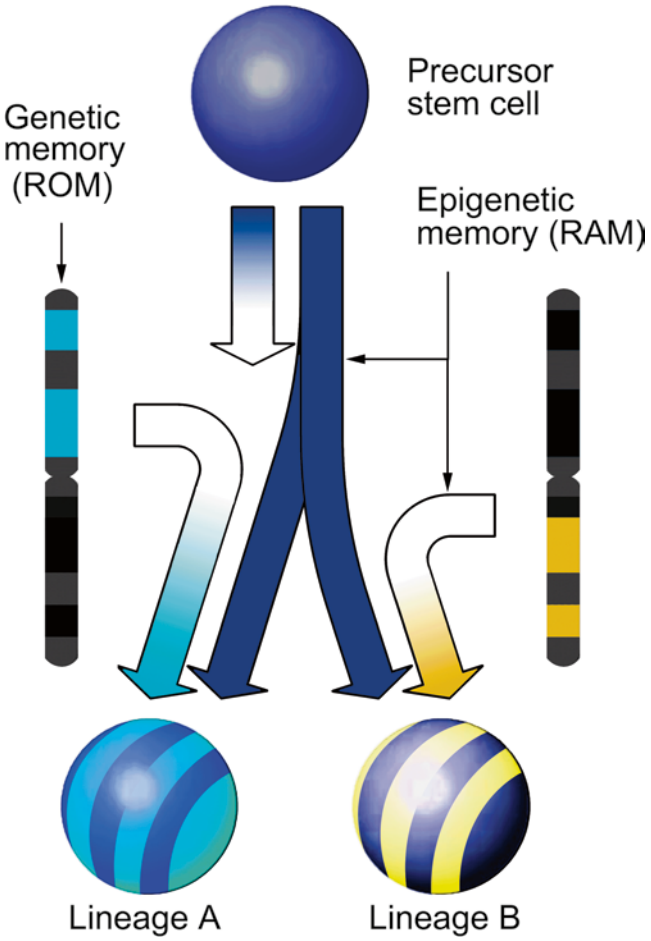

Fig. 8 Information flow in differentiation. Epigenetic memory may be defined as a set of gene product concentrations and gene activity levels in a cell. This information is analogous to random access memory of computers (RAM). Upon differentiation, some genes are shut down and part of the "memory" is erased; while new information is added to the system with newly activated genes, this new information is retrieved from the genome. The discrete genome information has analogy to read-only memory of computer devices (ROM). Gene activation and shutdown are shown in Fig. 4 by the color gradients. Differentiated cells typically "remember" their origins (see the striped patterns in lineages), which is essential to reprogramming

specific information from epigenetic memory may help to focus on the right candidate genes and reduce complexity of gene networks and the network-based quantitative models.

Attractor States and the Waddington Landscapes

Stem cells are capable of self-renewal and differentiating into terminal cell types. In a metaphorical way, Waddington [142] described the cells as marbles rolling down an epigenetic landscape containing "hills" and "valleys" during the development process: the "valleys" represent cell types separated by the "hills." From the network biology perspective, cells residing in the "valleys" are in the stable states, fixed-point attractors. Alternatively, cells may be in unstable states, characterized by oscillation around multiple "valleys." Different stable or transitive states are defined by characteristic gene expression patterns and epigenetic signatures. Certain experimental observations support this view. For instance, induction of differentiation of human leukemia cells (HL60) into neutrophils by different stimuli suggested the presence of two different attractor states. Based on global analysis of gene expression, L60 cells adopted two different trajectories of differentiation which finally converged into the same "end program" attractor state under the two different stimulating conditions [143]. Sometimes the attractor states are on the borderline between chaos and determinism. Mimicking cell fate commitment using RBNs produced such interesting borderline solutions [86]. Apparently, the model captured the generic properties of biological GRNs corresponding to "critical" dynamics, which seems to reflect an equilibrium between adaptiveness and robustness. From this point of view, stem cells would be expected to reside rather in semi-stable states in order to be ready for environmental changes, such as external differentiation signals. Sometimes, such semi-stable states on the borderline of chaos are called "primed" states, poised for differentiation [27, 144, 145] (see Fig. 6). Apparently, cells may travel from one semi-stable state to another driven by stochastic gene expression noise or deterministic cycling, characterizing limit-cycle attractors, damping oscillators, or strange attractors.

One interesting property of a Waddington landscape emerges from considering epigenetic factors and rewiring of GRNs in development and differentiation. These events may be represented by slow-changing variables, which may be considered as parameters in dynamic models describing the system at any given moment of time. Gradual drift of these parameters in time will change the phase spaces and the arrangement of solutions. This demonstrates that the dynamic attractors (see Figs. 4 and 6) are not still, they are born when the corresponding transcription factors come into the play, and the attractors can move across the phase space over time and give birth to new attractors or new stable states. This process is called bifurcation and it may reflect progression of development and sequential emergence of new lineages and new cell types. From this point of view, the Waddington landscape is a temporal order of stage-specific solutions and their bifurcations.

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Growth Dynamics of Fetal Human Neural Stem Cells

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Abbreviations

bFGF	Basic fibroblast growth factor (fibroblast growth factor 2)
DMSO	Dimethylsulfoxide
PBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
hNSC	Human neural stem cells
LIF	Leukemia inhibitory factor

Introduction

Neural stem cells isolated from human fetal and adult sources have been propagated and expanded in culture to provide research platforms for understanding control of nervous system development and their potential as therapies for neural injury and diseases [1–5]. When transplanted into diseased hosts, human neural stem cells (hNSC) interact strongly with the pathological microenvironment, migrating to loci of nervous system injury, differentiating to replace damaged neurons and glia, restoring homeostasis to damaged cells, and delivering chemotherapeutic agents [6]. Culture methods that facilitate biological mechanisms favoring engagement of hNSC with cues in the host-diseased microenvironment to enact migratory and regenerative programs are critical for realizing this therapeutic potential. Fostering retention and control of the inherent developmental properties of NSC crucial to the therapeutic potential of NSC—multipotency, the ability to differentiate to the different cell types of the nervous system, and self-renewal, the ability to undergo mitosis to multipotent daughter cells—is thus of intense interest. From a practical standpoint, producing large populations of hNSC is needed for understanding not only the therapeutic potential of hNSC but also how the cellular milieu influences expression of these developmental programs.

hNSC are cultured ex vivo in neurospheres [5, 7–10], monolayers [5, 11, 12], and multilayers [13]. Isolated cells introduced to a culture vessel aggregate and proliferate as free-floating suspended masses that exhibit minimal interaction with nonadhesive substrates [5]. These "neurospheres" may grow both by proliferation of individual cells within each sphere and by "fusion" of nearby spheres into masses populated by thousands to millions of cells that interact strongly with each other by the secretion of cell adhesion and extracellular matrix (ECM) molecules. Neurosphere culture remains a popular and standard technique for NSC propagation, but is criticized as being neither a unique property of neural stemness [10, 14] nor an indicator of clonal proliferation [15]. From a technical standpoint, the physical nature of the neurosphere mass of cells has led to difficulties in controlling stem cell differentiation by external factors in the bathing medium due to the nonuniformity of exposure throughout the cell mass. Furthermore, propagation is inherently limited by the consumption of nutrients by outer cells at the expense of inner cells, such that spheres eventually form dead cores that diminish culture yield as necrosis competes with mitosis [11, 16].

Electronic supplementary material
The online version of this chapter (doi:10.1007/978-1-4614-7696-2_5) contains supplementary material, which is available to authorized users.

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Monolayer culture is induced by preventing neurosphere formation through seeding at a low density to decrease intercellular interactions [11, 12]. Plastic or glass substrates are often coated with biomolecules enabling NSC adhesion to the substratum, including poly-L- or D-lysine, poly-L- or D-ornithine, and ECM constituents such as fibronectin or laminins [12, 17]. Monolayer culture has also been obtained without adhesion-enabling biomolecules on corona discharge- or other ion plasma-treated surfaces, termed "tissue culture-treated," when cells are seeded at low density [1, 7]. Monolayer culture is favored because all the cells have ostensibly unimpeded access to growth factors and nutrients in the medium. It has revealed that basic fibroblast growth factor (bFGF, FGF2) and leukemia inhibitory factor (LIF) are the only growth factors required to maintain self-renewal and multipotency of hNSC isolated from fetal telencephalon [7, 18, 19] such that they differentiate into neuronal and glial subtypes appropriate to the target brain region when engrafted into the nervous system of living mammals [1]. This has allowed formulation of chemically defined culture media lacking serum [1, 2, 19].

This laboratory has developed methods for culture of fetal telencephalon-derived hNSC in multilayers by seeding on tissue culture-treated but otherwise unmodified plastic surfaces at relatively high densities [13]. Multilayers are intermediate between monolayers and neurospheres—cells interact with each other in local aggregations or clusters but also with the substrate via lamellipodia extending toward adjacent cell clusters. Proliferation expands the cluster area horizontally along the substrate as well as vertically in each expanding cluster. Migration of cells between clusters along the lamellipodia and subsequent proliferation is hypothesized to eventually unify previously discrete clusters. This culture mode produces large numbers of hNSC compared to both neurospheres and monolayers with the resulting cells suitable for differentiation control and transplantation. As culture of hNSC in multilayers is much less studied than monolayers and neurospheres, the present work explores quantitative parameters for successful propagation and maintenance of hNSC in this culture modality. Understanding these parameters will further acceptance of this technique for the realization of these advantages.

Materials and Methods

hNSC Source and Derivation

The hNSC used in this study, HFB 2050, were isolated from the telencephalon of a 13-week-old human fetal cadaver [1]. NSC were selected by serial culture in serum-free DMEM/F12 culture medium supplemented with N2 (Life Technologies, Carlsbad, CA), 5 mM L-glutamine, and 10 ng/mL LIF. Culture media alternated between 20 ng/mL bFGF (FGF2) and 20 ng/mL epidermal growth factor (EGF) to induce reentry into the cell cycle and to maintain the cells in a state of early neuroglial multipotentiality [17]. This procedure required six enzymatic passages before cryopreservation in 10 % (v:v) DMSO, 50 % FBS, 40 % culture medium. Cells were maintained thereafter in defined, serum-free conditions as described [13, 20, 21].

NSC Culture

Culture Conditions

Procedures previously described for multilayer culture of these cells were followed [21]. For this study, cryopreserved aliquots of cells from the original derivation were thawed and diluted sevenfold in culture medium of the following defined composition: Neurobasal medium (Life Technologies), Vitamin A-free B27 supplement (Life Technologies, Carlsbad, CA), 5 mM L-alanyl-L-glutamine (GlutaMax, Life Technologies), 20 ng/mL bFGF (R&D Systems, Minneapolis, MN), 10 ng/mL LIF (Millipore, Temecula, CA), 8 µg/mL heparin (Sigma-Aldrich, St. Louis, MO), and 2 µL/mL Normocin (Invivogen) [13, 21]. After collection of the cells by centrifugation at 200 rcf for 4 min, cells were resuspended in medium and cultured at 37 °C in humidified air containing 5 % CO_2 in 6- and 12-well plates, and T25, T75, and T225 flasks fabricated of tissue culture-treated polystyrene (Cat. No. 430639, 430641, and 3001, respectively, Corning Costar).

Dissociation and Collection of Cells for Passage

Cells were passed by aspiration of medium and one wash with Dulbecco's phosphate-buffered saline (PBS) followed by incubation in a volume of Accutase equal to one-fourth of the normal culture medium volume at 37 °C for 45 s. Lifting and dispersion of multilayers was then observed by light microscopy with gentle agitation of the culture vessel as described [13, 21]. Cells were dissociated to single cells and small aggregates (≤5 cells) by triturating 2 or 3 times with a 5 mL disposable pipette, taking care to avoid undue shear stress, and transferred to a 15 mL conical polypropylene centrifuge tube. Total time in Accutase was confined to <2 min. Enzyme was quenched by dilution with 6 volumes of culture medium used to wash the culture vessel, and cells were pelleted by centrifugation at 200 rcf for 4 min at room temperature. The pellet was resuspended in 1.0 mL culture medium by 2 triturations with an Eppendorf 1.0 mL pipetter.

Quantitation of Viable Cells

At each passage, viable cell densities were quantified by counting on a Neubauer hemocytometer. Typically 2 µL of cell pellet resuspension was diluted 1:50 with PBS, and then an equal volume of 0.4 % Trypan Blue was added for a final counting dilution of 1:100. Cells exhibiting a bright white

boundary under phase contrast observation were scored as viable and used for seeding density and yield calculations.

Seeding New Culture Vessels and Feeding

New culture vessels were seeded with dispersed cells at a desired surface area density. Freshly seeded cultures were allowed to grow at 37 °C without mechanical disturbance (including movement for observation) for at least 3 and up to 5 days. The first feeding was a replacement of half the medium volume covering the cells with fresh medium containing twice the normal concentrations of supplementary growth factors, i.e., 40 ng/mL bFGF, 20 ng/mL LIF, and 16 μg/mL heparin. All medium replacements were performed by slow rotation of the vessel about its horizontal axis (e.g., taking 5–10 s) to move the liquid off the growth surface to avoid detachment of the cells by the meniscus and into a flask corner. After removal and replacement of the desired liquid volume, the vessel was subsequently slowly rotated back to allow the refreshed medium to cover the cells while avoiding meniscus shear. After this first feeding, cells were washed and the medium replaced every 3–5 days utilizing the same liquid removal and replacement maneuvers to avoid detaching the cells from the growth surface.

Coating Tissue Culture Vessels with Laminins

Culture vessels, including 6-well plates, T25, T75, and T225 flasks, were first coated by incubation in 10 μg/mL poly-l-ornithine (Sigma-Aldrich) in sterile PBS for 24 h at 37 °C. Unattached poly-L-ornithine was removed by three washes with sterile PBS at room temperature. The surfaces were subsequently coated with 10 μg/mL mouse laminins (Life Technologies) in PBS by incubation for 24 h at 37 °C. The incubation solution was removed, the surface washed once with PBS, and the flasks stored for use at 4 °C as described [11].

Time-Lapse Microphotography

Cells were plated on 35 mm diameter tissue culture-treated polystyrene Petri dishes for observation with a ×20/0.4 NA 4.5 mm working distance objective (Meiji Techno, Saitama, Japan), and plated on glass bottom 35 mm FluoroDishes (Cat. No. FD35, World Precision Instruments, Sarasota, FL) for observation with a ×40/0.65 NA 0.5 mm working distance objective. The seeding density was 10^5 cm^2 when the surface was untreated, and 4×10^4 cm^2 when the surface was coated with laminin. Cells were observed with a custom inverted fluorescence microscope having an integrated CMOS imager interfaced via USB 2.0 with a computer for display (Lumascope, EtaLuma, Inc., Carlsbad, CA). Cells were transilluminated in brightfield at an oblique angle through the plastic dish cover with a white LED on a flexible cable. After plating, the dish was placed on the stage of the microscope inside a 37 °C incubator, a field of view was brought into focus, and successive images were acquired and stored as bitmaps at time intervals of 5 or 10 min for a collection period of up to 14 days with custom software (LumaView). Image files were assembled into movies with Adobe Premier 6.5. In brief, each 4 MB bitmap image (1,280 × 800 pixels) in a time-lapse series was replicated into five successive video frames (760 × 480 pixels). The resulting sequence of video frames was compressed by an MPEG-1 codec into a movie for playback at 30 frames per second. Total time-lapse collection periods and movie durations are noted in each movie legend.

Results

NSC Growth in Multilayers and Monolayers: Intercellular Interaction with Continual Expression of Lamellipodia

Growth on Tissue Culture-Grade Polystyrene

When introduced to a fresh surface, dissociated hNSC grew into multilayers with a stereotypical pattern and time course. The seed of freshly dissociated cells shown in Fig. 1a, in which the plane of focus is located at the flask floor, consisted of single cells and small aggregates of ≤5 cells. After 3 days of undisturbed incubation at 37 °C, most cells had settled on the growth surface, aggregated into small colonies, and begun proliferating within these clusters (Fig. 1b, left). Some cells along the edges of each cluster extended lamellipodia along the growth surface. Lamellipodia extended from nearby colonies appeared to be directed toward each other, suggesting a chemosensory tropism. This surface exploratory behavior by elaboration of lamellipodia was critical for establishment of proliferating multilayer cultures. Cells remaining as singlets unassociated with any colony extended few, if any lamellipodia, suggesting minimal interaction with the surface. Settled cell clumps not expressing lamellipodia either did not proliferate or formed neurospheres that lifted away from the growth surface (Fig. 1b, right).

Between 3 and 5 days of incubation (Fig. 1c, left), colonies increased in mass by proliferation, resulting in the multilayer appearance. In addition, cells migrated, as if in "chains," along the exploratory lamellipodia established by cells near the peripheries of the colonies, especially lamellipodia that had come into contact with lamellipodia extended from adjacent colonies. Proliferation of these migratory cells resulted in establishment of "branches" or "chains." Continual migration and proliferation of cells both along and

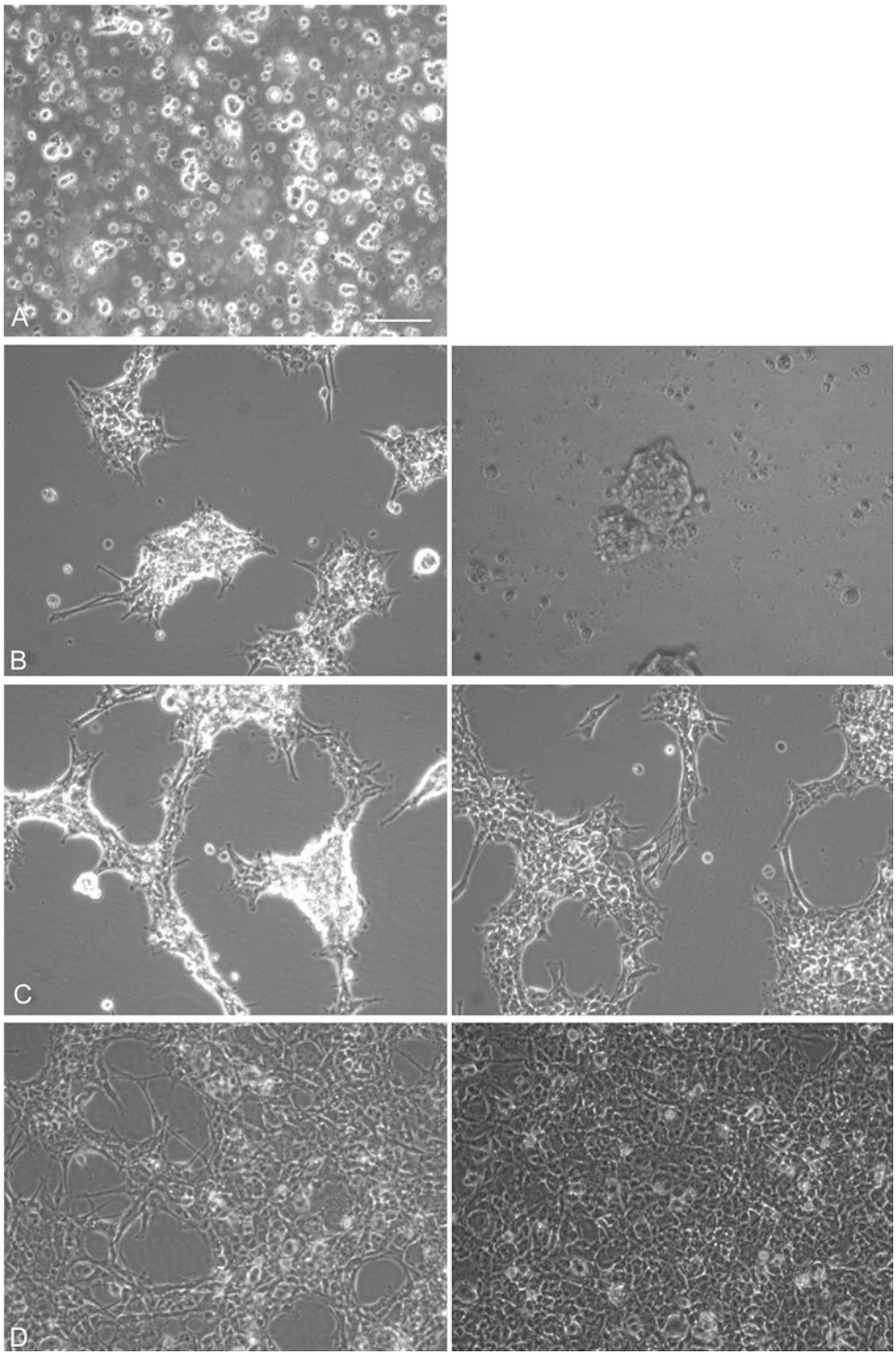

Fig. 1 Time course of fetal-derived hNSC multilayer growth on an uncoated, tissue culture-grade polystyrene surface. (**a**) Accutase-dispersed cells freshly seeded in a T25 flask in 6 mL medium at a surface area density of 1.3×10^5 cells/cm^2 (3.25 million cells total cells). While not all cells had settled to the flask floor, as seen by out-of-focus cell boundaries, their center-to-center spacing was consistent with an average separation of 15.6 ± 3.9 μm expected for the area density of the seed. (**b**) *Left*: after 3 days of mechanically undisturbed growth, cells have aggregated and begun to proliferate within each group. Cells have also begun to explore the growth surface by expression of lamellipodia. Lamellipodia from nearby groups appear to project toward each other. *Right*: cells 3 days after seeding at $<0.8 \times 10^5$ cells/cm^2 in a different T25 flask. These cells formed small clusters, did not extend lamellipodia, and died within 5 days of seeding, indicating that surface exploration was critical for survival. (**c**) *Left*: between 3 and 5 days of culture, cells migrate along exploratory lamellipodia and proliferate to form branches that connect adjacent colonies. *Right*: over days 5–7, cells continue to proliferate within colonies, and migrate and proliferate along branches established between colonies. (**d**) *Left*: By 7–10 days after seeding, expanding colonies merge into multilayers. *Right*: cells have grown to confluence by 2 weeks of growth. The plane of focus was positioned at the growth surface such that the boundaries of lamellipodia were the sharpest. Cells growing on top of surface-attached cells thus exhibit less sharp boundaries. Scale bar, 50 μm

within these branches and subsequent extension of new lamellipodia enabled further branch/chain migration and proliferation to increase coverage of the growth surface area.

Over days 5–7 (Fig. 1c, right), growth progressed by the colonies appearing to flatten, suggesting that daughter and sibling cells sought and established contact with the growth surface with the resultant crowding stimulating further area expansion. By 7–10 days of growth (Fig. 1d), many branches between colonies had merged creating a more uniform coverage of the growth surface by the cells to ~80 % confluence. Cells along colony peripheries continued to extend lamellipodia into unoccupied growth surface with concomitant chain and branch migration and proliferation. Growth continued in this highly stereotypical pattern until attaining confluence by 2 weeks (Fig. 1e).

This pattern of growth was observed independently of passage number for up to 43 passages, the greatest passage studied.

Time-Lapse Observation of hNSC Growth on Uncoated Surfaces

Movies of freshly seeded hNSC on glass surfaces revealed surprising dynamics underlying the stereotypical growth pattern time course sampled in static images. Over the first 24 h (Movies 1 and 2), cells were mobile in the fluid medium, suggesting Brownian motion [22] as is observed during the initial stage of neurosphere formation [5]. Cells jostled into one another to aggregate into colonies that eventually became stationary on the surface. During the subsequent 1–2 days, cells in these colonies became unipolar in shape, producing extensive lamellipodia along the surface in multiple directions from each colony. One of the most striking observations was that many lamellipodia extended by cells located along peripheries of colonies were temporary. These lamellipodia were extended relatively long distances and subsequently retracted to the cell soma over a period of ~30 min, suggesting chemotactile "sampling" of the surface. By the end of the third day, many lamellipodia became longer-lived—particularly those extending toward lamellipodia extending from an adjacent colony of cells. Lamellipodia from one colony that touched a smaller neighboring colony triggered a rearrangement of cells in the smaller colony along the apparently contacting lamellipodia, an event reminiscent of neurosphere fusion [5]. Individual cells often migrated away from colonies, and then migrated back to the original colony merging in a different location, or merging with a different colony. One notable point of contact was extended lamellipodia, such that as multiple cells migrated and grouped, a branch was created.

Growth on Laminin-Coated Polystyrene

When freshly dissociated hNSC were introduced to laminin-coated polystyrene, single cells and aggregates of cells grew as a discontinuous monolayer (Fig. 2). By 1 day after seeding, cells within clusters had begun flattening on the growth surface, adopting uni-, bi-, and multipolar shapes, and migrating with extensive lamellipodia (Fig. 2a). Over days 1–3, the flattening of cells increased their surface area occupancy, and cells migrated so as to disperse any initial clusters that had been previously formed (Fig. 2b). Cells continued to extend long lamellipodia and migrate resulting in prominent visible cytosol surrounding the nucleus of each cell with prominent lamellipodia (Fig. 2c). In contrast to growth as multilayers, cells on laminin underwent much less migration along lamellipodia established by other cells or clusters of cells grouping along pioneer lamellipodia. As a result, hNSC on laminin grew more as individual cells in small proliferation colonies, and clear unoccupied growth surface remained visible between adjacent cells for at least 12 days after seeding (Fig. 2d).

In time-lapse observation, cells remained as individuals immediately on seeding, settling on the growth surface and extending lamellipodia (Movies 3 and 4). Cells began rapid migration along the surface, continually making apparent contact with each other, but typically passing either over or under one another apparently to regain contact with the laminin-coated surface. As cells proliferated, nearby cells tended to remain close to one another resulting in small colonies of flattened adjacent cells. These colonies never resulted in the large extensive groupings of proliferated cells characteristic of growth in the multilayer pattern. Thus, cells seeded on laminin-coated surfaces began immediately to migrate along the surface, without undergoing extensive intercellular interactions as on uncoated surfaces.

Immunochemical Markers of Neuroglial Stemness

hNSC growing as multilayers on bare polystyrene and as monolayers on laminin stably expressed immunochemical markers of neural stemness and multipotency. All cells in cultures at 2 weeks stained positive for human nestin, the classical neural stemness marker whether cultured on uncoated (Fig. 3a) or laminin-coated polystyrene (Fig. 3c). Less than 5 % of cells stained for the neuronal marker doublecortin or the oligodendroglial marker O4 (not shown), indicating that these culture conditions did not favor differentiation to late, specific neuroglial lineages [13].

Cells throughout multilayer colonies expressed βIII-tubulin as indicated by positive staining with the monoclonal primary antibody clone TuJ1 (Fig. 3c). Staining was confined to long lamellipodia projecting from these peripheral cells. On laminin-coated surfaces, most cells stained positive for βIII-tubulin (Fig. 3d). While βIII-tubulin is generally regarded as a marker of neuronal commitment, it was

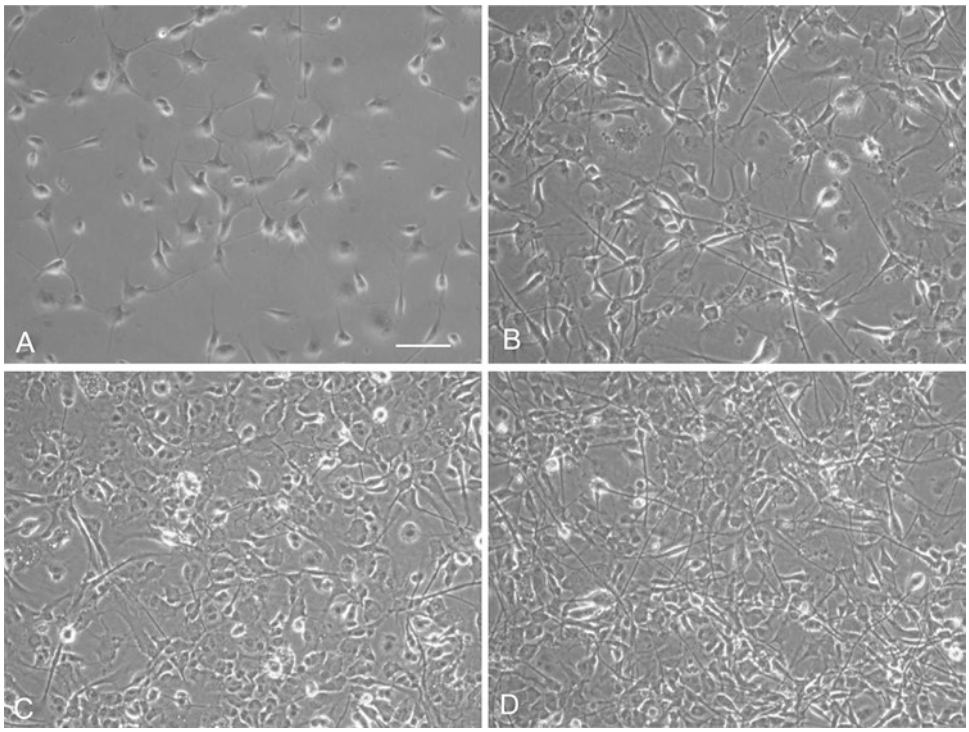

Fig. 2 Time course of fetal-derived hNSC growth on a laminin-coated tissue culture-grade polystyrene surface. (**a**) Cells 24 h (1 day) after seeding. Aggregates and colonies of cells expressed extensive lamellipodia exploring the growth surface. (**b**) Cells 3 days after seeding. Cells have migrated from clusters and attached to the laminin-coated surface to flatten. Each cell occupies greater surface area than comparative growth on uncoated polystyrene and expresses a greater profusion of lamellipodia. (**c**) Cells at day 7. The cells have established a discontinuous monolayer with unoccupied surface area between cells. (**d**) Cells at day 12 appear similar to cells at earlier times. Scale bar, 50 **μ**m

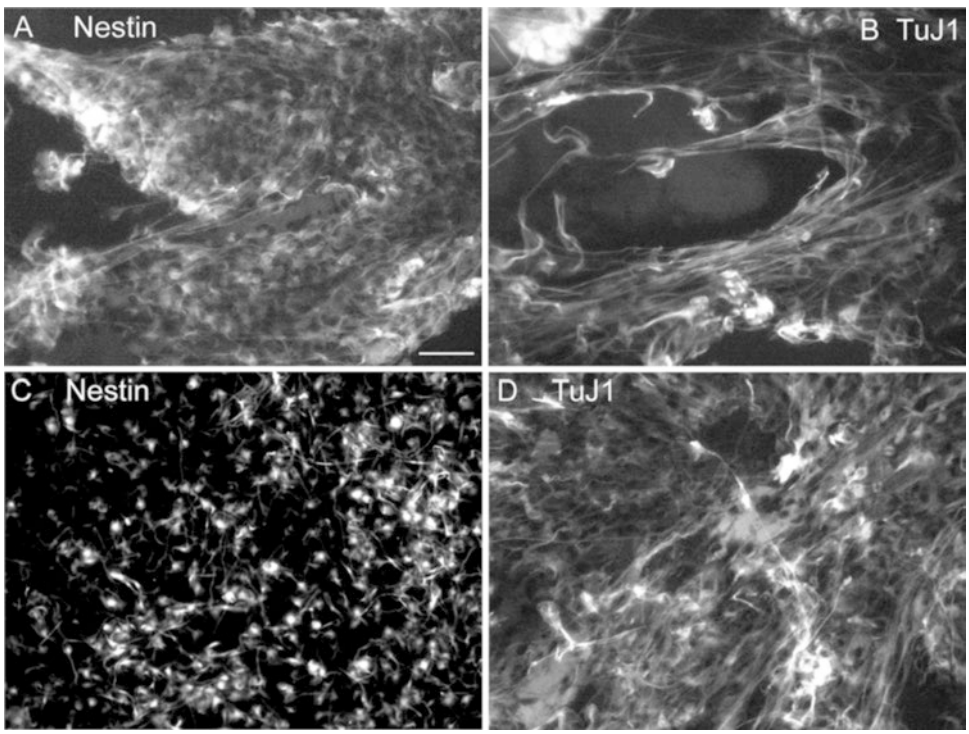

Fig. 3 Immunostaining of nestin (**a**, **c**) and **β**III-tubulin (TuJ1, **b**, **d**) of fetal hNSC grown as multilayers on uncoated tissue culture-grade polystyrene (**a**, **b**) and as monolayers on laminin-coated surfaces (**c**, **d**). Scale bar, 50 **μ**m

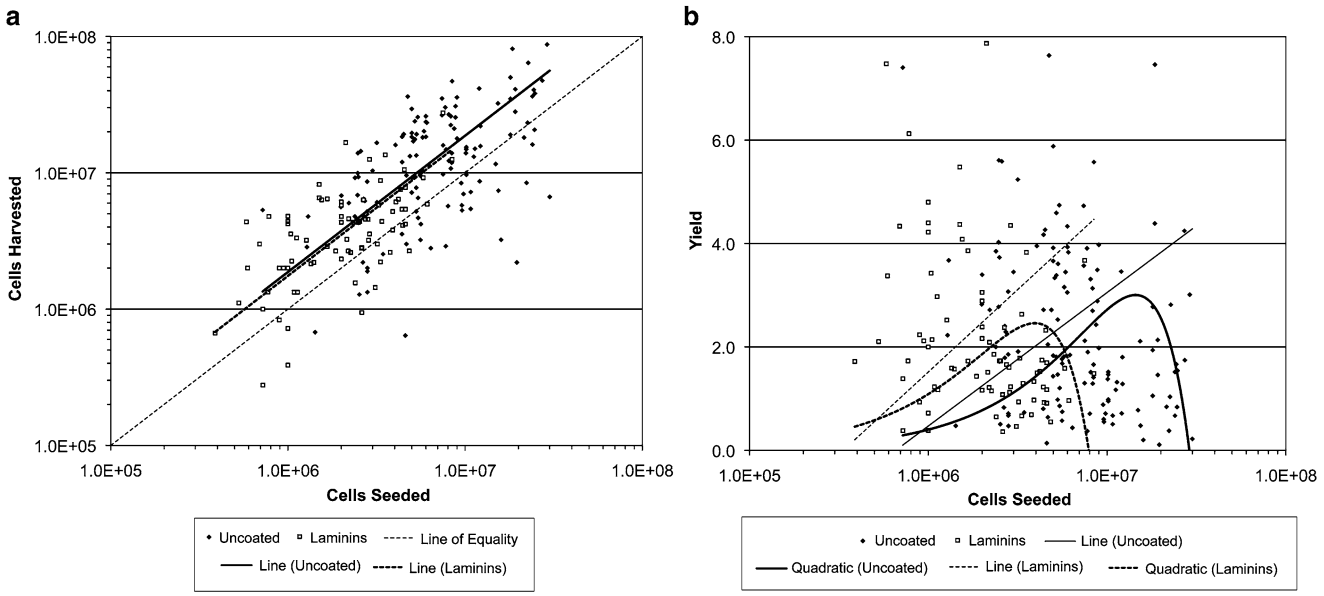

Fig. 4 Yields of hNSC cultured on uncoated and laminin-coated surfaces. (**a**) The number of viable cells harvested plotted as a function of the number of cells seeded into 242 uncoated (*filled diamond*) and 137 laminin-coated (*square*) culture flasks. Seed and harvest numbers ranged from 3×10^5 to 8.7×10^7. The abscissa and ordinate are represented with common logarithmic scales to give equal spatial representation on the graph across these orders of magnitude. The *dashed diagonal line* denotes the line of equality, in which the same number of cells were harvested as seeded. Lines through the data points (*solid line*, uncoated; finely *dashed line*, laminin-coated vessels) were obtained by linear regression of cells harvested on cells seeded, constrained by 0 cells harvested for 0 cells seeded. The slopes of these lines were not significantly different. (**b**) Yield, the number of cells harvested/the number of cells seeded, plotted as a function of the number of cells seeded for uncoated (*filled diamond*) and laminin-coated (*square*) vessels. The abscissa has logarithmic scale to provide equal graphical representation across of the range of seed size. The *upward sweeping lines* (*solid line*, uncoated; finely *dashed line*, laminin-coated vessels) were obtained by linear regression of yield on seed size, whereas the biphasic lines were least-squares minimization fits of a parabola to the data. Both fits were subject to the constraint of a yield of 0 for a seed size of 0. Parabolae provided significantly better description of yield as a function of seed size, indicating the requirement for an optimal number of cells to maximize yield

observed in nestin-positive NSC not expressing other markers of neuronal commitment. Therefore, βIII-tubulin may indicate an exploratory phenotype rather than specific lineage specification [23, 24].

Quantitative Parameters of hNSC Growth in Multilayers and Monolayers

Cell Yield

Seeding a vessel with a greater number of cells increased the number of cells harvested at passage. Each culture vessel was seeded with a counted number of viable cells, and viable cells were counted at passage. The number of cells at harvest is plotted in Fig. 4a as a function of the number of cells in the seed for 242 otherwise uncoated tissue culture-grade polystyrene vessels (*filled diamond*) and 79 vessels of the same type coated with laminin (*square*). Both axes of the plot are logarithmic to provide equal representation of the data across each power-of-ten of cell number. In general, fewer cells were seeded on laminin-coated surfaces compared to uncoated surfaces, due to the larger cell area on laminin at confluence. As denoted by points located above the dashed line of equality, representing the same number of cells harvested as seeded, more cells were harvested than seeded from 67.4 % of uncoated vessels and 82.3 % of laminin-coated vessels ($P<10^{-3}$, one-way ANOVA; $F=54.57$, uncoated; 19.45, laminin-coated). Linear regression of cells out on cells in (constrained by 0 cells out on the *y*-intercept for 0 cells in) yielded slopes of 1.8713 ± 0.1305 (uncoated, $R^2=0.2956$) and 1.7522 ± 0.4655 (laminin, $R^2=0.3287$). Within the modest goodness of fit, the slopes are not significantly different, indicating that these fetal hNSC grow similarly on both surfaces. More noteworthy were the strong correlation coefficients of 0.5554 (uncoated) and 0.5893 (laminin) between cells harvested and cells seeded. This indicates that >30 % of the variance in the number of cells harvested for the number cells seeded was accounted by the correlation between cells harvested and cells seeded, making the seed size the most significant predictor of cells obtained at harvest.

Yield, the number of cells at harvest per each cell seeded, was used to examine the roles of seed size and growth time in promoting cell growth. Cell yield was calculated as the

number of viable cells harvested from each vessel at passage divided by the original number of viable cells seeded in the vessel, i.e., yield = cells out at harvest/cells in at seed. Yields were plotted in Fig. 4b as a function of the number of cells seeded in uncoated (*filled diamond*) and laminin-coated (*square*) vessels. Average yield on laminin, 2.156 ± 1.546, was not significantly different than that on uncoated polystyrene, 2.308 ± 1.614 (*t* test, $P > 0.40$). The correlation coefficients between yield and seed size were –0.2252 for uncoated and –0.2826 for laminin-coated plastic, suggesting that seeding larger numbers of cells attenuated relative growth. Comparing fits of linear and parabolic functions to the data revealed that the biphasic parabola provided better goodness of fit between yield and seed size. Coefficients of determination R^2 increased from 0.0255 for the line to 0.4764 for the parabola for cultures in uncoated vessels and increased from 0.0654 for the line to 0.5421 for the parabola for cultures in laminin-coated vessels. Thus, yield was decreased both when too few or too many cells were seeded on either bare plastic or laminin-coated surfaces. While a linear relation between seed size and yield with slope >0 would suggest interactions between cells promoting proliferation, the better fits by parabolae indicate that an optimal number of seeded cells was necessary to maximize these interactions.

Period of Time in Culture and Doubling Times: No Effect of Laminin

The period of time elapsing between seed and harvest (growth time) resulting in greatest yield ranged between 7 and 21 days on bare polystyrene and between 5 and 14 days on laminins. Yield as a function of growth time is shown in Fig. 5a for uncoated (*filled diamond*) and laminin-coated polystyrene (*square*) for all vessels. Cells grown on laminin spread out such that each cell occupied greater surface area compared to growth as multilayers on uncoated polystyrene. Thus, cultures on laminin attain confluence earlier and were harvested after shorter growth periods. Yield initially increased the longer a culture was allowed to grow. For longer growth periods, yield decreased, consistent with contact inhibition of proliferation. Correlations between yield and growth time of 0.4463 for uncoated and 0.2037 for laminin-coated polystyrene made growth time the second most significant growth parameter after seed size.

To quantify growth dynamics of hNSC, the exponential growth function,

$$\frac{N_{\text{out}}}{N_{\text{in}}} = 2^{T/t_2} \qquad (1)$$

where T is the growth period, and N_{out} and N_{in} are cells harvested and cells seeded, respectively, was used to calculate the doubling time constant t_2 for vessels with yields >1. In this approach, the cells in each culture vessel are assumed to be a uniform population in which t_2 is the average time period elapsing between the birth of a cell by mitosis and its subsequent mitosis into two daughter cells and t_2 is stationary, that is, identical for each cell and constant during growth of the population. Yield, $N_{\text{out}}/N_{\text{in}}$, is thus predicted to be logarithmic with respect to growth time. Fitting exponential growth curves to the data of Fig. 5a decreased goodness of fit for cultures grown on uncoated surfaces ($R^2 = 0.1991$ for the line, 0.1838 for the exponential), and only slightly improved goodness of fit for cultures grown on laminin-coated surfaces ($R^2 = 0.0415$ for the line, 0.0503 for the exponential). More noteworthy, however, was that the slopes of linear relations between yield and growth time were not significantly different for the two growth surfaces (0.1151 ± 0.0203, uncoated; 0.0996 ± 0.0546, laminin-coated; $t = 0.2646$, $P > 0.40$). This suggests that cells on uncoated and laminin-coated surfaces proliferated at the same rate.

To test this hypothesis, it was necessary to determine that the assumptions underlying the simple doubling function model were applicable to hNSC growth on both surfaces. Doubling times t_2 calculated for each culture vessel were shown as frequency histograms for both growth surfaces in Fig. 5b. Dividing the growth time T of each harvested culture by the doubling time t_2 calculated for that culture results in a normalized growth time T/t_2. Plotting each culture's yield against its normalized growth time revealed that all points obtained on both growth surfaces were coincident along the same line having a slope indistinguishable from log(2.0) (Fig. 5c) with excellent goodness of fit ($R^2 = 0.9992$), indicating validity of the assumptions underlying the doubling model for growth on both uncoated and laminin-coated surfaces.

Distributions of t_2 for uncoated and laminin-coated polystyrene substrates shown in Fig. 5d revealed no effect of laminin on growth rate. Doubling time t_2 was not significantly greater for cultures grown on uncoated polystyrene (average ± SD, 13.660 ± 11.461 days) compared to laminin (13.193 ± 13.339 days) by both parametric (*t* test, $P > 0.8$) and nonparametric (Kolmogorov–Smirnov test, $P > 0.60$) tests. Furthermore, restricting the comparison to the 80 % fastest growing cultures in each group, to exclude cultures that might have experienced unknown anomalous conditions contributing to abnormally slow growth, did not result in a significant difference in t_2 (9.260 ± 3.387 days, uncoated; 6.939 ± 3.527 days, laminin; *t* test, $P > 0.30$). Thus, laminin does not act as a growth factor for hNSC.

Inverse Correlation Between Doubling Time and Yield

Yield and doubling time t_2 measured for individual cultures were strongly correlated on both types of growth surfaces. As shown in the plot of yield as a function of doubling time in Fig. 6, greater yields were obtained with shorter t_2.

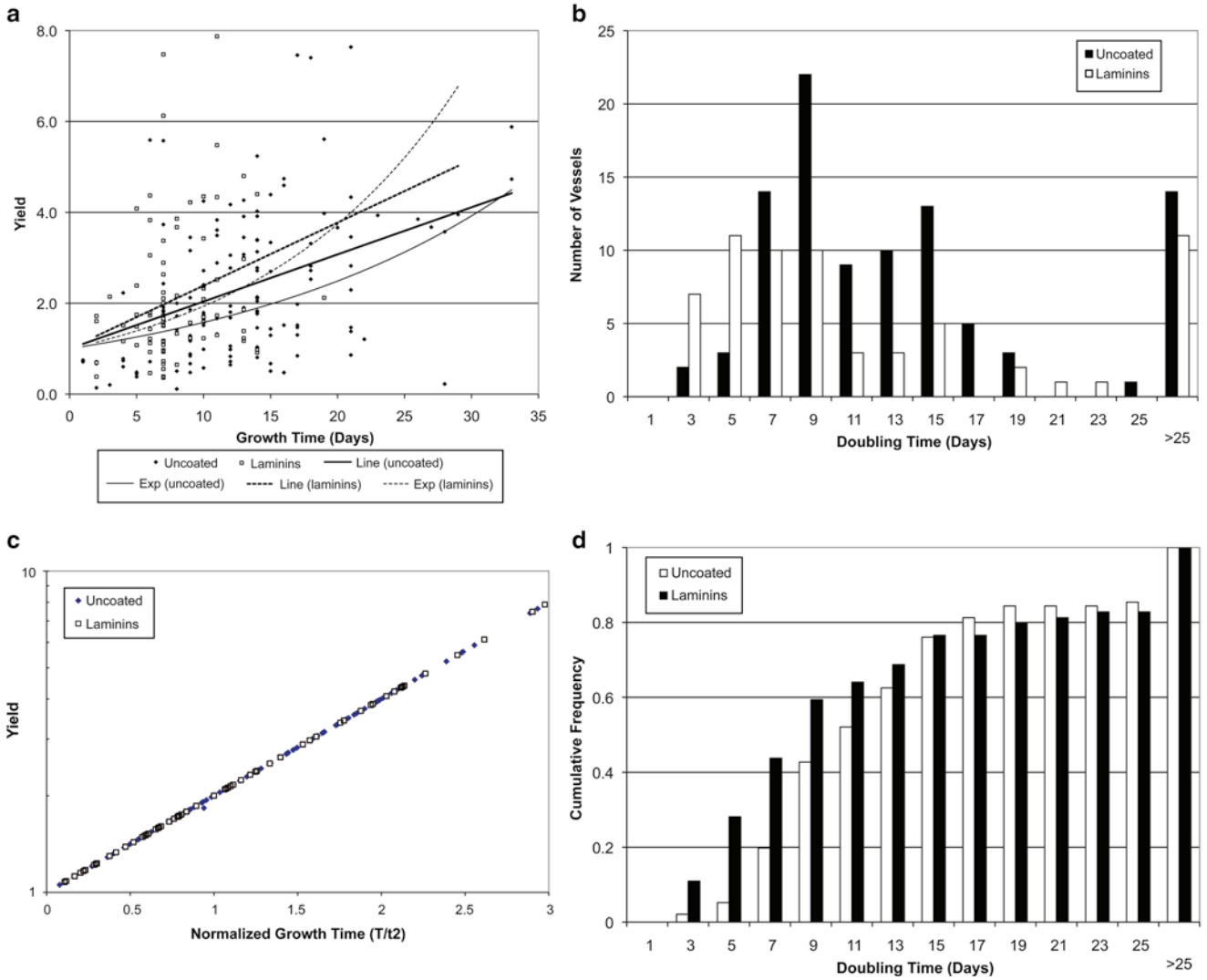

Fig. 5 Growth rates of hNSC on uncoated and laminin-coated surfaces. (**a**) Yield plotted as a function of time elapsing between seed and harvest (growth time) for hNSC seeded on uncoated (*filled diamond*) and laminin-coated (*square*) surfaces. *Straight lines* (*solid*, uncoated, yield = $0.1037T + 1$; *dashed*, laminin-coated, yield = $0.1386T + 1$) were obtained by linear regression of yield on growth time subject to the constraint of yield = 1.0 for a growth time of 0 days. The upwardly *curved lines* (*solid*, uncoated, yield = $2^{0.0656T}$; *dashed*, laminin-coated, yield = $2^{0.0952T}$) were obtained by least-squares fitting of the exponential doubling function, yield = $2^{\text{constant T}}$ to the data. (**b**) Frequency histograms of the doubling time t_2 calculated from (1) for hNSC grown on uncoated (*open columns*) and laminin-coated (*closed columns*) polystyrene vessels. (**c**) Validity of exponential growth model for proliferation of hNSC on uncoated (*filled diamond*) and laminin-coated (*square*) surfaces. For each culture, growth time T was divided by t_2 calculated from (1), and the culture yield was plotted as a function of this normalized growth time. The best-fitting line ($R^2 = 0.9992$) has slope $\log_{10}(2) = 0.30103$, indicating consistency of hNSC growth on both uncoated and laminin-coated polystyrene surfaces with the exponential growth model of (1). (**d**) Frequency distributions of doubling time t_2 on uncoated (*square*) and laminin-coated (*filled square*) surfaces

The correlation coefficients were –0.342 on polystyrene and –0.566 on laminins. The increased correlation on laminin is consistent with the relatively rapid surface spread of the cells into a monolayer within the 3-day period during which cells on uncoated polystyrene gradually underwent aggregation into colonies and attached to and explored the surface. This faster spread attenuated yields on laminin with respect to those obtained with uncoated polystyrene as cells proliferated horizontally on the laminin-coated surface and attained confluence with a decreased number of cells rather than both horizontally and vertically within and at the peripheries of the multilayer colonies formed on uncoated surfaces.

Growth dynamics of hNSC on uncoated and laminin-coated polystyrene were not significantly different by analysis of yield dependence on t_2. Least-squares analysis was used to fit exponential and polynomial functions to the data of Fig. 6. Single exponential functions (see Fig. 6) provided the best fits, $R^2 = 0.3962$ for polystyrene and 0.4630 for laminins.

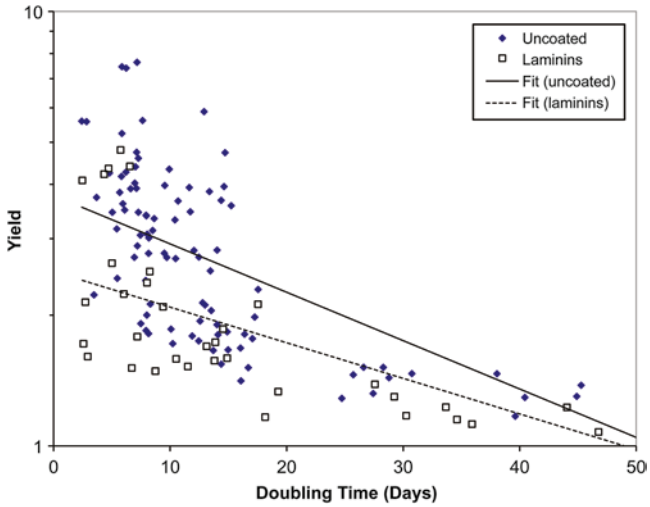

Fig. 6 Yield as a function of doubling time t_2 for hNSC growth on uncoated (*filled diamond*) and laminin-coated (*square*) surfaces. The curves (*solid*, uncoated; *dashes*, laminin-coated) are best-fitting exponential functions of the form $\text{yield} = a \times 2^{-b\,t_2}$. Best fitted parameters: uncoated polystyrene, $a = 3.7701 \pm 0.4414$, having units of yield which is dimensionless, and $b = 0.0369 \pm 0.0123$ days ($R^2 = 0.3962$); laminin-coated surfaces, $a = 2.5195 \pm 0.5215$ and $b = 0.0273 \pm 0.0242$ days ($R^2 = 0.4630$). The b or slope parameter did not significantly differ between the two surfaces (two-sided $P > 0.36$, $t = 0.3558$, 112.374 degree of freedom by the Welch–Satterthwaite equation). The yield intercepts a, where the fitted lines intersect the yield ordinate at 0 doubling time, tested significantly different (one-sided $P < 0.04$, $t = 1.8304$, dof = 156.27). This is consistent with decreased yield on laminin without change in the hNSC growth rate

More complicated functions did not improve goodness of fit. For example, double exponential functions resulted in $R^2 = 0.3749$ for polystyrene and 0.4372 for laminins. The best fits of polynomial functions of the form $\text{yield} = \sum_{i=1}^{n} a_i (t_2)^n$ were obtained with the highest order tested, $n = 6$, resulting in $R^2 = 0.366$ for polystyrene and 0.447 for laminins. Rate constants of the single exponential functions for uncoated (average ± SEM, 0.0369 ± 0.0123 days) and laminin-coated polystyrene (0.0273 ± 0.0242) were not significantly different (two-sample t test with unequal variances and sample sizes, $P > 0.36$). Thus, laminin did not change the proliferation rate of fetal-derived hNSC as measured by the dependence of yield on doubling time.

Surface Seeding Density Dependence of Yield and Doubling Time

Yield was strongly dependent on the number of cells seeded per unit surface area of growth surface (the seeding density) for both uncoated and laminin-coated vessels, having correlation coefficients of −0.2561 and −0.2042. The greatest yields of hNSC grown in multilayers on uncoated polystyrene surfaces were obtained by seeding within a range between 0.8 and 1.2×10^5 cm^2. For cultures seeded in this range, yields were >2.0 in >50 % of cultures for all growth periods (Fig. 7a). Seeding outside of this range resulted in decreased yields. At lower seeding densities, e.g., $< 0.6 \times 10^5$ cm^2, cells aggregated into small colonies that did not express exploratory lamellipodia (Fig. 1b, right). These colonies proliferated at rates too slow to overcome cell death, which decreased yield to <1.0. Seeding densities $> 1.2 \times 10^5$ cm^2 resulted in aggregation of cells into large neurosphere-like colonies within several days. While these colonies typically flattened on the growth surface and engaged in the multilayer pattern of proliferation, they sometimes detached from the substrate to form neurospheres. The attached colonies were large enough to attenuate access of interior cells to nutrients in the extracellular medium and decrease yield. Even greater seeding densities (e.g., $> 1.6 \times 10^5$ cm^2) favored more rapid aggregation into larger neurospheres, which would detach from the surface, never form multilayers, resulting in decreased yield, consistent with the negative correlation between yield and seeding density.

Growth on laminin-coated polystyrene shifted the optimum seeding surface density to a lower range such that fewer cells were necessary to evoke proliferation. Seeding densities as low as 5×10^3 cm^2 resulted in yields >1.0, and decreases in yield were not obtained until cells were plated at densities $> 1.2 \times 10^5$ cm^2 (Fig. 7a).

These biphasic functional relationships between seeding density and yield for both uncoated and laminin-coated surfaces revealed complexities in the interactions between cells determining yield. As these intercellular interactions are encapsulated formally in the seeding density, polynomials of the form $\text{yield} = \sum_{i=1}^{n} a_i (\text{density})^i$ were fitted to the data, where the a_i are coefficients indicating the strength of each order term in density, and omission of the zeroth order term ($n = 0$) constrained each fit to 0 yield for a seeding density of 0. Linear fits ($n = 1$) were interpreted as a simple paracrine interaction in which each cell secretes diffusible or surface-attached growth factors promoting proliferation of itself and neighboring cells by a non-saturable interaction. Lines exhibited the worst goodness of fit for both surfaces, however ($R^2 = 0.002$, uncoated; 0.0009, laminin-coated; see Table 1), indicating that the intercellular interactions underlying the surface density effect were more complicated than could be described by a simple linear model. Goodness of fit was improved most greatly by increasing the order of fitted polynomial to $n = 2$, which describes the biphasic relationship as a parabola. R^2 was 0.0400 for uncoated surfaces and 0.0286 for laminin-coated surfaces, denoting improvement of the parabola in describing yield as a function of seeding density compared to a line by factors of 20 and 32, respectively. Increasing the order of the fitted polynomials improved goodness of fit by a proportionally decreasing amount by providing local maxima most closely coinciding with the observed optimum seeding densities. While polynomials up to

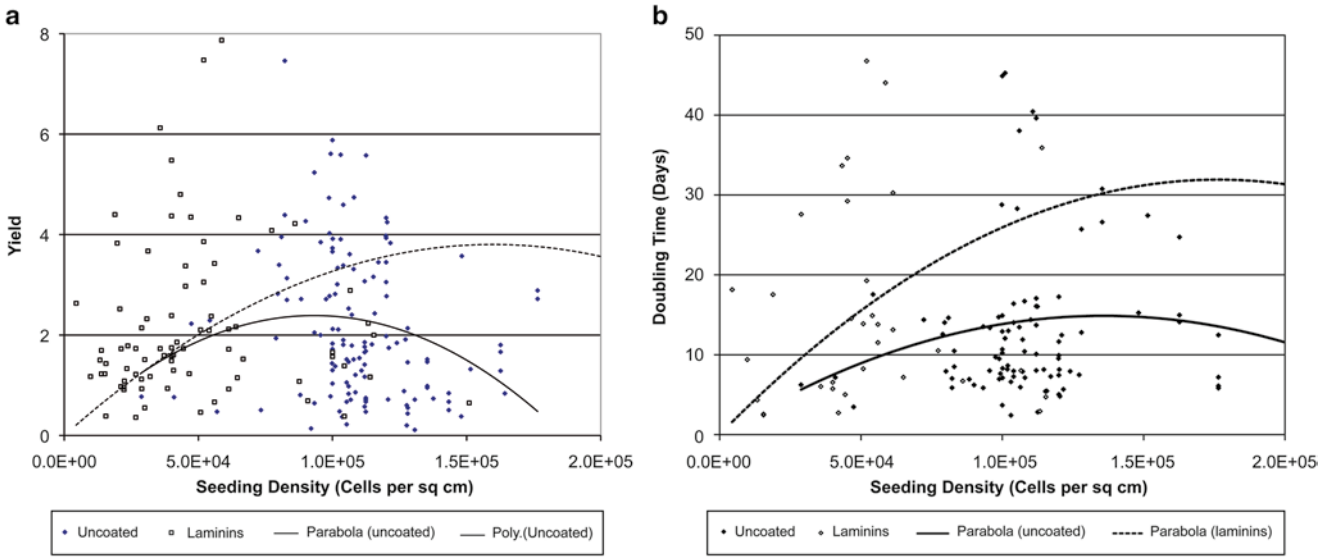

Fig. 7 Density of cells initially seeded on the growth surface. (**a**) Yield as a function of seeding density (cells/cm^2) for hNSC growth on uncoated (*filled diamond*) and laminin-coated (*square*) surfaces. The curves are fits of quadratic polynomials (parabolae) of the form yield $= a \times d^2 + b \times d$, where d is surface seeding density, to the respective data. Fits were constrained to yield = 0 at $d = 0$. Fitted parameters: uncoated surfaces (*solid line*) $a = -3.0\ (\pm 0.65) \times 10^{-10}$, $b = 5.0\ (\pm 0.47) \times 10^{-5}$; laminin-coated surfaces (*dashed line*), $a = -1.0\ (\pm 0.83) \times 10^{-10}$, $b = 5.0\ (\pm 2.33) \times 10^{-5}$. (**b**) Doubling time t_2 as a function of seeding density for hNSC growth on uncoated (*filled diamond*) and laminin-coated (*square*) surfaces. Fitted parameters for parabolae: uncoated surfaces (*solid line*) $a = -8.0\ (\pm 0.5) \times 10^{-10}$, $b = 2.0\ (\pm 0.9) \times 10^{-4}$; laminin-coated surfaces (*dashed line*) $a = -1.0\ (\pm 0.7) \times 10^{-9}$, $b = 4.0\ (\pm 3.0) \times 10^{-4}$

Table 1 Goodness-of-fit coefficients of determination R^2 for polynomials of different order fitted to yield as function of seeding density

Order	1	2	3	4	5	6
Uncoated	0.0020	0.0400	0.0853	0.1357	0.1367	0.1367
Laminin	0.0009	0.0286	0.0917	0.0983	0.1020	0.1027

$n = 6$ were fitted, R^2 did not improve beyond $n = 4$ for uncoated surfaces and $n = 5$ for laminin-coated surfaces, due to providing local maxima in the fitted curves most closely coinciding with the observed seeding density ranges producing greatest yields. The best-fitting parabola for growth on uncoated plastic was relatively sharp, extending through the data points on both upward and downward trajectories, and having its maximum at 1.2×10^5 cells/cm^2, the largest seeding density that did not result in a decrease in yield. The best-fitting parabola for laminin extended upward well beyond the actual data, peaking at 2.5×10^5 cells/cm^2, such that the observed data points were situated along the increasing portion of the curve. This suggests that plating density was less critical on laminin, consistent with the decreased tendency of cells to aggregate by enhanced interaction with the growth surface compared to uncoated surfaces. This increased surface interaction appeared to improve survival and enable growth at lower seeding densities.

Seeding surface density exerted minimal detectable effect on t_2 on both uncoated and laminin-coated plastic (Fig. 7b). Correlation coefficients between t_2 and surface density were only +0.079 on uncoated and −0.040 on laminin-coated polystyrene. This is consistent with the biphasic relationships between surface density and yield, given the inverse relation between yield and t_2 (Fig. 6). Fitting of the data to parabolae, however, while better than lines, resulted in relatively poor goodness of fit ($R^2 = 0.023$ uncoated, 0.156 laminins). Therefore, while the effect of seeding density is most pronounced on yield, it had much weaker influence on growth rate as measured by doubling time.

Discussion

Growth Patterns of fhNSC

Previous studies have established the minimal set of growth factors (basic FGF and LIF) and their concentrations necessary to sustain proliferation in chemically defined serum-free media [2, 8]. Within the context of these requirements, growth of fetal hNSC in multilayers and monolayers was studied in the present work to understand how these patterns emerge in culture and how culture parameters such as seed number and growth time determine cell yield. Each growth pattern was found to progress with a unique time course in a stereotypical series of events. These patterns were found to originate from differences between the extents to which cells

interact with each other and with the culture surface during the first few days of culture. Dynamics of fetal- and adult-derived hNSC have been quantified previously for growth in neurospheres [5] and monolayers [25]. They have also suggested extensive intercellular dynamics during growth of fhNSC in neurospheres, in which smaller aggregates of cells coalesce by "neurosphere fusion" into larger, smooth-surfaced aggregates [5]. The present study documents the extensive motile activity of fhNSC underlying dynamics of growth in multilayer and monolayer patterns to reveal how multilayers are capable of greater cellular production than either neurosphere or monolayer modes [13].

Cell Motions During Early Growth

Motions of cells both within the liquid medium and along the growth substrate were observed to be crucial for fhNSC survival, growth, and proliferation. Furthermore, elaboration of lamellipodia early in culture was found to be the most reliable indicator of cell survival and proliferation. hNSC are typically cultured on low-attachment surfaces to encourage formation of neurospheres [5], and on coated surfaces to promote monolayer formation [2, 7, 25] and to avoid serum-containing medium [11, 12]. The erratic mobility of freshly dispersed cells in the fluid phase above tissue culture-treated but otherwise uncoated plastic surfaces is reminiscent of "Brownian motion," in which the cells are randomly buffeted by water molecules and, more likely, microscopic fluctuations in convection aided by heat from the light source [22, 26]. This motion enables their coalescence into small (e.g., 20–100 cell) clusters during the first 24–72 h after plating, which is further reminiscent of neurosphere formation [5]. Instead of coalescence into larger aggregates, however, clusters were observed to attach to the culture surface resulting in the nucleation of multilayers, as though the clusters had "fused" with the surface rather than other clusters. Cells within the clusters or colonies elaborate lamellipodia within minutes of surface contact that repeatedly "sample" the substrate by rapid extension and retraction. These extension and retraction cycles occur in different directions until a fortuitous encounter with the proximate area surrounding lamellipodia extended by a neighboring cluster, which stabilizes both lamellipodia such that they are much less likely to retract. This "contact" appears to promote proliferation of cells within both clusters as well as adherence of cells to the substrate and enable exchange of cells between clusters by migration along the substrate. This early pattern explains the observations of Wakeman et al. [13] that multilayer formation is most successful by allowing freshly plated cells to remain undisturbed for 3–5 days before any movement of the culture vessel. It also explains their observed critical dependence of culture yield and proliferation rate on the surface area density of the seeded cells quantified in this study.

Substrate Attachment and Multilayer Versus Monolayer Growth

The comparative strengths of surface attachment and intercellular interactions appear to determine the subsequent pattern of growth as neurospheres, multilayers, or monolayers. Our time-lapse studies indicate that laminin coating of the surface induces a growth pattern characterized by extensive interaction with the substrate almost to the exclusion of the extensive intercellular interactions observed with neurospheres and multilayers. Freshly seeded cells were observed to attach to laminin surfaces, express lamellipodia, and begin migration along the surface within minutes of seeding. Apparent colonies form only after extensive migration and proliferation of the freely motile cells results in the diminution of free surface area, suggesting that some colonies arise as families of daughter cells lacking space to migrate away from the site of mitosis.

With weaker surface attachment, hNSC also move in the fluid phase above the substrate and readily interact with each other to create clusters. Then, depending on the strength of surface affinity, they either proliferate within the clusters to form neurospheres in the case of weakest surface attachment or simultaneously interact with both each other and the surface to create the flattened colonies that nucleate proliferation as multilayers. Cells migrate away from colonies with which they were originally associated to join either the central masses of other colonies or the cells extending along the multicellular branches that form along lamellipodia initially projected by surface-attached cells during their initial exploration of the growth substrate. Thus, the branch or chain migration originally documented by Imitola et al. [27] results from wandering cells recruited to already growing branches of their colonies of origin and other colonies.

Quantitative Parameters of Growth

Growth Time Course and the Exponential Doubling Model

One perhaps striking finding of the present study is the quantitative consistency of fetal hNSC growth dynamics in multilayers and monolayers. Earlier studies of hNSC derived from fetal telencephalon and subventricular zone between 5 and 20 weeks gestation found doubling times ranging from 7 to 21 days, dependent on cell isolate, duration of continuous culture in bFGF and LIF, and growth factor concentrations, but with averages of 12–15 days under the most optimal growth conditions [2, 25]. The present study found a similar wide range of doubling times for hNSC derived at 13 weeks, in optimally supplemented culture media, with an average of 13 days, indistinguishable from the earlier data. Therefore, these data represent proliferation dynamics characteristic of hNSC derived from this source.

Previous studies have generally used relatively constant growth periods in determination of yield, such that this effect could be masked [2, 8, 25]. By allowing growth time to vary in this study, however, it became possible to elucidate that hNSC proliferation was consistent with the simple exponential doubling model. In this model, each cell in a culture population undergoes mitosis with a constant doubling time across the entire span of culture, from seeding to increasing fluence. The results of the present study are consistent with proliferation dynamics being both uniform across the population of cells and stationary with respect to the duration of the culture. Nonetheless, given the morphological differences observed in the growth patterns of multilayers and monolayers, it is reasonable to conclude that more detailed examination of the timing of individual cell divisions, e.g., with fluorescently tagged vital nuclear markers, will be necessary to reveal the temporal fine structure of hNSC growth to test the model's assumptions.

Growth Dependence on Intercellular Interactions

Statistical analysis of growth data unsurprisingly shows that the number of harvested cells was most strongly determined by how many cells were seeded and the period of time they were allowed to grow. Absence of correlation between number of cells harvested per number seeded (yield) and the seed size, however, results from a biphasic (or higher order) relationship between yield and seed size. Since yield is ultimately determined by the proliferation rate of each cell during the culture period, this relation suggests that both positive and negative interactions between cells influence survival and proliferation.

A model incorporating competing influences is captured in an inverted parabolic relation. For example, a linear, additive model, in which each cell secretes a growth factor (at a constant rate), binding to a saturable receptor necessary for survival, produces an increased yield at greater seed number, i.e., a relation with a positive slope. If, instead, synthesis and secretion of the growth factor are dependent on the secreted growth factor, the relation becomes parabolic. As seed size increases, the cells become closer in proximity, the interaction is positive, and yield increases. Due to the saturable receptor interaction necessary for synthesis and secretion of the growth factors, however, at a critical seed size, the positive effect of increased cell density attains a maximum. Competition between the crowded cells begins to deplete the extracellular concentration of secreted growth factor, and synthesis and secretion rates become limited. Thus the effect of further crowding is a negative influence, which may include competition for nutrients and other growth modes favored by cell crowding, such as neurosphere formation that predominates at greater seed sizes [13], to decrease yield.

Wakeman et al. [13] observed that proliferation in a non-neurosphere, multilayer growth pattern in serum-free medium could be obtained by seeding fetal hNSC at a sufficiently large surface area density of ~10^5 cells/cm^2. The present work quantitatively defines the ranges of seeding densities around this optimum resulting in both maximum yields and shortest doubling times for both uncoated and laminin-coated surfaces. The optimal seeding density of 10^5 cells/cm^2 on uncoated plastic spaces each individual cell (or small aggregate) at mutual separation distances at which paracrine interactions by small diffusible growth factors are feasible. For closely packed cells (i.e., arranged on a hexagonal lattice), the average center-to center separation distance is 34 μm. For cells with an average diameter of 10 μm, the average edge-to-edge intercellular distance is thus 24 μm, which can be traversed by a 10 kDa growth factor with a diffusion constant of 1 μm^2/s in an average time of 2.5 min. As multilayers tend to form from aggregates, this intercellular separation is less important as cells within each colony are able to maintain high local concentrations of growth factors to sustain survival. With laminin coating, however, the minimum seeding density observed in this study of 10^4 cells/cm^2 places the cells with an average edge-to-edge separation distance of 98 μm, which increases the average diffusion time to 40 min. Therefore, laminin appears to relax the requirements for intercellular communication by diffusible growth factors.

Laminin

While laminins, other ECM molecules, and surface coatings are not required for culture of fetal hNSC as monolayers [1, 25], they had been regarded as necessary for growth in the absence of serum [11, 12] until discovery of the multilayer growth pattern [13]. The findings of the present study reveal that laminin-coated surfaces induce a monolayer growth pattern morphologically characterized by extensive exploration of the surface by lamellipodia almost to the exclusion of the attractive intercellular contacts that develop in multilayer and neurosphere patterns. Thus, laminin eliminates the early phase of interactions between cells necessary in multilayer culture, and intercellular interactions only appear later as proliferation decreases the available surface area. These surface interactions also appear to decrease the need for paracrine interactions between cells, as revealed by the shift in yield to greatly decreased area seeding densities and the widening of the yield curve to a much broader range of densities. Laminins and other ECM have been considered to be growth factors similar to bFGF, LIF, EGF, and others that sustain survival and proliferation, largely due to apparent sharing by integrins of signal effectors converging on mitogen-activated mitosis regulators [11, 17] sustaining NSC. The present study revealed, perhaps surprisingly, that the profound morphological effect of laminin on the fNSC growth pattern was accompanied by no increase in the proliferation rate compared to growth on uncoated plastic, as measured by the doubling time constant, or on the functional dependence of yield on the doubling time. Thus, our findings support the hypoth-

esis that laminin facilitates survival of fetal hNSC without changing cell cycle dynamics as has been observed for other artificial growth surfaces [28]. As elaboration of lamellipodia was found to be the key indicator of survival and proliferation in both monolayer and multilayer growth patterns, and laminin evokes very early expression of lamellipodia, this exploratory phenotype may promote essential metabolic processes that provide a necessary condition for proliferation on artificial substrates. It is thus noteworthy that transcriptome profiling of human neocortical germinal zones at the same period of fetal development coinciding with the isolation of hNSC used in the present study has revealed abundant expression of laminins as well as β-tubulins within areas of stem and progenitor cell self-renewal [29].

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Suggested Readings

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Immunobiology of Embryonic and Induced Pluripotent Stem Cell Transplantation

Eleanor M. Bolton, J. Andrew Bradley, and Craig J. Taylor

Introduction

Developments in embryonic stem cell (ESC) research and regenerative medicine over the last decade have led to widespread optimism that stem cells offer great potential for studying and treating human disease. Patients suffering from a range of degenerative and autoimmune diseases, such as Parkinson's disease and type 1 insulin-dependent diabetes, or genetic disorders such as alpha-1-antitrypsin deficiency or cystic fibrosis, would benefit from tissue replacement therapy to replace and replenish diseased tissues and to provide genetically corrected tissues [1]. While some diabetic patients are currently treated with pancreatic organ transplantation or isolated pancreatic islet transplantation, the major limitation for such therapies remains the inadequate supply of donor tissues. The potential for ESC and induced pluripotent stem cells (iPSC) to expand almost indefinitely and to differentiate into precursors of all the tissues in the human body generates optimism that these cells provide an untapped resource for such tissue replacement therapy [2]. Moreover, the use of stem cell lines to research the origins of human disease and to test corrective treatments is of great potential interest to the pharmaceutical and biotech industries.

Certain hematologic malignancies are routinely treated by ablative therapy and CD34+ stem cell transplantation, while graft versus host disease (GVHD) may be attenuated with the use of mesenchymal stem cell (MSC) transplantation [3]. One of the criteria for CD34+ stem cell treatment, like bone marrow and some forms of organ transplantation, is to ensure matching (for human leukocyte antigens, HLA) between donor and recipient, since poorly matched transplanted tissues are recognized by the recipient's immune system as foreign, and will be rejected.

A solution to this hurdle is to use HLA-identical or autologous tissue; while this may be possible for some cases of peripheral blood CD34+ stem cell, bone marrow, and pancreatic islet transplantation, it is not possible for most types of organ and tissue transplantation, except where the donor is an HLA-identical sibling. Similarly, it would not be possible to use autologous ESC-derived tissue unless an embryo was created by parthenogenesis or by somatic cell nuclear transfer. While embryos may be created for therapeutic purposes, current ethical considerations ensure that the embryo must be implanted and develop to full term, and that therapeutic tissues resulting from such embryos are limited to provision of normal bone marrow or hematopoietic stem cells (HSC) for treating HLA-identical siblings. The generation of HLA-selected ESC lines for differentiation into tissues for treating a sibling would overcome potential problems of rejection of HLA-nonidentical tissue but does not take account of the moral and ethical issues related to creation, and subsequent destruction, of embryos for purposes other than procreation [2].

In contrast, the development of autologous iPSC lines from each individual who requires a tissue transplant is technically feasible and ethically acceptable, although at present impracticable. iPSC are commonly generated by retroviral transduction of differentiated cells (e.g., fibroblasts from an autologous skin biopsy) with genetic factors that restore pluripotency to the cells. Such cells then have the properties of ESC in terms of their potential for self-renewal and differentiation into multiple tissues, and benefit from being genetically identical to the individual requiring cell replacement therapy who provided the original skin biopsy. The limitations of both ESC and iPSC in regenerative medicine include

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the practicalities of directed differentiation and the time required to produce sufficient differentiated, functional tissues for transplantation. This article makes the assumption, therefore, that ESC and iPSC used for therapeutic purposes will be made available from a repository, such as a stem cell bank [4].

While ESC and iPSC have been extensively characterized phenotypically and genotypically, there is surprisingly little information on their expression of molecules relating to the immune system and, in particular, HLA molecules. Even among those ESC lines that have been deposited in national stem cell banks for use by researchers, few have their HLA type listed. Yet it is clear from clinical studies using HSC that HLA matching is important and the contribution of HLA expression to rejection of HSC cannot be ignored [3]. This chapter draws on current understanding of the immunobiology of organ and tissue transplantation to examine the likelihood that differentiated tissues derived from ESC and iPSC may, at some stage, express HLA molecules and, following transplantation, will participate in an immune response that culminates in destruction of the transplanted tissues unless they are HLA matched or unless the recipient receives long-term immunosuppressive treatment. Much of our knowledge of stem cell biology and transplant immunology derives from basic research in animal systems, but the principal mechanisms and pathways mirror those in humans; for the purpose of clarity, this chapter is written from the perspective of stem cell transplantation in humans.

Immunogenicity and the Innate Immune Response

Immunogenicity is the term that describes the ability of a substance or tissue to elicit an immune response in the host individual; thus, for example, a protein, a virus, or a tissue is said to be immunogenic if the host raises a response that culminates eventually in destruction and elimination of the protein or virus or tissue. The property that immunogenic substances have in common is that they are regarded as "foreign" in some way to the host. The immune system, comprising both innate and adaptive components, has evolved so that an individual is unresponsive, or tolerant, to "self" substances (proteins, cells) but is able to respond quickly to protect itself against harm from "non-self" (or foreign, or dangerous) exogenous proteins, pathogens, and cells. The potential for ESC and iPSC to elicit an immune response has been little studied and the possibility that stem cell-derived tissues may be rejected remains a barrier to clinical transplantation of stem cells and their derivatives. While the development of iPSC raised optimism that this barrier could be ignored, because "self" stem cell-derived tissue could, in principle, be created from iPSC generated from the individual requiring the transplant, the practical limitations of this approach have reinstated the barrier. In addition, a recent paper that appeared to demonstrate the immunogenicity of autologous iPSC in mice emphasizes the need to study this subject in greater depth [5].

The innate immune system is the first line of defense and is highly effective at protecting against pathogenic infections but is not sufficient, on its own, to establish the exceptionally specific memory and rapid recall responses that are characteristic of the adaptive immune response. Adaptive immunity develops with time and is initiated primarily by "licensed" dendritic cells that are activated by the combined functions of innate immunity, including receptor–ligand interactions, the physiological components of physical barriers such as the skin (enzymes, pH), and inflammatory or chemotactic proteins such as fragments of the complement system, histamine, and tumor necrosis factor (TNF) [6–8]. The receptor–ligand compartment of the innate immune response consists of a system of molecules, strongly conserved among species, that initially distinguishes self from non-self (Table 1). A non-self signal to the immune system, sometimes called a "danger" signal, can be initiated by the presence of pathogens, or of proteins not normally expressed but induced by factors relating to tissue handling and transplantation, such as hypoxia and other cell stress-related proteins. The simple, repeating pathogen-associated molecular patterns (PAMPs) present on bacterial cell walls and many microorganisms are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed by most cells of the innate immune system, including macrophages, neutrophils, natural killer (NK) cells, and dendritic cells (DC). These cells typically respond by releasing inflammatory proteins such as interferon-γ (IFN-γ) and TNF.

An alternative set of molecules form recognition elements termed damage-associated molecular patterns (DAMPs) that are expressed on heat shock proteins (HSPs) and other molecules induced by cellular stress; they are recognized by additional TLRs which facilitate scavenging of harmful molecules such as reactive oxygen species (ROS) that can induce cell apoptosis. The outcome of the response to HSPs and ROS is an altered balance in the expression of pro-survival proteins including Bcl-2 and BclxL, and the pro-apoptotic protein Bax, such that the cells either succumb to apoptosis or are better able to survive the hypoxic environment [9, 10].

Local inflammation results in activation of vascular endothelial cells which then activate the complement and coagulation cascades, resulting in release of a range of components that have two main outcomes: induction of endothelial adhesion molecules and chemokine receptors promoting accumulation of inflammatory cell types, and increase in vascular permeability that facilitates entry into the tissues by cells of the adaptive immune system [11]. While the expression of markers of the innate immune

Table 1 Components of innate immunity

Stimulus for innate immune response	Molecular stimuli	Recognition by pattern recognition receptors (PRRs)
Tissue infection by bacteria, viruses, fungi	Pathogen-associated molecular patterns (PAMPs) on structural and nucleotide components of pathogens	PRRs expressed on dendritic cells, macrophages, granulocytes, NK cells, NK T cells, γδT cells
	Lipopolysaccharide (LPS), peptidoglycan, flagellin, yeast zymozan	TLR2, TLR4, TLR5, c-type lectins (e.g., mannose-binding proteins), NLRs
	Ss and dsRNA of viruses	TLR3, TLR7, TLR8
	CpG motifs of bacterial DNA	TLR9
Tissue stress: cell damage, heat stress, ROS induced by ischemia/reperfusion	Damage-associated molecular patterns (DAMPs)	
	Heat shock proteins	TLR2, TLR4
	High mobility group box 1 (HMGB1)	TLR2, TLR4, RAGE
	MICA, MICB	NKG2D
	Hyaluronic acid fragments, heparan sulfate fragments	TLR2, TLR4

RAGE receptor for advanced glycation end product, *MICA & B* MHC class I polypeptide-related sequence A and B, *TLR* Toll-like receptor, *NLR* nucleotide-binding, oligomerization domain-like receptor

system in ESC and iPSC has not been formally studied in any depth, it is likely that transplanted iPSC-derived tissues will be no less susceptible than any other foreign body to immunological surveillance by the innate immune system which, once initiated, proceeds via induction of pro- and anti-apoptotic gene transcription, and production of inflammatory cytokines, chemokines, and adhesion molecules. In particular, transplanted stem cells and their derivatives are likely to suffer hypoxic damage during the transfer, and there may be local tissue damage at the site of injection and implantation. The ensuing local inflammation results in enhanced vascular permeability with increased drainage of extracellular fluid into the lymphoid system, thereby transporting free graft antigens from the site of injection or cellular damage to the draining lymph nodes where they engage the adaptive immune response.

The first task of the adaptive immune response to stem cell transplantation is to determine whether the tissue is self or non-self. The purpose of stem cell transplantation is repair and replacement of diseased and damaged tissue, so a criterion for transplantation is that the stem cell-derived tissue should be functionally and phenotypically normal and, therefore, as nearly identical as possible to the original tissue that has become dysfunctional. A number of groups of molecules expressed by the stem cell-derived tissues are likely to be nonidentical and include the class I and class II molecules encoded by the Major Histocompatibility Complex (MHC) of genes, the ABO blood group antigens and some minor histocompatibility (mHC) antigens such as the male H-Y antigen or certain mitochondrial gene products—unless the stem cells were derived from the recipient itself. Expression of dissimilar MHC molecules, in particular, indicates that the tissue is non-self in origin and is a highly potent inducer of the adaptive immune response.

Tissue Compatibility

The likelihood that transplanted stem cells will induce an adaptive immune response resulting in rejection depends upon their ability to express molecules that will be recognized by the recipient as non-self or foreign, whether they be molecules tethered in the stem cell membrane or intracellular proteins that may be recognized as antigenic peptides when presented in the context of MHC class II molecules.

Blood Group Antigens

More than a century ago, Karl Landsteiner and other independent researchers revealed the existence of the ABO system of antigens expressed on the membranes of red blood cells that are responsible for a transfusion reaction when ABO-incompatible blood is infused. Human ABO antigens are a family of molecules that have a conserved glycoprotein and glycolipid core structure inserted in the cell membrane, with exposed oligosaccharides that form the H antigen, known as group O, or variant carbohydrates forming the A and B antigens. Humans also possess naturally occurring IgM and IgG antibodies against the non-self A or B antigens that are thought to arise during infancy through cross-reactive recognition of simple repeat-pattern structures on gut commensals (bacteria, viruses) and other environmental antigens including plants [12]. Thus, a blood group A individual develops anti-B antibodies, a blood group B person develops anti-A antibodies, group O individuals develop both anti-A and anti-B, and group ABs do not have ABO antibodies.

ABO antigens are expressed on other cell types besides erythrocytes, including some epithelial cells and also vascular

endothelial cells [13, 14]. ABO-matching for blood transfusion, bone marrow and organ transplantation is normally mandatory in clinical practice in order to avoid loss of the tissue through hyperacute rejection, and is most likely to be necessary also for stem cell transplantation and regenerative medicine. ESC express ABO antigens and differentiated cells including cardiomyocytes and hepatocytes, derived in vitro from ESC, display ABO blood types [15].

The Rhesus blood group antigens that are also expressed on erythrocytes elicit strong antibody responses following exposure to rhesus-incompatible blood, but there are no pre-existing anti-Rhesus antibodies and it is not generally necessary to match for Rhesus antigens in tissue and organ transplantation.

The MHC Antigens

The MHC is a large system of around 200 genes that encode widely expressed cell surface molecules whose principal function is to act as recognition elements and to present fragments, or peptides, of potentially threatening antigens to cells of the adaptive immune system. The MHC is located on chromosome 6 in humans (at 6p21.1–21.3) and encodes three major classes of molecules involved in immune defense, including the class I and class II genes which have multiple allelic forms, and the more conserved class III genes which encode proteins of the complement system and the cytokines, lymphotoxin, and TNF [16]. In humans, the class I and II gene products of the MHC are termed the Human Leukocyte Antigens, or HLA molecules, and are so-called because they were first described as being expressed at high density on white blood cells. They have variable distribution that relates to their function: class I molecules are expressed on virtually all nucleated cells of the body while class II molecules are expressed mainly on cells of the immune system that have an antigen processing and presentation function; the expression of both is enhanced and extended in an inflammatory milieu, mimicked in vitro by culturing in the presence of the pro-inflammatory cytokine IFN-γ.

The structure of the two classes of HLA molecules is broadly similar, reflecting their common function. The three principal groups of class I HLA molecules (HLA-A, -B, and -C) are heterodimeric, comprising the highly polymorphic α chain of around 44 kDa which inserts into the cell membrane, non-covalently bound to the conserved β2-microglobulin chain of around 12 kDa (encoded outside of the MHC) [17]. The α-chain has three domains, of which the distal α1 and α2 domains form the peptide-binding groove that presents peptides of 8–9 amino acids in length. The three polymorphic class II HLA molecules (HLA-DR, -DP, and -DQ) are also heterodimers, with a two-domain α-chain of around 34 kDa and a two-domain β-chain of ~29 kDa, the distal domain of each chain together forming the peptide-binding cleft that presents peptides of around 13–15 amino acids. The two chains are tethered by insertion into the cell membrane and are stabilized by the bound peptide. The extensive polymorphism of the HLA molecules together with the bound peptides confers massive potential for inducing immune reactivity when tissues and cells expressing non-self HLA molecules are transplanted. The MHC class II genes also encode a series of additional, relatively non-polymorphic proteins involved in antigen processing and presentation, including LMP genes encoding proteasome components, the TAP1, TAP2, and tapasin molecules responsible for class I-peptide assembly, and the DM and DO molecules that regulate a similar peptide loading function in the HLA class II molecules.

The Minor Histocompatibility Antigens

A third group of potentially immunogenic non-self antigens, called minor histocompatibility antigens (mHC), may be responsible for graft rejection responses even in individuals receiving a transplant from an HLA-identical sibling. These antigens are not expressed on the cell surface but instead are peptides derived from intracellular proteins, and include the male-specific H-Y antigen and certain mitochondrial proteins. The H-Y antigen, in particular, is known to invoke an immune response and causes GVHD in male recipients of HLA-matched female HSC, is responsible for skin graft rejection in MHC-identical mice, and is associated with reduced renal transplant function in patients [18–20]. The creation of autologous ESC lines by nuclear transfer may introduce additional and nonidentical mHC antigens derived from extranuclear components of the donor oocyte.

The Adaptive Immune Response

The innate immune response is highly effective at neutralizing the threat from pathogens and exogenous proteins but is not, on its own, effective at eliminating transplanted tissues. However, it does contribute to the adaptive immune response and facilitates the first interaction between allogeneic MHC molecules expressed by the transplant and T cells that initiate adaptive immunity.

A pivotal event in the immune response is activation of naive CD4 T "helper" cells when their T cell receptors engage with molecular complexes on antigen-presenting cells (APC) consisting of the distal domains of the α and β chains of MHC class II and their bound peptide [21] (Fig. 1). At the same time, the CD4 molecule engages with a conserved sequence of amino acids on the membrane proximal domain of the class II β chain to stabilize the interaction.

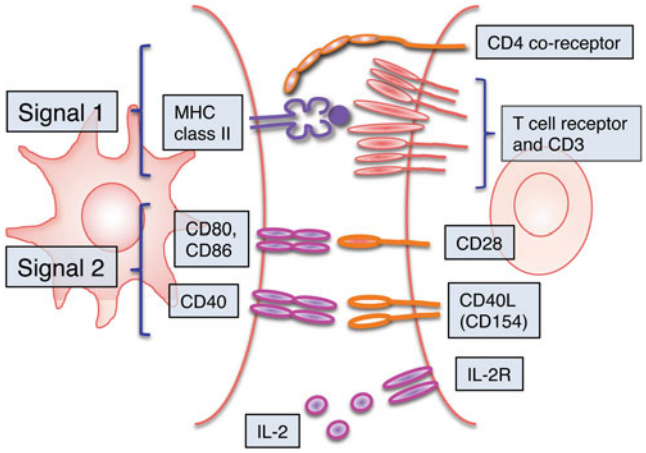

Fig. 1 T cell activation. T cell activation and the initiation of the adaptive immune response require two signals delivered from the antigen-presenting cell (APC) to the responding T cell. The initial low affinity interaction between the αβ T cell receptor and the MHC-peptide complex on the APC is stabilized by adhesion molecules and binding of the CD4 or CD8 co-receptor molecule. This initiates phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the CD3 molecule, providing Signal 1. Signal 2 is the result of interaction between the co-stimulatory molecules CD80 and 86 on the APC and CD28 on the T cell, which induces expression of CD40 on the APC and its ligand, CD40L on the T cell. The two signals initiate subsequent signaling pathways leading to expression of cytokine genes, particularly *IL-2*, whose gene product has an autocrine effect on the T cell and induces upregulation of the IL-2 receptor and T cell effector functions

Engagement of the T cell receptor, termed "signal 1," induces intracellular signaling pathways in the APC leading to transcription of genes encoding co-stimulatory molecules such as CD28 whose interaction with the CD80 and CD86 ligands on the CD4 T cell provides the "second signal" that is essential for full T cell activation, and "helper" cytokines such as interleukin-2 (IL-2). Activated CD4 T cells, through release of cytokines, are able to proliferate and provide help for activation and differentiation of CD8 T cells and B cells into cytotoxic T cells and antibody-secreting plasma cells, respectively. Engagement of the T cell receptor in the absence of signal 2 is likely to result in T cell deletion, anergy, or tolerance [22].

A number of subsets of CD4 T cells are recognized and are loosely defined both by their function and by the cytokines that drive them, although they exhibit significant plasticity [23, 24]. Regulatory CD4 T cells, or Tregs, arise in the thymus and protect the individual from making inappropriate responses to self proteins and peptides, but may also be induced in the periphery under certain conditions that prevent full T cell activation [25]. CD4 T follicular helper (Tfh) cells are present in B cell follicles and enable germinal center formation and antibody production. Activated CD4 T helper cells may be of the Th1 or the Th2 phenotype, distinguishable by the range of cytokines they secrete that determine their function. Th1 cells are driven by IL-12, produced by activated dendritic cells, and principally secrete IL-2, IFN-γ, and TNF-α that mediate endothelial cell activation, increased vascular permeability and recruitment, and activation of cells involved in delayed-type hypersensitivity (DTH) responses. Th2 cells secrete cytokines that help in B cell activation (IL-4, IL-5) as well as the inhibitory cytokines IL-10 and IL-13 that suppress macrophages and Th1 cells, respectively. CD4 Th9 cells secrete IL-9 that enhances mast cell activity and promotes Th2 cells, while CD4 Th17 cells are driven by IL-6, TGF-β, IL-21, and IL-23 to differentiate into IL-17-producing cells that are critical for supporting autoimmune responses but not Tregs.

Like CD4 T cells, naïve CD8 T cells are also activated only when they receive the two activation signals, beginning with engagement of CD8 T cell receptors with the α1/α2/bound peptide complex of MHC class I molecules together with binding of the CD8 co-receptor with a set of conserved amino acids on the membrane proximal α3 domain of the class I heavy chain to stabilize the interaction. CD8 T cells receive high levels of co-stimulatory signals from activated dendritic cells and are induced to produce IL-2 that promotes proliferation and differentiation into cytotoxic cells.

B lymphocytes function both as APC and as antibody-producing effector cells but, like CD8 T cells, require CD4 T cell help for differentiation into mature antibody-producing plasma cells. B cells are able to acquire and internalize exogenous antigen through recognition by the surface immunoglobulin receptors. Antigen is processed internally and presented at the cell surface as peptides in the binding cleft of MHC class II molecules where they engage with CD4 T cell receptors and receive help from the activated CD4 cell. Activated B cells are then able to proliferate and begin the processes of somatic hypermutation and affinity maturation, resulting in the production of high affinity antibody of the IgG class that is effective at neutralizing the initial antigen.

An important secondary effect of CD4 T cell help is the generation of memory T and B cells that have altered cell surface molecules and a reduced requirement for CD4 T cell help. Memory B cells are terminally differentiated plasma cells that have already undergone affinity maturation and are able to immediately produce high affinity, neutralizing antibody, while memory T cells exist at higher frequencies and are therefore more effective in their response to antigens, although their requirement for activatory cytokines persists. CD8 memory T cells, in particular, do not require co-stimulatory molecules on APC for differentiation into cytotoxic cells, thereby enabling them to respond rapidly to viruses that infect somatic cells.

During a typical, conventional immune response to exogenous antigen, the first step of antigen presentation is initiated when macrophages, dendritic cells, or B lymphocytes encounter and internalize the antigen (Fig. 2). The antigen

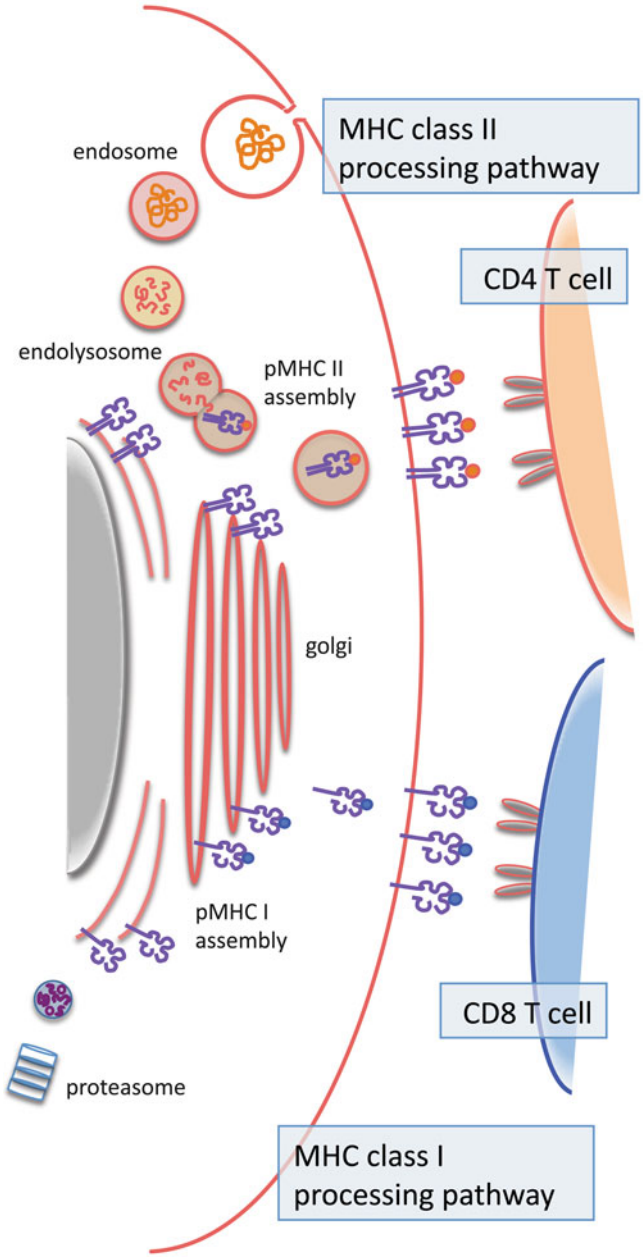

Fig. 2 Pathways of antigen processing and presentation. The MHC class II pathway processes and presents endocytosed extracellular proteins while the MHC class I pathway primarily processes and presents newly synthesized endogenous proteins and viral components. New proteins, including MHC class I molecules, are synthesized in the endoplasmic reticulum (ER), sampled, tagged with ubiquitin, and degraded by proteasomes in the cytosol, then further degraded to peptides in the TAP (transporter associated with antigen processing) molecules located in the ER. The correct folding of new MHC class I heavy chain with beta-2 microglobulin, and the loading of peptide, are mediated by the class I chaperone proteins, calnexin, calreticulin, and tapasin. The newly formed MHC class I-peptide complexes are transported via the Golgi apparatus to the cell surface where they embed in the cell membrane for presentation to CD8 T cells. New MHC class II molecules are also synthesized at the ER where the two chains are complexed with the "invariant chain" (that preserves the peptide-binding site) and pass through the Golgi apparatus to be released in lysosomal vacuoles within

may be a foreign, dangerous pathogen or protein, or it may be self protein that is continually sampled during normal immune surveillance. Internalized antigen is trapped in cell membrane vesicles that fuse with lysosomal vesicles within the cytoplasm where the proteins are broken down by proteolytic enzymes into shorter lengths of protein. These protein-rich vesicles then move towards the endoplasmic reticulum where they fuse with vesicles containing newly formed class II molecules. With the help of HLA-DM and the regulatory HLA-DO molecules, the proteins are trimmed to shorter peptides and transported by chaperone proteins that engage with the invariant chain occupying the peptide-binding cleft of the nascent class II molecules and exchange it for antigenic peptide. These class II molecules migrate via the Golgi body to the cell surface where they insert in the cell membrane and display the bound peptide for immune surveillance and recognition by CD4 T cells.

Immune surveillance is also required to identify and respond to unusual intracellular proteins, whether they are normal cell proteins that are misfolded, or altered by oncogenic processes, or viral components manufactured within infected cells. Foreign proteins located within the cytosol undergo degradation within the proteasome to produce short peptides that are transported by the TAP1 and TAP2 proteins to the peptide-binding cleft of MHC class I molecules. MHC class I molecules bearing antigenic peptides are similarly transported to and inserted into the cell membrane for presentation to CD8 T cells.

The T cell activation stage that follows depends upon the rules of MHC restriction dictating that T cells will normally only respond to APC-presenting antigenic peptides in the context of MHC class I or class II molecules that are identical to those expressed by the responding cells, i.e., "self" MHC. MHC restriction was demonstrated in elegant experiments with influenza-infected cells, where 'flu peptide-specific T cell lines could proliferate in vitro when cultured

Fig. 2 (continued) the cytosol. There, the invariant chain is shortened to become the CLIP (Class II associated invariant chain peptide). At the same time, extracellular protein antigens taken up by endocytosis are enclosed within endosomes and degraded to peptides under acidic conditions. The vacuoles fuse to form endolysosomes where the CLIP peptide is replaced by antigenic peptide to form the MHC class II-peptide complex, a process that is mediated and counter-regulated by the HLA-DM and HLA-DO molecules, respectively. This complex is transported and inserted within the cell membrane for presentation to CD4 T cells. Two additional pathways, termed autophagy and cross-presentation, enable presentation of endogenous (viral) proteins by MHC class II and exogenous proteins (endocytosed, virus-infected dead cells, for example) by MHC class I molecules. These strictly regulated pathways provide the initial help for generation of virus-specific cytotoxic T cells when viruses infect stromal cells that are not professional APC and therefore lack co-stimulatory molecules. The cross-presentation pathway may also be important for generation of alloantigen-specific cytotoxic T cells following transplantation

with virus-infected cells of the same MHC-type as the responding cells, but would not proliferate if the 'flu-infected cells were from a different MHC background. However, T cells are able to respond to APC with a different MHC background, and this is thought to be a result of cross-reaction where T cells with strong specificity for the bound peptide have a less stringent requirement for identical MHC. Similarly, when responder T cells have strong specificity for self-MHC, they may have less stringent specificity for the bound peptide which, in allogeneic transplantation, may be derived from the donor MHC molecules. Indeed, studies in naïve mice have shown that T cells able to respond to peptides derived from normal antigens exist at a frequency that is around 100-fold lower than the frequency of T cells able to respond to foreign MHC molecules.

Direct and Indirect Pathways of Allorecognition

Following allogeneic transplantation, there are three principle pathways for activation of T cells and generation of an immune response that culminates in rejection of the transplant [26]. The major stimulus of rejection is expression, by cells of the transplanted tissue or organ, of HLA molecules that are different from those of the recipient. Cell fragments bearing donor HLA molecules may be shed from the graft (as a result of the trauma of the donor operation) and are acquired by recipient APC, processed, and the resulting HLA peptides are presented in the context of recipient HLA molecules to the recipient's immune cells (Fig. 3). This is termed the indirect pathway and is analogous to the processing and presentation of normal antigens in a conventional immune response. In the direct pathway, recipient immune cells are able to recognize and respond to intact HLA expressed on donor cells, in contravention of the MHC restriction dogma. This is likely to be due to cross-reactive recognition of the donor HLA/peptide complex by recipient T cells. A third pathway, termed the semi-direct pathway, may operate under certain conditions where membrane exchange occurs between donor and recipient cells so that recipient APC present both processed HLA peptides and intact donor HLA; evidence for this pathway is limited to experimental models of transplantation and its contribution to clinical graft rejection remains to be studied [27].

The relative contribution to graft rejection by the direct and indirect pathways is unclear and may depend on the type of transplant. During the first hours and days following organ transplantation, donor dendritic cells in the grafted tissue are activated by the innate response to tissue injury and leave the graft, migrating via chemokine gradients to recipient draining lymph nodes where they encounter recipient lymphoid cells that are then able to mount a strong, acute rejection response via the direct pathway. At later stages, donor dendritic cells will have been replaced with recipient APC that are then likely to encounter donor HLA during normal immune surveillance, resulting in continual presentation of donor HLA peptides via the indirect pathway and contributing to a chronic rejection response [28].

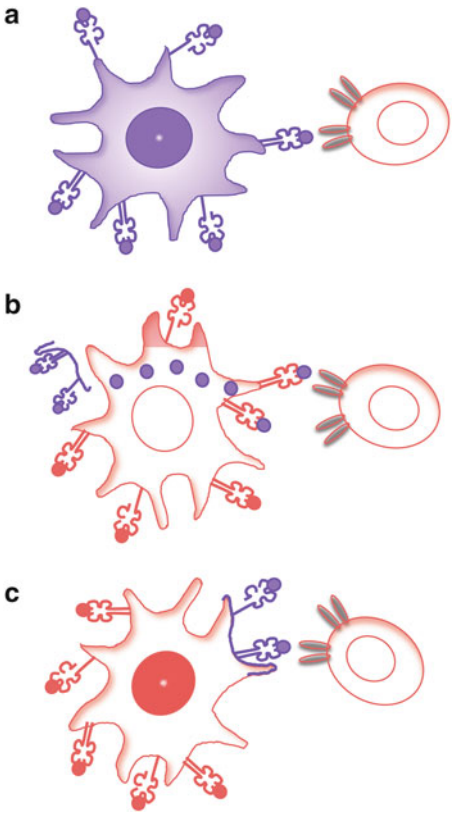

Fig. 3 Pathways of allorecognition. (**a**) In direct allorecognition, unique to transplantation, self-restricted recipient T lymphocytes (*pink*) recognize intact, donor MHC class I and class II molecules (by cross-reactivity) expressed on donor-derived APC (*blue*). (**b**) In indirect allorecognition, recipient CD4 T lymphocytes (*pink*) recognize and respond to donor MHC molecules that have been taken up and processed by recipient APC (*pink*), and presented as peptide fragments in the peptide binding cleft of recipient MHC class II molecules. (**c**) In the semi-direct pathway, donor-derived material in the form of secreted exosomes and membrane fragments bearing intact donor MHC molecules are acquired by recipient APC and presented intact to recipient T cells

In the case of regenerative medicine, the tissues differentiated from ESC or from iPSC will not, at early stages, be vascularized, will not contain dendritic cells, and are likely to express only low levels of HLA class I and no HLA class II molecules. This supports the widely held notion that ESC- and iPSC-derived tissues are not immunogenic. As the developing stem cell-derived tissue becomes vascularized following transplantation, the blood vessels and ensuing tissue dendritic cells will be host-derived; as such, they will neither initiate a rejection response nor will they act as a target. However, as the tissue differentiates and develops into

mature, functional tissue it will begin to express normal levels of HLA. Moreover, recipient APC infiltrating the transplanted stem cell-derived tissue will sample the tissues and process them, and present the resulting peptides which will include both normal tissue peptides to which the host will be tolerant, and HLA peptides of the stem cell donor type which, unless HLA-identical, are highly likely to generate a rejection response. Once rejection is initiated, the resulting inflammatory environment will contribute locally high levels of IFN-γ that have the capacity to upregulate the expression of HLA class I and II molecules, thereby increasing immunogenicity and enhancing rejection.

Mechanisms of Rejection

A wide range of cellular and molecular interactions collaborate in the immune response to an allograft and are characterized by a variety of pathological features identified in allograft biopsies, including perivascular and parenchymal cellular infiltration, complement deposition indicating the presence of alloantibodies, tissue infarction and/or fibrosis, and progressive vasculopathy with occlusion of the graft vasculature. In clinical transplantation these processes lead to acute rejection episodes over short time periods and require additional immunosuppressive therapy. They also initiate a chronic rejection process over a longer timeframe, with progressive attrition of graft function that is relatively resistant to current immunosuppressive strategies. In order to gain a better understanding of mechanisms leading to deterioration in graft function, research in transplant immunology makes use of experimental models of transplantation where a particular cell type or pathway has a dominant role. Such research has established that a pivotal component in graft rejection is recognition of graft antigens by CD4 T cells that are able to provide help for a range of mechanisms, the dominant ones being CD8 T cell-mediated cytotoxicity, B cell-mediated alloantibody production, and CD4 T cell-mediated inflammatory responses collectively termed delayed type-hypersensitivity (DTH) [29].

Delayed-Type Hypersensitivity

The DTH response, initiated by CD4 T cell activation following recognition of graft antigens, mirrors the nonspecific innate immune response with recruitment of macrophages, neutrophils and NK cells, and also additional lymphocytes. These cells secrete cytokines and chemokines that upregulate adhesion molecules, induce endothelial cell activation, and thereby increase both vascular permeability and coagulation, and leukocyte migration, promoting extensive tissue damage. Innate immune cells bearing Fc receptors engage in phagocytosis and antibody-dependent cellular cytotoxicity of target cells coated with antibody. NK cells are particularly likely to target stem cell-derived tissues because they express only low levels of HLA class I and may therefore be unable to engage the NK-inhibitory receptors, but instead may promote NK activity through engagement of the NKG2D family of activating receptors. CD4 T cells activated in response to organ transplantation may be directly activated and have specificity for intact HLA class II, or they may be HLA peptide-specific, as a result of encountering donor DC migrating from the graft that express high levels of HLA class II. The graft itself expresses class II target antigens on the vascular endothelium, and class II may be induced on other parenchymal structures. CD4 T cells themselves are not directly cytotoxic but they engage with target cells and it is easy to see how a localized nonspecific inflammatory response initiated by CD4 T cells can cause extensive graft damage. In the case of transplanted stem cell-derived tissues, it is harder to understand how CD4 T cells and the DTH response can play a direct role in rejection: stem cell-derived tissues do not harbor the highly antigenic DC and neither do they express class II target antigens. It is therefore appropriate to draw a distinction between the mechanism of tissue damage caused by nonspecific immune cells including macrophages, neutrophils, and NK cells as a result of the innate immune response, to which stem cells would be susceptible, and the almost indistinguishable DTH response mediated by the same cells but induced by class II-specific CD4 T cell activation.

Cytotoxic T Cells

CD8 T cell-mediated cytotoxicity is initiated, following conventional organ transplantation, when donor DC expressing high levels of both HLA class I and class II encounter recipient CD8 and CD4 T cells. The normal requirement for cognate CD4 T cell help for activation of CD8 T cells, however, is not fulfilled. Instead, help must be provided when CD4 and CD8 T cells directly recognize alloantigen through formation of a three-cell cluster comprising donor DC and recipient direct-pathway CD4 and CD8 T cells, so that help for CD8 T cells is non-cognate but linked [30]. Alternatively, donor-reactive CD8 T cells may be activated via the semi-direct pathway if recipient DC present donor HLA peptide to recipient indirect-pathway CD4 T cells, and on the same cell, present intact donor HLA class I acquired through membrane exchange; again, help is non-cognate but linked. Subsequently memory CD8 T cells may respond to class I expressed on the graft with reduced requirement for CD4 T cell help. Target cell death occurs when an immunological synapse, or supramolecular adhesion complex (SMAC) forms at the point of contact between the cytotoxic CD8 T cell (CTL) and the target cell [31]. The CTL undergoes structural alterations of its cytoskeleton with the result that

lytic granules within the CTL move to the SMAC. CTL perforins then puncture the target cell membrane, and granzymes enter the cell and, together with Fas ligand, activate caspase pathways which induce death of the target cell through apoptosis within around 20 min; the CTL is then released to kill further target cells.

In the case of transplanted stem cell-derived tissues, it is difficult to understand how, in the absence of donor dendritic cells, the initial CTL activation can occur. Indirect pathway CD4 T cells may be activated by presentation of donor HLA peptides derived from both class I and class II molecules, but since these peptides are unlikely to be present as conformational epitopes on the surface of the stem cells they do not present a target for CTL killing. Only the semi-direct pathway provides a plausible mechanism for generation of stem cell-specific CTLs.

B Cells and Antibody

Like CD8 T cells, B cells also require CD4 T cell help for maturation into antibody-forming plasma cells. In conventional transplantation, this may be provided in a non-cognate fashion when B cells interact with donor HLA on donor DC and receive linked help from CD4 T cells responding directly to donor HLA on the same DC [32]. Alternatively, recipient B cells may function as APC and internalize donor HLA acquired through their surface immunoglobulin B cell receptors. The B cell processes the donor HLA and presents peptides to indirect pathway CD4 T cells that respond by providing cognate help to the B cell. This second scenario, but not the first, is a possible mechanism for generation of antibodies to stem cells. B cell activation most likely takes place in the lymph nodes draining the transplantation site. Here, activated B cells undergo massive proliferation and antibody gene rearrangement in the lymphoid follicles of the secondary lymphoid tissue. This process of affinity maturation and isotype switching results in the production of high titer, high affinity antibody that is effective at binding to target tissue. On binding, the target tissue is then destroyed through complement activation and opsonization. A proportion of plasma cells migrate to and persist in the bone marrow, ready to respond rapidly to a repeat stimulus. Alternatively, some B cells, instead of becoming plasma cells, remain as memory B cells that can respond rapidly to antigenic challenge since they have already undergone affinity maturation.

Avoiding Allograft Rejection

Working on the assumption that stem cell-derived tissues will be susceptible to transplant rejection (unless they are derived from the recipient or from an HLA-identical donor), it is necessary to consider what strategies may be adopted to avoid the immune response [2]. One approach that has been used with some success in experimental stem cell transplantation is to implant the cells or tissues into immunologically privileged sites of the body. Such sites include the brain, protected by the blood–brain barrier, the cornea, the anterior chamber of the eye, and the testis, protected by the blood–testis barrier and by Sertoli cells. Tissues transplanted to these sites appear to be less susceptible to rejection than when transplanted to other sites of the body. Moreover, tissues from these protected sites are less rapidly rejected than other tissues when transplanted to alternative sites. Protective mechanisms include physical barriers provided by cellular tight junctions, reduced lymphatic drainage, the presence of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) produced by APC in the cornea, and protective proteins including the enzyme indoleamine 2,3-dioxygenase (IDO) that protects the developing semi-allogeneic fetus. Although several experimental studies have shown encouraging results with transplantation into the brain of cells differentiated from hESC, the potential for immune privilege at this site has been disregarded and, for the most part, researchers use immunodeficient or heavily immunosuppressed recipients [33, 34]. The degree of immunological privilege may be limited, as found in a study where additional intervention in the form of co-stimulatory blockade (CTLA4-Ig, anti-CD40L, anti-LFA-1) was required for survival of hESC transplanted into mouse testes [35].

The standard approach for avoiding rejection in clinical transplantation is twofold: where practically possible, tissues are closely HLA-matched, and recipients are treated with immunosuppressive drugs for the lifetime of the transplant. In many cases, the closer the HLA match between donor and recipient, the lesser the requirement for immunosuppression, although a study of transplantation of ESC-derived tissues in MHC-matched mice demonstrated that mHC antigens are sufficient to provoke graft rejection [36]. The challenge, for both organ transplantation and regenerative medicine, is to achieve a level of immunosuppression that prevents graft rejection but that maintains the individual's protection from infection and malignancy. In clinical transplantation, a wide range of approaches to immunosuppression are now available, which can be tailored according to the type of transplant and the level of immunosuppression required. Typically, organ transplant patients receive a calcineurin inhibitor (cyclosporine or tacrolimus) that may be combined with steroids and an anti-proliferative agent such as azathioprine or mycophenolic acid. Additional forms of immunosuppression include the m-TOR inhibitors, sirolimus and everolimus, and the so-called biological agents that include antibodies to lymphocyte cell surface molecules whose role is to deplete immune cell subpopulations (ATG or anti-thymocyte globulin, anti-CD3, anti-CD52, anti-CD20), to prevent T cell activation (anti-CD25), or to block

co-stimulatory activity (anti-CD40L, CTLA4-Ig). A range of these immunosuppressive agents is routinely used in recipients of bone marrow transplants or pancreatic islets, and it is likely that they might be used in a similar fashion in regenerative medicine.

An alternative approach to pharmaceutical immunosuppression is to harness the body's own resources for regulating the immune system to prevent inappropriate immune responses. Regulatory, or suppressor, T cells (Tregs) are CD4 T cells, which co-express the cell surface CD25 molecule (the IL-2 receptor α-chain) and the intracellular FoxP3 transcription factor, and maintain a state of immunological unresponsiveness to self tissues and proteins in normal individuals and prevent the development of autoimmune disease [25]. Naturally occurring Tregs arise in the thymus and appear to mediate their suppressive function by secretion of the immunosuppressive cytokine, IL-10, and also by the unusual, constitutive expression of CTLA-4 which binds to the ligands CD80 and CD86 on APC with higher affinity than the lymphocyte co-stimulatory molecule CD28, thereby transmitting regulatory in place of activatory signals to both DC and lymphocytes. Experimental studies have found that natural Tregs may be isolated, expanded in vitro in the presence of IL-2 and transferred to recipients of organ transplants where they are able to mediate donor-specific suppression [37, 38]. This approach is undergoing phase I clinical trials with the goal of inducing donor-specific unresponsiveness following tissue transplantation, thus avoiding the side effects associated with immunosuppressive drug treatment [39]. It is reasonable to suggest that this course of action may also be effective for regenerative medicine, although it is highly labor-intensive.

Alternative approaches for inducing donor-specific tolerance may be more practicable in stem cell transplantation and the potential to generate cells and tissues of different lineages from hESC and iPSC promotes the possibility of inducing stable tolerance by creating a state of mixed hematopoietic chimerism, where donor and recipient hematopoietic cells coexist and are unresponsive to each other's dissimilar HLA types. This approach is based on clinical evidence of tolerance to organ allografts in patients who have undergone myeloablation and hematopoietic reconstitution to treat a blood malignancy, and who have also received a renal transplant from the same (allogeneic) individual who donated the bone marrow [40]. A number of patients were able to discontinue immunosuppression with no detriment to renal transplant function, and this was associated with detectable hematopoietic chimerism in the peripheral blood. Current research in this field is focusing on clinically appropriate strategies for facilitating the induction of mixed hematopoietic chimerism in patients who require organ or tissue transplants but who are not suffering from blood malignancies [41]. In the field of regenerative medicine, this type of donor-specific tolerance might be achieved by creating both the replacement tissue, for example, pancreatic islets, to treat the patient's underlying disease, as well as hematopoietic tissue to induce mixed chimerism, from a single source of hESC or iPSC.

Genetic modification is an experimental strategy that may be used to minimize or reduce the immunogenicity of tissues for transplantation, or of hESC and iPSC. Proof of principle has been obtained in experimental organ transplantation where adenovirus or lentivirus-mediated overexpression of immunosuppressive gene products such as CTLA-4 and IL-10 has been shown to ameliorate graft rejection [42–44], while another promising pathway is inducing overexpression of protective, anti-apoptotic genes such as A20 and bcl-xL [45, 46]. Of more direct relevance to stem cell transplantation, genetic manipulation of hESC and iPSC is relatively straightforward and has enabled preliminary studies in attempting to reduce their immunogenicity by, for example, preventing expression of HLA class I using RNA interference. Mouse recipients of HLA class I-knockdown hESC showed reduced T cell activation and alloantibody production compared with recipients of wild-type hESC, but were not more susceptible to NK activity [47].

Immunogenicity of ESC and iPSC

There has been a long-held assumption, possibly based on the fact that semi-allogeneic fetuses normally survive in utero, that ESC are not immunogeneic but are, instead, immunologically privileged cells. It is only in the past decade that studies have formally questioned this assumption and have set out to determine whether ESC express immunogeneic markers or have immunosuppressive properties, and very few studies have considered the immunogenicity of iPSC. Since ESC and iPSC develop into normal mature tissues, it is reasonable to assume that they have the potential to express HLA, and will begin to express these markers as they differentiate into functional tissues. One of the first studies to look for HLA expression found that hESC express low levels of HLA class I and no detectable class II, and as they differentiate into embryoid bodies, only class I expression increases [48]. At the same time, Fandrich et al. found that rat ES-like cells injected into allogeneic hosts were not rejected and instead contributed to chimerism, resulting in long-term acceptance of a subsequent allogeneic heart graft [49].

While the absence of HLA expression is consistent with avoidance of the adaptive immune response, it raises the possibility that stem cell-derived tissues may fail to become established after transplantation because they are susceptible to immune surveillance by NK cells. It has long been acknowledged that NK cells kill target cells through recognition of the absence of HLA class I on the target cells, a mechanism that possibly evolved to protect against oncogenes and viruses that avoid the attention of CD8 T cells by downregulating HLA class I expression by the host cell. The mechanism

of recognition involves two sets of NK receptors and the absence of class I alone is not sufficient for NK activation [50, 51]. The inhibitory NK receptors include killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), both expressing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their intracytoplasmic tails, and their ligands on target cells are predominantly the classical HLA or MHC class I molecules and Ly49 molecules in mice. They also express numerous activating or coactivating NK receptors that induce common intracellular signaling pathways through immunoreceptor tyrosine-based activating motifs (ITAMs), the NKG2D receptor complexes, and the CD244 receptor system. The activating receptors initiate signaling when they engage with nonclassical HLA class I-like molecules including MICA, MICB, and HLA-G, but co-expression of classical HLA class I engages the KIRs and terminates intracellular calcium mobilization, cytoskeletal rearrangement of perforins and granzymes, cytokine production and proliferation of the NK cells.

Further evidence for the immunogenicity of ESC continues to accumulate [52–54], together with reports demonstrating their tolerogenic potential [33] and their reduced expression of MHC compared with adult cells [55]. Evidence that HLA expression by both ESC and iPSC is regulated by DNA methylation and epigenetic silencing is consistent with the possibility that differentiated stem cells have the potential to express levels of HLA normally found in mature, functional tissue, implying that strategies to improve the survival of stem cell transplants should continue to be explored [56]. Finally, of particular relevance to iPSC-derived tissues, the recent paper that demonstrated failure of both syngeneic and allogeneic iPSC to form teratomas in mice cannot be ignored. Rejection of teratomas appeared to be associated with localized immune responsiveness, while syngeneic (but not allogeneic) ESCs were tolerated and successfully formed teratomas [5]. The possible contribution of viral elements to immunogenicity was negated by the fact that iPSC created by a nonviral, episomal approach were also rejected. Yamanaka's group suggested that the non-differentiated iPSC expressed a different set of tumorigenic markers and were appropriately rejected by the host's immune defense mechanisms; non-differentiated cells would not be used for regenerative medicine for this very reason, and in any case, transplantation of HLA-matched ESC should instead be considered [57].

Stem Cell Banking for HLA-Matched Transplantation

A bank of HLA-typed, pluripotent stem cell lines will be an invaluable resource for research into heritable diseases and for regenerative medicine. Selection of a cell line from such a resource requires an understanding of HLA typing, and insight into HLA matching criteria appropriate to the purpose for which the cell line will be used.

HLA glycoproteins have evolved from common ancestral genes, through gene duplication, translocation of gene fragments, equal and unequal recombination and point mutation into a highly polymorphic system comprising multiple HLA loci and their constituent alleles. As a result, different HLA types (the combination of co-expressed maternally and paternally inherited HLA glycoproteins at each locus) have arisen, driven by environmental selection and survival mechanisms resulting in a high degree of diversity between individuals within and (more notably) between ethnic groups. Simple Mendelian genetics for codominant inheritance dictate that each individual possesses any two of the many hundreds of possible HLA alleles at each locus, having inherited one HLA haplotype from each parent. Siblings have a one-in-four chance of sharing two HLA haplotypes (HLA identical) or no haplotypes (HLA-mismatched). Occasionally, the parents may share a common HLA allele, in which case their offspring would be homozygous at that HLA locus and siblings would be HLA identical at one or more loci.

A wide range of epitopes at each HLA locus (e.g., HLA-A, HLA-B) can be recognized by alloantisera and monoclonal antibodies, and further polymorphism is identifiable at the DNA and amino acid sequence level that is indistinguishable using serological methods, but carries important biological significance. In general, alloreactive B cells exposed to alloantigen produce antibodies that recognize tertiary epitopes on the protein surface, whereas alloreactive T cells are able to distinguish small differences in amino acid sequence either through direct recognition of the conformational protein or indirect recognition of foreign peptide presented by self HLA.

Clinical experience gained over the last 50 years from allogeneic bone marrow, peripheral blood stem cell, umbilical cord blood stem cell, and solid organ transplantation has established the efficacy of reducing the immunogenic potential of transplanted tissue through minimizing the number of mismatched HLA alleles between donor and recipient. Close donor and recipient HLA matching correlates strongly with a reduced incidence of GVHD and graft rejection following allogeneic HSC transplantation, and rejection-free long-term graft survival following kidney transplantation [58].

HLA Matching

Recognition of the contribution of donor-recipient HLA matching to improved outcome following HSC and kidney transplantation has promoted the establishment of national and international strategies to address the logistics of providing HLA-matched tissue for unrelated donor-recipient pairs. National HSC donor registries have combined to form the international "Bone Marrow Donors Worldwide" (BMDW)

registry that now contains information for over 20 million potential stem cell donors, representative of all major ethnic groups. BMDW is thus able to identify suitably HLA-matched unrelated donors for the majority of HSC recipients. Furthermore, national and international registries, such as Eurotransplant, UNOS, and NHS-Blood and Transplant (UK), comprising algorithms and databases of HLA-typed potential recipients, have been established to facilitate organ allocation and improve transplant outcome.

Although achieving a perfect allelic donor-recipient match at all HLA loci (HLA identical) confers the greatest advantage on transplant outcome, in clinical practice ensuring a complete HLA match is not always possible. However, not all HLA locus mismatches contribute equally to the graft rejection response and for unrelated solid organ transplantation, matching at the HLA-A, -B, and -DR loci is considered practicable [59]. The degree of mismatch between donor and recipient is represented as "0.0.0" for a "zero mismatch" which is associated with a low incidence of graft rejection, while a donor that is mismatched for both HLA specificities at each locus is represented as "2.2.2" (or "six antigen mismatch") and this is associated with an increased incidence of graft rejection and post-transplant complications often associated with increased requirement for immunosuppression.

In contrast, HLA matching requirements for allogeneic HSC transplantation are more stringent because of the presence of immunocompetent alloreactive T cells in the transplanted donor tissue and in the recipient immune system that confer propensity for both GVHD and graft rejection, respectively. It is therefore essential to use high resolution typing techniques to ensure close HLA matching at the allelic level, with no more than a single mismatch permitted between donor and recipient at the HLA-A, -B, -C, -DR, and -DQ loci combined.

Many patients awaiting allogeneic tissue transplantation are immunologically sensitized to foreign HLA and harbor T and/or B memory cells acquired through previous exposure to HLA alloantigens. The most common route of exposure is through pregnancy, when paternal HLA antigens expressed on the semi-allogeneic fetus are recognized by the maternal immune system. Similarly, previous blood transfusion and tissue transplantation are common sources of HLA-specific allosensitization. HLA allosensitization is identified by the presence of circulating IgG HLA-specific antibodies in the patient's serum and this acts as a marker for the presence of memory T and B cells. While the majority of primary immune responses to HLA alloantigen are readily attenuated by modern immunosuppression, secondary responses attributed to memory T and B cells are often refractory to suppression using conventional agents, resulting in uncontrolled graft destruction. In the case of HLA-mismatched HSC and solid organ transplantation, it is therefore essential to avoid donor mismatches to which the recipient is sensitized.

Populating a Pluripotent Stem Cell Bank

The considerations of HLA matching and sensitization described for organ and HSC transplantation are likely to be relevant, to some degree, to regenerative medicine and stem cell therapy. Ideally, adult pluripotent stem cells would be derived directly from the intended recipient and used for autologous transplantation, but this is currently impracticable.

An alternative approach is to create national and international stem cell banks, containing a limited number of highly selected pluripotent stem cell lines that can undergo large-scale expansion and be used to provide sufficient differentiated tissue to treat large numbers of potential beneficiaries. Such tissue transplants would be allogeneic and therefore likely subject to allorecognition and rejection by the host immune system, necessitating strategies for overcoming the immunological barrier, including HLA matching and immunosuppression.

Pluripotent stem cells can be derived from surplus blastocysts donated after in vitro fertilization and ESC lines are established with capacity for massive expansion and differentiation into functional somatic tissue. Human embryonic stem cells (hESC) offer great potential for therapeutic use with the formation of stem cell banks containing HLA-typed cell lines to provide a resource with which to deliver large-scale therapy. However, the diversity of HLA types in the potential donor and recipient populations confers a practical challenge that would restrict their therapeutic efficacy. Taylor et al. undertook studies to estimate the number of hESC lines required to achieve a 0.0.0 HLA mismatch showed that a bank of 150 random UK donors would provide ABO compatible, zero HLA-A, -B, and -DR-mismatched tissue for only 18 % of the population and increasing the size of the bank to 1,500 cell lines would match 32 % of potential UK recipients. If the bank was restricted to include HLA-A, -B, and -DR homozygous cell lines, a relatively small panel of only ten HLA homozygous donors selected from a population of 10,000 donors would provide maximum clinical utility and zero HLA-mismatched tissue for 38 % of UK recipients [60].

The use of iPSC for populating such a stem cell bank would overcome ethical considerations of deriving cell lines through the destruction of human embryos. Taylor and colleagues have recently taken a practical approach to discovering the feasibility of establishing a stem cell bank comprising HLA-typed iPSC lines. Similar studies from Japan and the USA have recently confirmed Taylor's earlier findings that a relatively small number of clinically useful cell lines would be required to populate such an iPSC bank in Japan, where

the ethnic mix is limited, and in the USA, where there is extensive ethnic diversity [57, 61, 62]. However, all of the studies concluded that this would be a labor-intensive exercise, requiring the screening of many tens of thousands of volunteers in the hope of identifying by chance the required HLA types for populating the banks. In contrast, Taylor and colleagues' practical methodology was to first compile a list of all theoretical homozygous HLA types in the UK and rank them according to frequency, based on a representative population consisting of 10,000 consecutive UK deceased organ donors [4]. This list would provide an HLA match for all potential UK recipients. The list was then used to interrogate the BMDW database of around 20 million volunteer potential stem cell donors to find a minimum of ten donors that matched each of the homozygous HLA types; this limit was chosen to ensure a greater chance that volunteer donors would be of blood group O (universal donor blood type). This yielded a list of 236 ranked homozygous HLA types (out of 405 theoretical types), all of whom exist as potential donors who could be approached for consent to donate skin cells, for example, to populate the iPSC bank. The top 150 of these 236 donors would provide a zero HLA mismatch for 93.16 % of the UK population [4].

Recent research thus supports the anticipation that, providing the practical and technological hurdles of directed differentiation and expansion of ESC and iPSC, can be overcome; there are robust approaches that will minimize the immunological challenges of regenerative medicine.

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Part II

Pluripotent Stem Cells

Urodele Limb Regeneration: Mechanisms of Blastema Formation and Growth

David L. Stocum

Introduction

The ability of larval and adult urodeles to regenerate the complex spatial organization of amputated limb segments has been known since the experiments of Spallanzani [1] in the sixteenth century. Limb regeneration is accomplished by the histolysis of tissues at the amputation site to release resident stem cells, as well as differentiated cells that undergo dedifferentiation to progenitor cells. These cells accumulate under the wound epidermis to form a regeneration blastema that grows and self-organizes into the tissue patterns and morphological shapes of the amputated structures. Blastema formation and growth requires early signals mediated by amputation and by the wound epidermis that lead to histolysis, as well as subsequent interactions between regenerating nerve axons and wound epidermis that drive blastema cell accumulation and proliferation. These signals and interactions are the subject of this chapter.

Stages of Limb Regeneration

Figure 1 illustrates the stages of a regenerating urodele (larval Ambystoma) limb [2]. Within 24 h after amputation (depending on limb size), epidermal cells migrate over the wound surface to provide a thin epithelial sheet that thickens within 3–4 days to form an apical epidermal cap (AEC) several layers thick in the center of the amputation surface. Undifferentiated mesenchymal cells derived by the histolysis of dermal, nerve sheath, and muscle and skeletal tissues accumulate under the AEC to form the accumulation blastema or early bud. The outer layers of the AEC are protective, whereas its basal layers appear to be equivalent to the outgrowth-promoting apical ectodermal ridge (AER) of amniote embryonic limb buds [3].

Regenerating motor, sensory and sympathetic axons, as well as capillaries, penetrate into the forming blastema, with sensory axons reaching the AEC by the early bud stage. Eventually regenerating motor axons will innervate developing muscle, and sensory and sympathetic axons will innervate the skin, skeletal structures, and blood vessels. Following reinnervation of the AEC, the early bud grows rapidly to a conical medium bud stage. As the blastema continues to grow through late bud and redifferentiation stages, its cells self-organize the patterns of differentiation that replicate the amputated limb parts. While the growth and differentiation of the blastema appears similar to embryonic limb bud development, the requirements for mesenchymal proliferation in the two are not the same. Blastema cell proliferation is dependent on signals generated by interaction between the AEC and the regenerating nerves, whereas proliferation of limb bud mesenchymal cells relies solely on signals from the AER, the counterpart of the AEC in the regenerating limb.

The tissues of the new limb parts derived from the blastema redifferentiate in continuity with their parent tissues. Differentiation and morphogenesis of the blastema take place in a proximal to distal and anterior to posterior sequence, except that in the proximodistal (PD) axis, the digits begin differentiation prior to the carpals or tarsals. Differentiation in the dorsoventral (DV) axis appears to take place simultaneously across the axis. The remainder of the regenerative process consists of growth to match the size of the unamputated limb.

Light and electron microscopic studies have suggested that myofibers of the limb cellularize to produce mononucleate cells that dedifferentiate to form blastema cells [4]. Recently, using the satellite cell-specific transcription factor Pax-7 as a marker, satellite cells were shown to contribute to the limb regeneration blastema and develop into new muscle

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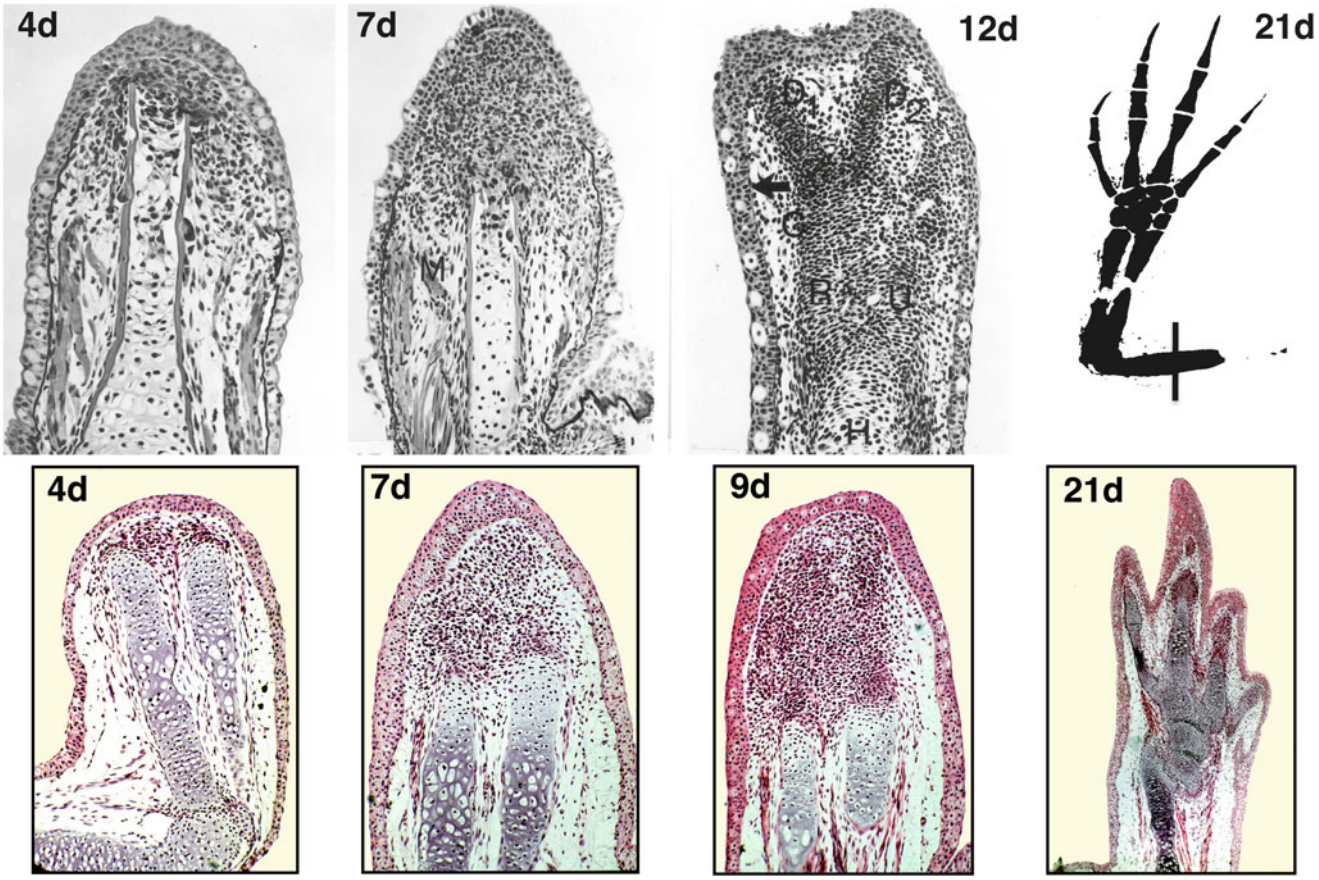

Fig. 1 H & E-stained longitudinal sections of larval Ambystoma forelimbs regenerating from (**a**) the mid-humerus and (**b**) the distal tips of the radius/ulna. Sections of upper arm regenerates are shown for 4, 7, and 14 days postamputation, along with a methylene-blue-stained whole mount of a 21-day regenerate (*line* indicates amputation level). Sections of distal R/U regenerates are shown for 4, 7, 9, and 21 days postamputation. The length of time required to regenerate from these two levels is approximately the same. The blastemas pass through an initial accumulation stage (4 days, early bud), then a conical (7 days, medium bud) stage, and followed by stages of progressive differentiation and morphogenesis (late bud and fingerbud)

[5]. By grafting individual limb tissues from transgenic GFP-expressing axolotls in place of their unmarked host counterparts, Kragl et al. [6] showed that blastema cells derived from muscle, fibroblasts, cartilage, and Schwann cells retained an epigenetic memory of their origin and redifferentiated into their preexisting parent cell types but that dermal fibroblasts also undergo transdifferentiation into chondrocytes and tenocytes, confirming earlier results [7–9]. Dermal fibroblasts contribute nearly half the blastema cells of the amputated axolotl limb and contribute the majority of the regenerated chondrocytes [10]. In the axolotl, dermis represents ~19 % of the limb tissue, while cartilage represents 6 %. However, grafts of triploid dermis to diploid limbs contributed an average of 43 % of the blastema cells and grafts of triploid cartilage only 2 %. Thus, dermal fibroblasts are overrepresented in the blastema by more than a factor of 2, and cells from cartilage are underrepresented by a factor of 3.

These results show that cartilage and muscle of the regenerate are each derived from two sources, cartilage from dedifferentiated chondrocytes and transdifferentiated fibroblasts and muscle from dedifferentiated myofibers and satellite stem cells. However, it is unclear what the proportional contributions of dedifferentiated mononucleate cells vs. satellite cells to regenerated muscle might be. Satellite cells could be the sole source of muscle in regenerating limbs, or the proportions of satellite cells and dedifferentiated myofibers might change with age, metamorphosis, or species of animal. Inducible genetic marking of myofibers and/or genetic ablation of satellite cells in transgenic animals might provide answers to these questions. Assuming that myofiber dedifferentiation is real, regeneration of the urodele limb involves the simultaneous use of four different mechanisms: dedifferentiation and redifferentiation, differentiation of adult stem cells, transdifferentiation, and regrowth of single cells, in this case axons of neurons.

Mechanisms of Blastema Formation

Early Signals: IP3, DAG, and Ionic Flux

Two early signals for blastema formation are inositol triphosphate (IP3) and nitric oxide (NO). IP3 and diacylglycerol (DAG) are the products of PIP2, which in turn is derived from inositol. IP3 synthase, a key enzyme for the synthesis of inositol from glucose-6-phosphate, is highly upregulated during blastema formation in regenerating axolotl limbs [11]. IP3 stimulates a rise in cytosolic Ca^{2+} that results in the localization of protein kinase C (PKC) to the plasma membrane, where it is activated by DAG and regulates transcription. During blastema formation, there is a general downregulation of proteins involved in Ca^{2+} homeostasis, which suggests that IP3 might signal a rise in cytosolic Ca^{2+} in regenerating limbs by this mechanism [11]. Other studies have shown that IP3 is generated from PIP2 within 30 s after amputation in newt limbs and that beryllium inhibition of IP3 formation prevents blastema formation [12]. PKC rises to a peak by the accumulation blastema stage [13]. The enzyme that catalyzes NO synthesis, nitric oxide synthase 1 (NOS1), is strongly upregulated in the wound epidermis of amputated axolotl limbs by 1 day postamputation [11]. NO has a wide variety of signaling functions [14]. It is produced by macrophages and neutrophils as a bactericidal agent and has a role in activating proteases known to be important effectors of histolysis in regenerating limbs.

Na^+ influx in the amputated newt limb and H^+ efflux in the amputated tail of *Xenopus* tadpoles generate ionic flow across the skin and wound epidermis. Na^+ influx is via sodium channels [15], while H^+ efflux is driven by a plasma membrane ATPase in the epidermal cells [16]. H^+ efflux is likely to be important in limb regeneration as well, since a gene encoding a v-ATPase was the most abundant clone in a suppressive subtraction cDNA library made from dedifferentiating axolotl limb tissue [17]. These ion movements are obligatory for regeneration, since drug-induced inhibition of either Na^+ in limbs or H^+ movements in tails during the first 24 h or so after amputation results in failure of blastema formation [16, 18].

The timing of IP3 and DAG synthesis, the probable rise in cytosolic Ca^{2+}, the upregulation of NOS1, and the subsequent movements of Na^+ and H^+ across the wound epidermis suggest that these molecules and ions may be the earliest signals that initiate blastema formation. The details of how their activity is translated into histolysis and dedifferentiation, however, are unknown. Campbell et al. [19] have carried out a comparative microarray analysis of gene activity between the epidermis that re-covers limb radial skin wounds and the epidermis that re-covers amputation wounds. They identified 125 genes with higher expression in the wound epidermis of amputated limbs, indicating that these genes are specific to a limb regeneration response as opposed to general wound healing. Quantitative PCR data showed significantly higher expression and changes in expression overtime for several genes, including a gene encoding an mRNA similar to a methyltransferase. Study of the function of genes revealed in this way will help further understand how the wound epidermis promotes the early events of regeneration.

Apoptosis May Be Obligatory to Initiate Limb Regeneration

Apoptosis is minimal in the axolotl and newt limb 24 h after amputation and beyond [20, 21], but observations have not been made earlier than this. A transient wave of apoptosis has been shown to occur in the first 24 h after amputation of Xenopus tadpole tails [22] and tails of the knifefish *Apteronotus leptorhynchus* [23, 24]. This apoptosis is obligatory for Xenopus tail regeneration because when prevented by caspase inhibitors, regeneration fails. Whether apoptosis is obligatory for knifefish tail regeneration is unknown, but apoptosis of neurons remains elevated at the regenerate/stump interface, suggesting that integration of new neurons into circuits at that level requires substantial cell pruning [23]. Whether there is a relationship between apoptosis and ionic currents is unknown. Apoptosis and its potential role in regeneration should be examined in regenerating urodele limbs.

Histolysis

The cells that form the blastema, whether stem cells or progenitors derived by dedifferentiation, are released from their tissue organization by degradation of the extracellular matrix (ECM) and cellularization of myofibers, a process called histolysis. The liberated cells undergo dedifferentiation to mesenchyme-like blastema cells with large nuclei and sparse cytoplasm that exhibit intense DNA, RNA, and protein synthesis. Histolysis and dedifferentiation are visible histologically within 2–3 days postamputation in larval urodeles and within 4–5 days in adults [4].

Degradation of tissue ECM is achieved by acid hydrolases and matrix metalloproteinases (MMPs) [25, 26]. Acid hydrolases identified in regenerating urodele limbs include cathepsin D, acid phosphatase, β-glucuronidase, carboxyl ester hydrolases, and *N*-acetyl-glucosaminidase. Osteoclasts degrade bone matrix via hydrochloric acid, acid hydrolases, and MMPs. Upregulated MMP transcripts include *MMP-2* and *MMP-9* (gelatinases) and *MMP-3*/*10a* and *b* (stromelysins) [27–29]. In the newt limb, the basal layer of the wound epidermis transcribes *MMP3*/*10a* and *b*, as well as a novel

MMP with low homology to the others [30]. Chondrocytes express *MMP-2* and *MMP-9* transcripts in the newt limb, and these enzymes are proposed to diffuse outward from the degrading skeletal elements [30]. The importance of MMPs to regeneration is underscored by the failure of blastema formation in amputated newt limbs treated with the MMP inhibitor GM6001 [31].

An important function of the MMPs encoded by the basal layer of the wound epidermis is thought to be the prevention of basement membrane reassembly beneath it, thus maintaining communication between the wound epidermis and subjacent mesenchymal cells. Loss of such communication, either by removing the wound epidermis [32] or conditions under which a pad of connective tissue becomes prematurely interposed between wound epidermis and blastema cells [33], inhibits regeneration. MMPs from the basal wound epidermis might also diffuse into the underlying tissues to participate in the degradation of other ECM components. Histolysis continues through the medium bud stage of blastema growth and then ceases due to the activity of tissue inhibitors of metalloproteinases (TIMPS) [34]. TIMP1 is upregulated during histolysis when MMPs are at maximum levels and exhibits spatial patterns of expression congruent with those of MMPs in the wound epidermis, proximal epidermis, and internal tissues undergoing disorganization.

The levels and temporal expression patterns of the MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, and MMP-13 proteins during blastema formation are different in regeneration-competent wild-type axolotls vs. regeneration-deficient *short-toe* axolotls and Xenopus froglets [35], suggesting that these differences play a role in the abnormal histolysis and thus availability of cells for dedifferentiation noted in regeneration-deficient Xenopus limbs [36].

Dedifferentiation

Dedifferentiation involves the epigenetic reprogramming of limb cells that alters their global pattern of transcriptional activity to produce a less differentiated state. The activity of differentiation genes is suppressed and genes associated with stemness (*msx-1*, *nrad*, *rfrng*, *Notch*) are activated [37]. Carlson [38] showed that inhibition of this transcriptional shift by actinomycin D does not affect histolysis, but does prevent or retard dedifferentiation, leading to regenerative failure or delay. This suggests that at least some of the protease expression involved in histolysis is not regulated at the transcriptional level, but that dedifferentiation is regulated primarily at the transcriptional level. Dedifferentiated cells express a more limb bud-like ECM in which type II collagen synthesis and accumulation are reduced, collagen I synthesis is maintained at a steady level, and fibronectin, tenascin, and hyaluronate accumulate [39].

Mammalian adult fibroblasts have been reprogrammed to pluripotency (induced pluripotent stem cells, iPSCs) equivalent to that of embryonic stem cells (ESCs) by transfecting them with four of six transcription factor genes (*Oct 4*, *Sox 2*, *c-myc*, *Klf-4*) [40] and *Oct 4*, *Sox 2*, *Nanog,* and *Lin 28* [41]. Three of these six genes (*klf4*, *Sox2*, *c-myc*), but not the others, are upregulated during blastema formation in regenerating newt limbs and also during newt lens regeneration [42]. Upregulation of the microRNA-processing protein Lin 28 was detected during blastema formation in regenerating axolotl limbs [11]. Further studies are needed to comprehend the role of these and other transcription factors, as well as understanding the changes in promoter and histone methylation, histone acetylation, and microRNAs that determine the extent and course of epigenetic erasure and rewriting involved in dedifferentiation, redifferentiation, and transdifferentiation. Studies on such changes have begun for the transdifferentiation of newt lens regeneration [43, 44] but have not yet been reported for limb regeneration beyond the observation that the long-range limb specific *shh* enhancer, a conserved sequence called mammals-fishes-conserved-sequence 1 (MFCS1), which is located in a noncoding region of the LMBR1 gene [45], is hypermethylated in Xenopus vs. moderately methylated in the axolotl and newt [46]. This hypermethylation is associated with the lack of *shh* expression on the posterior side of the blastema and regeneration deficiency of the amputated Xenopus limb.

Blastema Cell Accumulation

The AEC directs the migration of mesenchymal cells to form the accumulation blastema beneath it [4]. This was shown by experiments in which shifting the position of the AEC laterally caused a corresponding shift in blastema cell accumulation, and transplantation of an additional AEC to the base of the blastema resulted in supernumerary blastema formation. Nerves that innervate the AEC do not appear to physically guide blastema cells, since similar experiments on aneurogenic limbs also resulted in eccentric blastema formation. The redirected accumulation of blastema cells under an eccentric AEC may be due to the migration of the cells on repositioned adhesive substrates produced by the AEC. TGF-β1 is strongly upregulated during blastema formation in amputated axolotl limbs [47]. A target gene of TGF-β1 is fibronectin, a substrate molecule for cell migration that is highly expressed by basal cells of the wound epidermis during blastema formation [3, 11]. Inhibition of TGF-β1 expression by the inhibitor of SMAD phosphorylation SB-431542 reduces fibronectin expression and results in failure of blastema formation [47], suggesting that fibronectin produced by the AEC may provide directional guidance for blastema cells.

The Structure and Function of the Wound Epidermis Is Nerve-Dependent

Neither denervation nor deprivation of wound epidermis prevents histolysis, dedifferentiation, and entry of blastema cells into the cell cycle, but blastema cells do not accumulate under the wound epidermis and disappear (Fig. 2). Thus, transection of the brachial nerves at the level of the shoulder or preventing the formation of a wound epidermis by inserting the amputated limb tip into the coelom or grafting full thickness skin over the amputation surface prevents formation of the accumulation blastema [32, 48–51], showing that both nerve and AEC are required for its formation.

Maintenance of AEC structure and function is dependent on innervation by regenerating axons [52], but the nature of this dependency has not been clear. In experiments making a wound in the skin of axolotl limbs, the regenerated epidermis developed a thickening comparable to the AEC that subsequently regressed. However, if a nerve was deviated into the wound, the epidermal thickening was maintained, and a blastema-like growth was formed from the underlying tissues [53]. This growth is equivalent to a blastema formed by amputation in terms of morphology and expression of MMP-9, Msx-2, Hox A-13, Prx-1, and Tbx-5 [54]. In other experiments on amputated axolotl limbs, the nerves were shown to induce expression of the zinc finger transcription factor *Sp9* in the wound epidermis, which is associated with epidermal dedifferentiation [55]. Collectively, the results imply that in a normally innervated limb, the AEC forms independently of the nerve, but its structure and function are not maintained unless the AEC becomes innervated by regenerating axons, an implication that fits the timing of AEC formation and initiation of axon regeneration into the wound epidermis during the formation of the accumulation blastema.

A Neural-Epidermal Circuit Is Required for Blastema Cell Proliferation

A great deal of evidence indicates that blastema growth requires the action of a signaling circuit between limb nerve axons and the wound epidermis/AEC.

Effects of Denervation and Deprivation of Wound Epidermis/AEC

During formation of the accumulation blastema, the DNA-labeling index of blastema cells is high, indicating that a substantial percentage of dedifferentiating cells enter the cell cycle. However, the frequency of cells undergoing mitosis is very low (~0.4 %), suggesting that most blastema cells temporarily arrest in G_2 after completing DNA replication [50,

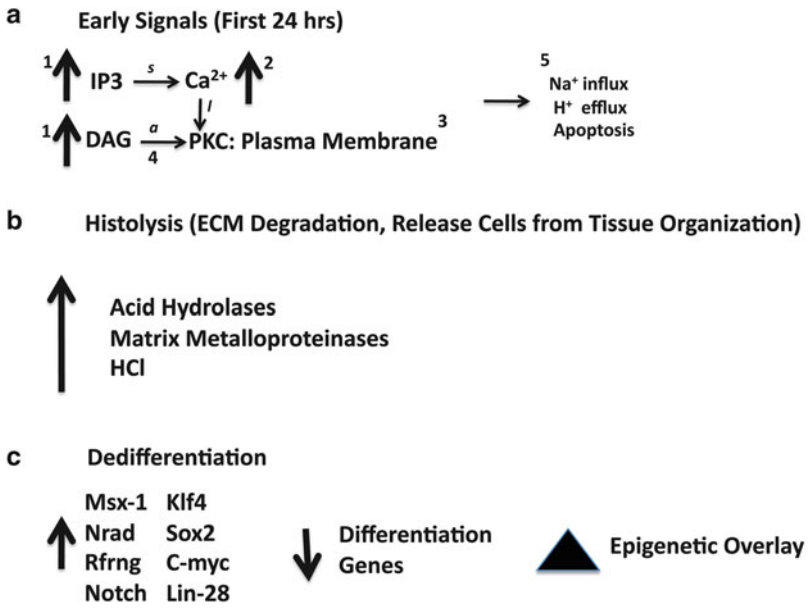

Fig. 2 Elements of the mechanism of blastema formation. (**a**) Early signals within the first 24 h postamputation. 1–5 are steps in signaling (see text for details). *s* stimulates, *a* activates, *l* localizes. (**b**) Molecules active in regulating the degradation of extracellular matrix (ECM) during histolysis of tissue organization. (**c**) Dedifferentiation of liberated cells. A group of genes associated with stemness (*left*) is upregulated, while differentiation genes are downregulated (*middle*). The *triangle* (*right*) represents change in the pattern of histone acetylation and methylation, DNA methylation, microRNAs, and Polycomb and Trithorax proteins constituting the epigenetic overlay that stabilizes transcription

56–60]. Further indirect evidence for G_2 arrest is the strong upregulation of the ecotropic viral integration factor 5 (Evi5) throughout formation of the accumulation blastema in regenerating axolotl limbs [11]. Evi5 is a centrosomal protein that accumulates in the nucleus during early G_1 in mammalian cells and, in concert with Pin1, prevents them from prematurely entering mitosis by stabilizing Emi1, a protein that inhibits cyclin A degradation by the anaphase-promoting complex/cyclosome [61, 62]. At G_2, Emi1 and Evi5 are phosphorylated by Polo-like kinase 1 and targeted for ubiquitin-driven degradation, allowing the cell to enter mitosis. The high levels of Evi5 during blastema formation, which takes significantly longer than the ~50 h cell cycle [63], may restrain dedifferentiated cells from entering mitosis until they have accumulated sufficiently to constitute a blastema [11]. While this hypothesis remains to be tested, it is significant that a high proportion of the fibroblasts of the ear tissue of the MRL/lpj mouse, which regenerates after punch injury, are arrested in G_2, suggesting that fibroblasts of this tissue are poised for mitosis upon injury [64].

Once the accumulation blastema has formed, it enters a growth phase where the mitotic index increases tenfold or more [51, 58]. In both larval and adult limbs, denervated growing blastemas are nerve-independent for morphogenesis and patterned differentiation. The regenerates formed by these blastemas, however, are much smaller than control regenerates [65–67]. This is because denervation at any stage of blastema growth leads to the reduction of blastema DNA synthesis [57, 68] and decreases the mitotic index to zero [69] due to disruption of the AEC mitogenic function. Direct evidence that proliferation during blastema growth stages requires the AEC is that DNA synthesis and mitosis of epidermis-free newt limb blastemas cultured in the presence of dorsal root ganglia are reduced three- to fourfold [70, 71]. These observations are compatible with the hypothesis that nerve axons induce and maintain a cell cycling function of the AEC that operates throughout regeneration.

The growing blastema may also be dependent on the AEC for proximodistal patterning and morphogenesis. Medium bud and later stage blastemas of larval *A. maculatum* denuded of wound epidermis and grafted into dorsal fin tunnels form smaller than normal skeletal elements with a distally truncated pattern, the degree of truncation being proportional to the developmental stage of the implant [72]. A complete proximodistal sequence of smaller than normal elements is formed by blastema implants positioned so that their distal tip becomes covered with fin wound epidermis. The small size of the skeletal elements in both types of implant is consistent with the lack of a nerve-dependent mitogenic function of the AEC. The PD truncation of implants denied regeneration of epidermis at their distal tip, however, suggests that the AEC may have a PD patterning function distinct from mitogenesis, although it remains to be established that the truncation is not due simply to death of apical cells destined to form distal structures.

Blastema Cell Proliferation Becomes Nerve-Dependent During Digit Stages of Limb Development

Amputated urodele limb buds are able to regenerate in the absence of innervation until they reach digital stages of development. At these stages, the limb bud becomes heavily innervated, whereupon regeneration becomes nerve-dependent and will not take place if the limb is denervated [73]. Nerve dependence is not acquired, however, if the limb never becomes innervated [74, 75]. This was shown by parabiosing two early embryos and excising the neural tube from one of them so that the fully differentiated limbs were aneurogenic. These limbs require only the wound epidermis/AEC to regenerate normally. Aneurogenic limbs can be oscillated between nerve-independent and dependent states. When grafted in place of innervated host limbs, they become innervated and nerve-dependent for regeneration by 10–13 days posttransplantation, but nearly half of the cases become nerve-independent again if re-denervated and maintained in a denervated state for 30 days [76].

These results can be explained by assuming that the outgrowth-promoting function of the limb bud apical epidermis [77] during limb development is either autonomous or depends on signals from the subjacent mesoderm as observed for chick limb buds [78]. As nerves grow into the limb, however, the epidermis becomes dependent on (“addicted to”) neural factors to maintain its outgrowth-promoting function during regeneration [79]. This dependency never develops in aneurogenic limbs, and the AEC maintains its original functional capacity after limb amputation.

What Are the Mitogenic Factors for Blastema Cell Proliferation?

A protein has been identified that can substitute for the nerves in denervated and amputated adult newt limbs [80]. The protein is the anterior gradient protein (AGP), a ligand for the blastema cell surface receptor Prod1. Prod1 is a member of the Ly6 family of three-finger proteins anchored to the cell surface by a glycosylphosphatidyl inositol linkage [81–83]. As assessed by co-expression of the Schwann cell marker HNK1, AGP is strongly expressed in the distal-most Schwann cells of regenerating newt limbs at 5 and 8 days postamputation, when histolysis and dedifferentiation are underway [80]. AGP expression is abolished by proximal nerve transection, indicating that it is induced in the Schwann cells by axons. The function of the Schwann cell AGP is not clear, however.

By 10 days postamputation, when the newt accumulation blastema is forming, AGP expression shifts from Schwann cells to subepidermal secretory gland cells of the AEC [80]. The wound epithelium of the axolotl does not have subepidermal gland cells, and here AGP expression is observed in the Leydig cells of the AEC. Both sets of gland cells appear to discharge secretions by a holocrine mechanism [84]. The expression of AGP by gland cells is also axon-dependent, as shown by the fact that it is abolished in denervated limbs. AGP has been shown to be a complete mitogen for blastema cells in vivo. When electroporated into denervated newt limbs at 5 days postamputation, the AGP gene supported regeneration to digit stages. Conditioned medium of Cos7 cells transfected with the AGP gene stimulated BrdU incorporation into cultured blastema cells, and antibodies to Prod1 blocked this incorporation [80]. Collectively, these results suggest that nerve axons induce the AEC to express AGP, which is then secreted and acts through Prod1 on subjacent blastema cells to stimulate their proliferation, thus giving the nerve dependence of AEC function a molecular basis. Further persuasive evidence for this idea is that AGP is downregulated in the apical epidermis of the limb bud during its acquisition of nerve dependence, but remains high in the apical epidermis of aneurogenic limbs throughout development and during regeneration [85].

Factors other than AGP that promote blastema cell proliferation in vitro and in vivo have been detected in the wound epidermis of the regenerating axolotl limb, primarily members of the fibroblast growth factor family (FGFs 1, 2, and 8) [86, 87]. Blastema cells express fibroblast growth factor-10, which is essential for maintaining FGF-2 expression by the AEC in regenerating Xenopus limb buds [88, 89]. The role these epidermal factors play in regeneration in vivo is not clear, but one hypothesis would be that FGFs, while not essential for blastema cell proliferation, synergize with AGP to augment their mitosis, or even that FGFs are the essential mitogens for blastema cells, but require AGP for their synthesis. Examining the effect of denervation on synthesis of these factors by the AEC would help to reveal their function. If their expression is eliminated by denervation and they fail to rescue regeneration after exogenous delivery to denervated blastemas in vivo, the hypothesis that they are essential mitogens would be unlikely. FGFs made by the wound epidermis/AEC might also play an essential role in axon and capillary regeneration into the blastema.

What Are the Axon Factors that Stimulate AGP Expression?

A major question is the identification of the factors produced by axons that induce AGP expression by Schwann cells and the AEC. Glial growth factor 2 (GGF-2, neuregulin) fulfills the criteria to be a candidate for the axon stimulus. It is expressed by neurons, is present in the blastema, and is lost from the blastema upon denervation [90, 91]. GGF-2, along with other growth factors produced by platelets and macrophages (FGFs, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), interleukins (IL) 1, 2, 6), has been shown to be mitogenic for Schwann cells in transected mammalian peripheral nerves [92]. The GGF-2 gene is expressed in newt dorsal root ganglia, and recombinant human GGF-2 infused into denervated axolotl limb blastemas was reported to maintain the DNA-labeling index at control levels and to support regeneration to digit stages [93], similar to the rescue of denervated blastemas by implants of spinal ganglia [94]. However, little detail was supplied in support of the ability of GGF-2 to promote complete regeneration in these experiments. Furthermore, there is no experimental data to show that GGF-2 actually induces the expression of AGP in the AEC. KGF (Fgf-7) has been shown to be expressed in axolotl dorsal root ganglion cells and to induce expression of the *Sp9* gene when administered in beads under a wound epidermis in the absence of the nerve [55]. KGF stimulates the mitosis of keratinocytes and thus the thickening of the wound epidermis in mammalian skin wounds [95], suggesting that it might also play this role in AEC formation.

Neurons synthesize other factors that directly promote the proliferation of blastema cells in vitro and in vivo. Denervation reduces protein synthesis by regeneration blastemas, but addition of neural extracts to blastema explants partially restored protein synthesis [96, 97]. The activity of the extracts was abolished by trypsin treatment and heating, but not by RNase, suggesting that the active molecules are proteins [97]. Spinal cord extracts from axolotls undergoing limb regeneration stimulated the mitosis of cultured blastema cells at twice the level of extracts from unamputated animals, and blastemas explanted next to cultured dorsal root ganglia or spinal cord segments that had regenerated many neurites had a mitotic index substantially higher than control cultures [70, 98]. Specific neural factors that promote blastema cell proliferation include transferrin, FGF-2, and substance P [99–101]. FGF-2 is the only factor shared with the AEC. With the exception of transferrin levels, which are reduced by 50 % in vivo, the effect of denervation on loss of these factors from the blastema has not been tested, and none has been shown to support the full course of regeneration. The function of these mitogens is thus unclear. They might synergize with GGF-2 in an augmentative but nonessential role to enable the function of the AEC, or along with the FGFs made by the AEC, they might be synergistic with AGP but be nonessential for mitosis. Singer [102] showed that the axon requirement for regeneration is quantitative and independent of the motor or sensory quality of the axons. It would be interesting to examine AGP synthesis in the AEC

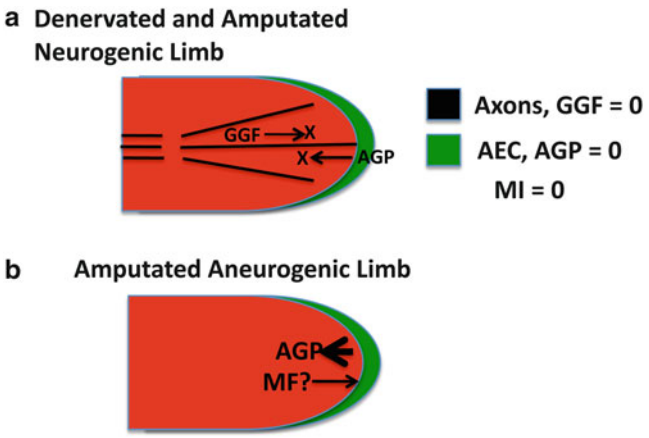

Fig. 3 The neural/epidermal circuit that drives proliferation of blastema cells. (**a**) Axons (*black lines*) secrete GGF-2 (hypothetical) that stimulates the AEC (*green*) to secrete the mitogen AGP, which binds to its ligand Prod1 on the surface of blastema cells to promote cell cycling. Upon denervation (*break* in lines), GGF-2 is no longer delivered to the AEC (X), and its secretion of AGP is terminated, causing a rapid fall in the mitotic index (MI) to zero. (**b**) The epidermis of a neurogenic limb acquires dependence on the nerve for AEC maintenance and AGP secretion during late stages of limb bud development when axons are ramifying throughout the limb tissues. The tissues of an aneurogenic limb never encounter axons, and the AEC formed after amputation maintains the capability of the limb bud apical epidermis to make AGP and blastema cells proliferate normally. An unresolved question is whether blastema cells undergo mitosis during their accumulation in an amputated aneurogenic limb or exhibit a very low level of mitosis like neurogenic limbs and whether blastema cells of a regenerating aneurogenic limb signal the AEC via a maintenance factor (MF) to secrete AGP

of regenerating limbs with selectively denervated motor or sensory components.

The relationships of the tissues and molecules that comprise the nerve/epidermal circuit are summarized in Fig. 3.

Blastema Cells Promote Axon Regeneration

As the blastema grows, axons must continually elongate to innervate differentiating tissues. Schwann cells provide most of the soluble factors (nerve growth factor, brain-derived growth factor, neurotrophic factors 3 and 4, ciliary neurotrophic factor, and glial-derived neurotrophic factor) and some adhesive factors required for neuron survival and axon elongation after transection of peripheral nerves [103]. Regeneration of axons from amphibian spinal cord neurons is promoted in vitro by co-culture with limb regeneration blastema mesenchyme [104]. Brain-derived neurotrophic factor, neurotrophic factors 3 and 4, glial-derived growth factor, and hepatocyte growth factor/scatter factor can substitute for blastema tissue in promoting this axon outgrowth [105]. Axon outgrowth was significantly more vigorous with blastema tissue, suggesting that blastema cells produce other factors that support neuron survival and axon outgrowth. One of these factors may be retinoic acid [106]. In cultures of newt spinal cord, retinoic acid added to the culture medium not only evoked the extension of a greater number of axons than in control cultures, the length of the axons was 4 times greater. Axon outgrowth was enhanced even more by co-culture with blastemas in the absence of exogenous retinoic acid. However, treatment of the co-cultured blastemas with the retinoic acid inhibitor citral reduced axon outgrowth, suggesting that retinoic acid is an axon outgrowth-promoting molecule made by the blastema. It would be of interest to know whether the wound epidermis/AEC also produces axonotrophic and angiogenic factors.

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Mechanism of miR-302-Mediated iPS Cell Generation

Shi-Lung Lin

Introduction

Stem cells are responsible for generating new cells to maintain our life; yet, once differentiated a stem cell naturally does not revert back to its earlier developmental stage. The mechanism underlying this one-way development is DNA methylation. During stem cell differentiation, methylation of cell genome like a lock sets up various tissue-specific gene expression patterns in all kinds of body cells and hence determines their final properties; however, this event also makes any change of the determined cell properties impossible. The pool of stem cells in a body is limited. In a person's life span, stem cells can be damaged by numerous environmental and pathogenic factors, such as pollutants, toxic materials, free radicals, stress, microbial/viral infections, and various illnesses. When the stem cell pool becomes exhausted, the body will grow old. This is why rejuvenation of life is almost impossible because reversing the ageing process requires generating new stem cells. In order to achieve rejuvenation, we need to unlock and revert the differentiated somatic cell properties back to a stem cell-like state. Therefore, it is understandable that the mechanism underlying DNA demethylation holds the key for stem cell generation.

Naturally, global demethylation of genomic DNAs occurs only in two developmental stages; one is during migration of primordial germ cells (PGC) into embryonic gonads (germline demethylation at approximately embryonic day E10.5 to E13.5) and the other is in the 2- to 16-cell-stage zygotic cells after fertilization (zygotic demethylation) [1–4]. The majority of parental imprints are erased and reestablished in germline PGCs but preserved in postfertilized zygotic cells [5, 6], suggesting that germline and zygotic demethylation mechanisms are not identical. However, the differences between these two natural demethylation mechanisms still remain largely unclear. Our recent finding of a novel reprogramming mechanism in induced pluripotent stem cells (iPSCs) established a new kind of global DNA demethylation mechanism comparable to the natural ones [7–9]. This man-made reprogramming mechanism triggers massive erasure of genomic DNA methylation sites but preserves many parental imprints similar to the effect of zygotic demethylation [9, 10]. Due to such high preservation of parental imprints, certain inheritable cell properties such as epigenetic memory and immunogenic compatibility have been observed in iPSCs [11–13].

The method for iPSC generation was first introduced by Takahashi and Yamanaka in 2006 [14]. Using retroviral delivery of four defined transcription factors (Oct4, Sox2, Klf4, and c-Myc) in vitro, they successfully reprogrammed somatic fibroblasts to embryonic stem cell (ESC)-like iPSCs [14, 15]. Later, Yu et al. also developed another method using a different set of four defined factors—Oct4, Sox2, Nanog, and LIN28 [16]. The development of iPSCs not only addresses the ethical concerns of using human ESCs but also may provide a patient-friendly therapy when used in conjunction with somatic cell nuclear transfer (SCNT) technologies. Due to these advantages, iPSC-based SCNT therapy has been tested for treating sickle cell anemia in a transgenic mouse model [17]. Nevertheless, the mechanism underlying the four-factor-induced iPSC formation is still elusive and a number of concerns have been found. First, oncogenic c-Myc is required to boost the success rate of iPSC formation. Without c-Myc, the success rate is less than 0.002 %. Second, iPSC population is heterogeneous due to the variable delivery efficiency of four factors. Until now, the best combination of four factors for reprogramming has not been identified. Last, the combined effect of the four defined factors seems merely to maintain the intrinsic network of embryonic gene expression [18, 19]; yet, how this effect leads to a global cancelation of somatic gene expression patterns remains largely unknown. According to these uncertainties, the safety of four-factor-induced iPSCs is questionable.

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Global DNA demethylation is essential for initiating epigenetic reprogramming of somatic cell nuclei to form pluripotent stem cells [10, 20–22]. Epigenetic reprogramming involves not only global cancelation of somatic gene methylation profiles but also activation of ESC-specific gene expression. In light of zygotic demethylation during early embryogenesis, we have known that exclusion of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) from zygotic nuclei causes passive demethylation of maternal DNA in 2- to 16-cell-stage embryos while paternal DNA is mainly demethylated by another undefined active mechanism before the first embryonic cleavage [3–6]. As DNMT1 functions to maintain inherited CpG methylation patterns by methylating the newly replicated DNA during the S-phase cell cycle, the deficiency of DNMT1 activity leads to passive global DNA demethylation in the daughter cells following early zygotic divisions [3, 4, 23, 24]. In this scenario, none of the previously defined reprogramming factors (either Oct4–Sox2–Klf4–c-Myc or Oct4–Sox2–Nanog–Lin28) can demonstrate such an important epigenetic mechanism in reprogramming. In fact, most of these defined factors are expressed after zygotic demethylation, showing no direct involvement in epigenetic reprogramming. Activation of these defined factors, therefore, is a consequence of global DNA demethylation rather than the cause. It is conceivable that another novel reprogramming factor likely plays a pivotal role in epigenetic reprogramming.

To address this unresolved question, our recent studies for the first time revealed that a group of noncoding RNAs (ncRNAs) rather than proteins are responsible for initiating global DNA demethylation in human iPSCs [7–9]. These ncRNAs belong to an ESC-specific microRNA (miRNA) family, miR-302, which is expressed most abundantly in human ESCs and iPSCs but not in differentiated somatic cells [25–27]. Native miR-302 consists of four familial sense homologues (miR-302b, c, a, and d) and three distinct antisense members (miR-302b*, c*, and a*), all of which are transcribed together as a polycistronic RNA cluster along with another miRNA, miR-367 [25]. It is noted that the elevation of miR-302 is highly coordinated with zygotic demethylation in 2- to 16-cell-stage embryos [25, 26]. In addition, another study found that mouse iPSCs also express an elevated level of miR-302 rather than miR-291/294/295, one of the regular markers for mouse ESCs [28]. These findings suggest that miR-302 likely serves as a major reprogramming factor in both human and mouse pluripotent stem cells. However, miR-302 is a gene silencer, not an activator. It is known that miRNA functions to suppress the translation of its target genes through complementary binding and formation of RNA-induced silencing complexes (RISCs) in the 3′-untranslated regions (3′-UTRs) of the targeted gene transcripts [29, 30]. Due to this unique function in gene silencing, miR-302 may play a role in suppressing DNMT1 to induce global DNA demethylation and hence initiating epigenetic reprogramming in both human ESCs and iPSCs.

Role of miR-302 in Global DNA Demethylation

Somatic cell reprogramming (SCR), a mechanism underlying reprogramming of somatic cells to iPSCs, starts from global DNA demethylation. The molecular basis of this mechanism was first reported by Lin et al. [7–9]. Lin's studies found that cellular miR-302 concentration determines the extent of genomic DNA demethylation in iPSCs [9, 10]. As shown in Fig. 1, miR-302 functions as a gene silencer to simultaneously down-regulate several key epigenetic regulators, including DNMT1, lysine-specific histone demethylases 1 and 2 (AOF2/1, LSD1/2, or KDM1/1B), methyl-CpG binding proteins 1 and 2 (MECP1/2), and histone deacetylase 2 and 4 (HDAC2/4). Concurrent silencing of these important epigenetic regulators induces global DNA demethylation, the first sign of epigenetic reprogramming required for formation of stem cell pluripotency [7, 9, 10]. DNA methylation often serves as a transcriptional block to repress gene expression and many ESC-specific genes are suppressed by DNA methylation in their promoter regions [20]. Through inducing global DNA demethylation, miR-302 removes these transcriptional blocks and hence reactivates an almost full spectrum (>91–93 %) of ESC-specific transcriptome expression, consequently leading to reprogramming of somatic cells to iPSCs.

When observing iPSC formation in a time-course fashion (Fig. 2), we found that the downregulation of AOF1/2, DNMT1, MECP1/2, and HDAC2 was most prominent at 3 days following the miR-302 elevation, while the expression of Oct4, Nanog, and Sox2 was increased to a maximal level at the fifth day [7, 9]. During this time, miR-302-transfected cells rested for a few days and then 1–2 cell divisions were detected in 4–5 days. This cell-division-dependent process highly resembles the zygotic demethylation of a maternal genome, and now it takes place in both parental genomes in iPSCs. Previous transgenic animal studies have shown that, in the absence of AOF1, germ cells fail to undergo de novo DNA methylation during oogenesis [31], while AOF2 deficiency leads to embryonic lethality due to a progressive loss of genomic DNA methylation and lack of cell differentiation [22]. Inhibition of either AOF1 or AOF2 also promotes methylation of histone 3 on lysine 4 (H3K4me2/3), a standard chromatin mark specific for ESCs and fully reprogrammed iPSCs [22, 31–33]. Therefore, silencing both AOF1 and 2 is sufficient to cause global DNA demethylation and chromatin modification required for reprogramming [9, 10]. Additionally, suppression of MECP1/2 further

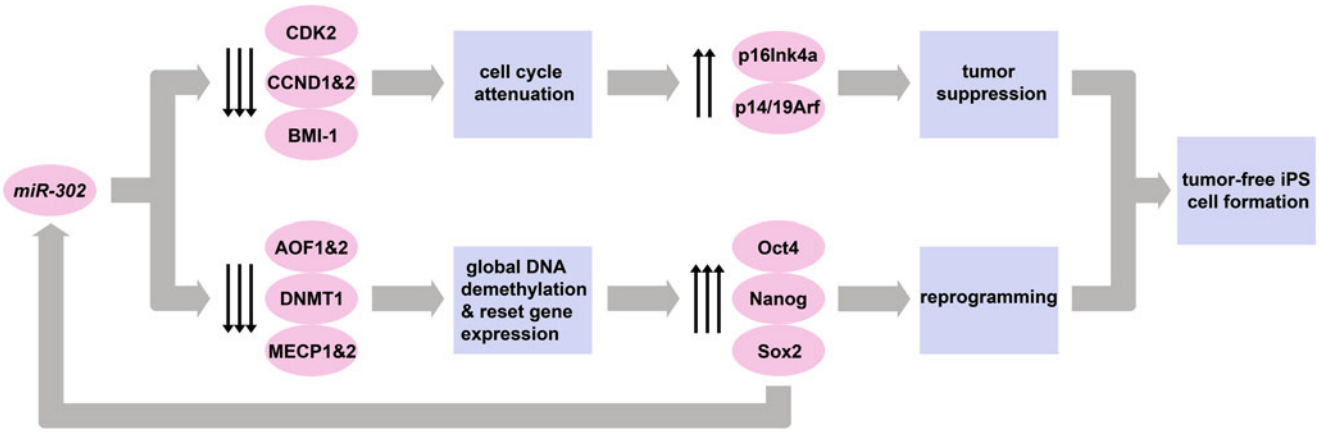

Fig. 1 Mechanisms of miR-302-mediated induced pluripotent stem cell (iPSC) generation and its related tumor prevention. Similar to zygotic reprogramming during early embryogenesis, miR-302 functions as a gene silencer directed against several major epigenetic regulators, including AOF1 and 2, MECP1 and 2, and DNMT1, to induce global DNA demethylation in transfected cells. Subsequently, global demethylation erases somatic transcriptional blocks (i.e., methylation sites) on the promoter regions of embryonic stem cell (ESC)-specific genes and activates these genes, which continue to execute the reprogramming process of iPSC formation. Meanwhile, other ESC-specific genes, such as Oct4 and Sox2, further stimulate miR-302 expression, resulting in a positive feedback loop cycle to maintain the full process of reprogramming. Accompanying this reprogramming process, miR-302 also serves as a tumor suppressor to silence multiple key cell cycle regulators, including CDK2, cyclin D1 and D2 (CCND1&2), and BMI-1, resulting in cell cycle attenuation at G1-phase checkpoint. Silencing BMI-1 further stimulates p16Ink4a and p14/p19Arf expression to prevent tumor formation. As a result, miR-302 reprograms somatic cells to tumor-free iPSCs

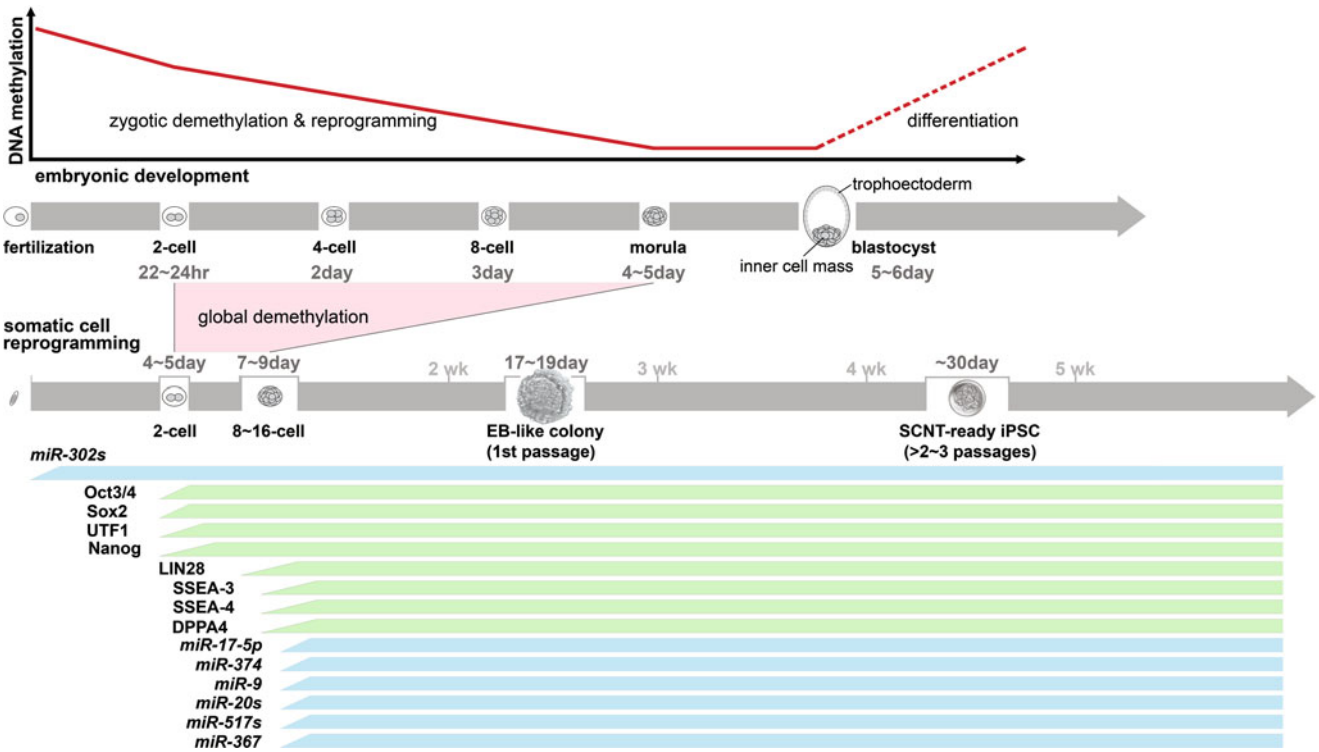

Fig. 2 Time-course scheme of ESC-specific gene and miRNA activation in miR-302-induced iPSCs. Approximately 4–5 days post-transfection of miR-302, Oct4, Sox2, and Nanog can be detected in the 2- to 4-cell-stage iPSCs. Subsequently, other ESC-specific genes, such as Lin28, SSEA3, and SSEA4, are activated in the 8- to 16-cell-stage iPSCs approximately 7–9 days post-miR-302 transfection. Most ESC-specific miRNAs are also stimulated at this time point, including members of miR-17–92, miR-93, miR-367, miR-371–373, and miR-520 cluster families. The reprogramming process between these two time points highly resembles zygotic demethylation occurring in the 2- to 16-cell-stage zygotes; yet, premature iPSCs take a significantly longer time (3–4 days) to activate Oct4 and Sox2 expressions. During this reprogramming process, cells not expressing all these ESC-specific gene markers will stop cell division and cannot form colonies. Only fully reprogrammed iPSCs form three-dimension cell colonies, which can be clearly observed approximately 17–19 days post-transfection

enhances the AOF1/2-mediated DNA demethylation effect in iPSCs [7].

DNMT1 is another important downstream target of miR-302 because AOF2 is responsible for stabilizing DNMT1 [9, 22]. In iPSCs, miR-302 silences AOF2 to reduce DNMT1 activity [9]. Alternatively, the analytic results of miRNA-target prediction program provided by the European Bioinformatics Institute EMBL-EBI (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/detail_view.pl?transcript_id=ENST00000359526) also suggest that miR-302 may directly inhibit DNMT1 translation. As a result, miR-302 strongly down-regulates DNMT1 via both translational suppression and posttranslational degradation. During early embryogenesis, DNMT1 inherited from oocytes is excluded from zygotic nuclei by an undefined mechanism while zygotic DNMT1 expression is very limited due to miR-302 overexpression [4, 34–36]. The function of DNMT1 is to methylate the newly replicated DNA in daughter cells during cell divisions; hence, diminished DNMT1 activity leads to a passive global DNA demethylation mechanism in early zygotes [4, 23, 24]. Similarly, miR-302-mediated DNMT1 silencing in iPSCs also elicits passive global DNA demethylation comparable to zygotic demethylation. However, since passive demethylation is unable to remove the methylated sites originally left in a somatic genome before reprogramming, this reprogramming model will generate two hemi-methylated cells in every single iPSC colony. Whether these hemi-methylated cells are degraded via programmed cell death (apoptosis) during reprogramming or further demethylated by another active mechanism remains to be determined.

A number of active DNA demethylation mechanisms have been postulated to play a role in reprogramming. First, activation-induced cytidine deaminase (AID) was found to initiate active DNA demethylation in early mouse embryos [3, 37, 38]. MiR-302 has also been shown to promote AID expression in iPSCs [9]. AID is normally expressed in B cells, PGCs, oocytes, and early stage embryos and functions to remove 5-methylcytosine (5mC) by deaminating 5mC to thymine (T), subsequently resulting in T-guanine (G) mismatch base pairing [39, 40]. To correct such T-G mismatch pairing, a base excision DNA repair (BER) pathway was proposed to replace the mismatched T with a C [41, 42]; yet, the enzyme required for this BER correction has not been identified in mammals. On the other hand, another theory involving the excision repairing of 5-hydroxymethylcytosine (5hmC) has also been proposed. In this model, Tet familial enzymes first convert 5mC to 5hmC that can be further converted to C by spontaneous loss of its formaldehyde group [43] or by a currently undefined DNA repair system. The formation of 5hmC in ESCs and iPSCs enhances passive DNA demethylation since DNMT1 does not recognize 5hmC as a substrate for replication [44, 45]. Nevertheless, several recent studies did not support this theory [45, 46]. First, Tet expression is subject to Oct4 regulation; hence, active DNA demethylation occurs after Oct4 activation [45]. Second, Tet depletion has no effect on Oct4, Sox2, and Nanog expression in ESCs [45]. Last, Tet depletion in mouse embryos only affects trophectoderm development and related developmental signaling, a stage much later than zygotic demethylation [46]. In view of these unsolved questions, the involvement of an active DNA demethylation mechanism in iPSCs is currently unclear.

Activation of ESC-Specific Gene Expression

Global DNA demethylation has been reported to promote Oct4–Nanog expression in early mouse embryos and mouse-human fused heterokaryons [37, 38]. Many ESC-specific genes are suppressed by DNA methylation in their promoter regions, particularly Oct4 and Nanog [20, 47, 48]. Our studies further showed that induction of iPSC formation requires a 1.1- to 1.3-fold higher miR-302 concentration than that found in human ESCs (approximately 0.9–1.0 million copies per ESC) [9]. As shown in Fig. 1, such a high cellular miR-302 concentration induces both global DNA demethylation and co-expression of Oct4, Sox2, and Nanog in human iPSCs [9, 10]. The expression of Lin28 and many other ESC marker genes was also observed 1–3 days following Oct4-Sox2-Nanog co-expression. In human ESCs, induced miR-302 expression over the normal level was also found to increase Oct4-Nanog expression by twofolds [49]. Further studies revealed that miR-302 directly suppresses the expression of nuclear receptor subfamily 2, group F, number 2 (NR2F2), a transcriptional repressor directed against the methylated Oct4 promoter, to enhance Oct4 expression [50]. Taken together, these findings strongly suggest that miR-302 triggers global DNA demethylation to remove transcriptional blocks on ESC-specific gene promoters and thus activates ESC-associated gene expressions.

Thus far, the activation of ESC-specific genes and their mutual interactions have only been studied in mammalian ESCs [18, 19], not in iPSCs. iPSCs generated by different induction methods from various somatic cell types all share a highly similar epigenetic and transcriptomic profile like the one found in ESCs, indicating the importance of global DNA demethylation in erasing and resetting different somatic epigenomes and transcriptomes into a unique ESC-like pluripotent state. Following the time-course formation of iPSCs as shown in Fig. 2, we found that iPSCs take longer time to reach the point of cell division than postfertilized zygotes, probably due to lack of germline elements such as paternal protamines and maternal oocyte transcripts. After the first cell division, the process of global DNA demethylation is almost identical between iPSCs and the 2- to 16-cell-stage

zygotes up to the morula stage. During this period, the expression of Oct4, Sox2, Nanog, and undifferentiated embryonic cell transcription factor 1 (UTF1) is gradually elevated to a maximal level within 1–2 days, while Lin28 and many other ESC marker genes are expressed 1–3 days later than the Oct4-Sox2-Nanog co-expression. Microarray analyses of the genome-wide gene expression patterns between iPSCs and human ESCs have indicated that they share over 91–93 % similarity [7, 9]. In addition, miRNA microarray analyses have further shown that many ESC-specific miRNAs are stimulated by miR-302 and likely functions together with miR-302 to promote and/or maintain the pluripotency of iPSCs [7, 8]. Based on these observations, the time-course activation pattern of ESC-specific genes and miRNAs following miR-302 induction in iPSCs is summarized in Fig. 2.

Inhibition of Differentiation Signals

In view of miRNA functionality in gene silencing, miR-302 serves as a major gene silencer in human ESCs and zygotes. Based on the analytic results of online miRNA-target prediction programs TARGETSCAN (http://www.targetscan.org/) and PICTAR-VERT (http://pictar.mdc-berlin.de/), the majority of miR302-targeted genes are transcripts of differentiation-associated genes and developmental signals, such as members of the RAS-MAPK, TGFß-SMAD, and Nodal-Lefty pathways, indicating its significance in inhibiting stem cell differentiation. Recent studies have started to investigate the miR-302-mediated inhibition of TGFß-SMAD and Nodal-Lefty pathways [51, 52]. In vertebrates, specification of anterior–posterior axis and left-right asymmetry depends on TGFß-related signal proteins, such as activin/inhibin, Nodal, Lefty, and bone morphogenetic proteins (BMPs) [53]. Lefty1 and 2 are also involved in neural cell induction [54]. Additionally, Nodal signals are responsible for patterning the visceral endoderm through SMAD2-dependent pathways [55]. Interestingly, both TARGETSCAN and PICTAR-VERT have predicted that SMAD2, Lefty1/2, and activin/inhibin are strong targets of miR-302. Silencing of Lefty1/2 by miR-302 leads to a significant delay in early ESC differentiation prior to germ layer specification [51], while miR-302 also suppresses BMP inhibitors TOB2, DAZAP2, and SLAIN1 to promote ESC pluripotency via preventing neural induction [52]. Since silencing either Lefty1/2 or BMP inhibitors results in the same inhibitory effect on neural differentiation, these findings indicate that miR-302 is able to simultaneously target multiple parallel signaling pathways to completely block a specific cell lineage differentiation.

MiR-302 is not the only miRNA involved in SCR. Using miRNA microarray analyses, we have also identified that the expressions of other ESC-specific miRNAs, such as members of miR-17–92, miR-93, miR-367, miR-371–373, miR-374, and miR-520 families, are elevated in iPSCs [7–9]. MiR-302 shares over 440 target genes with these miRNAs, suggesting their potential roles in iPSC induction and/or maintenance. These conserved target genes include not only many members of the RAS-MAPK, TGFß-SMAD, and Nodal-LEFTY signaling pathways but also numerous transcription factors, oncogenes and cell differentiation factors, such as E2F transcription factors, Myb-like transcription factors, HMG-box transcription factors, Sp3 transcription factors, NFkB activating protein genes, BMI-1 oncogene, Rho/Rac guanine nucleotide exchange factors, IGF receptors, protocadherins, CXCR4, EIF2C, PCAF, and many nuclear receptors and cell surface molecules. Given that these target genes are highly involved in embryonic development and/or cancer tumorigenicity, miR-302 may stimulate these homologous miRNAs to further enhance its function in preventing stem cell differentiation and tumor formation. Due to the complexity of these intricate miRNA-miRNA and miRNA-target interactions, understanding the full spectrum of these gene regulation mechanisms involved will be a great challenge.

Prevention of Stem Cell Tumorigenicity

Tendency in tumor formation is one of the major problems inherent in stem cell therapy. Developing tumor-free ESCs/iPSCs is critical in view of the current Food and Drug Administration (FDA) regulations regarding concerns of cancer stem cells. However, oncogenic factors such as c-Myc and Klf4 are frequently used to boost the survival and proliferative rates of the four-factor iPSCs, creating an inevitable problem of tumorigenicity in these cells. MiR-302 has been shown to induce reprogramming while preventing stem cell tumorigenicity [56]. Tumor-free pluripotency is one of the key advantages of miR-302-induced iPSCs compared to those induced by the four factors. As the mechanism underlying stem cell tumorigenicity is still poorly understood, elucidating the miR-302 function in tumor prevention may greatly benefit the development of safer and more reliable iPSCs or ESCs for stem cell therapy.

To prevent iPSC tumorigenicity, we first look into the natural tumor prevention mechanism during normal embryogenesis. Early zygotes before the morula stage (16–32 cell stage) often exhibit a relatively slow cell cycle rate (20–24 h/cycle), whereas such stringent cell cycle regulation is not found in later blastocyst-derived ESCs (15–16 h/cycle) [57]. Embryonic cells in early zygotes possess two unique features of stemness: pluripotent differentiation into almost all cell types and unlimited self-renewal in the absence of tumor formation. Clearly, these two features are also important for the clinical application of ESCs or iPSCs. As demonstrated in Fig. 1, our study

revealed for the first time that miR-302 is responsible for inhibiting human iPSC tumorigenicity through co-suppression of both cyclin E–CDK2 and cyclin D–CDK4/6 cell cycle pathways to block >70 % of the G1-S-phase transition [56]. Furthermore, miR-302 also silences BMI-1, a cancer stem cell marker, to promote the expression of two tumor suppressor genes, p16Ink4a and p14/p19Arf. p16Ink4a inhibits cyclin D-dependent CDK4/6 activities via phosphorylation of retinoblastoma protein Rb and subsequently prevents Rb from releasing E2F-dependent transcription required for S-phase entry [58, 59], while p14/p19Arf prevents HDM2, an Mdm2 p53 binding protein homolog, from binding to p53 and permits the p53-dependent transcription responsible for G1 arrest or apoptosis [60]. Taken together, the combined effect of reducing G1-S cell cycle transition and increasing p16/p14(p19) expression results in an attenuated cell cycle rate similar to that of 2- to 8-cell-stage embryonic cells in early zygotes (20–24 h/cycle). Hence, this attenuated cell cycle rate may reflect the timing required for iPSCs to fully pass the cell cycle checkpoint surveillances for preventing premature differentiation and tumor formation.

The mechanisms of miR-302-induced reprogramming and tumor suppression/prevention are parallel to each other. Through inducible miR-302 expression, we found that both events occur almost simultaneously at a miR-302 concentration over 1.1- to 1.3-folds the level found in human ESCs, indicating that this specific concentration is the minimal threshold for reprogramming somatic cells to iPSCs while preventing iPSC tumorigenicity [7, 9, 10, 56]. Based on this understanding, many tumor-free iPSC lines have been successfully generated from human normal skin keratinocytes and melanocytes as well as cancerous melanoma Colo-829, prostate cancer PC3, breast cancer MCF7, hepatocellular carcinoma HepG2, and embryonal teratocarcinoma Tera-2 cell lines [7, 9, 56]. Notably, normal and cancerous somatic cells respond very differently to the miR-302-mediated tumor suppression effect. MiR-302 overexpression often induces apoptosis in over 98 % of the fast growing cancer/tumor cells, while only a few remaining cells become iPSCs. On the contrary, the majority (>90 %) of the miR-302-transfected normal cells can tolerate this inhibitory effect on cell proliferation [7, 56]. It is understandable that tumor/cancer cells may not survive in such a relatively slow cell cycle rate due to their high metabolism and rapid consumption rates. Together, these results identify miR-302 as a tumor suppressor in iPSCs and hence provide a beneficial advantage in using miR-302 for not only reprogramming but also preventing tumorigenicity.

Conclusion

MiR-302 plays a critical role in four aspects of the iPSC formation mechanism, including initiation of global DNA demethylation, activation of ESC-specific gene expression, inhibition of developmental signaling, and prevention of stem cell tumorigenicity. Because of these important functions, the present approach of miR-302-mediated iPSC generation is simpler, safer, and more effective compared to the previous four-factor induction methods. The miR-302-induced pluripotent stem cells (mirPSCs) may also serve as a better choice of tumor-free iPSCs based on the current FDA regulations regarding concerns on tumor formation. Table 1 shows that several mirPSC-associated technologies have been developed and mainly led by two major research groups, such as Lin et al. (WJWU & LYNN Institute—4 filings) and Yamanaka et al. (Kyoto University—2 filings). The original concept of this novel methodology is derived from Lin's 2008 studies [7, 8], which demonstrated the first evidence of miR-302-mediated iPSC formation. Meanwhile, other miR-302 homologs, such as members of miR-93, miR-200c, miR-367, miR-371–373, and miR-520 families, were also found to possess a partial or similar function in reprogramming, which may be used to improve the efficiency of previous four-factor induction methods.

The development of mirPSC-associated technologies holds a great promise in discovering new therapies for regenerative medicine. Deciphering the mechanisms underlying mirPSC induction and prevention of stem cell tumorigenicity has led to the identification of new methods for improving the efficiency and safety of iPSCs. As a result, mirPSCs represent a new kind of tumor-free iPSCs capable of overcoming the two major problems in stem cell therapy: supply and safety. Compared to the previous four-factor induction methods, these mirPSC-based technologies have prominent advantages in both reprogramming efficiency and safety. Between delivering a single miRNA and four large transcription factor genes, it is not difficult to understand that the mirPSC-based technologies provide a higher success rate and less damages to the reprogrammed cells. Moreover, miR-302 is a tumor suppressor in humans, whereas c-Myc, one of the four defined factors, is a well-known oncogene. In fact, it has been reported that the optimal reprogramming efficiency for miR-302 and the four-factor induction method is >10 % and <1 %, respectively, showing at least a tenfold improvement [7, 9, 61].

Both four-factor and miR-302 induction methods lead to a forced epigenetic reprogramming mechanism similar to zygotic demethylation but completely bypassing germline demethylation. When compared to SCNT-iPSCs, four-factor iPSCs have been shown to present a less epigenomic and transcriptomic similarity to ESCs [11]. SCNT is a well established technology to generate ESC-like pluripotent stem cells by hybridizing a somatic cell nucleus into the oocyte cytoplasm [62, 63]. Due to the use of oocytic ingredients, SCNT also delivers a better reprogramming rate than four-factor induction. On the other hand, the cytoplasm of miR-302 iPSCs has been tested for SCNT and shown that most (93 %) of the hybrid cells were successfully reprogrammed to ESC-like

Table 1 Published patent applications related to miR-302-mediated iPSC generation

Patent application publication number and inventors	Filing year[a]	Description of novelty	No
WO 2009/058413 A1; LIN, Shi-Lung et al.	March, 2008	A composition and method for reprogramming mammalian somatic cells to induced pluripotent stem cells (iPSCs) using miR-302 and/or miR-302-like microRNAs/shRNAs, such as miR-93, miR-367, miR-371–373, and miR-520	1
US 2008/0293143 A1; LIN, Shi-Lung et al.	May, 2008	Same as WO 2009/058413 A1 to LIN et al.	2
WO 2009/091659 A2; LIN, Shi-Lung et al.	January, 2009	An inducible composition and method for generating tumor-free iPSCs using miR-302 and/or miR-302-like microRNAs/shRNAs	3
US 2009/0203141 A1; LIN, Shi-Lung et al.	January, 2009	Same as WO 2009/091659 A2 to LIN et al.	3
US 2009/0246875 A1; Yamanaka, Shinya et al.	February, 2009	A method for generating iPSCs or enhancing the reprogramming efficiency of iPSC generation using miR-302 and/or miR-302-like microRNAs in conjunction with the previously defined three or four factors (Oct4, Klf4, c-Myc, and Nanog but not Sox2)	4
EP 2 202 309 A1; Yamanaka, Shinya et al.	August, 2009	Same as US 2009/0246875 A1 to YAMANAKA et al.	5
US 2011/0189137 A1; Rana, Tariq M, et al.	November, 2010	A method for generating iPSCs or increasing the reprogramming efficiency of iPSCs using miR-302 and miR-302-like microRNAs	6

[a]Listed up to December (2010)

pluripotent cells [9]. This finding is coincident with the previous SCNT results using the oocyte cytoplasm, which contains several miR-302 homologs that may play a similar role as miR-302, such as miR-200c and miR-371–373. Given that miR-302 is a cytoplasmic effector whereas the four defined factors are all nuclear proteins, it is clear that miR-302 is responsible for initiating reprogramming through global DNA demethylation in the SCNT-iPSCs. Furthermore, miR-302 may also stimulate certain undefined factors similar to those in the oocyte cytoplasm to facilitate the completion of reprogramming. Therefore, mirPSCs are not exactly the same as the iPSCs induced by four defined factors.

It took almost 5 years from Yamanaka's discovery of iPSCs [14] to the revelation of its mechanisms by Lin et al. [9, 10]. The progress of modern iPSC technology is fast and becoming important for the development of regenerative medicine. When most of current research is still performed in isolated cells under in vitro conditions, it is noted that applying these findings for in vivo applications will be the next challenge. Several attempts in using iPSCs for in vivo therapy have been conducted in animals and many more continue to be found. Due to these efforts, the development of today's regenerative medicine has grown beyond simply iPSC generation; in fact, current applications have extended to a whole new level of using the mirPSC-associated reprogramming or tumor suppression mechanisms for developing new drugs and therapies. For example, direct stimulation of in vivo stem cell generation to enhance tissue regeneration and recovery is one of the leading trends that show great promise. Following the recent advances in understanding the details of the miR-302-mediated reprogramming mechanism, further development in this direction is highly expected.

Today, regenerative medicine is no longer just a concept of future medicine. It holds great potentials in curing aging-related illnesses by repairing and rejuvenating body cells. It was assumed in the past that, once differentiated, a cell cannot revert back to its earlier undifferentiated state. The discovery of iPSCs, however, totally changes this concept and proves that there is an internal mechanism capable of reprogramming differentiated cells back to the earliest ESC-like pluripotent stage. In recent years, the revelation of this reprogramming mechanism further advances the design and development of iPSC-based regenerative medicine to a whole new era for not only disease therapy but also body rejuvenation. Since numerous environmental and pathogenic factors, such as pollutants, toxic materials, free radicals, stress, microbial/viral infections, and various illnesses, can damage our stem cells to accelerate the aging process, the use of miR-302-mediated iPSC generation may provide us a sufficient amount of stem cells for maintaining our body rejuvenation. As now we have plentiful body cells to serve for stem cell generation, a variety of new regenerative medicine methods may be exploited in the near future for improving our quality of life.

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Bioreactor Expansion of Pluripotent Stem Cells

Jaymi T. Taiani, Mehdi Shafa, and Derrick E. Rancourt

The Advantage of Stem Cells

The field of tissue engineering came into the main stream in the early 1990s and was defined by Vacanti and Langer as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [1]. Since this time, there have been significant efforts worldwide to create "biological substitutes," with the majority of studies focusing on developing regenerative strategies for tissues that possess a limited natural repair process and which evoke detrimental and often life-threatening effects when compromised by disease or injury. These include, but are not limited to, cardiac, pancreatic, hepatic, spinal cord, and brain tissues. Through these efforts, we have made significant progress on many fronts, and the lives of nearly 50 million people in the USA alone have been saved as a result of artificial organ therapy [2].

Tissue engineering strategies typically rely on the combination of bioactive factors, such as growth factors, natural or synthetic scaffolds, and living cells. Although these three components demonstrate some efficacy when used individually, their combined use most commonly yields the greatest degree of tissue regeneration and repair [3–8]. A variety of cell sources have been explored for tissue engineering strategies, including autologous or allogeneic specialized cells, however much of the work in this field has focused on the use of stem cells.

Two Canadians first identified hematopoietic stem cells in mature animals in 1963. This group noticed that nodules formed on the spleens of irradiated mice after the animals received bone marrow injections [9]. Further, the group noted that the number of nodules changed in proportion to the number of bone marrow cells injected, and through further examination they found that each nodule in fact arose from a single marrow cell. Through this study, Becker, Till, and MacCulloch were the first to discover these stem cells and identified the defining properties of them as (1) the capacity for self-renewal and (2) the ability to differentiate into specialized cell types.

Stem cells have been derived from many sources including various adult tissues, fetal tissues, umbilical cord blood, amniotic fluid, and early embryo. In the adult organism, stem cells exist in a diversity of tissues, including bone marrow, fat, brain, skin, and blood, and these cells are responsible for tissue growth, maintenance, and injury repair. One feature that distinguishes one stem cell type from another is their potency or their ability to differentiate into functional specialized cell types of different lineages. The range of lineages that an adult tissue-derived stem cell can generate is typically quite restricted. For example, adult bone marrow-derived mesenchymal stem cells (MSCs) are characterized as multipotent cells since their differentiation capacity is limited to mesenchymal tissues including bone, cartilage, muscle, fat, tendon, ligament, bone marrow stroma, dermis, and other types of connective tissues [10, 11]. Conversely, stem cells derived from the early embryo, or embryonic stem cells (ESCs), can generate any cell type in the body, an attribute known as pluripotency. In addition to differentiation potential, the choice of stem cell line for a given clinical therapy will also be influenced by the expansion capability of the cells. Adult-derived stem cells generally have limited self-renewal capabilities, and thus the in vitro expansion of these cells is usually challenging. For example, MSCs exist in low numbers in the bone marrow, and their population is believed to decrease with age. In fact, Caplan [11] has suggested that MSCs are present in the bone marrow of a newborn at a frequency of 1 in 10,000 cells and that this number drops to 1 in 250,000 by the age of 30 and then to 1 in 2,000,000 by the

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age of 80. ESCs on the other hand expand quite readily in culture and can undergo long-term self-renewal.

Although adult stem cells have a restricted differentiation potential, are scarce in number, difficult to isolate, and typically possess a limited self-renewal capacity, these cells are not tumorigenic and do not evoke an immune response when used for autogenous implantation, two significant advantages over other stem cell sources, including Pluripotent Stem Cells (PSCs).

Pluripotent Stem Cells

ESCs were first cultured from mouse embryos in 1980 [12, 13] and later isolated from human embryos [14]. They have also been isolated from a variety of other animals including the rat [15], rabbit [16], dog [17], pig [18], and nonhuman primates [19, 20]. Importantly, since their original derivation, stringent regulations have been placed on the derivation and culture of human ESC lines in Canadian and American laboratories. In Canada, human ESC lines can only be derived with informed consent from couples undergoing treatment for infertility [21]. These embryos are obtained through in vitro fertilization techniques and no longer required for reproduction purposes.

As a result of their pluripotency and high capacity for self-renewal, ESCs have attracted a great deal of interest for potential use in novel tissue engineering and cell therapies, and many laboratories worldwide have focused their efforts on studying the expansion and differentiation of ESCs to achieve this goal. Despite these advantages, ESC researchers are faced with two significant challenges. As a result of their ability to generate any cell type in the body, ESCs possess tumorigenic potential when implanted in vivo. This is actually a hallmark characteristic of these cells [12–14]; an ESC line is typically defined as a cell line that can generate a teratoma following injection in vivo. This characteristic is not observed following implantation of adult-derived stem cells. Furthermore, ESCs are an allogeneic cell source and thus also present the challenge of immune rejection following implantation in vivo.

To overcome the obstacle of immune rejection, a Japanese group led by Dr. Shinya Yamanaka discovered that a specialized cell derived from adult tissue could be genetically reprogrammed to behave like an undifferentiated PSC [22, 23]. This was a very significant discovery since it is now possible to create an autologous PSC line. In addition to mediating the challenge of immune rejection, induced pluripotent stem cells (iPSCs) also avert the ethical concerns surrounding the use of embryo-derived PSCs. Yamanaka's discovery represents an important advancement in the field of stem cell research and has inspired tremendous international interest in the area of cellular reprogramming.

iPSCs are superficially identical to their ESCs counterparts and possess all of the essential criteria of a pluripotent line such as self-renewal, clonality, and potency [24–26]. The similarity between pluripotent iPSCs and ESCs in their global chromatin structure and gene expression profile [27], cell morphology, capacity to differentiate into three germ layers, teratoma formation, and tetraploid complementation [28–32] has suggested that these cells might hold a great potential for future cell therapies in regenerative medicine and also for the derivation of patient or disease-specific iPSCs [33, 34].

PSCs, like other types of stem cells, can be maintained in an undifferentiated state and expanded in vitro using a variety of culture systems, including static adherent culture, suspension culture, and different types of bioreactors. Differentiation of the stem cells into lineage-specific specialized cell types can be induced through the addition or removal of specific medium supplements, such as growth factors, exposure to mechanical loading, exposure to extracellular matrix molecules, or coculture with other cell types.

Expansion of Pluripotent Stem Cells

The use of stem cells for clinical therapies will require effective methods for the generation of large numbers of stem cells and their progeny. Traditionally, ESCs have been expanded in static adherent cultures and require coculture with murine embryonic fibroblast feeder layers (MEFs) or supplementation with growth factors. Leukemia inhibitory factor, or LIF, for example, is used to expand pluripotent murine ESCs but is ineffective for human ESC culture. In our laboratory, static culture methods generally yield approximately 0.5×10^6 cells/mL over one passage. Thus, in a 20 mL static culture vessel (a typical volume for a 75 cm^2 tissue culture flask), the resultant total number of cells attained at the end of the culture period is approximately 10×10^6. Normally, the culture period ranges from about 2 to 3 days.

Through advances in the field of biotechnology, bioreactors have been used for culturing various types of cells; however these applications have mainly focused on the large-scale production of antibodies, enzymes, vaccines, and viruses from the cultured cells [35]. More recently, stem cell researchers have begun to focus their efforts on establishing effective bioreactor expansion techniques to generate "clinically relevant" numbers of stem cells. It has been estimated that 10^{10}–10^{11} pancreatic islet cells would be required to treat one diabetic patient [36], and at least 10^9 dopamine-producing neurons would be required to treat one patient with Parkinson's disease [37]. Moreover, 10^9 cardiomyocytes are required to repair cardiac tissue in a patient who has

suffered a myocardial infarction [38], and approximately 10^{10} hepatocytes seeded on an artificial liver implant can support a patient with hepatic failure [39]. Using data from our laboratory, one thousand 75 cm^2 static tissue culture flasks would be required to generate 10^{10} ESCs. Clearly, the generation of enough cells to treat a large number of patients will require large-scale expansion techniques that are cost and labor effective. Furthermore, the target number of cells produced from the scale-up process will depend on the therapeutic strategy employed. For example, if therapies are developed on a "per patient" basis, a smaller target number of cells, on the order of 10^{10}, would be required. However, the creation of cell banks, for treating numerous patients at once, will require much larger cell numbers. The creation of bio-banks has been considered by some research groups as an alternative strategy to hamper the immunological graft rejection of transplanted allogeneic cells. Through the establishment of a clinical-grade HLA-haplotype bio-bank of pluripotent cell lines with enough diversity, cells would be available to all patients, to treat a multitude of conditions. The possibility of constructing a human ESC bank was first suggested for the United Kingdom [40]. It was estimated that only ten donors, who are homozygous for common HLA types, can provide a complete HLA-A, HLA-B, and HLA-DR match for 37.7 % of recipients and an acceptable match for about 67 % of the population in the UK. In Japan, a bio-bank size of only 50 iPSC lines would be adequate to match three loci in about 90 % of the population [41]. Another advantage of this kind of centralized bio-bank is the availability of reliable, robust, and well-characterized ESCs/iPSCs for researchers. Development of such banking systems necessitates the generation of adequate number of cells through a scalable and reproducible process.

To achieve clinically relevant cell numbers, suspension culture bioreactors were first considered for the large-scale culture of pluripotent ESCs nearly a decade ago [42–46]. However, these initial studies focused on the use of bioreactors for the differentiation of ESCs that had been expanded in static culture vessels. For example, Gerecht-Nir et al. were the first to culture human ESCs in suspension bioreactors. Using low-shear slow-turning lateral vessels (STLVs) and high-aspect rotating vessels (HARVs), they successfully cultured human embryoid bodies (EBs; aggregates of differentiated ESCs) [46]. Massive agglomeration of the cells was observed in the HARVs; however the differentiated cells grew 70-fold in the STLVs and remained viable.

Our group and one other laboratory were the first to report effective bioreactor expansion protocols for pluripotent murine ESCs [47, 48]. Since that time, we have also developed effective suspension bioreactor protocols for the large-scale production of human ESCs [49] and iPSCs [50, 51]. Similar procedures have also been established for neural stem and precursor cells [52–54], human mesenchymal progenitor cells [55], and hematopoietic stem cells [56–58].

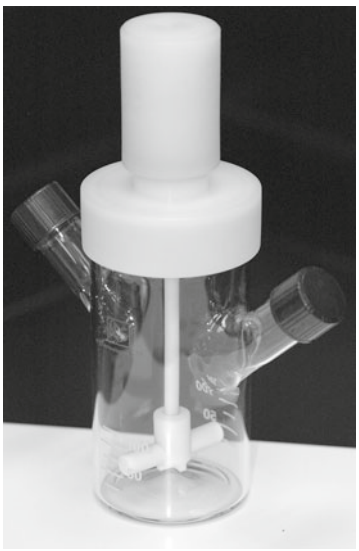

Fig. 1 Stirred suspension bioreactor [SSB] (125 mL)

Our large-scale expansion protocol relies on the use of a stirred suspension bioreactor (SSB) with a 100 mL working culture volume, although larger volumes are also available (see Fig. 1). For these systems, a magnetic impeller, controlled by a magnetic stir plate, is used to agitate the medium at a desired rate. In this type of culture system, the ESCs form spherical aggregates that remain suspended in the medium and only require the use of basic expansion medium supplemented with LIF, and not coculture with feeder cells, to remain pluripotent. Furthermore, these cultures can be serially passaged for long-term production, while maintaining ESC pluripotency attributes [59].

As demonstrated in Fig. 2, SSBs offer several important advantageous over the typical use of static tissue culture flasks including a homogeneous culture environment and large culture volume. In addition, a range of sizes are available, from 100 mL to 10 L or more, and the larger vessels permit computer control and monitoring of culture conditions, such as temperature, pH, and oxygen concentration. Clearly, the greatest advantage is the production of large cell numbers with limited batch-to-batch variability, in a time and labor effective process (see Fig. 2). In fact, under optimized bioreactor culture conditions, our laboratory can produce pluripotent ESCs at a concentration of 1×10^6 cells/mL, at a viability of over 90 % [47]. In a 100 mL working volume, the resultant total cell yield is 10^8 per bioreactor, after a culture period of only 5 days [47]. In terms of clinical usefulness, the benefit of SSBs is clear: two bioreactors could be used, as opposed to 1,000 static culture flasks, to generate more than enough cells to treat one patient.

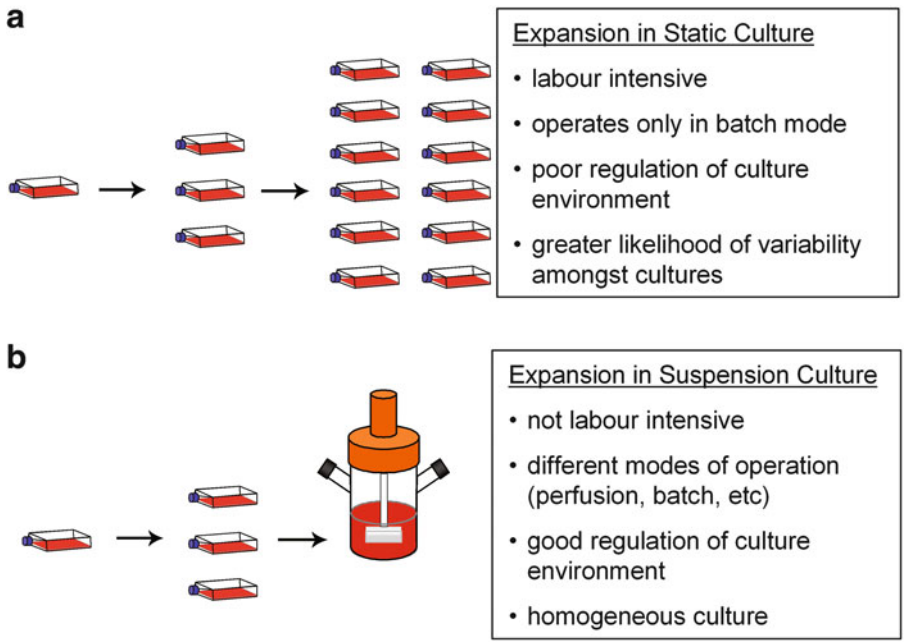

Fig. 2 Cell expansion capabilities in (**a**) static versus (**b**) stirred suspension culture systems (from [88])

Optimization of Bioreactor Culture Conditions

To develop effective bioreactor expansion protocols, a variety of culture conditions can be adjusted to optimize cell yield, including inoculation density, agitation rate, medium composition, medium viscosity, and oxygen concentration (through surface diffusion or perfusion). The agitation and aeration of the bioreactor culture are two critical issues and are the most difficult factors with respect to scale-up [60]. Mixing is generally implemented in order to avoid the formation of nutrient, pH, and dissolved oxygen concentration gradients and to increase mass transfer rates of nutrients and oxygen. Larger volumes lead to an increased mixing time and can require higher agitation rates to meet the oxygen demand of the cells. However, in achieving bulk homogeneity and adequate mass transfer rates, the hydrodynamic shear that is generated can often be detrimental to the cells. Thus, changes to each of these parameters needs to be carefully balanced in order to maximize cell viability and expansion.

To develop effective bioreactor protocols for murine ESCs, we initially assessed the influence of agitation rate and inoculation density on cell viability and expansion. We then characterized the oxygen uptake rate and metabolic activity of the cells at the optimal conditions. Our findings from these studies were presented in a report by Cormier et al. [47] and are summarized briefly below.

Three agitation rates—80, 100, and 120 rpm—were compared, which evoked maximum shear stresses on the cells of 4.5, 6.1, and 7.8 dyn/cm^2, respectively. We found that 100 rpm minimized aggregate diameter, which reached a maximum of approximately 125 μm by 5 days; an important factor in the diffusion of nutrients, waste, and oxygen to and from the center of the aggregate. Further, when cultured at 100 rpm cell expansion (31-fold) and viability (>90 %) were maximized. By comparison, at 80 rpm, aggregate diameters reached approximately 200 μm over the 5-day culture period, which significantly reduced cell expansion due to cell necrosis at the aggregate centers, and ultimately cell expansion was significantly reduced (16-fold, $p<0.1$). An agitation rate of 120 rpm evoked shear stresses that were intolerable, resulting in detrimental effects on cell viability and expansion. After identifying 100 rpm as the optimal agitation rate, we then compared various inoculation densities, 0.5, 1.0, 3.75, 7.5, and 10×10^4 cells/mL, for their effect on cell expansion and ultimately found that 3.75×10^4 cells/mL was optimal for the highest expansion.

We then measured the oxygen consumption rate of murine ESCs grown in suspension using a modified spinner flask. Under our optimized conditions, we determined that our culture system could support up to 3.75×10^6 cells/mL, and our maximum cell density of 1.07×10^6 cells/mL was well within these limits. By analyzing cell metabolism and pH, we determined that the accumulation of waste products, namely, lactic acid and ammonia, was likely responsible for limiting cell viability and expansion and that perfusion or fed-batch systems (where fresh medium is continuously being replenished) should be considered to achieve greater cell expansion rates.

Under optimal conditions, we found that the murine ESCs had greater expansion capabilities over one passage in bioreactors compared to static culture systems. Further, the cells retained high expression levels (>90 %) of the pluripotency markers Oct-4, Nanog, and SSEA-1, retained colony-forming potential and multi-lineage differentiation capability in vitro and in vivo.

Although we chose to expand murine ESCs as aggregates, other groups have optimized bioreactor expansion protocols using different types of microcarriers [48, 61, 62] and after encapsulation [63, 64]. For example, Fok and Zandstra (2005) showed that murine ESCs could be expanded on glass and Cytodex 3 microcarriers in stirred suspension culture [48]. Use of the glass microcarriers yielded population doubling times that were comparable to static culture at 17 ± 1.9 h (for the R1 cell line); however the Cytodex 3 microcarriers had reduced doubling times. In both culture systems, pluripotency marker expression remained greater than 80 %.

In addition to their potential use for pluripotent cell expansion, we have found that the use of microcarriers is highly effective for the differentiation of ESCs in SSBs. One of the benefits of using microcarriers for differentiation is that the material used can be customized to enhance differentiation efficiency into a given lineage. For example, we have used bioreactor-expanded murine ESCs to generate osteoblasts and chondrocytes. For these studies, CultiSphere-S microcarriers, made of highly cross-linked collagen, were added to murine ESC bioreactor cultures, and the maintenance medium was replaced with differentiation medium that contained appropriate inducing factors. After a 25-day differentiation period, approximately 80 % of the cells were positive for osteogenic (or chondrogenic) markers [65]. Importantly, these differentiated cells did not form tumors following ectopic or orthotopic implantation in vivo. Overall, we found that the use of microcarriers for murine ESC differentiation enhanced our ability to generate a population of specialized cells, improved the differentiation potential (compared to the use of no microcarriers), and facilitated transplantation of the cells in vivo.

Following the establishment of bioreactor expansion protocols for murine ESC studies, our research group adapted these expansion techniques for human ESCs and murine iPSCs. The bioreactor expansion of these cells posed some challenges since the protocols developed for mouse ESCs could not be directly applied. Optimization of the bioreactor protocols for human ESC and murine iPSC expansion largely relied on adjusting the medium composition.

One of the challenges faced in propagating human ESCs in suspension bioreactors is their sensitivity to dissociation. Typically, a dramatic reduction in cell viability is observed when traditional chemical dissociation methods are used. For the large-scale, long-term expansion of the cells, this posed a significant challenge since it severely limited our ability to culture the cells beyond one passage and impeded our ability to control aggregate diameter in order to prevent cell necrosis. It was recently discovered that supplementation with inhibitor of Rho kinase (ROCKi; Y-27632) increases the survival rate of dissociated human ESCs [66]. Although the use of ROCKi alone was not sufficient to allow for human ESC expansion in SSBs, we found that co-supplementation with rapamycin and ROCKi was successful. Rapamycin is a macrolide antibiotic that suppresses the phosphoinositide kinase family. The downstream target of rapamycin is mammalian target of rapamycin, or mTOR, a protein that is essential for cell growth and development and involved in regulating cell cycle progression, cell size, cell migration, and survival [67–69]. For expansion of human ESCs in SSBs, we used 100 mL of regular maintenance medium supplemented with ROCKi for the first 24 h of culture and supplemented continuously with 0.1 nM rapamycin. Cultures were agitated at 100 rpm. Under these conditions, the cells were expanded up to 12-fold over a 6-day passage period. Further, the expression of pluripotency markers Oct-4, Nanog, Tra-1-60, Tra-1-81, and SSEA-4 remained high, multi-lineage differentiation through teratoma formation in vivo was retained, and the cells exhibited a normal karyotype [49]. Serial passaging, for long-term bioreactor culture, was also achieved.

We have also developed effective bioreactor protocols for the large-scale expansion of murine iPSCs. These protocols were very similar to those used for the murine ESCs, with only subtle changes to inoculation density and medium composition [50]. Under these conditions, murine iPSCs grew as aggregates, similar to the murine ESCs, and retained their pluripotency. Specifically, they expressed hallmark pluripotency markers at levels comparable to their starting population and could be functionally differentiated into different lineages including cardiomyocytes, chondrocytes, and osteoblasts in vitro, as well as differentiated into three germ layers in vivo through teratoma formation. We also found that exposure to conditions in the SSBs did not induce any chromosomal aberrations in the iPSCs, which was again similar to our findings with the murine and human ESCs. In suspension culture, a 24- and 58-fold expansion of the highly viable and undifferentiated iPSCs was achieved for the RS-2 line and RS-3 line, respectively. Growth rates were consistent with those characteristic of mESCs cultured in suspension culture conditions [47, 59].

Several factors may explain the differences between two iPSC lines. The RS-2 line was generated using Oct-4, Sox2, Klf4, and c-Myc retroviral vectors while the RS-3 line was produced using equivalent lentiviral vectors. It is possible that the integration sites are different between two lines giving the RS-3 line faster growth characteristics by possible activation of genes involve in growth and cell division. Future studies are required to compare more cell lines with

different reprogramming protocols. While this first study focused on murine iPSCs, we are currently looking to adapt these protocols for use with human iPSC expansion.

Future Challenges

We have made appreciable progress toward the large-scale production of pluripotent cells, and these achievements may facilitate the future use of these cells in clinical therapies. Despite this important progress, some critical challenges remain.

In addition to the challenges associated with optimizing bioreactor culture conditions for a given cell type (i.e., medium composition, agitation rate, inoculation density), we have encountered some interesting yet somewhat alarming observations over the course of our studies on the bioreactor expansion and differentiation of PSCs. In our murine ESCs studies, we noted that the pluripotency characteristics of the cells remained high over the long-term culture of the cells in bioreactors. Specifically protein and gene expression of Oct-4, Nanog, and SSEA-1 were sustained, and the cells retained their colony-forming ability and multi-lineage differentiation potential. Prior to our experiments involving the induction of osteo- and chondrogenic differentiation of murine ESCs on microcarriers, we attempted to drive differentiation of bioreactor-expanded cells as aggregates (i.e., through medium supplementation alone). Overall, we found that the differentiation was inefficient in the bioreactors and, more interestingly, that a large proportion of the cells actually remained in an undifferentiated state. Specifically, the bioreactor-differentiated cells retained expression of the pluripotency marker Oct-4 as well as tumorigenic potential following implantation in vivo, even after being exposed to differentiation conditions for 30 days (Fig. 3). These findings were in stark contrast to the results obtained from static control cultures, where Oct-4 and tumorigenic potential were rapidly reduced within the first week of differentiation.

Further to this, our group has observed that iPSCs can be generated with a much higher efficiency in SSBs than in the traditionally used static tissue culture vessels. Typically, iPSC generation is an inefficient process, which requires several weeks before a cell line is established. In fact, the efficiency of reprogramming fibroblasts using four viral vectors (Oct-4, Sox2, Klf4, and c-Myc) in static adherent culture was originally demonstrated to be between 0.001 and 0.5 % [70]. However, our group and one other group have recently shown that the rigid cell substratum attachment is not an essential factor for reprogramming and demonstrated successful cellular reprogramming of terminally differentiated mouse somatic cells to iPSCs in matrix-free suspension bioreactors [51, 71]. For this study, our group transduced mouse embryonic fibroblasts (MEFs) with the four Yamanaka factors (Oct-4, Sox2, Klf4, and c-Myc retroviral vectors). Two days post-transfection, the cells were transferred to a 100 mL of medium in a SSB and agitated at 100 rpm. ESC-like aggregates appeared 3 days later, and positive expression of alkaline phosphatase (ALP) was detected from day 5 onward. By day 12, we obtained 50 million ALP^+ cells, while adherent culture only gave rise to about 4 million cells within the same period. The aggregates displayed ESC-like characteristics in vivo and in vitro as shown by gene expression profiling, differentiation and teratoma formation assays, and chimera and germline transmission assays. Importantly, SiPSC (suspension-derived iPSCs) aggregates remained stable after 18 passages in suspension culture, as determined by morphology, percentage of ALP^+ aggregates, and ploidy. We estimate our novel bioreactor reprogramming process to be 100- to 1,000-fold more efficient than conventional methods. The high efficiency of reprogramming in suspension bioreactors suggests that this culture environment plays an important mechanistic role in the regulation of pluripotency.

Taken together, the findings from our studies provoked an important question: what is the mechanism through which the SSBs regulate ESC and iPSC pluripotency? Since cells cultured in SSBs are exposed to fluid shear, and since fluid shear is known to regulate gene expression in a variety of cell types, it is likely that the activation of shear-sensitive mechanotransduction pathways was responsible for the expression of Oct-4 and maintenance of tumorigenic potential in the bioreactor-differentiated cultures. Hydrodynamic shear arises from the formation of small areas of intense turbulence called eddies, which develop as a result of the transfer of energy from the impeller to the medium during agitation [72].

Using a parallel plate flow system [73], we carried out a preliminary study to examine the influence of fluid shear on an adherent monolayer of murine ESCs. Following a 24-h differentiation period under static conditions, a 70 % drop in Oct-4 gene expression was observed in the parallel plate cultures. However after subsequent exposure to fluid shear for 24 h, Oct-4 expression was significantly increased (unpublished results). Although we have been unable to identify other published reports citing fluid shear as a potential regulator of Oct-4 expression, a member of Dr. Robert Nerem's group (Georgia Institute of Technology) also noted that Oct-4 expression could be rescued in differentiated ESCs through exposure to fluid shear using a similar parallel plate flow system (personal communication). Together, these observations may support the notion of fluid shear as a regulatory factor for Oct-4 expression.

A variety of signaling pathways are involved in the regulation of ESC pluripotency; however three pathways are critical for maintaining the cells in an undifferentiated state, the JAK/STAT pathway, the TGF-β pathway, and the Wnt pathway, and both the TGF-β and Wnt pathways are known

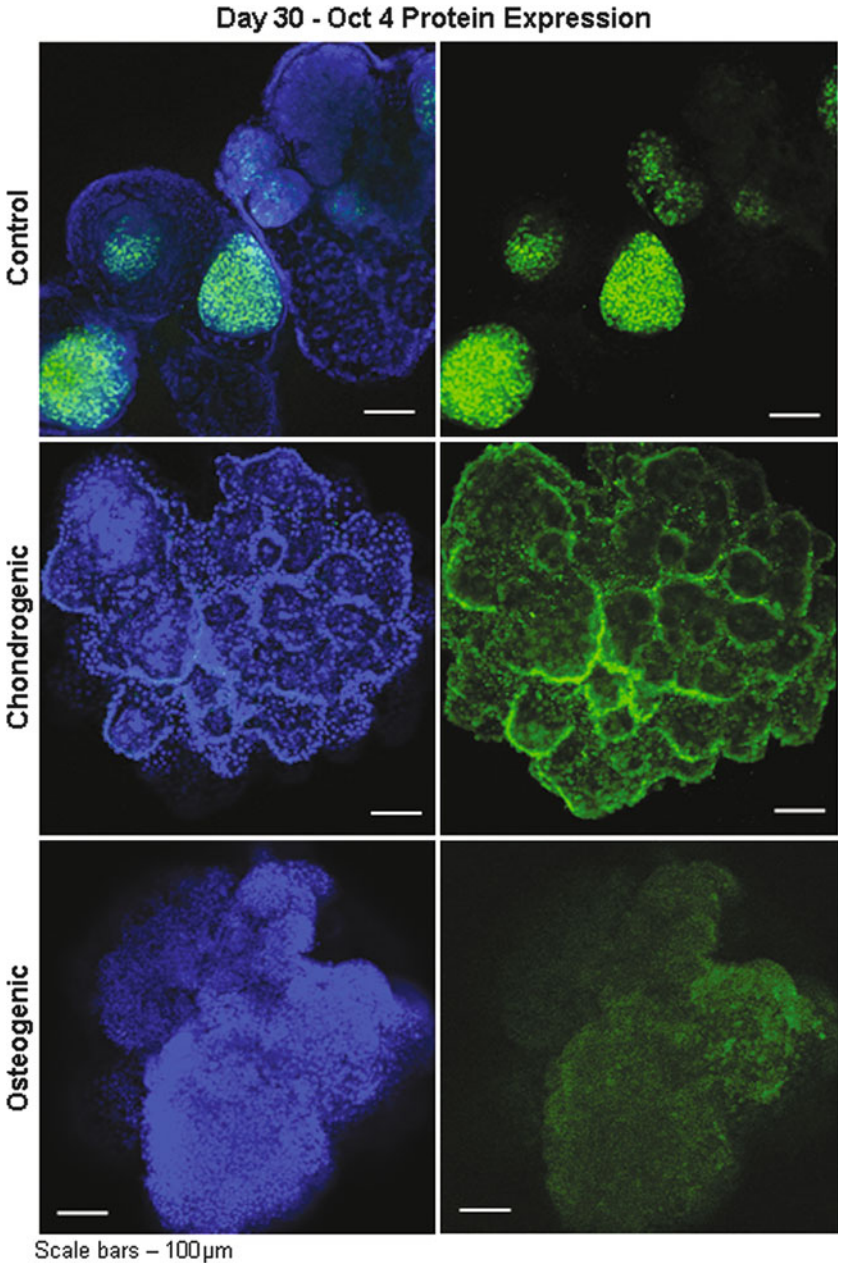

Fig. 3 Pluripotency marker expression. Immunofluorescence of Oct-4 (*green*) with nuclear marker Toto3 (*blue*) in aggregates from the bioreactor cultures on day 30 of bioreactor differentiation. The secondary antibody controls for each treatment group did not show nonspecific binding (inserts) (from [89], with permission from Mary Ann Liebert, Inc.)

to be activated through exposure to fluid shear. The TGF-β pathway regulates a diversity of cellular processes including growth, differentiation, migration, apoptosis, and tumorigenesis [74]. When the TGF-β cell surface receptor is activated, SMAD-2 is phosphorylated and forms a complex with SMAD-4. This complex then translocates into the nucleus and activates the expression of pluripotency genes. Activation of the TGF-β receptor through ligand binding causes phosphorylation of SMAD-2 at the C-terminal. In certain cell types, including endothelial cells, it has also been shown that fluid shear can activate the TGF-β pathway, but causes phosphorylation of SMAD-2 at the linker region [75]. Activation of the pathway under these conditions is thought to occur via surface receptors and/or through activation of other signaling cascades (including ERK/JNK). Under these conditions, it is thought that SMAD-2 associates with SMAD-4 and then translocates into the nucleus to regulate gene expression [75].

Several studies have found that the administration of the TGF-β inhibitor SB-431542 to ES cell cultures blocks the TGF-β receptor kinases and results in a decreased expression of pluripotency markers [76]. Through a separate study, we found that the addition of the SB-431542 TGF-β inhibitor to the suspension bioreactors reduced the tumorigenic potential of the ESCs by day 10 of differentiation, but did not affect Oct-4 expression (unpublished data).

Although there is some evidence to suggest the involvement of the TGF-β pathway in the regulation of ESC pluripotency, the wingless-type protein (Wnt) pathway is another central regulator of pluripotency, and this pathway can also be activated through fluid shear. Binding of the Wnt protein to the Frizzled receptor on the cell membrane activates the canonical pathway, causing inhibition of glycogen synthase kinase 3 (GSK-3) and nuclear accumulation of β-catenin [77]. This signaling cascade has been found to maintain a pluripotent phenotype in both human and mouse ESCs through the transcriptional induction of a variety of pluripotency genes, including Oct-4, Rex-1, and Nanog [78, 79]. Although there are currently no published reports regarding activation of the Wnt pathway in ESCs through exposure to fluid shear, flow-induced shear stress has been found to regulate gene expression in colon cancer cells [80], osteoblasts [81], and endothelial cells [82] through modulation of β-catenin signaling.

Further studies will be required to explore whether the TGF-β and/or Wnt pathways are involved in the regulation of ESC pluripotency in the bioreactors. It should also be noted that the mechanical regulation of Oct-4 expression in the bioreactor cultures may involve the combined action of several signal transduction pathways.

An alternative and equally plausible explanation for the upregulation of Oct-4 in the bioreactor cultures is that the ESCs were transformed into an oncogenic cell type as a result of exposure to the bioreactor culture environment. Interestingly, there are remarkable similarities between ESCs and oncogenic cell types, and most of the core characteristics used to identify pluripotent cells are also exhibited by cancerous cell types. Namely, both ESCs and cancer cells have the ability to undergo long-term clonal self-renewal, both cell types possess tumorigenic potential in vivo, and numerous studies have shown that ESCs and cancer cells share many of the same regulatory networks, including Oct-4, Nanog, and Sox-2 expression [83–86]. As well, seven major signaling pathways have been found to be implicated in both stem and cancer cells: the JAK/STAT pathway, the NOTCH pathway, the MAP-Kinase/ERK pathway, the PI3K/AKT pathway, the NFκB pathway, the Wnt pathway, and the TGF-β pathway [87].

Based on the findings from our studies, the enhanced expression of Oct-4 in the SSB cultures could very well be explained by the mechanical activation of pluripotency regulatory pathways, or by an oncolytic transformation of the cells. Due to the fact that most of the ESC attributes are shared by cancerous cell types, identification of the bioreactor cells as being truly PSCs, and not cancerous cells, will be very challenging. However, finding a definitive answer to this question will be a crucial part of developing effective bioprocesses for the generation of clinically useful PSCs.

In conclusion, effective protocols have been developed for the large-scale production of PSCs, and we are now at a point where the generation of clinically relevant numbers of pluripotent cells is possible. Despite the fact that we can generate large numbers of cells, it is imperative that we consider the effect of these bioprocesses on the genotype and phenotype of these cells. The impact of the bioreactor culture conditions on the cells should be clearly and completely elucidated prior to the clinical use of these cells, so that we can guarantee their safety and efficacy.

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Generation of Hepatocyte-Like Cells from Human Pluripotent Stem Cells

Fallon K. Noto and Stephen A. Duncan

Abbreviations

AFP	Alpha-fetoprotein
BMP	Bone morphogenetic protein
CYP450	Cytochrome P450
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HNF4a	Hepatocyte nuclear factor 4 alpha
iPSCs	Induced pluripotent stem cells
LDL	Low density lipoprotein
OSM	Oncostatin M
TGFb	Transforming growth factor beta
TTR	Transthyretin

Introduction

The liver is a vital organ that is responsible for a broad array of functions such as the production of bile, biotransformation, detoxification, and the synthesis of a myriad of secreted serum factors including Albumin, Alpha-1-antitrypsin, and several blood clotting factors. The liver also has various metabolic activities including, gluconeogenesis, glycogen synthesis, hormone production, urea production, and regulation of cholesterol and lipid flux. This diversity of activities associated with the liver equates with the complexity of pathologies caused by liver dysfunction and infection [1]. The hepatocyte is the major functional cell type of the liver and cultured primary hepatocytes have been used extensively for the study of metabolic liver disease. Unfortunately, under normal culture conditions, plated primary hepatocytes dedifferentiate and rapidly lose many of their characteristic metabolic functions, which limit their usefulness Repopulation of the liver with exogenous hepatocytes could be used to treat a subset of inborn errors of hepatic metabolism and provide an alternative to orthotopic liver transplantation [2]. Although hepatocyte transplant therapies could offer significant advantages over liver transplants, a number of challenges must be overcome before such therapies become routine [2]. Such challenges include the need to access large numbers, $>1 \times 10e^9$ cells per transplant [3], of highly differentiated functional hepatocytes that ideally are genetically matched to the patient. The need for an abundant supply of high fidelity primary human hepatocytes that can be used for both research and therapeutics is therefore substantial.

A potentially inexhaustible source of hepatocytes could be provided by human pluripotent stem cells, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). In addition, iPSCs derived from patients with metabolic liver disease may offer culture models to study the molecular mechanisms underlying hepatocyte dysfunction in these patients. Furthermore, if hepatocytes differentiated from iPSCs can rescue animal models of liver disease, not only would this provide proof-of-principle supporting the therapeutic use of pluripotent stem cells, but could provide patient-specific animal models to study drug toxicity, efficacy, and metabolism.

Differentiation and Characterization of Hepatocyte-Like Cells Derived from Pluripotent Stem Cells

An overwhelming number of protocols have been published that describe the generation of hepatocyte-like cells from human pluripotent stem cells. We have recently provided through open-access publishing a step-by-step procedure that has been used successfully by many labs to produce hepatocytes from both human ESCs and iPSCs (http://www.stembook.org/node/721) (Fig. 1).

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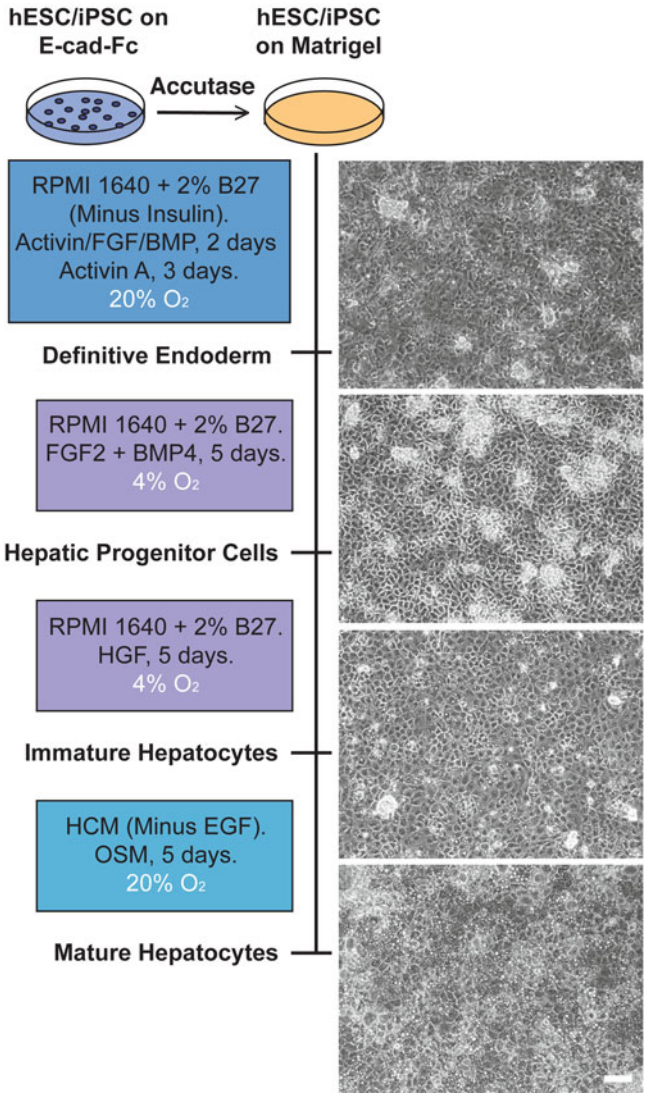

Fig. 1 Overview of the protocol used to differentiate human pluripotent stem cells into hepatocyte-like cells. Pluripotent cells are maintained on an E-Cad-IgG Fc substrate before being passaged to Matrigel-coated dishes for differentiation. Cells are exposed to growth factors to differentiate the cells in a stepwise fashion that recapitulates hepatogenesis. Images on the *right* show phase contrast micrographs of the cells during each stage of the differentiation (scale bar = 100 μm) (figure modified from Cai et al. 2012. Protocol for directed differentiation of human pluripotent stem cells toward a hepatocyte fate. 2012. Stembook. Harvard Stem Cell Institute. http://www.stembook.org/node/720)

Differentiation by Embryoid Body Formation

The first studies attempting to produce hepatocytes from human ESCs were based on protocols developed using mouse ESCs, which were classically differentiated by growing the cells in small clusters as suspension aggregates on non-adherent dishes [4, 5]. The resulting clusters are called embryoid bodies because they generate cell types from all three germ layers in a process that recapitulates aspects of gastrulation. Lavon et al. showed that formation of embryoid bodies without any exogenous factors could produce a population of cells of which ~6 % expressed albumin, and culturing the cells with media conditioned by mouse primary hepatocytes increased the number of albumin-expressing cells dramatically [6]. These results suggested that exogenous factors could direct differentiation of human pluripotent cells to a specific cell fate and as a consequence many protocols now use growth factors to enhance differentiation after generating an embryoid body intermediate [7–19]. The choice of growth factors used in these various protocols differ; however, most were selected because of their known effects during hepatogenesis and include bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), oncostatin M (OSM) and/or dexamethasone. Given the substantial number of variations in the cocktails of growth factors added following embryoid body formation and the observation that no single protocol is uniquely efficient, it appears that there is flexibility in inducing hepatocyte-like cells using embryoid body-based approaches. While cells generated by these procedures all express at least a subset of hepatocyte markers it is important to realize the heterogeneous nature of embryoid body-mediated differentiation. Although, contaminating non-hepatic cell populations may well contribute factors that positively affect hepatocyte differentiation, the heterogeneity associated with embryoid body formation can be problematic if pure populations of hepatocytes are needed for downstream applications. In this regard, procedures have been developed to facilitate the purification of hepatocyte-like cells from mixed cell populations that include using hepatocyte transcriptional regulatory elements to drive expression of reporter constructs or selectable markers or the isolation of hepatocytes by FACS [6, 9, 20, 21].

Directed Differentiation by Cell Signaling Factors

Because pluripotent stem cells are reminiscent of early progenitor cell types, such as those found in the inner cell mass or epiblast, it seems logical that human iPSCs or ESCs could be directed toward a specific cell fate in culture by recapitulating the environmental cues normally encountered during embryonic development. The study of liver development has revealed several signaling factors that have integral roles in regulating hepatogenesis and several recent reviews have described advances in the field in detail [22–24]. The parenchymal components of the liver originate from the ventral portion of the anterior endoderm after the endoderm is produced during gastrulation in response to Nodal signaling [24]. At around embryonic day (E) 8.0 in the mouse (2–4 somites), morphogenesis of the foregut positions the presumptive hepatic endoderm next to the developing heart

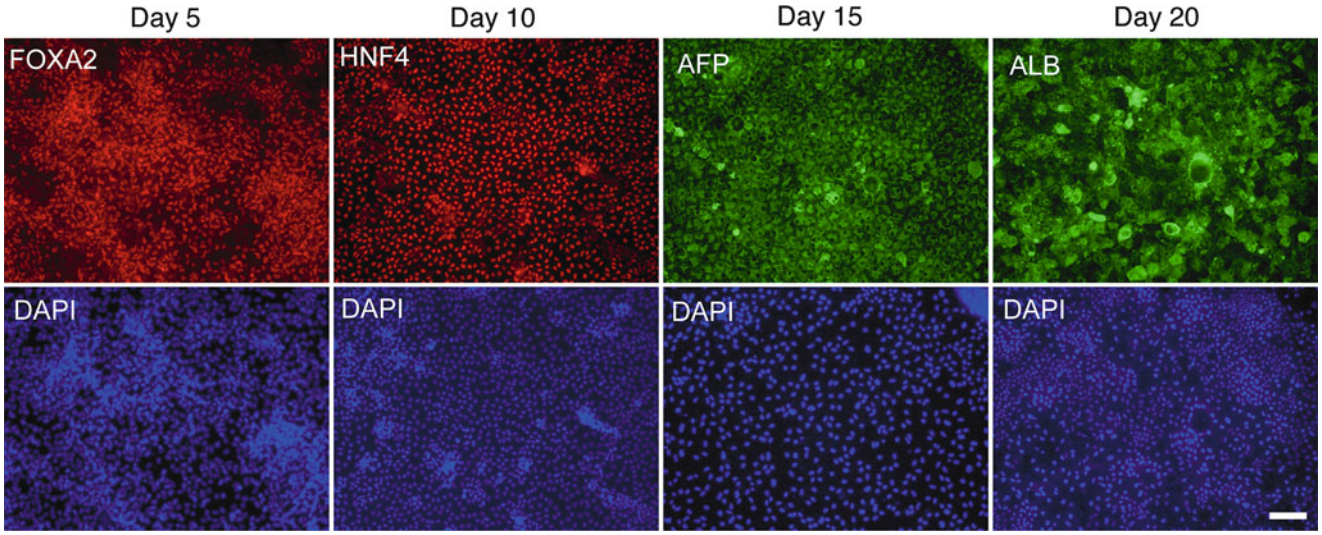

Fig. 2 Characteristic proteins expressed at each stage of differentiation. Immunocytochemistry was used to identify definitive endoderm cells expressing FOXA2 expression after 5 days of differentiation. After 10 days, specified hepatic cells express HNF4a. Hepatoblasts express alpha-fetoprotein after 15 days, and finally, after 20 days of differentiation, albumin expression can be detected in the cells (scale bar—100 μm)

and in close proximity to the mesoderm that will form the mesothelial linings of the intraperitoneal cavity. BMPs and FGFs, that appear to originate from the mesoderm, instruct the endoderm to follow a hepatic fate. By E8.5, an anatomical expansion of the ventral endoderm, called the liver bud, can be identified and shown to express several characteristic hepatic mRNAs including albumin, alpha-fetoprotein (AFP), Ttr and Hepatocyte nuclear factor 4 alpha (HNF4a). At E9.5, the hepatic progenitor cells delaminate and invade the surrounding mesenchyme. The liver undergoes a tremendous amount of growth from this point until E15.0. Signals from the mesenchyme, including FGFs, BMPs, HGF, and WNTs stimulate hepatoblast migration and proliferation [25] and by mid-gestation stages of development the liver becomes the major site of hematopoiesis within the fetus. The hematopoietic cells within the liver secrete the cytokine OSM, which is required for maturation of hepatocytes [26].

A significant advance in using human pluripotent stem cells to generate endoderm-derived lineages was provided by D'Amour et al. [27]. Using hESCs, the authors demonstrated that definitive endoderm could be induced with impressive efficiency by treating the cells with high concentrations of Activin A, a Nodal mimic, under conditions that inhibited PI-3 kinase activity [27, 28]. Under these conditions 80–90 % of the cells express proteins, such as CXCR4, FOXA2, SOX17, and FGF17, that together define endodermal character (Fig. 2) [20, 28, 29]. Marker analyses suggested that the formation of the endoderm from hESCs was preceded by the transient production of mesendoderm, which reflects the process through which definitive endoderm is normally formed in the embryo [27, 28]. Profiling mRNA expression also suggested that the endoderm specifically exhibited foregut characteristics, expressing GATA4, HHEX, and CER1 all of which are enriched in the ventral foregut endoderm in pre- to early-somite stage embryos [30–32]. The ventral foregut nature of this specified endoderm is important because key endodermal organs, including the liver and pancreas are derived from this specific portion of the endoderm, suggesting that endoderm produced by the D'Amour approach could be ideal for further differentiation to produce hepatocytes and pancreatic islet cell types [33]. Although the majority of directed differentiation protocols rely on induction using Activin A, some modifications have been described that may increase the efficiency of endoderm production such as inclusion of WNT3A, BMP4, or HGF along with Activin A treatment; however, the increases in efficiency that are reported appear to be relatively modest [34–36]. More recently, modifications to the addition of Activin A, including inhibition of TGF-β and BMP activity or inclusion of WNT3A and FGF4 after initial formation of the definitive endoderm, can also impact the character of the endodermal cells causing them to express anterior or posterior markers, respectively [37, 38]. This may have important consequences for the generation of other endodermal-derived cell lineages such as those that generate the lung or gastrointestinal tract.

In most cases the efficient generation of endoderm from the pluripotent stem cells is crucial for the successful production of hepatocyte-like cells. Once the definitive endoderm is produced most protocols rely on the removal of Activin A and the subsequent addition of FGF2 and BMP4 to induce the endoderm to adopt a hepatic fate. The choice of adding FGF and BMP was informed by studies in the mouse predominantly by the Zaret laboratory, who demonstrated, using ex vivo embryo culture models, that addition of these

factors was sufficient to induce hepatic specification in isolated ventral endoderm [39, 40]. The efficiency of induction of the endoderm can again be measured by examining the identification of markers that are known to be expressed in the early liver bud including several liver transcription factors such as HNF4a, HNF1b, TBX3 and HHEX all of which have known roles in controlling early development of the hepatic progenitor cells (Fig. 2) [41–46]. Again the efficiency through which the endoderm is converted to a hepatic fate is generally very high, with the more robust protocols generating upwards of 80 % of cells expressing early hepatic progenitor markers.

A variety of factors have been used to induce the hepatic progenitor cells to further differentiate including addition of BMPs, a variety FGFs, HGF, and Dexamethasone to complex media commonly containing Insulin, Transferrin, and Selenium [21, 47–53]. The consequence of these factors is to produce cells that express proteins that are commonly enriched in the fetal hepatocytes including AFP, fibrinogen alpha chain, angiotensinogen, and transferrin (Fig. 2) [29]. As with previous stages, the conversion of the hepatic progenitor cells to immature hepatocytes is upwards of 80 % when using the most efficient protocols.

Finally, several groups add OSM at various steps of the differentiation process to induce maturation of the immature hepatocytes [12, 34, 36, 54–68]. The choice of adding OSM again was the result of original studies by the Miyajima laboratory that examined regulation of mouse liver development by cytokines [26, 69, 70]. This work led to a model in which factors from hepatic mesenchymal cells including HGF and Integrin signaling pathways converge with OSM, which is secreted from hematopoietic cells that are abundant within the fetal liver, to drive the fetal hepatocytes toward a mature phenotype. While the role of OSM during hepatocyte maturation in the mouse is supported by several genetic experiments, many of the protocols used to differentiate hepatocytes from human ESC/iPSCs circumvent the addition of the OSM suggesting that under some conditions this factor is dispensable.

In general, these protocols lead to populations of cells that have many features associated with hepatocyte function. The differentiated cells express a large number of proteins that reflect key activities of the liver, including albumin, tyrosine aminotransferase, several apolipoproteins, orosomucoids, coagulation factor VII, and the asialoglycoprotein receptor (Fig. 2) [8, 29, 55, 63]. In addition to secreting albumin, the resulting cells have also been shown to be capable of synthesizing glycogen, internalizing and secreting vLDL/LDL, metabolizing indocyanine green, producing urea, storing lipid, and in a subset of studies the cells were capable of engrafting into the parenchyma of the mouse liver, at least in short-term assays [8, 9, 34, 36, 53–55, 63, 71, 72].

While many protocols can achieve populations of cells that are highly enriched in liver-specific markers, all protocols have one major pitfall in common: no protocol has generated cells that are functionally equivalent to primary human hepatocytes. Most protocols produce cells that retain expression of fetal proteins that are normally silenced in adult liver cells such as AFP and commonly lack expression of a subset of proteins necessary for full hepatocyte function. Global gene expression studies that compare expression profiles of human ESC/iPSC-derived hepatocytes to adult or fetal liver samples reveal a substantial overlap in mRNA distribution [29, 63]. However, quantitative studies using qRT-PCR show that the levels of many genes normally expressed in adult livers or primary hepatocytes are significantly lower in hepatocytes derived from pluripotent stem cells [51, 58, 59, 63, 64, 73, 74]. Unfortunately, the majority of protocols describing the differentiation of hepatocytes from human ESCs or iPSCs fail to compare mRNA levels to those found in fresh primary hepatocytes or liver samples and instead report the relative increases in mRNA that occur during the differentiation process. The failure to compare mRNA levels to accepted standards makes it difficult to judge the quality of cells produced by different protocols and leads to considerable confusion in interpreting differentiation efficiency. Among the genes whose mRNA levels are commonly significantly lower in stem cell-derived hepatocytes compared to adult hepatocytes are those encoding phase I, II, and III enzymes, which have critical roles in detoxification [49, 63, 73]. The reduced levels of these enzymes is important because hepatocytes are the principal site for the metabolism of xenobiotics and pharmaceuticals and so human ESC/iPSC-derived hepatocytes could be extremely useful for drug toxicity testing if the cells recapitulated expression of detoxification enzymes, such as CYP3A4. A number of groups have tried to improve the fidelity of the metabolic response of iPSC-derived hepatocytes by introducing exogenous factors known to regulate expression of phase I, II, and III enzymes. Takayama et al. demonstrated that by sequentially increasing the expression of transcription factors during differentiation using adenoviruses, they could increase the levels of CYP3A4, CYP2D6, and CYP7A1 [68]. In a separate study, increasing expression of the constitutive androstane receptor (CAR) also resulted in increased expression of detoxification and metabolic mRNAs, including several encoding Cytochrome P450 (CYP450) proteins [57].

While forced expression of exogenous transcription factors provide insight into how to overcome the lack of maturity of stem cell-derived hepatocytes, less invasive methods will likely be necessary for such cells to be useful in a therapeutic setting. It could be argued that the culture of hepatocytes in a 2D environment is too simplistic and to produce hepatocytes that more closely resemble those in the liver and it will be necessary to generate a culture environment that more closely recapitulates the structure of the liver. Indeed, modifying the extracellular matrix and using sandwich

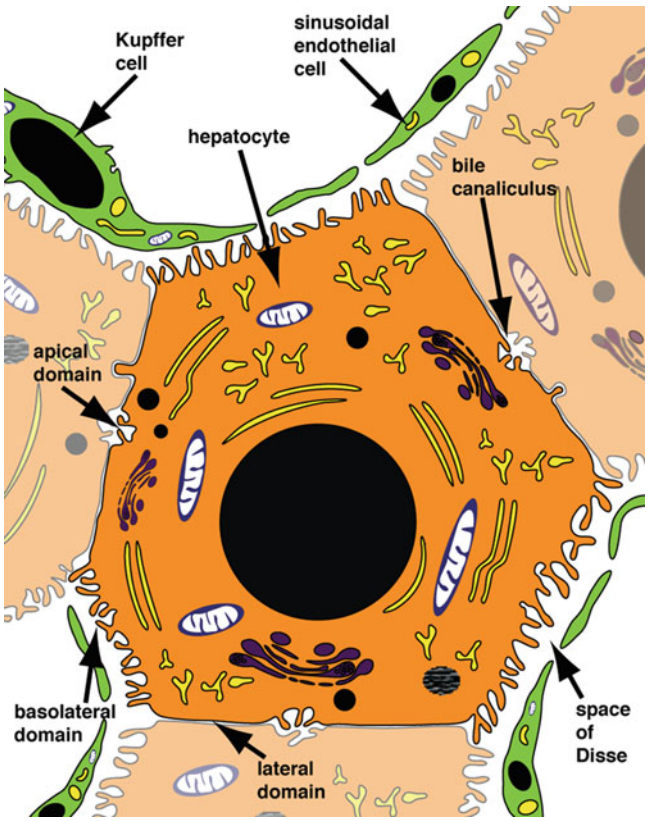

Fig. 3 Illustration showing the relationship between hepatocytes and the sinusoidal endothelium. Hepatocytes are polarized epithelial cells with apical domains that generate the bile canaliculi, lateral domains that face neighboring hepatocytes, and basolateral domains that face the sinusoids. The basolateral surface directly contacts the peri-sinusoidal space (also called the space of Disse) which contains matrix proteins. The close association with Kupffer cells, sinusoidal endothelial cells, and extracellular matrices are believed to influence hepatocyte function and maturity (illustration by Cameron B. Duncan, interpreted from Bloom and Fawcett, 1994. A textbook of histology, 12th edition. New York, NY, Chapman & Hall)

cultures has been shown to improve the quality of primary hepatocytes [75, 76]. Although hepatocytes are responsible for the majority of liver functions, hepatocytes closely interact with several other cell types found in the liver. Biliary epithelial cells, also known as cholangiocytes, form the bile ducts, sinusoidal endothelial cells and Kupffer cells, which are resident hepatic macrophages, form the hepatic capillaries, and stellate cells, which resemble pericytes found within the peri-sinusoidal space, collaborate to form the basic architectural unit of the liver known as the lobule [77]. The architecture of the hepatic lobule is integral to the liver's function (Fig. 3). The basolateral side of the hepatocyte is lined by sinusoidal endothelial cells, which facilitates absorption of toxins and metabolites by the hepatocytes and the secretion of serum factors into the blood stream. Adjacent hepatocytes are connected by tight junctions, generating a canaliculus that transports bile acids and salts to the bile duct. Within the lobule the hepatocytes are supported by a fine extracellular matrix consisting primarily of collagen type III with small amounts of collagen type I. Since complex interactions between several cell types and matrix components in the liver are likely to influence the activity of the hepatocytes, several laboratories have attempted to use culture conditions that more closely resemble the in vivo environment such as coculture with supportive cell types including human fetal liver stromal cells (hFLSCs) and Swiss 3T3 cells [13, 78]. A number of groups have also used 3D formats including culture in dynamic 3D perfusion bioreactors [79]. A combination of tissue engineering, 3D culture, and matrix optimization, may well be important because several studies have shown that such variables can affect the maturity of cultured primary hepatocytes [80–83].

Although the differentiation protocols discussed above have produced cells that promote hepatocyte maturation, the resulting cells still fall short of the activity associated with fresh human hepatocytes. A limitation of the hepatocytes derived from human ESCs and iPSCs is the failure of the cells to extensively repopulate a damaged liver in the long term. In contrast, extensive, upwards of 80 %, repopulation of the hepatic parenchyma is routinely achieved when using primary human hepatocytes in several different animal models of liver damage [84–88]. A number of groups have demonstrated that human ESC/iPSC-derived hepatocytes can integrate into the hepatic parenchyma in short-term analyses [8, 9, 18, 34, 36, 53–55, 63, 72, 89, 90]. In the limited cases where long-term engraftment has been claimed, the level of human albumin that can be detected in the serum appears to be vanishingly low [72]. When primary human hepatocytes are transplanted into the FRG mouse, if 3 % of the liver contains human hepatocytes, ~1 mg/mL of human albumin can be detected in the serum of the transplanted mouse and this level rises to 15 mg/mL when repopulation approach 90 % [84, 86]. In the study by Liu et al., which examined mice after 8-weeks of engraftment, the maximum level of albumin detected in the serum was ~40 ng/mL [72]. These results would suggest that the human cells are either very poorly differentiated or that the levels of engraftment are much lower than has been estimated using antibody staining techniques, which can be prone to artifact.

Why human ESC/iPSC-derived hepatocytes fail to repopulate damaged mouse livers with high efficiency is unclear. It seems unlikely that human pluripotent stem cells are inherently unable to generate functional hepatocytes because several studies have demonstrated that mouse iPSCs are capable of forming fully functional livers in vivo [63, 91, 92]. It would seem more plausible that the differentiation procedure requires improvement, animal models need to be optimized, and efficient transplant techniques need to be employed to enhance the implantation and survival of the exogenous cells [93].

Using Pluripotent Stem Cells to Model Liver Disease and Hepatocyte Differentiation

Although current differentiation protocols generate hepatocytes that retain fetal characteristics, it is important to acknowledge that pluripotent stem cell-derived hepatocytes also display many of the activities normally associated with the adult liver [17, 29, 48, 49, 63, 65, 94]. The ability to generate cells with the majority of hepatocyte function intact, particularly from iPSCs, raises the possibility of using such cells to study inborn errors of hepatic metabolism and a number of groups have generated iPSCs from patients with inherited liver disease [11, 62, 95, 96]. The Vallier laboratory demonstrated that hepatocyte-like cells derived from patients with a variety of metabolic disorders displayed pathologies characteristic of their respective liver deficiencies [62]. For example, cells derived from patients with alpha 1-antitrypsin deficiency accumulated misfolded alpha 1-antitrypsin protein in the endoplasmic reticulum and cells derived from patients with glycogen storage disease had significantly elevated glycogen accumulation. In addition, the group demonstrated that it is possible to genetically repair the causative mutations in alpha 1-antitrypsin deficient iPSCs using a combination of zinc finger nucleases (ZFNs) and piggyBac transposon mediated gene targeting [97].

Many heritable liver metabolic disorders are highly complex where the dysfunctional response of the hepatocyte to a given mutation involves multiple pathways. An example of such a disease is familial hypercholesterolemia that is primarily caused by mutations in the low density lipoprotein receptor (LDLR). In familial hypercholesterolemia patients, the hepatocytes not only fail to correctly internalize and clear low density lipoprotein (LDL)-cholesterol, but also secrete extraordinarily high levels of VLDL/LDL into the serum [98, 99]. To determine the feasibility of modeling such complex metabolic disorders our laboratory recently generated iPSCs from a familial hypercholesterolemia patient with well characterized mutations in the LDLR [100]. Hepatocytes derived from these iPSCs were found to reproduce key aspects of the pathophysiology associated with familial hypercholesterolemia including a failure to internalize LDL-cholesterol, a dramatic increase in LDL secretion, and an inability to respond to lovastatin treatment [95]. Such results are encouraging because they suggest that patient iPSCs could be useful for determining the contribution of specific allelic variations to control lipid metabolism.

The use of iPSC-derived hepatocytes is not limited to the study of metabolic disease, since such cells could potentially be used to study infectious liver disease including hepatitis virus infections and malaria. Two groups have independently demonstrated that iPSC-derived hepatocytes are capable of supporting the entire lifecycle of hepatitis C virus [101, 102]. Such findings open up the possibility of using iPSC-derived hepatocytes from individual patients to examine the role of host genetics in modifying viral replication. Treating HCV infected iPSC-derived hepatocytes with antiviral drugs block viral replication, which suggests that iPSC-derived hepatocytes could also provide a platform to identify novel pharmaceuticals that could be useful in blocking HCV infection. If iPSC-derived hepatocytes are capable of supporting the lifecycle of other liver pathogens, such cells are likely to make a significant contribution to our understanding and treatment of infectious liver disease.

Pluripotent Stem Cells as a Model for Human Hepatocyte Differentiation

The process through which factors that control cell differentiation are identified has historically been extremely laborious, relying heavily upon the generation of transgenic and knockout mice. The use of mice has been necessary because a robust model to study hepatocyte differentiation in culture has been lacking. One can deplete target mRNAs in hepatoma cells quite easily; unfortunately, the data generated through this approach have relatively little relevance to the differentiation of hepatocytes in vivo. This is because (1) hepatoma cells are pathologically abnormal and have lost many hepatic functions as well as normal control of gene expression, and (2) hepatoma cells at best represent a snapshot of a specific developmental stage, or more accurately an abnormal dedifferentiated state caused, in part, by loss of appropriate transcription factor networks and genomic rearrangements. The static state of hepatoma cells is a serious limitation because the developmental process is dynamic, with many factors being essential at specific developmental stages. Metaphorically, using hepatoma cells as a model to study differentiation is akin to attempting to understand how to construct a skyscraper by examining only the fifth floor! However, the observation that the stepwise differentiation of pluripotent stem cells toward hepatocytes appears to mimic the process that occurs during hepatogenesis suggests that human ESCs or iPSCs could offer a model system that would facilitate the study of the fundamental molecular mechanisms that control hepatocyte differentiation. Our laboratory is exploring the usefulness of this system to determine the role of specific transcription factors in controlling the specification of hESC-derived hepatocytes. Oligonucleotide array analyses throughout the differentiation procedure have established mRNA profiles that are characteristic of each stage of differentiation [29]. In addition, these analyses identified mRNAs encoding proteins with potential roles in regulating differentiation of hepatocytes from the hESCs. HNF4a is one such protein that is initially expressed coincident with specification of hepatoblasts from hESC-derived

endoderm [29, 63]. We generated hESCs that expressed an shRNA that efficiently depleted HNF4a following differentiation and found that when HNF4a was depleted the differentiating cells were incapable of adopting a hepatic fate. These findings determined that human pluripotent stem cells can be used to efficiently probe the molecular basis of hepatocyte differentiation and that, in comparison to using the mouse as a developmental model, the use of human ESCs was extremely efficient.

Summary

Substantial progress has been made in the effort to generate high quality functional hepatocytes from human pluripotent stem cells. Although many protocols have been described, most attempt to mimic the native signaling events that occur during hepatogenesis. Although the efficiency of the many different procedures varies significantly, in the better protocols upwards of 80 % of the cells express hepatocyte mRNAs and proteins and display several activities associated with liver function. Although the cells produced by these procedures are extremely useful in providing models of liver disease and cell differentiation, these hepatocyte-like cells are not identical to freshly isolated hepatocytes. Current protocols are unlikely to produce cells that would be useful in drug toxicity testing nor could they supply cells to be used in cell transplant therapy for the treatment of liver disease [103]. With this in mind, the effort to improve the quality of hepatocytes derived from human pluripotent stem cells continues aggressively and new procedures that use 3D culture of mixed cell populations are particularly promising. In addition, several groups are attempting to repopulate livers of animals with human pluripotent stem cell-derived hepatocytes including mice, rats and pigs with genetic lesions that facilitate humanization of the hepatic parenchyma [8, 84, 104, 105]. Similar approaches have allowed the maturation of pancreatic endocrine progenitor cells in diabetic mouse models [106, 107]. If successful, such transplantation models could provide a limitless supply of patient-specific, highly differentiated hepatocytes.

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Neural Differentiation from Pluripotent Stem Cells

Mahendra Rao and Nasir Malik

Introduction

Significant progress has been made over the last 2 decades in understanding the molecular basis for neural development. This accrued knowledge has been translated into improved methodologies for generating subtypes of central nervous system (CNS) and peripheral nervous system (PNS) neurons and glia from pluripotent stem cells (PSCs). PSCs are thought to be equivalent to cells of the inner cell mass (ICM) of the early mammalian embryo which gives rise to the three germ layers and the entire embryo. All of the cells of the CNS and PNS originate from the neural tube, which is derived from the ectodermal germ layer. CNS development follows an orchestrated chain of events controlled by signaling cascades and region-specific transcription factor codes that convey spatial and identity to multipotent neural stem cells (NSCs). The PNS is more spatially diffuse than the CNS and, therefore, its development is not as tightly orchestrated. All PNS neurons and glia arise from neural crest stem cells (NCSCs) migrating out of the neural tube. Because embryonic stem cells (ESCs) and induced PSCs (iPSCs) are equivalent to the ICM, it stands to reason that PSCs could be differentiated into the various neural lineages under the proper culture conditions. Years of research in stem cell biology and neural development have buttressed this reasoning and yielded experimental protocols for the generation of neural cells from PSCs. As is the case in vivo, the protocols involve the serial diminution of differentiation potential beginning with PSCs being differentiated to multipotent NSCs, which in turn are differentiated to region-specific progenitors that are matured to terminally differentiated neural cells (Fig. 1). Positional information and maturity is governed by culturing cells with compounds that can activate or repress key signaling pathways that are active (or inactive) during neural development. The availability of markers specific to a particular neural lineage allows one to track what cell types are being produced during the in vitro differentiation protocols.

Generating Neural Stem Cells from Pluripotent Stem Cells

Several methods may effectively generate NSCs from PSCs with the first step involving dissociation of PSCs and growth in media lacking either fibroblast growth factor 2 (FGF2) for human PSCs or leukemia inhibitory factor (LIF) for mouse PSCs. The most common step following disaggregation involves either growing the cells in suspension culture as embryoid bodies (EBs) or in adherent monoculture with some protocols including the addition of bone morphogenetic protein (BMP) inhibitors to inhibit mesodermal differentiation while guiding PSCs along the neural default pathway [1–5]. Each of these procedures requires culture on substrates and in media formulations that will favor differentiation to the neural lineage. Once human or mouse NSCs form, culture in the presence of FGF2 allows for their proliferation and expansion [2, 3]. The ability to expand NSCs, freeze and thaw them, and grow them for 10–20 passages makes these cells very powerful tools in neural differentiation protocols.

Differentiation of Pluripotent Stem Cells to Generic Central Nervous System Neural Lineages

Early studies differentiating embryonic carcinoma cells to neural lineages suggested that retinoic acid (RA) induced posterior CNS markers [6]. The availability of more specific markers for neuronal subtypes revealed that this is indeed the

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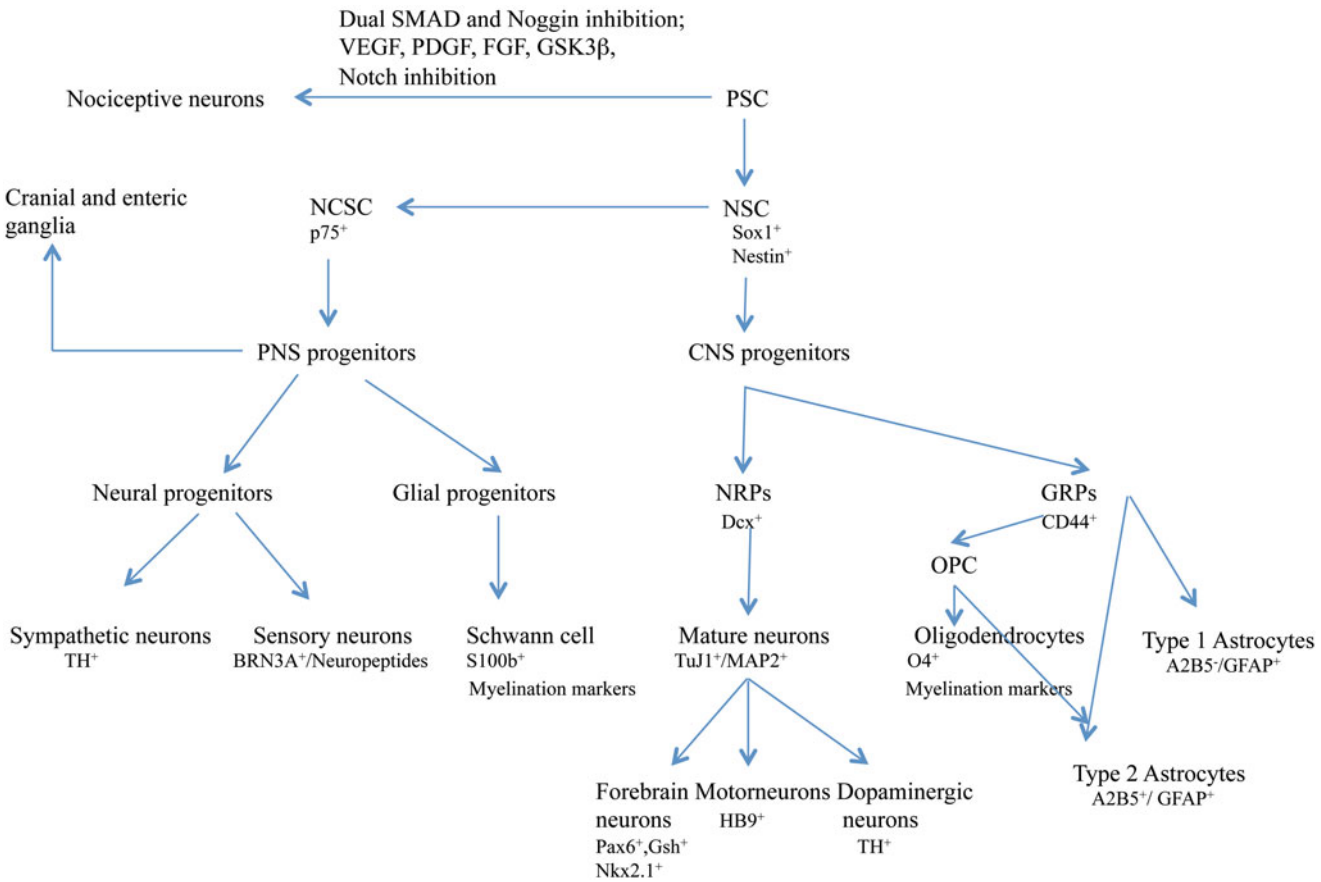

Fig. 1 The hierarchy of differentiation from a pluripotent stem cell (PSCs) to the neural lineages. Neural stem cells (NSCs) arising from a PSC can give rise to all of the neuronal and glial cell types of the peripheral nervous system (PNS) and central nervous system (CNS). The CNS progenitors can be subdivided to neuronal- and glial-restricted precursors (NRPs, GRPs). CNS GRPs also giving rise to an oligodendrocyte progenitor cell (OPC) that is bipotential in vitro. The PNS also has neuronal and glial progenitors that differentiate into mature neurons and glia. Markers used to identify a cell type are noted

case [7]. The original EB protocol enriches for the mesodermal lineage at the cost of ectoderm, but with the proper culture conditions this process can enrich for neural progenitors [8]. Bain et al. [9] added 500 nM RA during EB formation and found that it drove mouse ES cells towards a neuronal fate. Further characterization indicated that functional inhibitory and excitatory neurons were produced by RA induction [10, 11]. Some studies suggested that RA had the capacity to induce differentiation to both neurons and glia with an increased efficiency of glial differentiation upon the addition of 1 % fetal calf serum (FCS) and that the sequence in which the neural cell types appeared recapitulates in vivo development with neurons appearing first followed by glia [12–14]. RA appeared to act by inhibiting mesoderm specific genes and activating neuronal genes [15]. A detailed investigation of the effects of RA on ESC differentiation revealed that at 1–10 nM RA was permissive for mesodermal differentiation. However, at concentrations greater than 10 nM, more caudal progenitors started to be produced with spinal positional identity occurring at 1–10 nM RA [16]. As researchers gained new insights about cell culture medium additives and substrates for attachment that promoted neural differentiation of PSCs, RA was used only in those situations where caudal positional identity was desired [2]. All of the work mentioned above was performed in mouse PSCs, but RA has been found to have a similar affect in human cells.

Neural and Glial-Restricted Progenitors

Immunopanning was incorporated into the EB differentiation protocol to isolate neuronal-restricted precursors (NRPs) with the E-NCAM antibody [17]. These progenitors could be expanded with FGF2 and NRPs could be differentiated to neurons with FGF2 withdrawal and RA addition. Glial-restricted precursors (GRPs) were induced with the addition of platelet-derived growth factor (PDGF) and immunopanned with the A2B5 antibody. GRPs could be differentiated to mature glia with FGF2 withdrawal and addition of PDGF and triiodothyronine (T3). As is the case in vivo, the

differentiation potential of NRPs and GRPs was restricted to the neuronal and glial lineages, respectively. The ability to isolate NSCs and restrict their differentiation potential to neurons and glia opened up new opportunities for the directed differentiation of PSCs to specific neuronal and glial cell types.

Differentiation of NSCs to Specific CNS Neurons

Signaling pathways and transcription factors required for positional identity as well as many neuronal subtype-specific markers have been characterized to such an extent that it is possible to differentiate NSCs to many different neuronal types and then identify the subtypes that are present in the differentiated populations. CNS neurons develop from an NRP that has the capacity to generate a neuron at any dorsoventral or rostrocaudal position if cultured with the appropriate signaling molecule(s). The mature neurons can be identified with one or more markers that are unique to that subtype (schematized in Fig. 1). Differentiation of NSCs to forebrain, midbrain, and hindbrain/spinal cord neurons is described in the following sections.

Forebrain Neuron Differentiation

Dorsal/ventral patterning in the developing forebrain generates the dorsal-most pallium and the lateral, medial, and caudal ganglionic eminences (LGE, MGE, CGE) going from a dorsal to ventral direction. Wnts and BMPs are signaling molecules that dorsalize the forebrain while Sonic hedgehog (SHH) and FGF ventralize it [18]. Most neurons in the forebrain are excitatory pyramidal neurons derived from the pallial subventricular zone. Cortical inhibitory interneurons originate primarily from the MGE and CGE. Although neural progenitors derived from NSCs tend to be biased towards a rostral/cortical fate, additional knowledge about signaling molecules active in the neocortex has been used to develop differentiation protocols for more efficient production of specific forebrain neurons (Fig. 2).

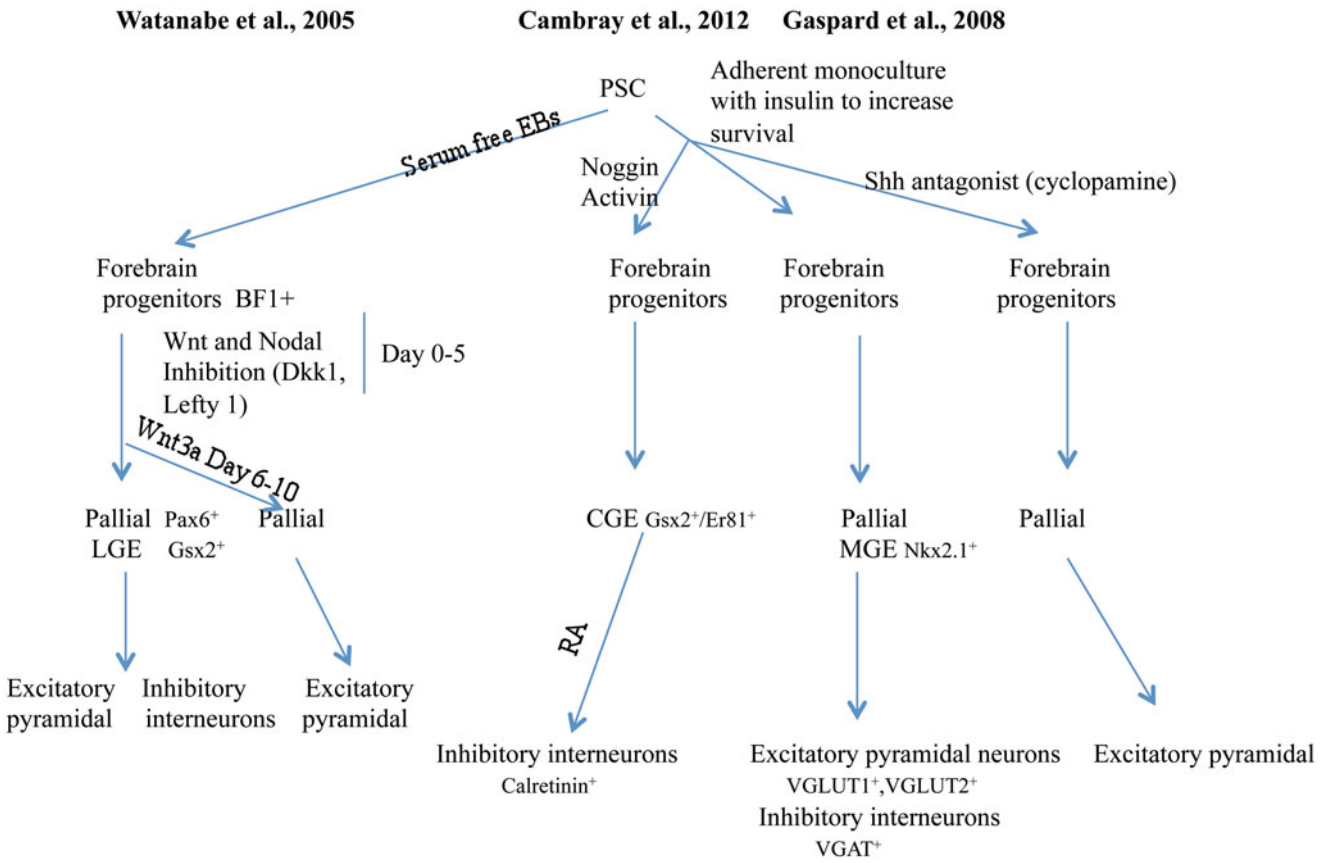

Fig. 2 Differentiation of forebrain neurons. Culture in serum-free medium as an embryoid body or in adherent monoculture can generate progenitors of the lateral, medial, and caudal ganglionic eminences (LGE, MGE, CGE). Upon further differentiation these precursors can become inhibitory interneurons or excitatory pyramidal based upon the factors in the culture medium. Publications which are summarized are listed at the *top* of the figure

Watanabe et al. [19] used a serum-free EB protocol to make NSCs from mouse ESCs that were enriched for forebrain progenitors. As Wnt and BMP signaling have an inhibitory effect during early neuralization, the Wnt inhibitor Dkk1 and the Nodal inhibitor Lefty1 were used to enrich for NSC. The resulting NSCs appeared to be biased towards forebrain progenitors. In contrast to its inhibitory role during the early stages of forebrain specification, Wnt signaling can promote pallial specification during later phases of this process [20]. Treatment with WNT3A at later stages of the differentiation protocol increased the number of pallial neurons (most likely pyramidal neurons) at the expense of LGE and MGE neurons. Conversely, SHH treatment at later stages of the differentiation protocol increased the number of MGE neurons while sharply diminishing pallial neurons. The temporal specification of neurons and effects of manipulating WNT, BMP, and SHH pathways were very similar to what occurs in vivo.

Adherent monocultures to produce NSCs from mouse ESCs yielded similar results [21]. As would be expected from developmental studies of the mouse neocortex, the number of excitatory pyramidal neurons could be increased in this protocol with the addition of the SHH inhibitor cyclopamine. The neurons were generated in a manner that faithfully reproduced the normal developmental profile in vivo and were functional as determined by transplantation studies in neonatal mice. A recent study with mouse and human ESCs found that in the monoculture protocol, addition of activin during progenitor differentiation and RA during neuron maturation resulted in the generation of calretinin$^+$ inhibitory interneurons that are derived from the CGE in vivo [22]. The authors claimed that activin promotes differentiation of NSCs by inhibiting an SHH-mediated mitogenic effect on forebrain precursors. Although RA caudalizes NSCs, its effects are context-dependent in progenitors derived from NSCs, and during the later stages of forebrain neuron development, it appears to synergize with activin to differentiate progenitors to specific mature forebrain neurons. In principle, forebrain neurons should be the easiest positional type to differentiate from NSCs and, as described above, much progress has been made in producing specific cortical subtypes from PSCs. However, because of the vast number of different cortical subtypes present, additional work remains to develop better methods for the controlled differentiation of PSCs to homogeneous subpopulations of these neurons.

Midbrain Dopaminergic Neuron Differentiation

Parkinson's disease (PD) is caused by a loss of midbrain dopaminergic (DA) neurons. Because of the clinical importance and interest in PD, there is a large body of work on differentiation of DA neurons from PSCs. DA neurons appear at the intersection of SHH and FGF8 signaling in rat neural plate explants [23]. The addition of ascorbic acid (AA) to rat midbrain cultures promotes the development of DA neurons [24]. This information along with general knowledge about neuronal differentiation was leveraged to design several protocols to differentiate PSCs to DA neurons (Fig. 3). Lee et al. [25] started with mouse ESCs, selected and expanded nestin positive NSCs from EBs, and induced differentiation through FGF2 withdrawal in a neuronal medium on polyornithine/laminin-coated plates. They observed the development of TH$^+$ DA neurons in 7–8 % of TuJ1$^+$ neurons. This frequency was doubled with the addition of SHH and FGF8 and further doubled if AA was also added. Kawasaki et al. [26] applied similar principles but with a different methodology to generate DA neurons. They grew mouse ESCs on PA6 cells, a stromal cell line derived from skull bone marrow. A very high proportion of the ESCs became NSCs and when differentiated further in culture medium supplemented with ascorbate ~30 % of the cells became TH$^+$ DA neurons. The stromal cell-derived inducing activity responsible for DA neuron differentiation has yet to be fully characterized. Growth on a PA6 stromal cell layer is also effective in generating DA neurons from human ESCs [27]. NSCs produced from human ESCs grown on a different stromal cell line, MS5, could be differentiated to DA neurons at very high efficiency with SHH and FGF8 treatment followed by culture in AA and brain-derived neurotrophic factor (BDNF) [28]. An EB method was also effective in generating dopaminergic neurons from human ESCs [29]. The one modification in this method from the similar mouse protocol was early addition of either FGF2 or FGF8 before FGF8/SHH combination treatment was used to generate midbrain DA progenitors. Similar protocols have been shown to be effective for scalable DA neuron production in defined xeno-free conditions from human ESCs and iPSCs [30, 31]. Importantly, a large proportion of the DA neurons generated in these studies were of the A9 subtype missing in PD patients. The ability to produce homogeneous populations of A9 DA neurons from human PSCs in defined xeno-free conditions will be critical for the effective development of cellular transplantation therapies for PD.

Spinal Cord Motor Neuron Differentiation

Pathways necessary for specification of spinal motor neurons (MNs) have been well defined for many years. MNs exhibit a columnar organization with subsets grouped according to the muscles they innervate. Diseases of MN dysfunction can differentially affect subtypes resulting in disease-specific spectra of movement disorders. The development of MNs can be divided into several steps including generation,

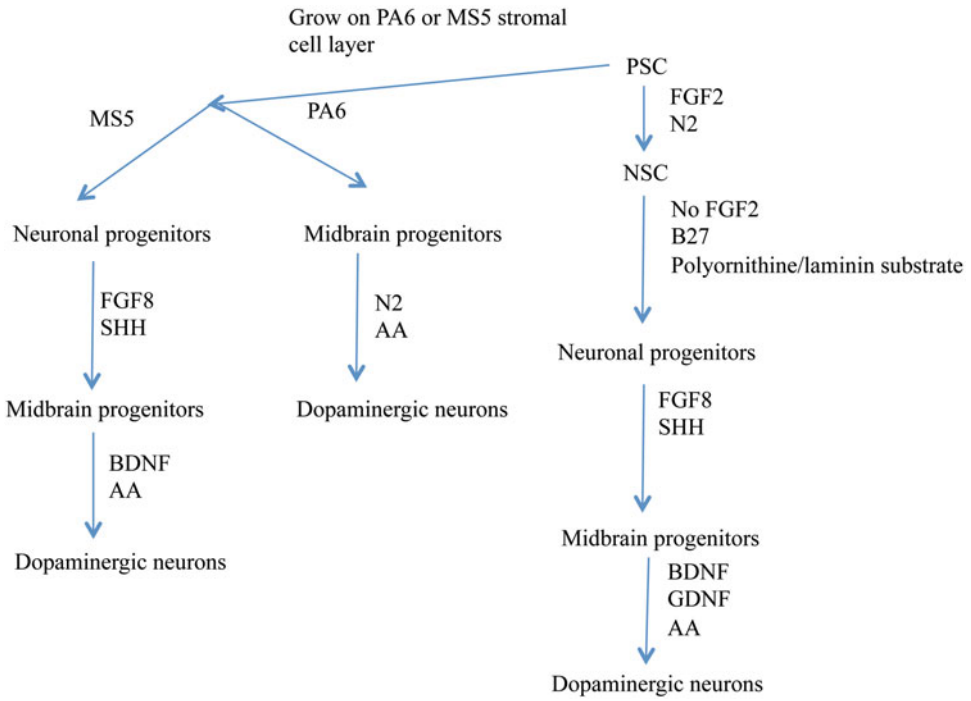

Fig. 3 Dopaminergic neuron differentiation from NSCs. The scheme for dopaminergic (DA) neuron differentiation from PSCs is shown with the two major protocols in which PSCs differentiate on a stromal cell layer or go through an embryoid body (EB) stage. The EB stage requires subsequent SHH/FGF8 treatment whereas one of the stromal co-culture protocols produces DA neurons without addition of FGF/SHH

subtype specification, apoptosis, and synaptogenesis. A dorsoventral SHH gradient specifies MNs in the ventral spinal cord early during vertebrate development. The gradient is translated into a transcription factor code that is required for the initiation of MN formation [32, 33]. Early pools of MNs pass through an apoptotic phase to fine-tune target neurons to the proper muscles. The final destination of the projections is under the control of a combinatorial LIM-homeodomain found within a particular MN. [34]. Protocols incorporating these principles have been utilized to successfully generate MNs from mouse and human PSCs.

PSCs are driven towards neuroectoderm by activation of the FGF pathway and inhibition of BMP signaling. These neuroectodermal cells are regionalized towards a caudal CNS position to become spinal progenitors, and these progenitors are ventralized by SHH to become MNs [32, 33]. This information has been utilized by several groups to differentiate mouse and human ESCs to MNs (Fig. 4). Wichterle et al. [35] treated EBs with RA and SHH to differentiate mouse ESCs to spinal motoneurons and ventral interneurons. The cells were directed towards MNs under high SHH concentrations and developed into ventral interneurons in moderate concentrations of SHH just as is the case in the developing embryo. The resulting MNs were restricted to a rostrocervical positional identity possibly because RA signaling pathways induced by the initial RA exposure continue to be active at later stages of differentiation and RA signaling is known to favor rostrocervical positions over thoracic and lumbar regions [36].

A similar differentiation paradigm was used to generate MNs from human ESCs except that FGF2 was used to derive NSCs from hESCs [37]. During differentiation of ESCs to NSCs, it was discovered that human NSCs, unlike mouse NSCs, have an early $Pax6^{+}/Sox1^{-}$ stage 8–10 days post neural induction protocol followed by a $Pax6^{+}/Sox1^{+}$ stage 14 days post-induction. The double positive cells were refractory to RA- and SHH-mediated MN induction. However, if $Pax6^{+}/Sox1^{-}$ NSCs were treated with RA/SHH, they developed into HOXC8 thoracic MNs and $ISL1^{+}/ISl2^{+}$ interneurons. This suggests that caudal identity is established at a very early stage in human NSCs and the possibility that FGF2 in combination with RA further caudalizes MNs. An enrichment protocol in which an HB9 enhancer drove GFP was used to purify MN progenitors with an RA/SHH differentiation protocol [38]. In addition to the enrichment step, this protocol differed from the others by growing human ESCs to confluence to form neuroepithelial rosettes. EBs were then made in the presence of RA and SHH and sorted for GFP^{+} MN progenitors which could be matured in the presence of neurotrophic factors. The result is a quicker protocol for the generation of functional, mature MNs. However, if MNs need to be generated from patient PSC lines, then routine use of reporters becomes cumbersome and expensive.

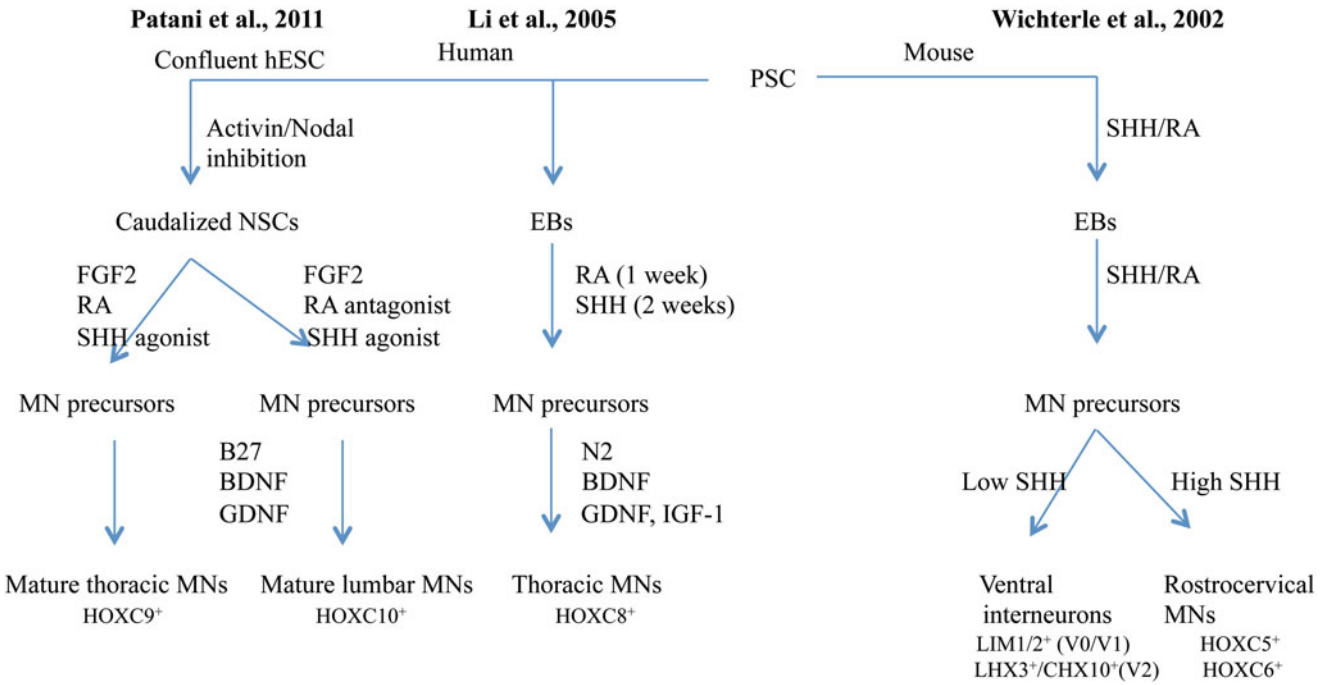

Fig. 4 Differentiation of motor neurons (MNs) from pluripotent stem cells (PSCs). Three standard protocols for generating MNs from mouse and human PSCs are outlined with a list of factors added to culture medium and markers used for analysis. Publications which are summarized are listed at the top of the figure

Several studies have shown that activin/nodal inhibition at the EB stage prevents differentiation to the mesendodermal germ layer and accelerates NSC formation with activin imparting caudal regionalization to the progenitor cells [39, 40]. Patani et al. [41] differentiated NSCs to MNs in the presence of the ALK4/5/7 inhibitor SB431542 with FGF2, the SHH agonist purmorphamine, and RA signaling antagonists. In terms of positional identity, there were significantly more HOXC10 MNs in the RA-independent group than in the RA treatment group, indicating that an RA-independent pathway leads to a more caudal lumbar positional identity. A significantly greater number of OLIG2$^+$ MN precursors and postmitotic MNs were found in the RA treatment group, indicating that work still needs to be done with additional small-molecule combinations to efficiently generate the entire suite of spinal MNs. Towards this end the overexpression of MN-specific transcription factors in the differentiation protocol has been used in an attempt to generate all spinal neurons. Adenoviral infection of human ESC- and iPSC-derived NSCs with the MN transcription factors LHX3 and ISL1 along with the neuronal specification factor NGN2 resulted in the generation of cervical and thoracic MNs 11 days post-infection [42]. The efficiency of MN neuron generation was similar to other protocols but the process occurred at a faster rate. Many research groups have now generated MNs from PSC with various modifications to the RA/SHH protocol, but improvements are still needed for production of the entire spectrum of hindbrain/spinal cord neurons.

CNS Glia Differentiation

Analogous to the way in which neurons are derived from NRPs, GRPs give rise to all the glia of the CNS [43]. The GRP can differentiate into either Type 1 or Type 2 astrocytes or to an oligodendrocyte progenitor cell (OPC). The OPC in turn has the capacity to differentiate into either Type 2 astrocytes or oligodendrocytes in vitro [44]. However, it is not clear if OPCs are bipotential or can only differentiate into oligodendrocytes in vivo [45, 46]. Studies with rodent fetal NSCs and cultures of rodent glial cells have yielded insights into how glia are formed. Knowledge gained from these studies has been transferred to protocols for the differentiation of CNS glia from PSCs.

CNS Astrocyte Differentiation

Astrocytes are the most abundant cell type in the CNS and fall into two classes. A2B5$^-$ Type 1 astrocytes arise only from tripotential GRPs, whereas in vitro A2B5$^+$ Type 2 astrocytes are derived from either GRPs or bipotential oligodendrocyte precursor cells (OPCs) [43, 44]. Immunopurified A2B5$^+$ cells from the rat spinal cord generated mature non-process bearing astrocytes if grown in medium with FCS [43, 47]. Exposure of GRPs to ciliary neurotrophic factor (CNTF) resulted in less mature process

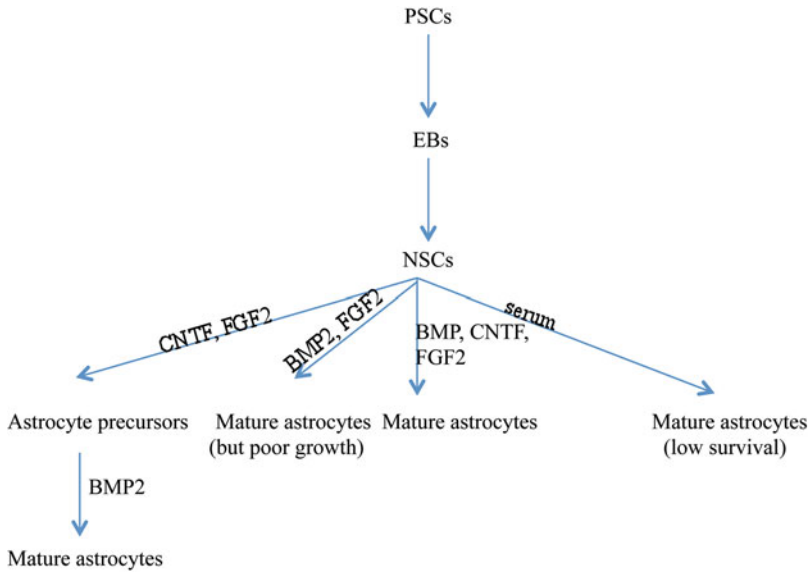

Fig. 5 Differentiation of PSCs to astrocytes. Once NSCs are produced from PSCs, the addition of various factors can lead to the generation of either astrocyte precursors or mature astrocytes. CNTF addition produces astrocyte precursors but mature astrocytes can be made if BMP2 is also added to NSCs

bearing astrocytes. Treatment of primary cells from mouse astrocyte precursors or GRPs from rat spinal cord with BMPs also resulted in the generation of a mature astrocyte population [47, 48]. Cardiotrophin-1 promotes astrocyte differentiation of fetal mouse neuroepithelial cells through the signal transducer and activator of transcription 3 (STAT3) which is also downstream of CNTF signaling [49]. CT-1 synergizes with BMP2 in the differentiation of astrocytes from neuroepithelial precursors [50]. STAT3/CNTF-independent induction of astrocyte fate occurs when the Notch pathway is activated in adult rat multipotent progenitor neurons [51].

The wealth of information regarding factors promoting commitment to the astroglial fate has resulted in the development of several protocols for the differentiation of PSCs to astrocytes (Fig. 5). Krencik and Zhang [52] developed a method in which NSCs were generated from PSCs and then grown in serum-free medium with supplements until the progenitor stage. At this point EGF, FGF2, and CNTF were added until the cells were committed to the astrocyte fate, at which point the cells were matured with CNTF. This method requires ~4 months at the end of which most of the cells are mature, functional $GFAP^+$ astrocytes. Emdad et al. [53] were able to differentiate PSCs to astrocytes with similar culture conditions using only CNTF over a 5-week period. Approximately 80 % of the astrocytes were $GFAP^+$ and there were no additional benefits to including CT-1 or Notch activators in the culture medium. Newer protocols incorporating CNTF, FCS, BMPs, and other factors shown to be important for astrocyte development need to be tested so that pure populations of mature astrocytes can be generated in a timely manner.

Oligodendrocyte Differentiation

Oligodendrocytes are one of the last CNS lineages to develop in vivo. Oligodendrocyte precursor cells (OPCs) arise from the caudal portion of the neural tube with ventralization by SHH driving OPC production [54]. In vivo OPCs rapidly migrate throughout the brain and initiate myelination. PDGF and T3 are important factors in OPC proliferation and differentiation. PDGF is required to maintain OPCs in a proliferative state and T3 promotes differentiation towards mature oligodendrocytes [55–58]. OPCs isolated from rat optic nerve differentiate to oligodendrocytes at the expense of astrocytes if grown in serum-free medium [44]. Generation of oligodendrocytes from PSCs generally involves making NSCs and then transiting NSCs through an OPC phase by addition of the appropriate growth factors. Growth factor removal allows progenitors to develop into mature oligodendrocytes (Fig. 6).

The first report of the differentiation of CNS glia from mouse ESCs found that growth in serum-free medium with PDGF resulted in the production of a mixed population of glial cells after short-term withdrawal of PDGF although prolonged withdrawal promoted greater differentiation to oligodendrocytes [59]. Oligodendrocytes were also derived from mouse ESCs as “oligospheres” in a suspension protocol using serum in the culture medium with RA to caudalize NSCs and T3 to differentiate towards the oligodendrocyte lineage [60]. Both studies showed that when transplanted into rats or mice that were deficient in myelination, the transplanted cells could myelinate host axons. Another oligodendrocyte differentiation protocol from mouse ESCs used SHH and serum-free medium with both PDGF and T3 to induce differentiation to oligodendrocytes [61].

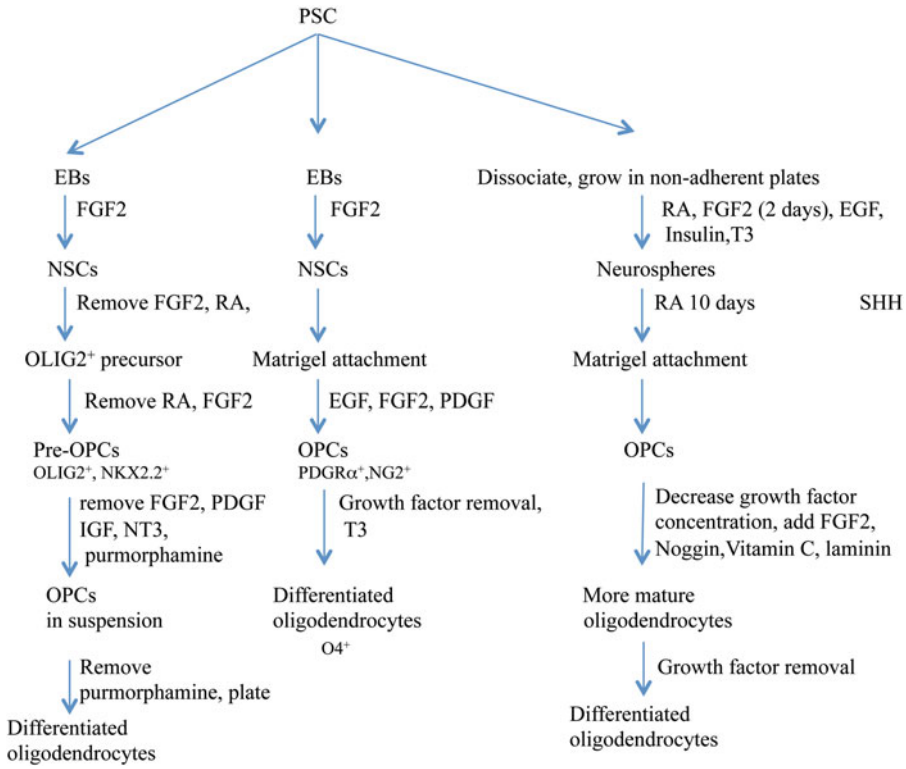

Fig. 6 Oligodendrocyte differentiation from PSCs. Three oligodendrocyte differentiation protocols are summarized with key steps, compound and growth factor additions to cell culture medium, and markers at key stages on route to mature oligodendrocytes identified

One of the first reports demonstrating differentiation of human ESCs to oligodendrocytes also made "oligospheres" in serum-free culture conditions with RA as a caudalizing factor and T3 to differentiate to mature cells over a ~40-day time period [62]. The resulting oligodendrocytes had the capacity to myelinate axons when transplanted into a myelination-deficient mouse model. Although the oligodendrocytes generated in this study matured when transplanted into a myelination-deficient mouse model, the authors found very few mature cells in vitro. Izrael et al. [63] used a similar sphere-based protocol but added noggin after RA treatment to induce expression of Sox10, which is required for terminal differentiation of oligodendrocytes. Exposure of OPCs to noggin at the appropriate time frame increased the yield of mature oligodendrocytes. Because OPCs are derived from OLIG2 precursors, a more recent protocol first generated these precursors from NSCs with RA and SHH treatment and then followed the in vivo development profile for oligodendrocytes by using previously described growth factor combinations to produce differentiated oligodendrocytes over a ~100-day differentiation protocol [64]. One of the critical steps in this protocol is the removal of FGF2 when transitioning from the pre-OPC to OPC stages. The inconsistent results seen from growing OPCs in suspension and the length of time needed to produce oligodendrocytes from PSCs highlights the need to develop new methods to differentiate human PSCs to mature oligodendrocytes.

Differentiation of Neural Stem Cells to PNS Neurons and Glia

PNS neurons mediate communication between organs and the CNS. The PNS is derived from NCSCs which themselves originate in the dorsal neural tube before undergoing an epithelial-mesenchymal transition and migrating throughout the embryo. As is the case for the CNS, there is some rostrocaudal positional identity linking the region from which the NCSCs migrate (cranial, vagal, trunk) to the types of cells that they can become [65]. Trunk NCSCs generate autonomic sensory neurons and Schwann cells and nearly all of the work performed to date has been on differentiating PSCs to these PNS lineages.

Several labs have developed methods to isolate NCSCs from PSCs and differentiate them to peripheral neurons and glia (Fig. 7). p75 has been established as a good marker for NCSCs and Lee et al. [66] observed extensive p75 staining at clusters surrounding the central NSC rosettes of cells differentiated from human PSCs. p75 FACS sorting isolated cells that were positive for multiple NCSC markers and could be

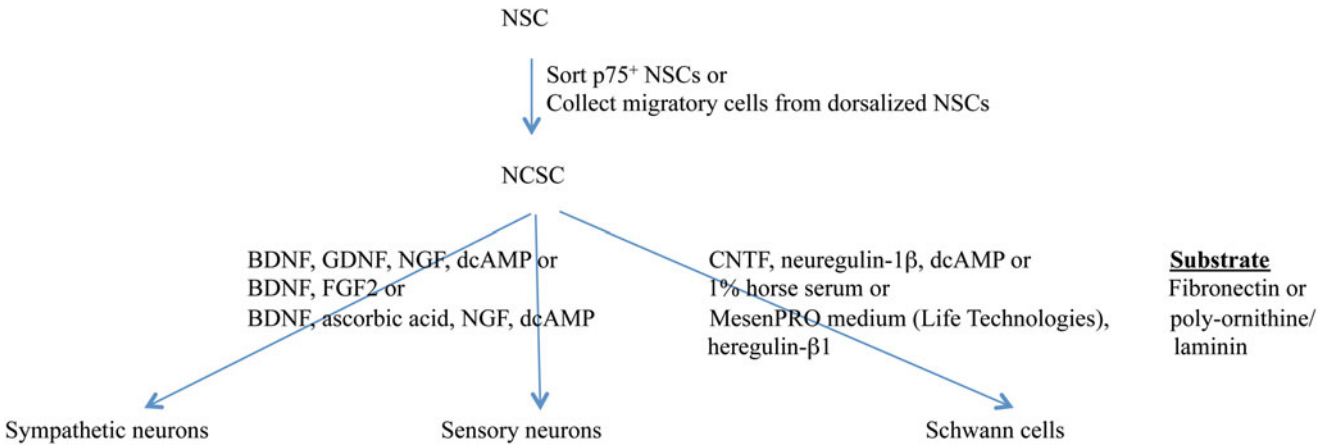

Fig. 7 Differentiation of neural crest stem cells (NCSCs) to peripheral nervous system (PNS) neurons and glia. The methods to isolate NCSCs from NSCs and factors used to differentiate them to PNS neurons and glia from three studies are summarized with substrates for neuronal and glial differentiation listed on the *right side*

differentiated to various neural crest lineages including PNS neurons and glia. The number of $p75^+$ cells increased significantly if rosettes were cultured in medium with FGF2 or BMP2. Withdrawal of FGF2 and EGF and addition of BDNF, GDNF, NGF, and dibutyryl cAMP (dbcAMP) resulted in the generation of PNS sympathetic and sensory neurons. Alternatively, if CNTF, neuregulin, and dbcAMP were added to the medium, Schwann cells developed. A similar p75 sorting strategy was used to isolate NCSCs except that EBs were grown in medium with FGF2 and AA and 50 % medium conditioned by the PA6 stromal cell lines to enrich for NCSCs prior to sorting [67]. PNS neuron differentiation was achieved with BDNF, AA, NGF, and dbcAMP and Schwann cells were generated when MesenPRO medium (commercially available from Invitrogen) and heregulin-β1 were used.

Another study enriched for NCSCs by culturing PSCs in FGF2, EGF, insulin, and nicotinamide and subsequently plating the cells on a fibronectin matrix [68]. This dorsalized the resulting NSCs with a migratory population of cells that were positive for NCSC markers. The migratory cells could be propagated on a Matrigel substrate and replated on a fibronectin substrate for differentiation to PNS neurons with FGF2 and BDNF and Schwann cells with 1 % horse serum [69]. PNS nociceptive neurons could be produced directly from NSCs grown in adherent monolayer culture with dual SMAD inhibition and three additional inhibitors [70]. These three compounds inhibited vascular endothelial growth factor, PDGF, and FGF (SU5402), glycogen synthase-3β (CHIR99021), and Notch signaling (DAPT). The differentiation occurred at a much faster rate than what happens in vivo with the neurons still exhibiting the functional properties of their in vivo counterparts. The results of this work suggest that small-molecule screens offer great promise in facilitating quick, high efficiency neural differentiation of PSCs.

Very little work has been done in differentiating to other PNS ganglia of cervical or vagal origin. Several studies have shown that enteric ganglia (vagal origin) can be differentiated from mouse or human PSCs. Treatment of EBs with BDNF resulted in the generation of cells that were positive for receptors and ligands expressed by enteric ganglia [71]. These neurons had a calcium signaling profile reminiscent of in vivo enteric ganglia. There have been no reports of differentiating PSCs to cranial ganglia, although differentiation to non-PNS cells from NCSCs migrating from cranial and vagal regions has been shown [68, 69]. These will not be discussed in any detail as they are beyond the scope of this chapter.

Differentiation to Retinal Pigmented Epithelium: Organoids from PSCs

Diseases of the visual system affect a significant percentage of the population. Because these diseases are often the result of degeneration of retinal pigmented epithelium (RPE) cells, the ability to generate RPE from PSCs offers hope for the treatment of these disorders. The earliest reports of generating RPEs from PSCs found that ~8 % of primate ESCs differentiated to RPE [72]. In the first paper describing RPE differentiation from human ESCs, the ESCs were grown to confluence in the absence of FGF2, and RPE appeared spontaneously if the cells were continuously grown for 6–9 months [73]. Generation of EBs from mouse ESCs with WNT and Nodal inhibitors and subsequent addition of activin and serum resulted in the appearance of retinal progenitors [74]. However, these progenitors did not differentiate into more mature RPE cells unless co-cultured on retinal cells. A defined differentiation medium was developed in which the addition of RA and taurine resulted in more efficient conversion of mouse, monkey, and human PSCs to

mature RPE [75]. WNT and nodal inhibitors are critical requirements for RPE differentiation as NSCs generated without these inhibitors failed to generate RPE. An exciting new development has been the ability to produce optic cups from both mouse and human PSCs using the medium described above in a three-dimensional cell culture system [76, 77]. The capacity to generate complex tissue structures with PSCs as a starting material provides hope for the development of a new class of regenerative therapies.

Conclusions

In this chapter we present a large database of knowledge demonstrating that differentiation of PSCs to neural lineages is technically feasible. Researchers can now generate CNS neurons found throughout the brain and spinal cord, and many studies show that these neurons are functional electrophysiologically in vitro and when transplanted into animal models in vivo. Great advances have also been made in differentiating CNS astrocytes, but further improvements need to be made in producing mature oligodendrocytes in vitro. Although differentiation to PNS neurons has not progressed as far as that in CNS, advances are being made. In contrast, it appears the protocols for making PNS glia (Schwann glia) are near full optimization. The capacity to generate such a wide array of neural cell types offers hope that cellular transplantation may be an achievable therapeutic objective for many nervous system disorders. One of the most promising tissue types under investigation is the eye, with the possibility that both cells and tissues can be produced from PSCs. Future work needs to focus on fine-tuning existing methods to increase purity and yields of the neural populations of interest, mining the available knowledge on neural development, and utilizing chemical screens to develop protocols for the differentiation of neural cell types and subtypes which have not been successfully derived from PSCs.

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Generation of Anterior Foregut Derivatives from Pluripotent Stem Cells

Hans-Willem Snoeck

Introduction

Embryonic stem cells (ESCs) are a model for the study of tissue function and a source for cellular replacement therapies. Derived from the inner cell mass of the blastocyst of mammals such as mouse and human, they can be maintained in a pluripotent state in vitro and give rise to every cell type in the organism [1]. The ability to reprogram somatic cells into a pluripotent state (induced pluripotent stem cells, or iPSCs) opens the way for the generation of patient-specific pluripotent cells, which would overcome rejection problems associated with the use of allogeneic hESC-derived tissues [2–7]. We will refer collectively to ESCs and iPSCs as pluripotent stem cells (PSCs).

Ultimately, it is hoped that human (h)PSCs will be valuable in cell replacement therapy for regenerative medicine. Several hurdles remain, however, including efficient and reproducible differentiation, production in GMP standard, large-scale conditions, safety concerns with respect to tumor formation, possible immunological rejection because of the use of genetically modified cells, and the complex architecture of the mature target organ, which may not be easily amenable to cell replacement therapy [2, 4, 8]. For these reasons, there are currently no clinical trials involving hPSCs, and this situation will likely not change until these issues are addressed satisfactorily. The biggest short-term challenge in the field is currently the development of strategies to differentiate hPSCs into functional, mature cell types with high purity and with minimal contamination from undesired lineages. The problems facing this endeavor are twofold. First, while lineage specification has been achieved for all three germ layers and for many cell types derived from these germ layers, the generation of functionally mature cells has proved to challenging. A prime example is the efforts to generate pancreatic β cells, where the best differentiation protocols yield polyhormonal cells that are likely developmental intermediates (called first transition progenitors) that may not be direct precursors of insulin-producing β cells and are poorly glucose responsive [9–11]. Transplantation into immune deficient mice however did induce further differentiation and yielded functional β cells, suggesting that in vitro generated, endocrine pancreas-specified cells may contain functional β cell precursors (or so-called second transition cells) [12]. These findings illustrate how the profound complexity of developmental processes is not readily recapitulated in vitro and how a deeper understanding of normal development is required to guide differentiation of PSCs in vitro. The second problem is that the specification of some lineages has been very difficult. While efforts at differentiation into mesodermal and neuroectodermal and some endodermal tissues, such as pancreas, liver, and intestine, have been relatively successful [1, 13–20], specification of one region of the endoderm, the anterior foregut endoderm (AFE), has been challenging. This region is nevertheless of major clinical and translational importance, as lungs and airways, thymus, parathyroid, and thyroid are derived from the AFE. Here we discuss the relevance of pursuing differentiation of AFE-derived tissues, the development of AFE, and the current state of the field in the generation of AFE-derived tissues from PSCs.

Relevance of Generation AFE-Derived Tissues

Lung and Airways. A severe shortage of donors, in addition to surgical, medical, and immunological complications, limits the use of transplantation for many end-stage lung diseases [21], which kill 100,000–200,000 people in the USA every year [22–25]. Stem cell-based approaches to regenerative medicine for lung and airway disease may provide alternative therapeutic options. However, convincing evidence of

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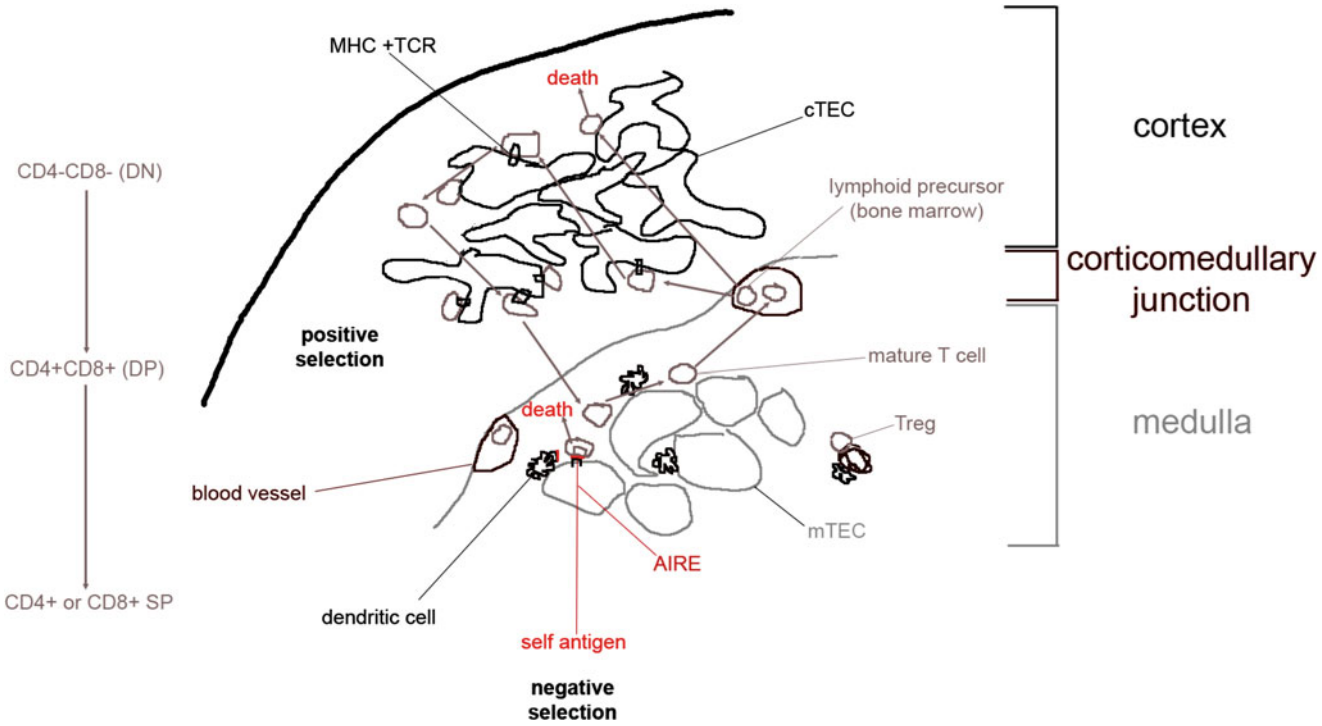

Fig. 1 Schematic representation of the structure of the thymus and of the processes of positive and negative selection

engraftment of any type of stem cell or its progeny in the lung in animal models of lung injury is lacking or at the very least controversial [26–29]. One reason may be the complex architecture and cellular composition of lung and airways. The respiratory system consists of a branched system of progressively smaller airways that terminate in alveoli where gas exchange takes place and contains multiple cell types with a specific regional distribution. In humans, pseudostratified epithelium consisting of ciliated, mucus (goblet), secretory (Clara), neuroendocrine, and basal cells lines trachea and bronchi. The proximal airways also contain submucosal glands. In the alveoli, alveolar epithelial type I (ATI) cells are essential for gas exchange, while type II (ATII) cells produce surfactant and are critical for the maintenance of alveolar integrity [30, 31]. A second reason for the absence of models of stem cells engraftment in lung and airway is likely the extensive inflammation and subsequent fibrosis and destruction of the supporting structures caused by injury and disease [32]. Transplantation of artificial or decellularized native lung matrices seeded with autologous, iPSC-derived cells appears a valid option, though its implementation is still far away [33]. Although proof-of-principle for this approach has been provided in rat models using fetal or neonatal lung cell suspensions [34, 35], the challenges to clinical application of this approach are still enormous. These include, in addition to challenges related to the process of decellularization, the design and generation of appropriate artificial scaffolds and biomechanical properties of the resulting graft [33], the generation from autologous iPSCs of sufficient numbers of the appropriate types of mature epithelial cells, which selectively home to their correct niches in the bronchoalveolar tree, and of regionally distinct postnatal stem and progenitor cells to provide endogenous regenerative capacity. Furthermore, for adequate mucociliary function planar polarity must be established correctly and uniformly, so that ciliary motion is not random. In vitro generated mature lung and airway epithelium from PSCs is also an excellent platform for the study of congenital and acquired human lung disease [36, 37], such as cystic fibrosis, tracheoesophageal fistulas, surfactant deficiency syndromes, and infectious disease caused by agents with specific tropism for human respiratory cell types, and for high-throughput drug screening [38]. Finally, the availability of sufficient numbers of cells at well-defined and controlled stages of development will allow unprecedented insight into human development [1, 13].

Thymus. The thymus is the site of production of T lymphocytes from hematopoietic precursors that seed this organ from the bone marrow [39, 40]. Thymic epithelial cells (TECs) of two predominant, but still heterogenous subtypes, cortical (cTEC) and medullary (mTEC), are of endodermal origin and are critical for the establishment of a self-tolerant adaptive cellular immune system through processes of positive and negative selection (Fig. 1) [39, 41–44]. Early CD4–CD8– lymphoid precursors enter the thymus at the corticomedullary junction and develop into double positive (CD4+CD8+) thymocytes in the cortex. Interactions with cTECs are important for positive selection. Only those cells that are able to

interact with self-antigens presented on major histocompatibility complex (MHC) molecules on cTECs (approximately 2 %) survive and proceed through further differentiation. The cells then move to the medulla, become single positive (CD4+ or CD8+), and undergo negative selection. Here, self-antigens that are normally only expressed in peripheral tissues (tissue-restricted antigens) are presented to the cells either on MHC of mTECs, where they are transcriptionally induced by the transcriptional regulator, autoimmune regulator (AIRE), or on MHC of dendritic cells, onto which they can be transferred from mTECs. Strong recognition induces apoptosis and, therefore, deletion. In this way, strongly autoreactive T cells are eliminated [39]. Furthermore, regulatory T cells (Tregs), which are capable of suppressing antigen-specific T cell responses, are selected in the medulla in a process that involves dendritic cells and, at least in humans, Hassall's corpuscles, distinct structures composed of mTECs and associated with dendritic cells that occur at much higher frequency in human than in mouse thymus [45]. While the purpose of negative selection in the medulla, deletion of autoreactive T cells, is obvious, the purpose of positive selection is less clear. One hypothesis holds that T cells with weak autoreactivity display stronger responses to foreign antigens [46]. As such, T cells respond best to antigen presented by cognate MHC on antigen-presenting cells. Another hypothesis, which is not mutually exclusive, posits that positive selection is required to regulate peripheral T cell homeostasis. T cells, in particular CD8+ cells, require continuous exposure to the MHC on which they were selected to survive, and it is likely that the antigen presented by MHC in the periphery is autoantigen [47]. Thus, selection of T cells with low but not negligible autoantigen responsiveness (positive selection), but deletion of T cells with strong responses to tissue-specific antigens (negative selection) creates a self-tolerant T cell repertoire, and a pool of T cells that survives best and is optimally functional in the host where they were positively selected.

A major reason to generate TECs from hPSCs is the improvement of so-called human immune system (HIS) mice, where the HIS is modeled in the mouse, a major challenge in immunology [48, 49]. HIS mice are immunodeficient *Rag1*$^{-/-}$*Ilr2g*$^{-/-}$ or NOD-SCID*Ilr2g*$^{-/-}$ (NSG) mice reconstituted with human hematopoietic stem and progenitor cells [50, 51]. While in particular β cell reconstitution was efficient, myeloid reconstitution was low, human T cell responses were weak, and peripheral T cell homeostasis was abnormal [52, 53]. Better T cell reconstitution and T cell-mediated immunity was achieved by co-transplantation with a human fetal thymus under the kidney capsule, providing proof-of-principle that the presence of human thymic tissue is critical [54, 55]. The presence of myeloid antigen-presenting cells derived from HSCs was furthermore shown to be important for T cell homeostasis in the periphery [56]. A subsequent improvement was the development of personalized immune (PI) mice [57], in which immune systems are generated from HSCs of adult patients with disease, allowing analysis of the role of genetic diversity in HSC-determined disease susceptibility and immune responses. In this model, the thymus tissue and HSCs, which also generate the myeloid cells that present antigens to T cells during immune responses, are from different donors but share at least one human leukocyte antigens (HLA) molecule. Antigen-specific T cell responses and maintenance of newly generated T cells, which also requires recognition in the periphery of cognate HLA (the human MHC) on which T cells were selected by TECs [58], would be optimized by the addition of or substitution of autologous thymus for allogeneic thymic tissue in the PI mouse model. In such a model, a full complement of "self" (i.e., HSC donor) HLA molecules on thymic tissue would positively select T cells that interact with all of these HLA molecules on autologous antigen-presenting cells in the periphery. Using iPSCs, HIS mice can be constructed where thymus and hematopoiesis are autologous and patient specific, thus better capturing genetic diversity in disease susceptibility and immune responses. Furthermore, co-transplantation of autologous, iPSC-derived tissues, for example, pancreatic β cells from type I diabetes patients, will allow study of tissue-specific immune responses.

Medical conditions where autologous thymus replacement would be beneficial are congenital diseases where a thymus is lacking, such as the FOXN1-deficient nude/SCID syndrome [59, 60] and complete DiGeorge syndrome (DGS) [61–63]. It is furthermore widely hypothesized that improving thymic function in the elderly, which have undergone severe thymic atrophy, will increase immunological and perhaps overall health [64–68]. Thymic involution, which begins during adolescence and leads to a decrease in the production of naïve T cells [64], also complicates allogeneic hematopoietic stem cell transplantation (aHSCT), a potentially curative treatment for many leukemias and lymphomas, in middle-aged and older adults. In these patients, the pre-transplant conditioning regimen and posttransplant graft-versus-host disease superimposed on physiological age-associated thymic involution severely affect thymic function, resulting in delayed T cell reconstitution where the full T cell repertoire is rarely restored [69–71]. Impaired cellular immunity in these patients results in increased relapse of the primary tumor, chronic viral infection, secondary malignancy, and vaccine failure [69]. Co-transplanting iPSC-derived thymus from the same donor as the donor of the HSCs would very likely improve the outcome aHSCT in these patients. It is also important to note that orthotopic transplantation is not required, as the thymus can be transplanted intramuscularly in the quadriceps muscle [60, 62, 63].

Parathyroid. Parathyroid replacement can be envisaged in several conditions associated with hypofunction of the parathyroids, collectively called hypoparathyroidism, characterized by hypocalcemia and hyperphosphatemia. Hypocalcemia causes muscular irritability, tetany, seizures,

paresthesias, congestive heart failure, long QT, calcification of the basal ganglia, altered mental state, and pseudotumor cerebri but can also be asymptomatic, in particular when slowly developing and long standing [72]. The most common form of hypoparathyroidism is iatrogenic. One to six percent of total thyroidectomies are complicated by inadvertent excision of all parathyroids or interruption of their blood supply. In addition, radical neck dissection for cancer and selective parathyroidectomy for primary or secondary hyperparathyroidism can lead to hypoparathyroidism [72]. Genetic diseases associated with hypoparathyroidism include the 11q22 deletion or DGS [61], activating mutations in the calcium sensing receptor (*CaSR*) and mutations in *PTH*, *GCMB*, and *SOX3*. Hypoparathyroidism can also be part of complex genetic syndromes, such as heterozygous GATA3 mutations [73]. Finally, autoimmune disease can lead to isolated hypoparathyroidism or to multiple endocrine deficiencies that include hypoparathyroidism [72]. Hypoparathyroidism is one of the few endocrine deficiencies where hormone replacement therapy is not FDA approved. Hypocalcemia is treated with vitamin D and calcium supplementation, which needs to be taken lifelong [72, 74]. Importantly, even though serum calcium and Pi are within physiological levels, calcium/Pi homeostasis is non-physiological. Furthermore, the incidence of kidney stones and cataracts is increased, while quality of life is compromised in patients with postsurgical hypoparathyroidism [75]. Interestingly, although endogenous PTH mobilizes calcium from bone, PTH treatment appears effective in the treatment of osteoporosis, likely because of the intermittent nature of subcutaneously administered PTH (1–34). Further complicating PTH therapy is the fact that regulation of PTH activity is also achieved by the secretion of C-terminal fragments, which may act as receptor antagonists and may in addition have as yet unknown biological functions [74]. Replacing parathyroids with iPSC-derived, patient-specific tissue would be the best therapeutic option. For genetic syndromes where parathyroid development is defective, gene correction will be required or the genetic defect will need to be bypassed to achieve appropriate parathyroid development in vitro. In iatrogenic hypoparathyroidism, the most frequent form of hypoparathyroidism, this is not necessary, however. Similar to the thymus, orthotopic transplantation is not required, as parathyroids can be transplanted subcutaneously [76].

Thyroid. Congenital hypothyroidism, autoimmune disease, and thyroid resection for malignancy are prime causes of hypothyroidism [77]. Current thyroid replacement therapy is very effective and economical. However, the capability to differentiate hPSCs into thyroid follicle cells would allow the generation of in vitro models of normal and abnormal thyroid function that currently do not exist [78].

Development of the AFE and Derived Structures

Differentiating PSCs exhibit remarkable degrees of similarity with their developing in vivo counterparts [1, 10, 11, 13], and recapitulating sequential steps in development lies at the basis of the relative success in the differentiation of PSCs into, for example, cardiac myocytes [15], pancreatic cells [9, 12], hepatocytes [14, 79], neural tissue [16, 17, 19], intestine [20], and hair cells of the inner ear [18]. Development proceeds through sequential cell fate decisions, whereby cells adopt distinct transcriptional regulatory states. These confer a unique susceptibility to various inductive cues, which guide and determine subsequent cell fate decisions. Inductive cues include interactions with extracellular matrix and with neighboring cells, as well as autocrine and paracrine signaling by soluble molecules. Many of these soluble molecules are morphogens, which have dose-dependent effects and direct position-specific differentiation events by inducing distinct sets of genes at different concentrations thresholds. In addition, the responsiveness of cells to these signals shows spatial and temporal restriction. Therefore, not only the nature of morphogens but also their concentration and timing are critical to direct differentiation [80, 81]. Both the signal transduction pathways and transcription factors that guide differentiation of cells though development are remarkably conserved throughout evolution. Thus, examining the patterning events driving murine AFE development will provide valuable information to be applied to subsequent derivation of human AFE from human ESCs. An overview of the development of the AFE in the mouse is shown in Fig. 2.

Development of the definitive endoderm. The earliest lineage restriction of the embryo proper occurs during gastrulation, in which the three germ layers, ectoderm (brain, skin), mesoderm (heart, muscles, blood), and endoderm (gut tube, lungs) are established. During this process, which occurs at around E7, cells move through a posterior structure in the early embryo called the primitive streak, in a complex and still incompletely understood process that involves signaling by fibroblast growth factor (FGF) 8, bone morphogenetic protein (BMP) 4, Wnt, and the transforming growth factor (TGF)-β family member, nodal [82]. Specification into mesoderm versus endoderm depends on the strength of nodal signaling. Cells exposed to the highest agonism become definitive endoderm (DE, marked by *Sox17*, and *Foxa1-3*), while cells exposed to lower levels of nodal signaling are fated towards mesoderm [83, 84]. The DE, which at this point is a single layer of cells, folds at E8–E8.5 at its proximal and distal ends and subsequently along the lateral borders. As the embryo turns, the endoderm, which was orig-

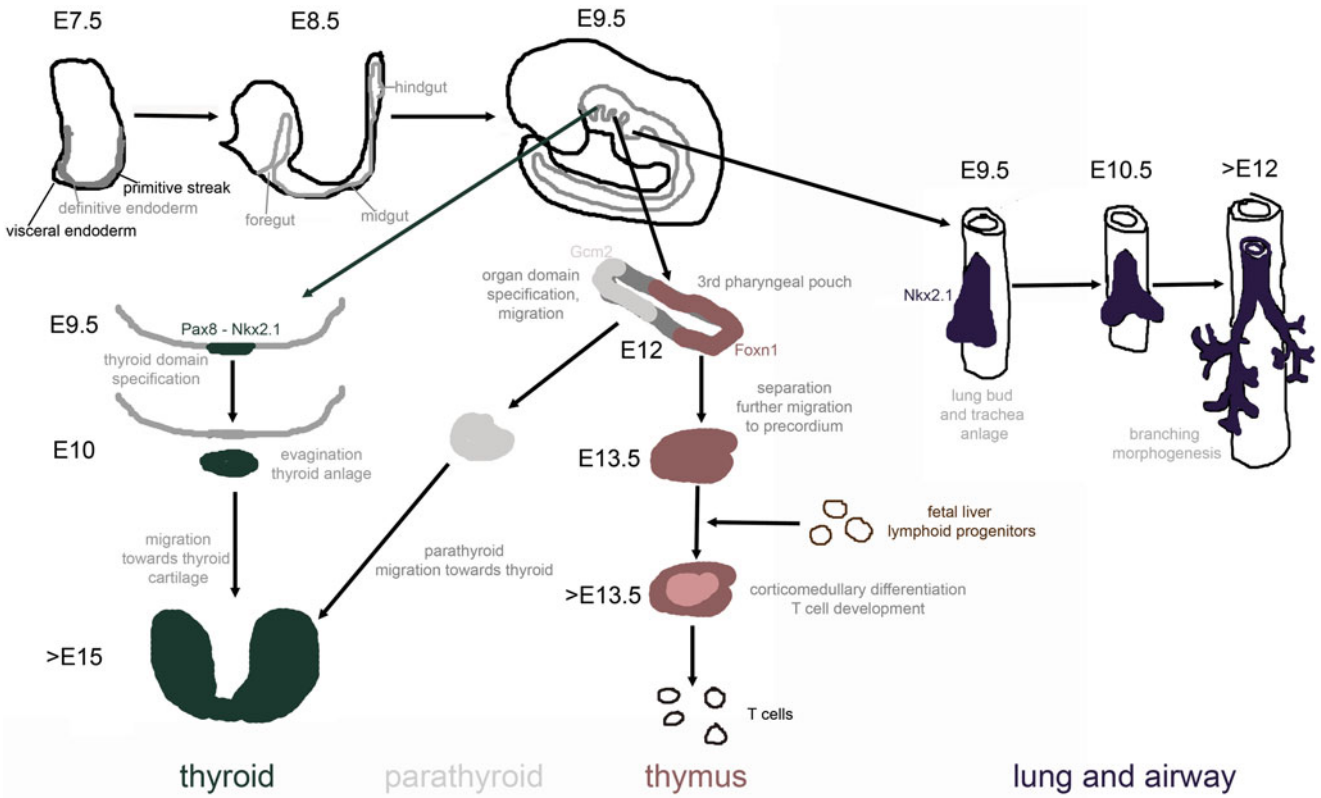

Fig. 2 Schematic representation of the development of the AFE and its derivatives in the mouse

inally at the perimeter of the embryo proper, becomes the innermost layer and forms a tube where organ domains are progressively defined through transcriptional and epigenetic mechanisms along the anterior-posterior axis. The anterior aspect is marked by the transcription factors *Sox2*, *Hhex*, and *Foxa2*, while the posterior half is marked by *Cdx2* [85]. Permissive and instructive signals from the primitive streak, the node and subsequently from surrounding mesoderm, the visceral endoderm, and the dorsally located notochord determine the fate of individual segments of the embryonic gut tube [80, 81]. Developmental and mES differentiation studies have implicated Wnt and FGF4 agonism as important for posterior endoderm specification [20, 86–88], while the morphogens important for anterior endoderm patterning remain elusive. The initial patterning of the endoderm may at least in part be established during gastrulation, however, and as timing of the exit from the primitive streak appears to determine anteroposterior identity [89].

Patterning of the AFE. Further organ domain specification leads to separate zones in the AFE. Most proximal is the pharyngeal endoderm, which forms lateral extensions, called pharyngeal pouches. The thyroid gland properly develops from the floor of the pharynx, from an anlage that expresses *Nkx2.1* and *Pax8* [81]. The lung field, which arises distal to the pharyngeal pouches, is characterized by the expression of *Nkx2.1* but not *Pax8* [81]. An integrated view of how specific organ domains of the AFE are established is largely lacking. Signals emanating from within the endoderm itself and instructive and permissive signals from the surrounding cardiac mesoderm, the notochord, and neural crest play a critical role in organ domain specification and migration [41, 42, 80, 81]. Organ domains may also be prepatterned through epigenetic mechanisms during the development of DE and of the gut tube, as has been shown for pancreas and liver [90]. A better understanding of the mechanisms involved in organ domain specification will be essential to achieve directed differentiation of AFE-derived tissues.

Thymus and parathyroid development. The pharyngeal endoderm forms four outcroppings, called pharyngeal pouches, at E9.5 in the mouse. These develop into specific organs: eustachian tube and inner leaflet of tympanic membrane (first pouch), palatine tonsils (second pouch), thymus (ventral third), parathyroids (dorsal third and, in humans but not in mice, fourth pouch), and parafollicular C cells of the thyroid (fourth pouch) [91, 92]. The pharyngeal endoderm expresses *Tbx1*, *Pax1*, *Pax9*, *Six1* and *Eya1,* and, restricted to the region caudal of the second pouch, *Hoxa3*. Together, these factors likely form a transcriptional cascade that is essential for specifying the third pharyngeal pouch and its derivatives [42, 43, 93–98]. Loss of *Tbx1* is the most likely

cause of the DGS [99–102]. At E9.5 in the mouse, *Gcm2* (called *GCMB* in humans) is expressed, defining the dorsal domain of the third pharyngeal pouch that will become the parathyroid [103]. Upstream of *Gcm2* is *Gata3* [38]. Deletion or mutation of *Gcm2* or *Gata3* causes parathyroid aplasia [73, 104]. The ventral domain begins to express *Foxn1* at E11.5 and is fated to develop into the thymus [103]. It is unclear which transcription factors, morphogens, and growth factors are essential in thymus and parathyroid specification. While *Gcm2* and *Foxn1* are required for the development of parathyroid and thymus, respectively, neither are essential for specification of these organ primordia, however [42, 43]. Furthermore, how the *Tbx1-Pax1/9-Six/Eya* network connects to *Gcm2* and *Foxn1* regulation is unclear, and it is very likely that not all transcription factors involved in thyroid and parathyroid development are currently known. BMP4 and sonic hedgehog (Shh) may play a major role in the delineation of the thymus and parathyroid domains of the third pharyngeal pouch. BMP-4 is expressed in the *Foxn1* domain, while its antagonist, Noggin, is expressed in the *Gcm2* domain. Sonic hedgehog (*Shh*), which is expressed in the parathyroid domain, likely negatively regulates BMP4. In the absence of *Shh*, the *Foxn1*$^{+}$ thymus domain expands at the expense of the parathyroid domain [105–107]. Other factors that are potentially required for pharyngeal pouch patterning are FGF8 [108] and Wnt5b [109], which are expressed by the pharyngeal endoderm and in the presumptive thymus domain of third pharyngeal pouch but not in the parathyroid domain. Several other markers have recently been shown to be expressed in the presumptive thymus domain prior to Foxn1 expression. These include *Isl1* and *Foxg1 and Il7* [110, 111]. The chemokine receptors, CCL21 and CCL25, which are critical for attracting T cell precursors to the thymus, are expressed early in the third pharyngeal pouch as well. The former is expressed predominantly by the parathyroid primordium, the latter predominantly by the thymus primordium at E10.5 in the mouse [112].

The third pharyngeal pouch first separates from the pharynx, followed by separation of parathyroid and thymus domains. The parathyroid anlage migrates towards the developing thyroid, while the thymus migrates towards its precordial location [41, 42]. Neural crest plays a critical role in the migration, in particular of the thymus [113], but not in the specification of these anlages. This was suggested by the fact Splotch mutant mice (Pax3 deficient), which have severe neural crest defects, are athymic. However, parathyroid and thymus primordia are present, and an increase in the thymus domain at the expense of the parathyroid domain of third pharyngeal pouch was observed [114]. The contribution of neural crest-derived cells to the postnatal thymus appears limited to pericytes [115]. The thymus is colonized by hematopoietic cells, and T cell development is initiated. This process begins while the thymic lobes move towards the precordial location and before the organ is vascularized, implying that the earliest T cell precursors move into the thymus through the surrounding mesenchyme and neural crest tissue [116]. Further development of the thymus beyond the stage of a FOXN1^{+} thymic primordium likely requires positively selected T cells [41], as well as, at least during embryonic life, lymphoid tissue inducer cells (LTis). The latter are CD4+CD8− CD3− hematopoietic cells that express CD40 and RANKL and are critical for the development of mTECs [117]. In addition, dendritic cells, which are also of hematopoietic origin, are required for appropriate thymic function [118]. FGF7 and FGF10 are expressed in the neural crest surrounding the thymus [119, 120]. They play a critical role during later thymic development as deletion of *Fgfr3b* blocks thymic growth at E12.5 but appear dispensable for differentiation of TEC progenitors into cTECs and mTECS [120].

Lung. Just distal from the tracheal anlage, the lung buds evaginate and connect with the nascent trachea. At the level of the developing trachea, dorsoventral patterning differentiates the gut endoderm into the dorsal esophagus (marked by *Dlx3* and high expression of *Sox2*) and a ventral trachea (marked by *Nkx2.1* and *Nkx2.5*, as well as lower expression of *Sox2*) [30, 31, 121]. Nevertheless, *Sox2* plays a critical role in tracheal development and in the maintenance of tracheal and proximal airway epithelium, where it is subsequently more highly expressed [122]. Hedgehog signaling from the endoderm to the mesoderm is required for the correct separation of trachea and esophagus, among others through induction of *Foxf1* [80, 123]. Canonical WNT, BMP, bFGF, and FGF10 signaling from the ventral mesoderm to the AFE are essential for the specification of the lung field [124–129]. A BMP signaling gradient appears critical for the establishment of dorsoventral identity, as BMP4 is expressed in the ventral mesenchyme, while its antagonist, Noggin, is expressed in the dorsal mesenchyme and in the notochord [30, 130]. It has been shown that BMP4 signaling represses *Sox2*, which in turn, through mechanisms that are currently unclear, allows induction of *Nkx2.1*, establishing a reciprocal dorsoventral *Sox2*/*Nkx2.1* gradient [131]. Reporter gene lineage tracing studies have shown that the lung field, but not the pharyngeal pouches, experiences retinoic acid (RA) signaling [132]. Consistent with these observations, RA is required for lung bud formation, though not for lung field specification [133–135]. One role of RA, secreted from the mesoderm, is inhibition of the expression of Dkk1, a Wnt inhibitor. Wnt2/2b, essential for lung bud development, induces FGF10. In addition, RA induces FGF10 through alleviation of TGF-β-mediated inhibition of FGF10 expression [135].

The lung buds grow and branch in a stereotyped pattern, driven by proliferating progenitors in the tips of buds [136], while the cells that are left in the stalks adopt an airway

epithelial cell fate with the emergence of basal, goblet, Clara, ciliated, and other cell types [30, 137]. Ultimately, the distal aspect of the buds gives rise to alveolar progenitors [136]. The alveolar progenitors slowly mature in the perinatal period to give rise to ATI and ATII cells. Alveolar morphogenesis and functional maturation proceed in part postnatally and go through defined pseudoglandular, saccular, and vesicular stages [137]. The regulation of branching morphogenesis and subsequent specification and terminal differentiation of specific cell types are extremely complex and involve interactions of multiple signaling pathways that signal between the pulmonary endoderm and the surrounding mesenchyme. Among the critical factors secreted by the mesenchyme are FGF10 and BMP4, while Hedgehog signals from the endoderm to the mesenchyme [30]. Canonical and non-canonical Wnt signaling as well as BMP4 are involved in promoting a distal fate [129, 138, 139], while RA and Notch signaling promote a more proximal fate [140, 141]. Notch furthermore plays a major role in the cell fate decisions in the developing airway epithelium. Notch signaling enhances differentiation of tracheal basal cells into secretory and goblet cells but not into ciliated cells or neuroendocrine cells [142, 143]. During early postnatal development, lung and tracheobronchial stem cells that provide extensive regenerative capacity are laid down as well [29].

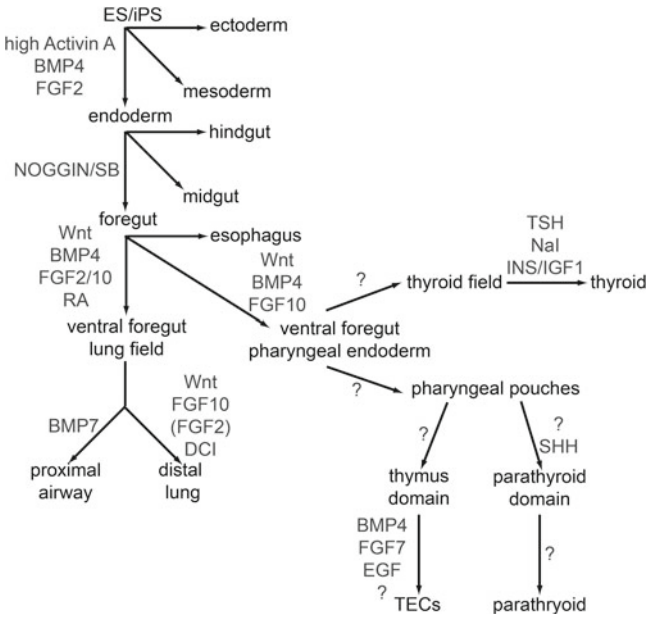

Fig. 3 Stepwise differentiation strategy for AFE and AFE derivative from pluripotent stem cells. Developmental progenitor fates that hPSCs must pass through to differentiate into tissues derived from the AFE are depicted together with critical signaling pathways (indicated in *grey*), based on current literature, required for the conversion between developmental progenitors

Thyroid. The thyroid domain is specified at E8.5 and expresses *Nkx2.1*, *Pax8*, *Hhex1,* and *Foxe1* [144]. The mechanisms involved in thyroid specification show partial overlap with those involved in lung bud specification. For example, FGF10 derived from the mesenchyme plays a critical role. However, while in the lung field, FGF10 expression depends on RA signaling, FGF10 expression if not dependent on RA in the thyroid field [134]. Furthermore, in contrast to the lung field, Shh is excluded from the thyroid domain of the AFE [145]. The thyroid evaginates from the floor of the pharynx and migrates caudally to its final position ventral of the thyroid cartilage [144]. Thyroid-stimulating hormone (TSH) signaling through its receptor, TSHR, only plays a role after thyroid morphogenesis has been completed [146].

Generation of Anterior Foregut Endoderm Derivative from Pluripotent Stem Cells

Directed differentiation. Current literature suggests that recapitulating development is the best approach to achieve differentiation of PSCs into a given cell type, a strategy referred to as directed differentiation [1, 10, 11]. However, while mechanisms underlying germ layer specification are quite well understood and could be applied successfully to the specification of DE from PSCs [10, 11, 83, 84, 147], the factors involved in the specification of organs domains, of specific mature cell types, and of postnatal stem cells within these organs are less well understood. It is, for example, not known how thymus and parathyroid are specified [42, 43]. Directed differentiation of PSCs combined with more unbiased approaches, such as morphogen and small molecule screens, may therefore reveal novel mechanisms underlying tissue specification and development and provide an accessible model for mechanistic studies, in particular in the human. To generate AFE derivatives, primitive streak and DE have to be induced first. Subsequently, anteroposterior patterning of the DE has to be achieved to generate AFE. Next, dorsoventral patterning of AFE is required to induce specific organ domains, which can be further differentiated. An overview of the current status of the field in directed differentiation of AFE derivatives is shown in Fig. 3.

Generation of definitive endoderm. ES cells are maintained in an undifferentiated state in specific culture conditions. Removal from these conditions results in the formation of embryoid bodies, spheres of cells where spontaneous gastrulation takes place, leading to the random generation of derivatives of all germ layers [1]. Several protocols have been developed to induce DE from PSCs in mouse and human. It has been shown that highly enriched DE can be generated by exposing ES cells, after withdrawal from conditions that maintain self-renewal, in either EB or monolayer format to serum-free conditions in the presence of high concentrations

of Activin A, which mimics nodal signaling [14, 83, 84, 147, 148]. A potential alternative to Activin A is small molecules. One such small molecule screen showed that two putative HDAC inhibitors, Ide1 and Ide2, can also induce DE [149]. DE induction from hPSCs in monolayer culture may be faster (3 instead of 5 days) but typically requires low amounts (0.2 %) of serum, however, although it has recently been shown that addition of BMP4, bFGF, and VEGF obviates the requirement for serum [150]. Addition of low concentrations of BMP4 and FGF2 have also been shown to enhance DE formation in EBs [151]. However, high BMP signaling in the absence of FGF signaling promotes primitive ectoderm and trophectoderm formation [84], while BMP exposure after endoderm induction favors a hepatic fate [14]. The appropriate concentrations and duration of exposure to these factors vary between laboratories [150–153] and need to be determined empirically. Wnt signaling is also required for primitive streak induction [82, 147] but is often not included in differentiation protocols [150, 151, 153]. It is likely that endogenous Wnt signaling in the cultures is sufficient for DE induction in the presence of high concentrations of Activin A. We have observed, however, that some variation among hPSC lines in the induction of DE can be alleviated by adding low doses of Wnt3a or a GSK inhibitor on the first day of DE induction (unpublished data). Use of an inhibitor (Y-27632) of the Rho kinase, ROCK, during the first day of differentiation has been shown to better maintain viability of dissociated hPSCs [154] and increases the yield of DE cells [152]. It is likely however that the strategy used to specify DE will determine its ultimate differentiation potential. For example, the length of induction of DE by Activin A is important in determining the subsequent potential of the cells. It has been shown that longer exposure to Activin A (7 days instead of 5 days) makes hESCs more amenable to differentiation into pancreatic endocrine cells when using the EB system but not when DE is induced in a monolayer [150]. In vitro generated DE expresses Epcam [85], c-KIT [14], and CXCR4 [84], so that the quality of DE induction can be assessed by flow cytometry, in addition to immunofluorescence and qPCR for the DE markers SOX17 and FOXA2. Up to 90 % pure DE, as judged by expression of Epcam, c-KIT, and CXCR4, can be achieved in hPSCs, although significant variability exists among lines in their capacity to generate DE.

Generation of AFE. Green et al. [153] observed that DE induced from hPSCs for 4.5 days in the presence of high Activin A and low concentrations of BMP4 and FGF2 shows a posterior bias, likely explaining why specification of hepatic, pancreatic, and intestinal fates has been reported [9, 12, 14, 20, 79, 150], while, until recently, there were no reports of the efficient specification of AFE or of AFE-derived tissues. A morphogen screen revealed that exposure of DE to a combination of NOGGIN, a physiological inhibitor of BMP signaling, and SB-431452, a pharmacological inhibitor of Activin A/nodal and TGF-β signaling (NOGGIN/SB-431542 or NS), led to expression of the foregut marker *SOX2*, suppression of the posterior marker *CDX2*, and maintenance of the endoderm marker *FOXA2* (Fig. 2) [153]. When transplanted under the kidney capsule of immunodeficient mice, these cells generated growths containing predominantly AFE-derived tissues, including GCM2+ parathyroid, AIRE+ putative TECs, and SP-C+ tubular structures indicative of lung and airway potential. There is developmental precedent for the importance of these compounds in anterior axial patterning. The most anterior endoderm is derived from cells that move through the primitive streak first and then leave the node. Hence, this part of the endoderm is the farthest removed from the nodal signaling area of the epiblast for the longest time. Furthermore, these cells are exposed to the nodal inhibitors, Lefty and Cerberus-like, from the anterior visceral endoderm that surrounds the forming DE [155, 156]. During its anterior migration, the cells fated to become AFE also pass through a zone where the BMP inhibitor Noggin is expressed in the anterior mesoderm [157]. This may explain why blocking TGF-β and BMP signaling after exposure to Activin A is required to specify this part of the endoderm. As such, these in vitro differentiation studies suggest a possible mechanism for AFE specification that is operative in vivo.

Generation of lung progenitors. There were, until recently, no reports of specific induction of a respiratory fate from mouse or human PSCs. Some studies reported lung differentiation from stochastically differentiating mPSCs but failed to show the efficiency of the protocol or depletion of other lineages [158–161]. An alternative approach has been the generation of a mES line where the neomycin resistance gene is expressed under the control of the promoter of the ATII marker, *Sftpc* (SP-C) [162]. Selection for neomycin-resistant cells from differentiating ES cells yielded cells with an ATII-like phenotype. However, genetic modification and selection carries the risk of mutations in the resulting cells. Green et al. attempted to specify the lung field from in vitro generated AFE by applying factors that are known to play a role in ventralization of the AFE in vivo. Treatment of hPSC-derived AFE, generated after 4.5 days of DE induction followed by 2 days of anteriorization with NOGGIN and SB-431542, with combinations of Wnt, FGF10, FGF7, EGF, and BMP leads to an increase in *Nkx2.1* expressing cells and a decrease in *Sox2* expression [153]. These data suggested ventralization and are consistent with the notion that FGF, BMP, and Wnt signaling are critical in the dorsoventral patterning of AFE [124–127, 131]. It is likely, based on these in vivo studies, that Wnt, BMP, and FGF10 are essential. The ventral AFE contains both pharyngeal and lung field endoderm, however. Reporter gene lineage tracing studies have

shown that the lung field, but not the pharyngeal pouches, experiences RA signaling [132, 163] and that RA is required during lung bud formation, through induction of FGF10 and Wnt2/2b [135]. In addition, RA represses TBX1, which is essential for the proper development of the pharyngeal endoderm [164]. Consistent with these in vivo findings in the mouse, addition of RA to the Wnt, FGF10, FGF7, EGF, and BMP cocktail decreased expression of the anterior pouch marker PAX1 but increased *FOXP2*, *NKX2.1*, *GATA6*, and the ciliated cell marker *FOXJ1*, a constellation of markers suggestive of a lung field fate and depletion of pharyngeal fate [153]. Together, these findings illustrate the value of following developmental paradigms discovered in the mouse model to direct differentiation of hPSCs and were the first demonstration of specific and quantified directed differentiation of the lung field from hPSCs.

Longmire et al. subsequently used a similar strategy with an Nkx2.1:GFP genetic reporter for pulmonary epithelium [165]. There were several differences with the data of Green et al. in the human model however [153]. The efficiency of lung field generation was lower. Furthermore, ventralization required FGF2. Finally, some of the Nkx2.1:GFP$^+$ cells showed evidence of thyroid specification, which was not observed in hPSCs [153]. This may be a reflection of interspecies variation and of the differences in the rate of development between humans and mice. Identifying and targeting the optimal developmental time window where cells are responsive to appropriate inductive signals may be more challenging in PSCs from the mouse, where development proceeds much more rapidly than in humans. In another report, Mou et al. used monolayer, as opposed to embryoid body differentiation, and observed that only inhibition of TGF-β was required for the specification of AFE in mouse ES cells [166]. This produced, after ventralization in the presence of Wnt, BMP, and FGF2 signaling, Nkx2.1$^+$ putative lung field epithelial cells from hPSCs with an efficiency of 15 % and from mPSCs with an efficiency of 10 %, again strikingly lower than what was reported in hPSCs. These authors also observed that addition of BMP4 was sufficient to induce NKX2.1, but that endogenous Wnt and FGF signaling was required. These observations were reproduced using iPSCs from cystic fibrosis patients, suggesting that human disease modeling could be undertaken using this strategy.

Terminal maturation of lung and airway. Green et al. showed that continued culture after ventralization in the absence of BMP-4 and RA but in the presence of Wnt, FGF10, and FGF7 induced expression of SP-C, a marker not only of ATII cells but also of early lung progenitors at E11 in the mouse [153]. These findings are in accordance with the observation that within the developing mouse lung, RA signaling is most pronounced proximally, while constitutively active RA signaling favors a proximal fate [140, 163]. Furthermore, Wnt and FGF signaling have been shown to promote a distal fate [138, 167]. Mou et al. found that a minority of mESCs-derived Nkx2.1 cells could be induced to express proximal markers (*p63*, *Sox2*) after addition of BMP7 and FGF7 and that this effect was enhanced by Wnt inhibition [166]. Further differentiation in vitro was not documented however. Some of the cells did form spherical structures expressing proximal airway markers after subcutaneous transplantation in vivo. Unfortunately, similar maturation studies in the cystic fibrosis iPSCs failed to show any mature cell types in vivo.

In the presence of FGF2 and FGF10, purified Nkx2.1:GFP$^+$ mESC-derived cells induced a variety of airway and alveolar markers, while addition of established maturation components for fetal lung explants cultures, consisting of dexamethasone, butyrylcAMP and isobutylmethylxanthine (DCI) [168], upregulated expression of SP-C, SP-B, and the Clara cell marker (CCSP). Furthermore, Nkx2.1:GFP$^+$ cells could home to a decellularized lung matrix and in some cases generate cells with marker expression and morphology consistent with ATI cells [165]. While this work showed that it is possible to differentiate mESCs into several lineages of lung and airway epithelial cells, the requirement for an Nkx2.1:GFP reporter is a drawback, as this approach cannot be used routinely in hPSCs.

While it is now established that lung bud epithelium can be generated from PSCs, many challenges remain. Specification protocols with terminal maturation steps will be needed to generate each of the different cell types of the proximal airway and alveoli. The lung and airways are also endowed with postnatal stem cells [29]. As postnatal lung and airway stem cells can regenerate airway and alveolar epithelium after severe injury in vivo, seeding decellularized lung matrices with these cells will likely achieve the most physiological locoregional distribution of appropriate cell types. However, although several populations have been identified in the mouse that, based on lineage tracing experiments, display regenerative capacity in vivo or are able to form colonies containing mature cells in vitro, none have been purified to homogeneity, and except for tracheal basal cells, their physiological role, phenotype, and physical location are unclear [169–174]. In particular, it is not known which cells are responsible for regeneration of alveoli [172, 175], although a newly identified p63+ distal airway cell [176] and a population of distally located a6b4 integrin expressing cells [172] are good candidates. A focus of the field should therefore include identification and generation of postnatal stem cells of the human lung. Directed differentiation hPSCs may represent a "forward" strategy to elucidate the nature and function of postnatal human lung and airway stem cells. Finally, functional assays are required to test the function of hPSC-derived lung and airway epithelial cells populations.

Generation of TECs. Of critical importance for efforts at generating TECs is that it is likely sufficient that the stage of a specified fetal TEC progenitor, equivalent to E11.5 in the mouse, is reached. The thymus domain of the third PP at E11-12 stains with the MTS24 antibody in the mouse [177, 178]. Using this antibody, which recognizes Plet1 (unfortunately, humans do not have a Plet1 orthologue) [179], it was possible to purify TEC progenitors, which can reconstitute a fully functional thymus after transplantation under the kidney capsule of adult mice [177, 178]. Although MTS24 was subsequently shown not to uniquely identify all TEC progenitors in the third PP [180], these findings do show that purified fetal TEC progenitors are sufficient to establish a functional thymus after transplantation. It is likely that other supporting cells required for thymic growth are recruited from the host. Lineage tracing experiments have furthermore shown that single fetal TEC progenitors can give rise to both mTECs and cTECs [181]. It is therefore likely that hPSC-derived fetal TEC progenitors will establish a functional thymus and support development of human T cells after transplantation into immunodeficient mice engrafted with human HSCs.

There are no reports of the generation of TEC progenitors from hPSCs. One report described the generation of TECs from mESCs [182]. In this report, differentiating mESCs were exposed to a combination of factors, FGF7, FGF10, BMP4, and EGF, that are known to play a role in terminal differentiation of TECs or of epithelial cells in general. A minor population of Epcam+ cells arose that was enriched for the expression of *Krt5*, *Krt8*, *Pax1*, *Pax9*, *Foxn1,* and *Plet1*. After reaggregation with thymocytes and transplantation under the kidney capsule, these cells formed structures resembling thymus where evidence of ongoing T cell development was present. Furthermore, these mice displayed higher numbers of peripheral T cells. Although naïve T cells appeared increased, there were no data on the number of memory T cells, which should decrease, as T cell numbers are determined by peripheral homeostatic mechanisms [47]. Remarkably, these ES-derived putative TECs engrafted after intrathymic injection into allogeneic hosts. These findings are remarkable for several reasons. First, it appeared unnecessary to direct differentiation along established developmental stages (endoderm, AFE, pharyngeal endoderm, pharyngeal pouch endoderm, TEC progenitors). Rather, adding factors that play a role in the final stages of thymic development appeared sufficient. Second, the cells did not appear to be rejected by an irradiated allogeneic host. The same authors showed in subsequent paper that mES-derived TECs could prevent graft-versus-host disease after allogeneic bone marrow transplantation [183]. While potentially very exciting, these observations require independent confirmation. Another report demonstrated that EBs developing in serum-free conditions contain cells that express markers of pharyngeal endoderm and of thymus. These EBs were able to support T cell development when reaggregated with CD4−CD8− fetal thymocytes but did not generate functional thymic tissue after transplantation under the kidney capsule [184]. As EBs are ESC-derived structures that arise after conditions conducive to self-renewal are withdrawn and where a process equivalent to gastrulation takes place, it is not entirely surprising that several lineages, including TECs, are detected. Finally, one report describes the appearance of thymic markers after exposure of mESC-derived DE to FGF7, FG10, and BMP4. No functional studies were performed, and the depletion of other lineages was not reported [185]. Taken together, a reliable, reproducible protocol to generate a functional and pure thymic organoid from hPSCs has not been reported, although some progress has been made in the mouse model.

Parathyroid. The generation of cells expressing markers consistent with parathyroids has been reported [186, 187]. These studies were based on differentiation of mES cells into endoderm, followed by extended culture in the absence of Activin A in varying concentrations of serum. However, rigorous quantification, assessment of depletion of alternative lineages, or functional analysis in vivo of these cells is not available. We observed in a medium throughput screen that hPSC-derived AFE, ventralized in the absence of RA and then exposed to either FGF8, SHH, or FGF8 and SHH expressed GCM2 in a large fraction of the cells [153]. However, PTH expression was not detected. Furthermore, GCM2 was almost exclusively cytoplasmic (unpublished data), likely explaining the lack of PTH expression. The reason for the cytoplasmic location of GCM2 is unclear. These data however again illustrate the difficulties facing efforts to generate functionally mature cells.

Thyroid. Rigorous protocols to induce thyroid have not yet been established, and there are no studies in hPSCs. Typical protocols using mESCs involve inducing formation of EBs and exposure of the cells to thyroid-stimulating hormone [188]. Although some cells co-expressing the Na/I symporter (Nis) and TSHR were observed, the fraction of cells co-expressing *Nkx2.1* and *Pax8*, a signature expression profile of thyroid progenitors, was very low and or not quantified. Using cell sorting for Tshr:GFP in a TSHR reporter mES line induced to form EBs, structures resembling thyroid follicles, where uptake of I was documented, were generated. Furthermore, TSHR, thyroid peroxidase, and NIS, but not thyroglobulin, were expressed [189]. TSH stimulation was not necessary to achieve expression of these thyroid markers in Activin A-induced cells cultured for up to 21 days [190], consistent with the notion that TSH is not required for thyroid specification and early thyroid development [146]. Combining sequential induction by Activin A and TSH, followed by TSH, insulin and IGF1 resulted in some expression

of thyroglobulin, indicative of further maturation [191]. Similarly, culture of EBs in the presence of insulin and potassium iodide yielded some expression of thyroglobulin [192]. No data on efficiency of differentiation or on the fraction of cells expressing these markers were given however. Longmire et al. observed, in their more developmentally guided directed differentiation approach using Nkx2.1:GFP reporter cells, that Nkx2.1:GFP$^+$ cells isolated from culture induced to generate ventral AFE expressed thyroglobulin, Pax8, and TSHR after further culture in the presence of FGF2 and FGF10 and that thyroglobulin and TSHR expression increased after exposure to "thyroid maturation media" containing TSH, IGF1, and NaI [165]. No data were provided on the frequency and function of putative thyroid-specified cells, however. There are currently no data on the generation of thyroid cells from hPSCs. Overall, no rigorous protocol to generate functional thyrocytes from PSCs have been developed yet, although some progress had been made in mouse. None of the protocols currently available strictly recapitulated development other than induction of DE using Activin A.

Conclusion

Generation of tissues and organs derived from the AFE, including lung, thymus, parathyroid, and thyroid, is a scientifically and medically highly relevant area of research. Although the specification of AFE from PSC-derived DE has been challenging, significant progress has been made recently, in particular in the specification of AFE and in the generation of progenitors corresponding to the lung field and the lung buds. Major challenges remain however. These include approaches to specify thymus, parathyroid, and thyroid; strategies to achieve differentiation into functional, mature cells as well as postnatal stem cells; and the development of in vitro and in vivo assays to probe the function of PSC-derived cells.

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Commercial Opportunities for Induced Pluripotent Stem Cells

Amy Lynnette Van Deusen and Rania Nasis

Introduction

The successful reprogramming of fibroblasts into induced pluripotent stem cells (iPS cells) was first reported using rodent cells in 2006 and human cells in 2007 [1, 2]. This innovation by Dr. Shinya Yamanaka and his colleagues at Kyoto University in Japan represents a major scientific achievement, such that in 2012, Yamanaka was awarded the Nobel Prize in Medicine, solidifying the importance of his pioneering efforts and resulting iPS cells in the history of science. The successful reprogramming of human fibroblasts into pluripotent cells reinvigorated the stem cell community at large by providing a promising alternative to human embryonic stem cells (hESCs) and an extraordinary opportunity to revolutionize modern medicine. The life sciences marketplace responded by developing an array of new products and services tailored to researchers' efforts with iPS cells, resulting in an ever-expanding commercial market for innovative stem cell research tools. With hundreds of billions of dollars in potential revenue and a customer base that includes over one million stem cell researchers in 179 countries (see Table 1) the market for new stem cell technologies has the potential to be very lucrative [3].

The market for stem cell products flourished over the last decade with global revenues currently estimated at $3.8 billion for 2011 [4]. This figure takes into account all stem cell-related tools, reagents, cell products, and adult cell therapies currently on the market but excludes indirectly associated market segments such as antibodies and cryopreservation. Such a rapid escalation in product revenues over the next few years is supported by the potential of stem cell treatments for macular degeneration [5], lysosomal storage disorders [6], Pelizaeus–Merzbacher disease [7], amyotrophic lateral sclerosis (ALS) [8], and other more common diseases as well as an expanding global market encouraged by recent advancements in iPS cell technologies.

The market for iPS cell research products is estimated at $873 million for 2012 and currently growing at a rate of 14.7 %. Continued growth in the iPS cell technologies market is substantiated by an expected increase in adoption and availability of iPS cell applications over the next decade as well as the expansion of product pipelines of companies already established within the market. With iPS cells being sold by 53.4 % of biological research product companies in the United States and 38.7 % globally, it is clear that the monetary potential of the stem cell market has not been lost on current market players [9]. Long-term revenue forecasts take into account the increasing global burden of an aging population, higher incidence of life-threatening diseases, opportunities for improvement of medical treatments, and the increasing costs of drug development [10]. iPS cell technologies are particularly suited for the task of addressing these challenges and in this chapter we will discuss how the commercial features of iPS cells convey opportunities for significant profits stemming from a diverse array of technologies.

The stem cell market is even larger when we consider the numerous associated or ancillary products required for successful implementation of this technology, such as antibodies. Antibodies are utilized as biomarkers to characterize both the pluripotency of stem cells and the identity of differentiated cell populations. Since antibodies are an expensive, but indispensable tool for stem cell researchers, it is no surprise they command significant market revenues. The antibody sector of the stem cell market alone was estimated at almost $2 billion bringing the total market value for all stem cell products and therapies to a staggering $5.72 billion for 2011 [3].

In the clinic, autologous iPS-derived cell therapies could potentially eliminate the need for immunosuppression and reduce the risk of graft vs. host disease. In addition, using a

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Table 1 Distribution of stem cell researchers throughout the world

Country	Number of researchers	Percentage of world total researchers
United States	187,317	18.6
Japan	97,100	9.6
India	81,641	8.1
UK	64,279	6.4
Australia	63,509	6.3
Germany	43,698	4.3
China	37,887	3.8
Italy	30,869	3.1
Canada	18,637	1.9
South Korea	16,840	1.7
France	16,377	1.6
Brazil	16,321	1.6
Russia	15,893	1.6
The Netherlands	15,251	1.5
Finland	8,678	0.9
REST OF WORLD	292,682	29.1

Source: BioInformant Worldwide, L.L.C., Research Analysis Group, Stem Cell Research Products: Opportunities, Tools & Technologies, May 1, 2012

patient's own cells opens up the possibility of correcting disease-causing mutations by genetically modifying and then replacing cells. Perhaps of even greater therapeutic promise is the ability to use iPS cell technologies for disease modeling and drug discovery. Primary cell-based disease models currently employed by researchers have many limitations when investigating mechanisms of disease. For the first time, researchers have the ability to create patient-specific cells lines to examine a variety of disease phenotypes, allowing for better insight into disease progression and more robust lead candidate identification studies for disease-modifying drugs. In addition, iPS cells provide researchers with the ability to test promising compounds on cells from a variety of patients, the so-called clinical trial in a dish, without ever having to risk a single patient's safety.

In this chapter, the commercial potential of iPS cell technologies will be explored. We will begin by discussing the fundamental characteristics of iPS cells that make them a valuable tool in the marketplace. Then, the commercial market itself will be surveyed and we will consider market players in context of their specific roles. Next, we will explore the relevance of iPS cells in modeling disease and discuss current market efforts to capitalize on this unique function of these cells. Further, we will consider the most important applications of this technology, from drug discovery tools and reagents to clinical cell-based therapies. Finally, we will discuss challenges for utilizing iPS cell technology for commercialization purposes and how modern science and industry can work together to overcome these challenges to fully realize the potential of iPS cells.

Commercial Features of Induced Pluripotent Stem Cells

In order to understand the potential commercial uses of iPS cells, it is first important to consider the features of this technology that make it both *suitable* and *valuable* in the market. While iPS cells are a relatively recent discovery, they have demonstrated the potential to convey all applications described in the last 30 years for embryonic stem cells with a few unique benefits. The basics of stem cells are covered in detail in other sections of this textbook; therefore, we will focus upon features of human-derived iPS cells that are most applicable in a commercial setting. We will begin by comparing iPS cells to hESCs in terms of their fundamental biology and commercial applicability. Next, we will examine the ability to reprogram adult cells and contrast this against existing adult-derived cell therapies. Finally, we will finish with a discussion of how iPS cells have changed the ethical discussion surrounding the use of stem cells in research and regenerative medicine.

Self-Renewal and Pluripotency in iPS Cell Technologies

The two most important features of hESCs and iPS cells are their capacity for self-renewal and potential to develop into numerous cell types, otherwise known as pluripotency. While a large body of work exists describing this potential in hESCs, a number of recent studies have clearly demonstrated that iPS cells share a commensurate capacity for self-renewal and pluripotency [11–18]. The developmental potential of iPS cells is similar to that of hESCs as researchers have been able to differentiate reprogrammed cells into ectoderm, mesoderm, and endoderm cell lineages representing all three embryonic germ layers [2, 19, 20]. Indeed, it is the ability of iPS cells to generate virtually any cell type that makes their use feasible and suitable for all of the diverse applications envisioned for embryonic stem cells.

The developmental potential of iPS cells was first demonstrated by tetraploid complementation studies where tetraploid blastocysts (formation of 70–100 cells that contain four copies of each chromosome instead of two, rendering them incapable of further embryonic development) were rescued using an injection of murine iPS cells. Researchers were then able to develop mice from these rescued blastocysts demonstrating that full developmental potential was indeed restored [21–23]. The tetraploid complementation strategy has subsequently been employed to verify the developmental potential of human iPS cell lines [1, 20]. However, because of the time-consuming nature of this method, it is inefficient for manufacturing and drug discovery purposes.

Another feature that makes iPS cells a valuable resource is their capacity for self-renewal that allows them to be generated in vitro virtually in perpetuity. This provides several advantages for academic and industrial uses, most prominently the ability to generate large amounts of cells from a relatively small amount of source material. Because iPS cells have the developmental potential to generate adult cells of almost any type, expansion and differentiation from a single sample could supply a limitless amount and array of cell products.

Since many human cells are difficult to source (some can only be obtained from human cadavers) or culture in vitro, iPS cells represent a novel and valuable resource for regenerative medicine. Further, the virtually unlimited availability of cells from a single donor negates potential complications arising from pooling donor cells and limited tissue accessibility. Hepatocytes, highly functional liver cells responsible for the majority of drug metabolism and detoxification, are a great example of a cell type that is difficult to source and culture and therefore it would be of great utility to be able to produce unlimited quantities of this cell type from iPS cells. iPS-derived hepatocytes present a valuable opportunity for researchers and entrepreneurs alike and will be examined in more detail later in this chapter.

The mechanisms underlying the differences between hESCs and iPS cells are currently not well understood. Many researchers believe the p53-p21 pathway, often referred to as "guardian of the genome," plays a distinct role in these processes [24]. Its primary function is to prevent the occurrence of genetic mutations and abrogation of p53 resulted in more successful reprogramming of cells in vitro, regardless of their level of DNA damage [25]. Others have found inhibition of the p53-p21 pathway suppresses subsequent generation of iPS-derived cell types while simultaneously ensuring genome integrity in these cells [24]. Normal, cancer-free mice have been developed from cells in which the p53 pathway was inhibited to enhance reprogramming [21–23]. However, the balance of the p53 system is delicate and downstream effects need further study [26, 27].

Another plausible explanation for the differences observed between iPS cells and hESCs is variation in their respective genetic profiles. Small variations in gene expression indicate that iPS cells generated by retroviral integration methods exhibit an miRNA expression profile that is slightly different from that of hESCs, but this profile gradually evolves to be more similar after extended culturing in vitro [28]. Regardless, control over self-renewal and pluripotency in both hESCs and iPS cells in vitro remains an ongoing challenge for researchers and product manufacturers.

For commercialization purposes, self-renewal and pluripotency translate into the ability to develop a diverse range of iPS cell products and applications. However, understanding the mechanisms underlying these most fundamental aspects of stem cells will help standardize experimental and manufacturing procedures.

Commercializing the Ability to Reprogram Adult Cells

The ability to reprogram adult somatic cells into pluripotent stem cells is what distinguishes iPS cells from hESCs and other sources of stem cells with regard to their potential use in research, commercial, and therapeutic applications. Numerous types of adult human cells have been successfully reprogrammed into iPS cell lines and differentiation protocols exist to create virtually any adult cell type, though quality and purity of these may vary greatly. Established methods to generate iPS cells from adult somatic cells are covered in detail in other chapters in this textbook and in an excellent review by Dr. Nimet Maherali and Dr. Konrad Hochedlinger [29]. Therefore, we will cover this area only briefly and instead focus on how reprogramming adult cells is valuable in the commercial stem cell marketplace.

Several adult tissue sources are capable of reliably generating iPS cell lines [1, 30, 31]. While the ability to generate patient-derived cells avoids the potential for immunological incompatibility between cell-based therapies and their recipients [1, 2, 20], the "stemness" and developmental potential of these adult tissue sources remain largely uncharacterized. Further, generating mature adult cells from these progenitors currently requires extensive in vitro and in vivo culturing [32, 33]. The success of making cells suitable for engraftment is variable between cell types and fully dependent upon generating pure populations of cells at a specific maturation stage [34]. Before full commercialization of adult cell reprogramming and iPS cell technologies can take place, researchers must refine current methods to generate clinically relevant cell populations and validate various patient tissue sources for their suitability in these paradigms.

In humans, keratinocytes (epidermal cells found in the outermost layer of skin) are a favorable source material for generating iPS cells as the process to obtain samples from patients is noninvasive and these cells have a demonstrated capacity for reprogramming. For clinical and cell banking purposes, this results in small patient samples that are easy to store and capable of producing virtually unlimited amounts of cell products. However, more research is needed to qualify which sources are best for which purposes, since data suggests that not all patient tissue sources are created equal. For example, keratinocytes harvested from a single strand of human hair have been successfully reprogrammed into iPS cell lines using a retroviral method to drive expression of Yamanaka's four reprogramming factors. When protein

expression was examined using a panel of stem cell biomarkers, including the transcription factor Oct4 and stage-specific cell-surface antigens SSEA-3/4 and TRA-1-60/81, iPS cells generated from keratinocytes appeared more stem-like than those obtained from adult human fibroblasts reprogrammed using the same method. Pluripotency was also confirmed by differentiating keratinocyte-derived iPS cells into dopaminergic neurons and rhythmically beating cardiomyocytes, representing ectodermal and mesodermal lineages, respectively. It was observed that iPS cells generated from keratinocytes were obtained with 100 times more efficiency and could be produced in half the time when compared to fibroblast-derived cells [30]. For commercial purposes, the difference translates into significantly decreased production costs and reduced time to turn around patient-specific products.

Peripheral blood cells [31] and adipose stem cells have also demonstrated an increased capacity for reprogramming [35] and similarly represent a relatively accessible patient source material. Although, the process to obtain these cells is slightly more invasive than for keratinocytes or fibroblasts, it is possible that the genetic condition of these cells remains more stable over a human lifetime because of reduced exposure to environmental factors such as UV light [36]. Other adult cell types, such as mesenchymal-like cells harvested from dental tissue, have also been successfully reprogrammed into iPS cell lines. By obtaining adult cells that are similar in origin to the desired final product, researchers hope that dental tissue-derived iPS cell will be particularly adept at developing into odontogenic, oral, and craniofacial tissues that would be particularly advantageous in studying and treating craniofacial disorders such as cleft lip and palate [34]. These congenital deformities that occur during gestation affect 1 out of every 500–700 children born today and currently require surgical treatment to correct. Though the various sources and methods used to derive iPS lines from those sources have yet to be fully evaluated for clinical suitability, it demonstrates that iPS technologies have been recognized by a broad spectrum of life science and medical disciplines.

The greatest advantage of iPS cell-based strategies compared to other types of adult stem cells is their pluripotent potential. Cord blood stem cells harvested from umbilical cords and mesenchymal stem cells found in bone marrow are only capable of producing hematopoietic cell types. Limbal stem cells found in the eye are currently being tested as a treatment for corneal disease [37], yet their suitability for other purposes appears limited. Though many sources of adult stem cells exist, it is important to keep in mind that the quality and safety of all adult stem cell treatments remain to be established. Regardless, the ability to reprogram adult cells that iPS cell technologies offer researchers presents a better platform for modeling human disease in vitro than other available methodologies.

The Ethics of iPS Cell Technologies

Beyond presenting a novel avenue for patient-specific medicine, the ability to generate iPS cells from adult sources also resolves many ethical barriers surrounding the use of embryonic stem cells. Soon after the first derivation of embryonic stem cell lines from human embryos by Dr. James Thomson at the University of Wisconsin, in Madison [38], controversy began to surround the use of these cells for any purpose. The basis for most objections concerned the origin of these cells, human blastocysts, and the manner in which they are obtained from donor sources.

In 1996, the United States Congress signed the Dickey-Wicker Amendment banning government funding for creation or destruction of human embryos into law. For 15 years, an ideological battle was waged between researchers, politicians, ethicists, and religious organizations. In 2009, US President Barack Obama issued an executive order that removed restrictions on federal stem cell funding, though this was contested with an injunction for 2 years [39] before the US District Court for the District of Columbia finally lifted the injunction in 2011. In July 2012, a panel of judges upheld this decision in the US Circuit Court of Appeals for the District of Columbia thereby affirming the legality of embryonic stem cell research in the United States. However, policies governing embryonic stem cell research remain restrictive throughout most of the world including Western Europe, with the exception of the UK, Sweden, and Switzerland where researchers are less limited in their ability to pursue hESC-based work.

The use of iPS cells has the potential to even the stem cell playing field across the globe as it opens up new applications and funding opportunities previously unavailable to thousands of researchers. Bioengineering cells through reprogramming offers an innovative strategy for embryo-independent creation of autologous cell therapies and avoids ethical and political issues surrounding embryonic stem cell work. While much research is still required to validate the use and safety of iPS cells and to fully characterize them in comparison to "gold standard" embryonic stem cells [40], many recent discoveries have brought the goal of regenerative medicine closer than ever to becoming a reality. Indeed, the speed with which iPS technologies could potentially deliver clinical therapies roused officials at the National Institutes of Health to thoroughly examine and codify the informed consent process for iPS cell research in coordination with the US Food and Drug Administration (FDA) in 2012 [41].

Disease Modeling

The concept of using stem cells to model human disease in vitro is based on their capacity for self-renewal and pluripotent potential [42]. The most distinct advantage of using iPS

cell-based models for studying disease is that they appear to be more reflective of actual pathophysiology than traditional biochemical assays, genetically modified animal models, or transformed cell lines [43]. Additionally, unlike embryonic stem cells, iPS cell technologies present the unique ability for researchers and physicians to reprogram patient cells into iPS cell lines, conveying several major advantages that we will examine in this section. We will consider the implications of generating disease-specific and patient-specific cell lines in the context of modeling disease and survey the market for disease-relevant stem cell products. Finally, we will discuss the utility of iPS cell technologies for researching various forms of cancer.

Disease-Specific iPS Cell Models

The ability to modify specific genetic or epigenetic parameters of cells allows researchers to more accurately reproduce disease states in the laboratory. Transformed cell lines and animal models currently utilized are limited by genetic variations and, in many cases, fail to reproduce certain aspects of human pathophysiology. Additionally, these models are insufficient for analyzing complex disorders or recapitulating transient physiological disease states in vitro. For these reasons, the generation of more accurate in vitro human disease models that allow researchers to thoroughly examine pathophysiology has long been a goal of academia and industry alike.

Numerous other iPS cell lines have been developed that replicate human disease phenotypes, including "diseased" lines for spinal muscular atrophy (SMA) [44], fragile X syndrome [45], Dyskeratosis Congenital [46], LEOPARD syndrome [47], Long QT syndrome [48], Rett syndrome [49], multiple liver diseases [50], Parkinson's disease (PD) [51], and schizophrenia [52]. See Table 2 for a list of iPS cell lines carrying disease-specific genetic mutations. Considering the variety of diseases these lines encompass, this strategy has the potential to be applied to almost any human disease for which there is a genetic link. The first report of functional

Table 2 List of registered iPS cell lines carrying disease-specific genetic mutations

Disease	Mutation	Cell lines
Amyotrophic lateral sclerosis	ALS (L144F [Leu144>Phe] dominant allele of the superoxide dismutase (SOD1) gene)	A29a A29b A29c 29d 29e 27b
	G85S [Gly85>Ser] dominant allele of the superoxide dismutase (SOD1) gene	27e
Crigler–Najjar syndrome	UGT1A1, p.Leu413Pro	CNS-hiPSC10 CNS2-hiPSC7
Cystic fibrosis	Homozygous DF508 mutant CFTR genotype	CF-RiPS-1.2 CF-RiPS-1.3 CF-RiPS-1.4 DF508 2 DF508 4 DF508 5 DF508 6 RC2 202 2 RC2 202 4
Diabetes, Type 1		DiPS-H1.5 DiPS-H2.1 DiPS-H2.4
Down syndrome	Trisomy 21	DS1-IPS4 DS2-IPS1 DS2-IPS10
Emphysema	AAT deficiency, PiZZ phenotype	RC2 100 3 RC2 100 3 Cr-1 RC2 100 3 Cr-6 RC2 102 37 Cr-1 RC2 102 37 Cr-3 RC2 103 43 Cr-1 RC2 103 43 Cr-3

(continued)

Table 2 (continued)

Disease	Mutation	Cell lines
Gaucher disease type III	GD (AAC>AGC, exon 9, G-insertion, nucleotide 84 of cDNA, GBA gene)	GD-IPS1 GD-IPS3
Huntington disease	HD (72 CAG repeats, huntingtin gene)	HD-IPS1 HD-IPS4 HD-IPS11
Juvenile diabetes mellitus	Multifactorial	JDM-IPS1 JDM-IPS2 JDM-IPS4
Lesch–Nyhan syndrome, carrier	Heterozygosity of HPRT1	LNSC-IPS2
Long QT syndrome, Type 1		LQT 1
Mucopolysaccharidosis, Type 1 (Hurler syndrome)		MPS-KC-iPS 1 MPS-KC-iPS 2 MPS-MSC-iPS 2
Muscular dystrophy, Becker	BMD, unidentified mutation in dystrophin	BMD-IPS1 BMD-IPS4
Muscular dystrophy, Duchenne		DMD-iPS (DYS-HAC) 1 DMD-iPS (DYS-HAC) 2 DMD-iPS (DYS-HAC) 3 DMD-iPS (DYS-HAC) 4 DMD-iPS (DYS-HAC) 5 DMD-iPS (DYS-HAC) 6 DMD-iPS (DYS-HAC) 7 DMD-iPS (DYS-HAC) 8 DMD-iPS (DYS-HAC) 9 DMD-iPS1 DMD-iPS2
	DMD (deletion of exon 45–52, dystrophin gene)	DMD-iPS1 DMD-iPS2
Myeloproliferative disorder (Polycythemia vera)	JAk2 gene, V617F mutation	iMPD183.C1 iMPD183.C2 iMPD183.C3 iMPD183.C5 iMPD183.C6 iMPD183.C7 iMPD183.C8 iMPD183.C10 iMPD183.C11
Myeloproliferative disorder (primary myelofibrosis)	JAK2 gene, V617F mutation	iMPD562.C.3 iMPD562.C2
Parkinson disease	Multifactorial	PD-IPS1 PD-IPS5
Progressive familial hereditary cholestasis	Multifactorial	HER-hiPSC1
Schizophrenia	DISC1 mutation	D1-iPSC-1 D2-iPSC-1
Severe combined immunodeficiency	ADA-SCID, adenosine deaminase deficiency-related (GGG>AGG, exon 7, ADA gene)	ADA-IPS2 ADA-IPS3
Spinal muscular atrophy, Type 1		IPS-SMA-3.5 IPS-SMA-3.6
Spinal muscular atrophy, Type 1 (carrier)		IPS-WT
Shwachman–Bodain–Diamond syndrome	SBDS (IV2+2T>C and IV3 − 1G>A, SBDS gene)	SBDS-IPS1 SBDS-IPS2 SBDS-IPS3
Tyrosinemia, Type 1	FAH gene, GLN64His mutation	TYR1-hiPSC1
X-linked Adrenoleukodystrophy, Adrenomyeloneuropathy (AMN)		AMN iPSC-3
X-linked Adrenoleukodystrophy, Childhood Cerebral ALD (CCALD)		CCALD iPSC-2 CCALD iPSC-10

Source: University of Massachusetts Medical School International Stem Cell Registry. Accessed 3 Dec 2012. Available: http://www.umassmed.edu/iscr/GeneticDisorders.aspx

cells being generated from patient-derived, disease-specific iPS cell lines occurred in 2009. A group at the Center for Regenerative Medicine in Barcelona, Spain, derived iPS cells from patients with Fanconi Anemia, a rare genetic blood disorder. Researchers were subsequently able to develop disease-corrected cells demonstrating the potential of iPS cell technologies for both research and cell therapy applications [53].

ReproCell (Yokohama, Japan) launched the first commercially available, disease-specific iPS cell derivatives onto the market in June 2012. Researchers at this company were successfully able to incorporate a gene related to Alzheimer's disease (AD) into neurons derived from iPS cells. These cells reportedly express a hallmark Alzheimer's phenotype that includes Amyloid β plaque accumulation and are the first human neurons on the market targeted specifically to this disease. Considering the market for Alzheimer's therapies was valued at $5.4 billion for 2010 and is expected to triple by the year 2020, models to improve the accuracy of safety and efficacy data for potential treatments are of great value to researchers and industry [54].

Several other companies have since launched disease-relevant iPS cell lines and products including non-virally transduced human iPS cell lines for diabetes, Parkinson's disease, ALS, and muscular dystrophy from System Biosciences, Inc. (SBI) (Mountain View, CA). SBI uses non-viral methods for reprogramming that incorporate phiC31 integrase [55], minicircle transfection [56], or recombinant proteins [57]. The benefit of non-virally transduced iPS cells is that products generated downstream are considered more suitable for clinical use. American Type Culture Collection (ATCC) (Manassas, VA) has also made available several human disease cell lines including cell lines for Down syndrome, cystic fibrosis, and Parkinson's disease. Though generating commercially available disease cell lines is a potentially lucrative venture, identifying effective and relevant stressors that accelerate cell processes driving late-onset disease phenotypes is not a straight-forward process.

We must also consider the current limitations of iPS cell-based disease modeling. First, as previously mentioned, iPS cells have yet to be fully validated against hESCs for use and reliability. Second, factors such as age and sex of patients from whom iPS cells are generated may critically affect how closely those cells reflect pathophysiology and/or how efficiently transgene integration occurs such that cells are capable of differentiating into disease-relevant phenotypes [58]. Beyond addressing existing issues with the genetic fidelity of iPS cells [59], the suitability of each adult tissue source, reprogramming process, and derivation scheme must be examined. Regardless, iPS cells still retain the potential to become one of the most valuable tools in replicating human disease in vitro.

Patient-Specific

One of the most profound advantages of iPS cell technologies is that they provide a strategy for cell lines to be generated from an individual in the context of his or her own genetic and epigenetic identity. This is especially important for individuals with sporadic forms of disease or for patients whose pathophysiology is idiopathic or complicated by polygenic origins, as is the case for autism spectrum disorders [60], Type 1 diabetes [61], and insulin-dependent Type 2 diabetes [62]. Patient-derived iPS cells would also provide a complimentary high-throughput approach to current human pharmacological studies by expanding the ability to compare diseased and healthy cell lines from various parental lineages [63].

Several proof of principle experiments have demonstrated that patient-derived iPS cell lines possess the potential to differentiate into "disease-relevant" phenotypes. In 2008, researchers reprogrammed iPS cells from patients diagnosed with a genetic form of SMA, a neurodegenerative disease that leads to loss of lower motor neurons. These iPS cells were differentiated into motor neurons and compared to cells similarly derived from wild-type iPS cells. Initially, the patient-derived motor neurons demonstrated a normal phenotype and morphology in culture. However, after 8 weeks, these cells exhibited decreased expression of survival of motor neuron (SMN) protein aggregates [44], similar to the deficiency of this protein observed in the disease progression of SMA in humans [64]. This characteristic phenotype of SMA was subsequently reversed in vitro by certain small molecules demonstrating the potential usefulness of this methodology as a discovery platform for novel therapeutic strategies [44].

The value of using patient-derived iPS cells to model disease pathogenesis was further demonstrated by researchers investigating familial dysautonomia (FD) where disease-specific phenotypic changes, including tissue-specific splicing defects due to a mutation in the IKAP gene, were observed in vitro using iPS cells derived from patients with the disorder. The generation of these cells allowed researchers to examine disease-specific defects in neurogenesis and migration of neural crest cells, tissues that heretofore were unavailable due to the early lethality associated with the disease. These cells were subsequently utilized for compound testing and phenotypic changes were somewhat alleviated by a single small-molecule candidate in a drug assay screen. Several other examples of patient-specific iPS cells and their use in pharmacologic testing and diagnostics have also been reported [65].

The relative simplicity with which patient-specific iPS cell lines can be generated also presents a lifeline to the hundreds of thousands of patients suffering from orphan diseases/disorders. The Orphan Drug Act of 1983 gives orphan status to diseases affecting less than 200,000 people in the United States. The high cost of drug development coupled with a

small market size reduced the potential for profits, thus relatively few financial resources are dedicated to finding treatments or cures for these patients. However, the ability to conduct patient-specific drug screens is currently being developed and there are substantial efforts to make the cost less than prohibitive, even for rare diseases. One such example is the neurodegenerative disorder ALS, more commonly known in the United States as Lou Gehrig's disease. This disease is characterized by a sustained loss of nerve cells that innervate muscles leading to neuropathy, weakness, and difficulty breathing. ALS is generally fatal within 3–5 years and there are few treatments to relieve symptoms. In 2008, researchers were able to differentiate motor neurons from iPS cells from an 82-year-old ALS patient [66]. While these cells may not yet be suitable for transplantation, they offer an improved research tool and may lead to the discovery of a cure for this disease that affects over 350,000 people worldwide.

Since it is virtually impossible to create patient-specific disease lines from embryos, as this would require cells removed from the embryo of that patient prior to implantation, providing iPS cell line generation for research or clinical uses is becoming a viable commercial service, even for individual consumers. The difficulty in creating disease lines from embryonic cells (except in rare cases of embryos with known genetic disorders being donated by patients undergoing IVF) has limited the available cell types that could be generated from patient samples. The commercial creation of patient-specific iPS cell lines and banks is already being undertaken by several companies in the United States and abroad, though currently these products are intended for research purposes only. In 2012, Cellular Dynamics International (CDI) (Madison, WI) and Lonza Group (Basel, Switzerland) launched services that include novel reprogramming of iPS cells from customer samples, as well as genetic engineering and differentiation of those cells into several progenitor cell types. More recently, in March 2013, the California Institute of Regenerative Medicine (CIRM) awarded CDI a $16 million grant to create and bank three iPS cell lines for each of 3000 healthy and diseased donors. This initiative intends to establish a state-of-the-art iPS cell bank in California that will be broadly accessible by researchers worldwide.

Cancer Modeling

Beyond the ability to generate cell lines that replicate disease from adult patient cells, iPS cell technologies present an extraordinary opportunity to study the progression of disease states in varying forms of cancer. Since cancer is fundamentally a genetic disease, the superiority of iPS cells as an oncologic research tool is that they do not require species extrapolation and may provide more accurate physiological responses to potential cancer therapies. Reprogramming adult cancer cells into iPS cells has so far proven a more difficult process than reprogramming other normal cell types, possibly because these cells are genetically compromised in some manner [67–70]. However, cancer patient-derived cells could provide valuable insight into processes of oncogenesis and metastasis, therefore presenting an enormous potential for researchers and investors alike.

Several studies indicate that there are significant mechanistic similarities between the process of oncogenesis and cell reprogramming [71]. Indeed, since the initial reporting of Yamanaka's four reprogramming factors, one in particular, c-Myc, has been criticized for its role as a well-documented oncogene. The manipulation of this gene during the reprogramming process negates the use of cells generated for diagnostic or clinical purposes [71, 72]. Even though certain strategies have removed c-Myc from reprogramming processes, these methods have yet to demonstrate the efficiency of c-Myc containing methods [73].

However, the other three reprogramming factors in Dr. Yamanaka's cocktail still remain viable targets for commercially exploiting the similarity of cancer and iPS cells. For example, Oct4, one of the main transcription factors required for cell reprogramming, is also overexpressed in a variety of oncopathologies, including bladder cancer [74], prostate cancer [75], primary colon cancer, ovarian carcinoma, lung adenocarcinoma, pancreatic cancer, chronic lymphocyte leukemia, and certain forms of glioblastoma, among many others [76]. As Oct4 is typically expressed in only embryonic stem cells and germ cells, this presents a good target for potential therapies.

Another reprogramming factor, Sox2, is also overexpressed in a variety of malignant cells including those associated with pancreatic cancer [77], breast cancer [78], brain tumors [79], and several carcinomas [76]. However, Sox2 is not necessarily required for successful generation of iPS cells, indicating the Sox transgene may be less vital to reprogramming or that reprogramming factors may vary from cell type to cell type [80].

Researchers examining the epigenetic differences between iPS cells, embryonic stem cells, and cancerous fibroblasts noted that the DNA methylation patterns observed varied between these cell types. Further, iPS cells had enriched or reduced methylation in CpG island shores previously identified as being cancer-specific methylation regions [81]. Another group observing similar results believe that studying these epigenetic changes will give great insight into mechanisms of gene silencing that lead to human tumorigenesis [82]. In short, the potential overlap in reprogramming processes and oncogenesis makes iPS cells a valuable tool in the battle against cancer.

Induced Pluripotent Stem Cells in Drug Discovery and Development

iPS cell technologies have enormous potential for applications in drug discovery research and development [83]. In fact, these technologies are currently being sought out and incorporated by all of the world's leading pharmaceutical corporations including Johnson & Johnson (New Brunswick, NJ), Pfizer (New York, NY), Roche (Basel, Switzerland), GlaxoSmithKline (GSK) (London, UK), Novartis (Basel, Switzerland), and Sanofi (Paris, France). This is in large part due to the potential of this technology to generate substantial savings in drug development costs by making the discovery process more efficient and reliable. Considering the cost to bring a single drug candidate to market is currently estimated at $1.2–1.7 billion, it is understandably a high priority of this industry to find more cost-effective strategies [84]. Embracing iPS cell technologies will help diversify global pharmaceutical product portfolios and drive more personalized approaches to medicine [58]. In this section, we will describe how this technology can be exploited for drug discovery research purposes and examine how using iPS cells in drug discovery has the potential to generate billions of dollars in revenue over the next decade. We will also use current products to illustrate this potential and identify emerging leaders in this valuable market segment.

iPS Cell Technology in Lead Generation

iPS cell screening platforms can be developed to identify small molecules, biologics, and other modulators that are efficacious in reverting disease-related phenotypes to that of healthy controls. Immediately, these cells have the potential to reduce development time and cost, which is a major incentive to big pharmaceutical companies who must prioritize their resources. iPS cell-based platforms have the ability to increase our understanding of disease progression as well as the fundamental biology of many cell types [49]. In addition, iPS cell screening libraries would give researchers the opportunity to observe efficacy in a large and varied preclinical human patient set.

The utility of stem cells in early drug discovery was recently illustrated in a publication by researchers at Sanford-Burnham Medical Research Institute in San Diego, CA. By chance, a high-throughput screen to examine the effects of thousands of small molecules on mouse embryonic stem cells resulted in the discovery of a chemical compound, ITD-1, that efficiently differentiates stem cells into cardiomyocytes [85]. This compound works by blocking transforming growth factor-beta (TGF-β), one of the key factors regulating cellular proliferation and differentiation. Results obtained in early studies suggest that ITD-1 may be useful for reducing scarring observed in tissues after heart failure and promoting the formation of new heart muscle. This group intends to further capitalize on this discovery by examining ITD-1 as a potential therapeutic drug for cardiovascular disease and recently partnered with ChemRegen, Inc. (San Diego, CA), a biotechnology company that specializes in developing small-molecule regenerative medicine strategies for the treatment of heart disease and cancer.

The ability to more cost-effectively screen human cells earlier in the discovery process will allow researchers and companies to better focus their time and resources. Panels of stem or adult cells that represent population variations will help better predict the safety of a drug candidate for global distribution. In conclusion, iPS cells have the potential to greatly reduce the time from hit to lead through more faithful and cost-effective in vitro recapitulation of human health and disease.

iPS Cell Technology in Toxicity Studies

The commercial properties of iPS cells provide for a range of novel applications in predictive toxicology, which is increasingly important as costs for the entire drug discovery process continue to escalate [84]. Currently, the success of drug development during early preclinical phases relies almost entirely on animal models to establish safety and efficacy [86]. Since these models are not genetically identical, they are incomplete representations of human disease pathophysiology and have frequently proven inefficient for this use. The high failure rate (estimated at >90 %) of new drug candidates that enter later clinical phases due to safety issues or poor efficacy demonstrates the inefficiency of this process and is a very expensive problem for the pharmaceutical industry [87]. However, iPS cells are poised to revolutionize the way toxicity is assessed in vitro as a more diverse collection of human iPS cell lines become available to researchers.

Hundreds of millions of dollars could be saved if iPS technologies are able to render more efficient and accurate elimination of toxic compounds earlier in the discovery process, but this potential can only be realized if these processes are reliable. Accuracy is extremely vital for predictive toxicology as failure in this respect can be not only expensive but also lethal. In 1957, a German pharmaceutical company began marketing the drug thalidomide as a "safe sedative" for the treatment of cough, cold, flu, and the nausea/vomiting associated with pregnancy. Clinical trials commenced in the United States two years later, but thalidomide had already earned approval in over 20 European countries, Canada, and Africa. By 1961, serious concerns about peripheral nerve damage and birth defects began to rise in Europe. It was the tenacity of one Medical Officer at the FDA, Dr. Frances

Oldham Kelsey, that led to halting these trials and thalidomide's eventual removal from the global market because of serious side effects. It is unknown how many patients received the drug during its lifetime worldwide, but it is estimated that more than 10,000 children in 46 countries were born with birth defects due to the teratogenic effects of thalidomide [88]. Indeed, following the thalidomide tragedy, Congress passed the Kefauver-Harris Amendments that require all drug components be proven safe and efficacious before marketing and further compel distributors to disclose accurate information about potential side effects. Perhaps most importantly, it strengthened the authority of the US FDA by establishing a precedent for an approval process for new drugs.

While teratogenic effects are of grave concern, the most common mechanisms for drug toxicity occur in the heart and the liver. Unsurprisingly, hepatotoxicity and cardiotoxicity are also the two major reasons why potential drug candidates fail during the discovery process. However, obtaining and culturing functional human liver and heart cells in vitro can be a prohibitively expensive undertaking. For commercial purposes, iPS cell technologies that faithfully recapitulate these cells represent an extraordinarily valuable market segment, not in small part because the primary customer base comprises multibillion dollar pharmaceutical companies examining millions of drug candidates every year. For these reasons, we will dedicate some time to discussing these two cell types in greater detail.

iPS Cell-Derived Hepatocytes

The majority of drug compounds are metabolized by the liver through numerous enzymatic pathways which makes it vital to screen drug candidates for toxicity in fully functional human hepatocytes. The current "gold standard" accepted by the US FDA requires evaluating inhibition or induction of several cytochrome P450 (CYP) metabolizing enzymes as well as examining effects on uridine diphosphate glucuronosyl transferases and other various hepatic transporters (FDA Guidance for Industry: Drug Interaction Studies, February 2012). There are 57 CYP isoforms in total, though three in particular (CYP1A2, CYP2B6, and CYP3A4) are responsible for most drug interactions. Because these cells are highly functional and a poor interaction with even one CYP can fail a drug candidate, there are rigorous standards for the source and quality of hepatocytes used in toxicity studies.

Currently only one product on the market satisfies these stringent requirements—primary human hepatocyte cells derived from healthy individuals. These cells are extremely fragile in culture, expensive because of their source, and require extensive analyses to qualify them for use in toxicity studies. Thus, researchers often implement an intricate sequence of biochemical evaluations to analyze toxicity prior to examining hepatic enzyme inhibition using in vivo rat models and in vitro human primary cells. For drug developers, this translates into a prolonged and expensive process that, in most instances, still fails to guarantee that a drug candidate will make it through the entire approval process. For entrepreneurs in the iPS cell market, this represents an extraordinary opportunity to revolutionize the manner in which the global pharmaceutical industry evaluates toxicity.

The year 2012 saw the launch of several iPS cell-derived hepatocyte products for use in in vitro drug discovery research. ReproCell (Yokohama, Japan) launched both frozen human iPS cell-derived hepatocyte cells and a CYP assay service that examines enzymatic function in the presence of drug compounds at their facility. Two other companies, Cellectis (Gothenburg, Sweden) and CDI (Madison, WI) have also launched frozen vial products containing human iPS cell-derived hepatocytes. With a consumer base that includes billion dollar pharmaceutical companies, it is easy to understand the enthusiasm market players are bringing to this particular area.

Since generating mature cell types from stem cells is still an imprecise science, creating functional adult cells as complex in nature as hepatocytes is an impressive achievement [89]. However, these products have yet to be fully validated against the FDA "gold standard" primary hepatocytes in a large compound screen.

Cardiomyocytes

The second most common reason a drug candidate fails is because of unforeseen cardiotoxic effects. Cardiotoxicity occurs when a drug impairs the ability of the heart muscle to pump blood normally. Substantial efforts are made during the discovery process to elicit this potential, yet current models remain inadequate to identify and measure the full spectrum of human cardiac responses. The complexity of cardiac physiology derives from dual innervation of the heart by the sympathetic and parasympathetic nervous systems. This results in a complex signaling cascade involving multiple types of cell receptors within and between cardiac cells.

Evaluating cardiac responses in vitro often produces an incomplete picture of how a drug affects an entire beating human heart. Evaluating the cardiotoxic effects of a drug compound involves reporter assays and electrophysiology performed on cardiomyocytes obtained from primary rodent and human sources, as well as transgenic models both in vitro and in vivo. Because the number and type of cardiac cell receptors may vary slightly between species, animal in vivo studies often fail to accurately predict human responses. Research methods to examine cardiac responses are discussed in an excellent review by Dr. Ralf Kettennhofen published in 2008 [90]. There are several studies demonstrating the effectiveness of iPS cell-derived cardiomyocytes in generating accurate electrophysiological data and responses to several cardiac and noncardiac drugs [91–93]. A leader in

this field is ReproCell (Yokohama, Japan) who launched the QTempo™ in vitro cardiotoxicity assay service in 2008. This service includes a comprehensive analysis of cardiac ion channels using human iPS-derived cardiomyocyte clusters that beat rhythmically in culture. The following year, the company launched a frozen cardiomyocyte product, ReproCardio™, for direct use by researchers. In 2009, CDI (Madison, WI) launched iCardiomyocytes™ frozen cell product intended for use in toxicity screening.

The reduced cost of examining cardiotoxicity and hepatotoxicity would potentially allow pharmaceutical companies, who currently possess millions of compounds, to significantly reduce their overall number of primary screening candidates by first eliminating all toxic compounds from these expansive libraries. This approach is currently economically unfeasible due to the aforementioned high cost of toxicity studies in general, but the long-term fiscal benefits of compiling such data would be significant. Regardless, iPS cell technologies have the potential to revolutionize in vitro toxicological studies.

Use of iPS Cells for Assessing Efficacy

Making more accurate determinations of drug efficacy in humans during preclinical development is another method for rendering the drug discovery process more cost-effective. In 2001, over 30 % of drug candidates that entered clinical trials were later abandoned because of poor efficacy data, even though preclinical studies demonstrated alleviations of disease symptoms in relevant animal models [94]. This highlights the importance of using human cells for determining efficacy for all potential drug candidates, as genetic variations resulting from species differences require extrapolation and may confound experimental results [89].

The utility of iPS cells for evaluating the efficacy of drug candidates can be demonstrated by the results of a recent clinical trial for a new treatment for ALS. ALS affects motor neurons in the brain and spinal cord to cause progressive muscle atrophy that subsequently leads to deficits in mobility and respiratory function resulting in death. The drug candidate being tested was found to be highly efficacious in animal models of ALS, yet the beneficial results observed were not reproduced in humans and yielded poor efficacy data [95]. While unfortunate, it is often the case that impressive preclinical animal results cannot be replicated in humans resulting in the loss of hundreds of millions of dollars and years of research wasted. These researchers may have benefitted immensely from screening compounds against patient-derived iPS cells from ALS patients prior to performing a clinical trial in humans.

Current preclinical testing strategies are also inadequate to measure the variability of individual patient responses to drug candidates and this presents a substantial problem for developing new treatments in a global marketplace [89]. iPS cells allow for the creation of cell screening libraries that can be tailored for a specific disease state or individual in context of their unique genetic identity [58]. This personalized approach to medicine is not economically feasible yet, but there are already services on the market to develop iPS cells and derive various cell types from customer samples, such as MyCell™ Services from CDI (Madison, WI) and Lonza Group's (Basel, Switzerland) Pluripotent Stem Cell Services.

Therapeutic Applications of Induced Pluripotent Stem Cells

iPS cells hold great promise for regenerative medicine because they can potentially provide a renewable source of cells without the risk of immune rejection and associated immunosuppression regimes necessary with allogeneic therapies. While there is some evidence that iPS cells may cause some degree of immune rejection, it is not expected to be as severe as compared to allogeneic methods and production strategies could be optimized as the field progresses to reduce immune response. Recently, there is concern that genetic and epigenetic abnormalities within iPS cells could result in immunogenicity of differentiated cells [96–98]. The oncogenic potential of induced pluripotency is another major concern that needs to be addressed before clinical application. Therefore, reprogramming techniques need further optimization to minimize or eliminate genetic and epigenetic abnormalities associated with iPS cells before they are ready for clinical application [99, 100].

iPS cells perhaps will provide the most utility in drug discovery and development because they will provide a window into diseases with a known or suspected genetic link that is not available through other methods. They also hold great potential for true personalized, regenerative medicine. Unlike adult stem cells which are multipotent and limited in their proliferation and differentiation potential, virtually any cell type can be differentiated from iPS cells, thereby opening the door to the treatment of a multitude of diseases or injury states. While there are some major challenges to overcome, they are not insurmountable, and given the fast pace of the field, it is likely only a matter of time before clinical translation of iPS cell-based therapeutics is brought to fruition.

Clinical Applications

iPS cells can be differentiated into most cell types and have the exciting potential to be applied to an unlimited number of disease and injured states. iPS cell-based products are largely

expected to restore function or ameliorate disease by cell replacement or by the secretion of factors that support the endogenous environment by either activating or altering the behavior of surrounding cells. In addition, there is the potential to correct genetic defects in the patient's cell line via gene manipulation, combing both cell and gene therapy in one product.

Some of the earliest therapeutic targets include CNS disease and injury states such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), Huntington's disease (HD), traumatic brain injury (TBI), stroke, spinal cord injury (SCI), ALS, SMA, age-related macular degeneration (AMD), and epilepsy. Other targets include endocrine disorders, such as diabetes, and skin ailments, such as burns and would healing. While there are currently no clinical trials using iPS cell-based products, Phase I clinical trials with hESC-derived products have commenced. Similar to allogeneic adult stem cell therapies, a key challenge in using allogeneic hESC-derived therapies is the requirement for immunosuppression. iPS cells therefore hold great hope for personalized medicine as an autologous cell line could be produced for each intended patient, obviating the need for chronic immunosuppression. Additional research is currently ongoing to address potential safety concerns unique to the clinical application of iPS cells such as potential for mutations due to genomic insertion, risk of tumor formation due to upregulation of oncogenes such as c-Myc, and inactivation of tumor suppressor gene p-53, as well as the potential for incomplete genetic reprogramming.

Because hESCs and iPS cells are both pluripotent, we will briefly cover hESC-based products that have entered the clinic, as the same approach could be taken using patient-specific iPS cells. To date, three clinical trials have been initiated in the United States, and one in the UK, using hESC-based products. The first ever approved product for clinical trials was GRNOPC1, composed of hESC-derived oligodendrocyte progenitor cells, for the treatment of acute SCI, developed by Geron Corporation (Menlo Park, CA). Oligodendrocytes are a type of cell that support and insulate axons in the CNS. Geron initially received FDA approval in January 2009 to commence clinical trials; however, the trial was put on hold shortly thereafter to address issues in manufacturing processes with subsequent re-approval in June 2010. The Phase I study was open to patients with a neurologically complete (ASIA Impairment Scale A) traumatic SCI to the thoracic region between T3 and T11. GRNOPC1 was administered once to patients between 7 and 14 days after the injury. Unfortunately, Geron decided to pull the plug on its Phase I study in November 2011, citing financial constraints as the reason [101].

Advanced Cell Technology, Inc. (ACT) of Marlborough, MA, was the second company to receive FDA approval to commence clinical trials using an hESC-derived product. In November 2010 the company FDA received approval to initiate a Phase I/II multicenter clinical trial using retinal pigment epithelium (RPE) cells derived from hESCs to treat patients with Stargardt's (juvenile) macular dystrophy (SMD), one of the most common forms of juvenile macular degeneration. Their product, MA09-hRPE, consists of PSC-derived RPE cells that are injected into subretinal space [102–104]. Soon after in January 2011, they received approval to initiate a Phase I/II multicenter clinical trial to treat patients with dry age-related macular degeneration (AMD), the most common form of macular degeneration in the world. In September 2011, ACT received approval to initiate a Phase I/II trial in the UK for the treatment of SMD—this marked the first ever European approval for a clinical trial using an hESC-derived product. While these clinical studies will only enroll a small number of patients (10–12 per study), and only a limited number of patients have received the investigational PSC-based products, there have been no serious adverse events reported thus far. Indeed, there are early signs of efficacy reported in two patients with Stargardt's disease, demonstrating improved vision following treatment [5].

The hESC-based therapeutic products previously described all require immunosuppression as part of their clinical protocols. While suppressing the immune system is desirable to prevent rejection of cell product, immunosuppressive drugs carry many untoward side effects. They render patients more susceptible to opportunistic infections and cancers and can cause dangerous side effects such as kidney or liver damage. RPEs and OPCs have been successfully generated from iPS cells [105–107], suggesting that the same clinical approach can be taken using iPS cells. The major advantage of using iPS cells over hESCs is the ability to create patient-specific therapeutic products, thereby potentially eliminating the need for immunosuppression and minimizing the risks of immune rejection. Promising research is currently underway to develop PSC-derived treatments for other diseases as well, such as diabetes, SMA, ALS, and sickle-cell disease.

Commercial Potential of iPS Cell-Based Therapeutics

With increased federal funding, a more supportive political environment for stem cell research, and some early signs of clinical successes, investor interest in regenerative medicine has been gaining momentum. While there was significant excitement around hESCs when they were first discovered, advancements in hESC research got off to a slow start due to the political and ethical controversy that surrounds its source material, the embryo, subsequent restriction and then easing of federal funding, and court proceedings (briefly discussed

Table 3 Publicly listed stem cell companies

Symbol	Company name	Market cap (in US dollars)	Share price, % change YTD
ACTC	Advanced Cell Technology, Inc.	134.33	−25
ASTM	Aastrom Biosciences, Inc.	56.48	29
ATHX	Athersys, Inc.	56.24	6
BTX	BioTime, Inc.	176.49	−2
CUR	NeuralStem, Inc.	84.99	30
CYTX	Cytori Therapeutics, Inc.	246.50	110
ISCO	International Stem Cell Corporation	15.82	−18
KOOL	Thermogenesis Corporation	11.57	–
MEDS	Medistem, Inc.	6.15	–
MSB.AX	MesoBlast Limited	1,770	−14
NBS	NeoStem, Inc.	100.43	24
OPXA	Opexa Therapeutics, Inc.	9.91	–
OSIR	Osiris Therapeutics, Inc.	315.60	79
PSTI	Pluristem Therapeutics, Inc.	192.87	68
STEM	StemCells, Inc.	67.39	118
Total market cap: $3.244 billion			
Average increase in stock price YTD: 27 %			

Source: Regenerative Medicine Strategy Group, LLC

in section "The Ethics of iPS Cell Technologies") which have only recently been settled. Even though iPS cells are not plagued by the same controversy that surrounds hESC research, the protracted legal battles served to deter big pharma and venture capitalists from entering the fray.

Federal funding for all types of stem cell research has increased under President Obama, despite ongoing litigation. In addition, several states jumped into the fold to fill the gap left by traditional venture capital investors. Most notably, the California Institute of Regenerative Medicine (CIRM), established in 2004 to provide $3 billion in funding to California institutions carrying out stem cell research, dedicated towards helping promising projects move from bench to bedside. In addition, CIRM started a Strategic Partnership Awards Initiative in order to attract industry engagement in stem cell research, with biotech companies already receiving funding. Viacyte Inc. of San Diego, CA, recently received a $10.1 million Strategic Partnership Award for the development of an hESC-based treatment for insulin-dependent diabetes, which is in addition to a $20 million Disease Team grant the company was previously awarded. Bluebird Bio Inc. was awarded $9.3 million to fund a Phase I/II study of LentiGlobin for the treatment of the inherited beta-thalassemia, expected to start in 2013. Stem Cells Inc. has also received a $20 million award to fund preclinical development of its neural stem cell product for the treatment of Alzheimer's, which is in addition to another $20 million for its SCI program [108, 109].

There is growing investor confidence in the stem cell field, as evidenced by the increase in stock price for publicly listed stem cell companies. The average increase in share price in the year to date for the 15 publicly listed stem cell companies was 27 % with a total market cap of $3.24 billon, see Table 3. While most of the companies are adult stem cell companies, the increase is expected to positively impact investor perception of stem cell research across the board. This boost has been aided by promising preclinical data, positive results in early clinical trials, and the recent approval in Canada of the world's first manufactured stem cell product, Prochymal, an allogeneic stem cell treatment for GVHD in children, by Osiris Therapeutics Inc. This approval led to an almost doubling of the company's stock price [108]. Excitement was also spurred when positive results were published in the Lancet showing that two patients with Stargardt's disease, an inherited form of macular degeneration, had improved vision following treatment with ACT's hESC-derived RPE cells [5]. Early data from StemCells Inc. also suggests that patients had regained function following stem cell treatment, in this case patients with SCI regained sensation below the level of injury. Positive data from this and another trial, along the awarding of significant grants from CIRM, has helped the shares of StemCells Inc. rise 118 % in the year to date. While these positive results were only reported in a limited number of patients due to the small enrollment numbers in early phase clinical trials, they are nonetheless very encouraging and help increase confidence in the therapeutic utility of stem cell therapeutics and their commercial potential.

Adult stem cell technologies have already proven to be a commercial success with the sales of allograft-derived stem cell products or systems used to concentrate stem cells estimated to have been $139 million in 2011. These include products derived from cadaveric or living donors and cover a wide range of indications from orthopedic to cardiovascular

to anti-inflammatory to nerve repair, with orthopedic indications making up the largest component. While there are no FDA-approved stem cell treatments, Prochymal by Osiris has completed Phase III studies and has been approved in Canada for the treatment of GVHD in children. The market for stem cell therapeutics is expected to experience significant growth over the next decade, with estimated sales of $6 billion by 2020 as more products move further along the development pathway [110].

Regulation

A major challenge in the development of iPS cell-based therapeutics is navigating the regulatory process. As a new and unproven technology, the regulatory requirements for demonstrating safety of IPS cell-based therapeutics will be high and potentially very costly, particularly for the first companies out of the gate, as was the case for Geron Corporation with its first-in-human hESC-based therapy. While there has been rapid growth in the adult stem cell sector, this is due in large part to differences in the regulatory classification of adult stem cell products vs. pluripotent stem cell products. Many autologous adult stem cell treatments are classified as human cells, tissues, and cellular and tissue-based products (HCT/Ps) and are regulated solely under section 361 of the Public Health Service Act (PHS Act) and do not require FDA approval. Establishments that provide products regulated under section 361 must follow good tissue practices for HCT/Ps, perform donor screening and testing, have procedures in place to prevent spread of communicable disease, and maintain proper records. While they are required to register and list their products with the FDA, the products are not considered drugs and therefore do not require a drug application or the conduct of clinical trials prior to use. There are several criteria, as defined by 21 CFR 1271.10, that must be met in order for a product to be eligible for regulation solely under section 361, the most important of which is that the product be minimally manipulated. Due to these diminished regulatory barriers to establishing autologous adult stem cell clinics, there has been a flurry of activity in this sector, with states like Texas quickly taking center stage [111]. However, the efficacy of many of these autologous stem cell treatments is up for debate as blinded clinical studies are rarely conducted. Further, the exact definition of "minimally manipulated" has been called into question with the FDA stepping in to regulate adult stem cell clinics in cases where it feels that product offerings are more than minimally manipulated and therefore should be treated as drugs. A court ruling in July 2012 reaffirmed the FDA's right to regulate such stem cell therapies [112]. This ruling has several implications for the adult stem cell field because drugs require extensive clinical studies and regulatory approval prior to being marketed to patients. This would result in a much longer and costlier development process, making the establishment of such clinics more cost prohibitive and perhaps driving some overseas.

iPS cell-based therapeutics require extensive manipulation due to derivation procedures and prolonged duration in culture and therefore require strict regulatory oversight. An investigational new drug (IND) application must be submitted to the FDA prior to the start of clinical trials. iPS cell products are classified as biologics and require the approval of the FDA's Center for Biologics Evaluation and Research (CBER) and its office of Cellular, Tissue and Gene Therapies (OCTGT). If the cell product will be used along with a medical device (e.g., matrix and encapsulation device) or the additional of a drug (e.g., growth factors), it would be considered a combination product and therefore also be reviewed by the Center for Devices and Radiological Health (CDRH) and the Center for Drug Evaluation and Research (CDER), respectively. In addition, if gene transfer via vector delivery is used in reprogramming, iPSCs could also be considered a gene therapy product and subject to review by the NIH Office of Biotechnology Activities program on Recombinant DNA (RAC).

Challenges for Commercializing iPS Cell Technologies

While rapid progress has been made during the 7 years since iPS cells were first discovered, specific recent advancements in nuclear reprogramming have brought bioengineered cells closer than ever to the safety and efficacy benchmarks necessary for clinical iPS applications. However, there remain distinct issues within the technology as well as obstacles presented by the surrounding regulatory and legal environments that require addressing before the commercial potential of iPS cells can be fully realized. In this section, we will discuss the challenges stem cell researchers and entrepreneurs must confront and present modern approaches to overcome these issues.

Safety Issues

As previously discussed, the ability to generate patient-derived iPS cells would enable clinicians to generate autologous cell therapies for a diverse range of medical applications. Since the risk of immune rejection for autologous cell therapies is generally lower than for non-autologous cells, this may overcome some of the immunological concerns that accompany any transplant therapy. (Immunological considerations concerning stem cell transplantation are covered in more detail in another chapter of this textbook.) However, when

considering clinical application of iPS cells, we must examine the cell manipulations required during the reprogramming process and all subsequent cell differentiation procedures. Even though cells are autologous, the methods used to manipulate them in culture remain largely uncharacterized for use in clinical settings and may present additional safety risks.

Early efforts to generate iPS cells incorporated genome-integrating retroviruses and lentiviruses, which by their nature present the potential for uncharacterized insertional mutations, thus rendering cells unsuitable for diagnostic and clinical uses [113]. Additionally, iPS cells created by viral methods could pose substantial clinical safety risks resulting from the potential for erratic reactivation of viral transgenes that would substantially increase the potential of cells transforming and becoming oncogenic to the patient [15, 73, 114]. Researchers have also observed that the reprogramming process itself may pose potential safety hazards [90].

Adult cells derived from iPS cell progenitors injected into immunocompromised mice resulted in tumors in some animals soon after transplantation [15]. If the injected preparations were truly free of stem cells, this provides evidence that the genetic integration that occurs during the reprogramming process may itself be oncogenic [114]. Two well-established recombination systems known for their ability to efficiently modify the genetic expression of mammalian cells, the piggy-bac transposon [115, 116] and the Cre/loxP system [16], were also successfully utilized to generate iPS cell lines. However, the use of these systems for generating clinically relevant cells remains uncharacterized.

The potential use of iPS cells for cellular replacement has recently benefited from the work of several groups dedicated to defining strategies for creating clinically relevant, non-oncogenic cell lines using non-integrating reprogramming methods. In 2008, an adenoviral approach was successfully implemented to reprogram mouse fibroblasts and liver cells into iPS cells [117]. The following year, alternative methods were developed using episomal vectors [118] and recombinant proteins, clearly demonstrating that random insertional mutagenesis is not required for successful somatic cell reprogramming [57, 119]. Most recently, researchers have described a process using zinc-finger nucleases to reprogram adult somatic cells into iPS cell lines [120].

Intrinsic variability appears to occur regardless of the reprogramming method [119, 121, 122]. There is additional evidence supporting the necessity for qualification of various adult cell sources as iPS cells derived from mouse tail-tip fibroblasts showed a much higher oncogenic propensity than similarly derived cells obtained from mouse embryonic fibroblasts and gastric epithelial cells [123]. Finally, there are strong correlations reported between gene expression signatures and specific laboratory facilities, suggesting that small differences in in vitro microenvironments have the potential to affect the overall genetic quality of stem cells [124]. For this reason, life science companies have committed substantial resources to developing standardized, component-defined products that will make cell culture conditions and downstream processing more consistent.

There is still much work to be done to validate the clinical use of iPS cells including determining methods to minimize abnormal expression of imprinted genes [59] that may result from various methods employed during the reprogramming process [125]. Since efficiently and reproducibly generating clinically relevant target cell populations is a primary goal of the iPS cell field, there is an immediate market for products that provide innovative solutions to this issue. Accordingly, academic researchers and industry alike are racing to define strategies for reliable and efficient creation of iPS cell lines using alternative methods. Yet, the effectiveness of various reprogramming strategies developed in the short time since iPS cells were discovered suggests that the modern challenge for reprogramming iPS cells is no longer how to make this process free of oncogenic elements, but rather how to make this process more efficient and reliable [58].

Establishment of Efficacy

Beyond establishing methods for creating clinically relevant iPS cell lines, the functionality and safety of products derived from these cells need to be characterized. In congruence with the FDA approval process, the efficacy of these cells in creating a functional benefit for patients must clearly be demonstrated prior to marketing that therapy for any particular clinical indication. For researchers, this entails demonstrating the functional equivalence of these cells to their in vivo counterparts and proving that placing these cells in the body alleviates symptoms caused by a defined disease or disorder, whether directly or indirectly.

While some degree of success has been achieved for stem cell replacement therapies in the laboratory and the clinic [6–8], there is also evidence supporting the need for long-term studies of this methodology, as only a tiny minority of transplanted cells appears to survive in multiple studies using various cell types. For example, in a primate model of Parkinson's disease, transplanted neuronal cells showed only 1 % survival after 14 weeks [126]. Though these cells were differentiated from hESCs, not iPS cells, there is currently little evidence to suggest this would provide any survival benefit to transplanted cells.

As previously described in this chapter, iPS cell therapies may function not only through cell replacement strategies but also through mechanisms of cell nursing or effects on the endogenous environment mediated by the secretion of various factors. For this reason, establishment of efficacy may prove more difficult as such mechanisms of action are often subtle.

Manufacturing Process Standardization

Large-scale manufacturing systems for the bioproduction of human cells have existed for decades. Yet, there are several important obstacles that keep engineers from standardizing and scaling manufacturing processes in a manner such that large quantities of stem cells or their derivatives can be produced. While modern efforts to use bioreactors for creating industrial-scale batches of stem cells are described in detail in another section of this textbook, it is important to remember the production of cell products is not limited to scaling up cell expansions. It requires a long process of cell manipulations and evaluations to generate quality cell products and many of these processes are not suited to high-throughput strategies necessary for large-scale production.

For example, current protocols for determining the potency of stem cells are time-consuming, expensive, and rely on consistent availability and quality of products such as antibodies to provide accurate results. For various reasons, it is often necessary to use several different antibodies to verify cell identity and this process must be repeated for each lot of cells produced. A more extensive qualification process, first established in Dr. Yamanka's laboratory, includes teratoma formation, differentiation of stem cells into three embryonic germ layers, confirmation of Oct4 transcription factor expression, and formation of embryoid bodies in in vitro culture [1]. Though these may be the best standards for defining the overall "quality" of stem cells, the time required would cripple most development processes.

Current differentiation methods are also insufficient for creating derived cell populations with 100 % purity without incorporating time-consuming sorting processes such as fluorescence-activated cell sorting, magnetic-based sorting [86], or high-content analysis [127, 128]. The time required for these processes renders them unfeasible for large-scale production methods and these methods are also dependent on the availability of specialized biomarker products. Additionally, researchers often have difficulty observing disease phenotypes in iPS cell lines derived from disease patients.

The rigorous standards that must be met when manufacturing cell products for diagnostic or transplant purposes require high levels of purity. Because many features of this process could be described as an "art," there are substantial challenges in completing the approval process and subsequent technology transfer that must occur between researchers and manufacturing facilities. Thus, standardizing culture procedures and manufacturing paradigms is key to bringing nearly all iPS cell technologies to the marketplace.

Intellectual Property and iPS Cell Technology Licensing

The immense potential of iPS cell technologies and their subsequent usefulness in a wide variety of research schemes makes them an extremely valuable commodity in today's marketplace. Therefore, it is of particular importance for researchers and entrepreneurs to protect their inventions and organizations across the globe are racing to secure rights to use and distribute these technologies. In a nascent environment where smaller iPS cell companies frequently demonstrate a more refined expertise in specific technologies, even the largest organizations appear willing to partner. Thus, it is important to establish scientific ownership of iPS cell technologies as early as possible in the discovery process.

iPS Academia Japan was established to manage the patents and technology rising from Dr. Yamanaka's discovery of iPS cells at Kyoto University after the institution was granted a patent for iPS cells by the Japan Patent Office in 2008 [129]. Since its founding, numerous institutions and companies have applied for licenses to use this technology. In 2011, iPierian (San Francisco, CA) licensed this technology and in 2012, CDI signed a new agreement to use Yamanaka's technology and cell lines.

Intellectual property laws vary globally, especially with regard to the applicability of patents to stem cell technologies, making establishing ownership tedious and difficult in some cases. For example, in 2011, the European Parliament courts banned patents on stem cell products citing an "unethical industrial" use of stem cells after a German researcher tried to patent a method of turning hESCs into neurons [130]. This placed the UK and Europe in a precarious situation with regard to commercializing any stem cell technology, including iPS cells, and was considered "a blow to years of effort to derive biomedical applications from embryonic stem cells" by leaders in the field [131].

Areas for Commercial Opportunity

We previously described the potential that iPS cells have to revolutionize the drug discovery process and generate a diverse range of cell therapies. Now, we will examine how these features translate into commercial opportunities. Frequently, the successful translation of this technology into commercial products requires a spectrum of expertise including basic life scientists, clinicians, and engineers. Thus, the commercial market for these technologies encompasses an extensive array of products and services targeted at a diverse audience.

Defining simple and cost-effective strategies to manipulate iPS cells is a valuable goal to both the stem cell field and the life sciences industry at large. While many challenges still need to be addressed, each potential solution represents a commercial opportunity within this sector. From relatively simple chemical compounds, such as growth factors, to highly evolved services including genetic cellular analyses and iPS cell reprogramming, global commercial opportunities are expanding.

In this section, we will consider global infrastructures that support stem cell markets and discuss examples of

international collaboration within the stem cell community. To illustrate the growing commercial potential of iPS cell technologies, we will examine major iPS cell technology market segments and provide innovative examples of products and services that have helped support ongoing research and clinical development investments.

Global Market Infrastructure

The scale at which stem cell research is conducted largely depends on the infrastructure and funding available from government sources. In the last decade, substantial initiatives have led to the creation of numerous regenerative medicine research centers focused on the translation of stem cells into commercial and clinical applications across the globe. While iPS cells may pose fewer ethical and legal concerns, the support systems and funding that allow this research are closely tied to government policies concerning all stem cells.

In the United Sates, federal stem cell research funding has been enhanced by many state initiatives. In 2006, the CIRM was created to oversee grants and loans for stem cell research totaling $3 billion over 10 years. As of July 2012, 528 awards totaling over $1.5 billion had been dispersed to institutions throughout California (CIRM Website). Other states, including Connecticut, Illinois, New York, and Texas, have made similar investments in regenerative medicine research by establishing state agencies and educational institutions to support stem cell research efforts within their state.

Europe, most especially the UK, has also demonstrated a commitment to the development of regenerative medicine research and education. In 2008, the Oxford Stem Cell Institute (OSCI) was established to combine the expertise of basic researchers and clinical professionals. Now encompassing over 40 research groups, the success of OSCI has encouraged similar initiatives within the UK. In 2012, the University of Cambridge and Harvard University announced the creation of a new $12.5 million stem cell research center underwritten by the UK Medical Research Council and Wellcome Trust. In Scotland, the Scottish Centre for Regenerative Medicine and other foundations providing business and research expertise in addition to financial support, such as Scottish Enterprise, Scotland Stem Cell Network, and the UK Stem Cell Foundation, help stimulate a vibrant regenerative medicine research environment. Additional funding through EuroStemCell partnerships by the European Commission's Seventh Framework Programme serves as a prime example of government/private partnerships working to progress regenerative medicine research throughout Europe.

In Asia, where policies on stem cells are generally permissive towards therapeutic research, Yamanaka's discovery of iPS cells immediately elevated Japan to a prominent position in the field and grew financial support in the region. In Singapore, public and private funding currently supports over 40 regenerative medicine research groups, led by investigators from all over the world. Large investments in manufacturing may help strengthen Singapore's position as a potential major exporter of iPS cell technologies and goods in the next decade.

One of the largest publishers of peer-reviewed stem cell research in the scientific literature is currently China. With a population of over one billion, the Chinese market represents one of the largest potential consumer bases for stem cell therapeutics. However, in December 2011, the Chinese Ministry of Health tightened regulations on all stem cell research by announcing that companies must register clinical activities involving stem cells and subsequently closed down several operations allegedly performing unapproved testing.

Other countries have also recognized the potential of regenerative medicine research and are working to establish a presence in the global stem cell market. In 2007, the Canadian Institutes of Health Research released Guidelines for Human Pluripotent Stem Cell Research outlining a framework for the uses of stem cells in Canada. This country hosts several successful stem cell companies, including Stem Cell Technologies (Vancouver, Canada), producers of popular stem cell culture medias. In Israel, several clinical trials using stem cells have commenced in recent years. In India, a flourishing industry exists for stem cell banking even after the Council for Medical Research banned these banks from being used for reproductive purposes in 2007. It is a policy shared by many countries, including Belgium, Switzerland, and South Africa, to allow and support the use of stem cells for therapeutic purposes, but ban their use for reproductive purposes.

The global infrastructure that supports all stem cell technologies is growing. In places where government policies regarding use of embryonic stem cells once prevented researchers from entering the field of regenerative medicine, iPS cells are finding new and enthusiastic audiences. Out of the 293 researchers who identify iPS cell technologies as a primary focus in their work, almost 40 % were outside the United States [9]. With an expanding global consumer base that includes individual academic researchers *and* large pharmaceutical companies, there are growing opportunities in this market for a range of international contributors.

Research Tools Market

The global market for iPS cell research products is expected to exceed $1 billion annually by 2015. Growing global infrastructure and increased adoption of iPS cell technologies in existing and new markets substantiate a continued increase in market value in the upcoming decade. Over 293 principal investigators now identify iPS cell research as a core focus of

their laboratory [9]. Further, a large number of these researchers are working on new applications for drug discovery and clinical development research purposes.

The tools and services that comprise the iPS cell research product space can generally be broken down into several market segments, each of which encompassing its own spectrum of products. Each segment described below commands a fair share of the market, with sectors focused on production, reprogramming, and differentiation of cells taking a slightly larger market share than those offering analytical or engineering services [132].

Cells-Based Tools

iPS cell lines and products, associated with the culture, maintenance, reprogramming, and derivation of these lines, command a majority share of the iPS cell research market space. The ability to generate a variety of functional cells, including cardiomyocytes, hepatocytes, and neurons for predictive toxicology, is an extremely useful and valuable aspect of iPS cell research technologies. Substantial commercial opportunities are created by the ability of these cells to generate nearly limitless amounts of stem cells and stem cell-derived products [133].

The requirement for every new drug candidate to have proven efficacy for each use indicated on the product label compels a substantial market containing a wide variety of human cell types and associated products. For example, CDI (Madison, WI) launched their iCell™ endothelial cell products as a tool for researchers to evaluate drug candidates in the fields of angiogenesis, atherosclerosis, inflammation, and wound healing. This is an extremely large market with potential mega-customers like the US Department of Defense, who recently signed a $200 million deal with Osiris Therapeutics Inc. (Columbia, MD) for a stem cell-related treatment for radiation poisoning.

Chemicals and Reagents

Since process standardization is a substantial challenge for both individual researchers and large-scale manufacturing facilities alike, there is a substantial opportunity for the development of more refined culture systems and reagents that reduce variability. However, there are several complex signaling pathways that contribute to the "stemness" of cells, including those involving WNT [134], BMP/Activin [135], FGF [136], and Notch signaling [137, 138], which complicates the development of stem cell culture products and reagents.

One of the most successful examples of a stem cell media is Stem Cell Technologies' (Vancouver, Canada) mTESR range of stem cell medias. Used by researchers worldwide, this media formulation was recently updated to reduce the total number of components in a new E8 version. Beyond creating reagents that are defined, the utility of xeno-free products for the maintenance and derivation of clinically relevant stem cell products represents a major opportunity for product developers [39].

Several groups across the globe are researching the use of small molecules to enhance the efficiency of the iPS cell reprogramming process, a task that entails large-scale screening efforts [139]. Chemical-based strategies to generate consistent cell culture conditions are a lofty goal, but some success has already been made.

The use of epigenetic strategies incorporating DNA methyltransferase inhibitors [14] and the histone deacetylase inhibitor valproic acid has also been effective in enhancing the overall efficiency of the reprogramming process. Indeed, use of valproic acid appeared to negate the requirement for Klf4 entirely in certain reprogramming paradigms [140]. Since 1978, valproic acid has been approved for use in the United States for the treatment of epilepsy, and though it has several mechanisms of action other than epigenetic modulation, gaining approval for methods utilizing valproic acid would be markedly less difficult than for completely new drug compounds.

Cell Engineering

Many of the experimental paradigms defined for hESCs, such as differentiation protocols employing various methods, have not been optimized for cells that have undergone reprogramming. For example, researchers differentiating neuronal cells from iPS cells applied a standard protocol previously validated using hESCs. While some success was demonstrated by producing iPS-derived cells expressing neuronal biomarkers, these cells were produced with less than half the efficiency of similarly derived hESCs [141].

Similar results were also observed by researchers attempting to differentiate hemangioblasts (multipotent cells that give rise to hematopoietic and endothelial cell types) from more well-characterized iPS cell lines generated by Dr. Yamanaka's laboratory in Japan. Hemangioblasts generated from iPS cells were created with less efficiency and appeared to age more rapidly when compared to cells similarly derived from hESCs. These cells also exhibited signs of programmed cell death after a short time in culture [142], highlighting the small, but significant differences that exist between these two cell populations and further demonstrates the need for optimizing current cell culture strategies for iPS cells.

Because of the time and resources required to optimize iPS cell culture strategies, there are numerous commercial opportunities to engineer cells for researchers in a variety of biomedical disciplines. StemGent (Cambridge, MA), a pioneer in lentiviral-based iPS cell reprogramming, began offering custom iPS cell line generation services and Cellular Reprogramming Training Courses in 2010. The following year, this company established a partnership to distribute cells with ATCC (Manassas, VA), a private, nonprofit biological resource center with an expansive collection of cell types.

More recently, CDI announced MyCell™ Services that include novel iPS cell line reprogramming. This service uses an episomal method to generate "footprint-free" human iPS cells at a manufacturing capacity of over two billion cells daily. Other features of this service include genetic engineering and differentiation of iPS cells into cardiomyocytes, neurons, and endothelial cells using CDI's previously optimized protocols to generate pure populations suitable for research. Users can provide iPS cells or choose from one of over 250 normal and diseased lines currently available through CDI.

Cell Characterization

The final segment of the research tools market includes products and services to characterize iPS cells and derived adult cell types. This includes validating stem cells to confirm pluripotency and assessing maturity levels of cells derived from iPS cell lines. While many of these procedures are currently performed in-house by researchers, outsourcing this analysis can be cost-effective and removes the burden from researchers who may not be familiar with such techniques.

Commercial opportunities exist in this area to develop tools that quickly and accurately identify cell characteristics both in the form of products and services. Currently available products incorporate the use of primary antibodies to qualify the identity of cells as well as other methods to analyze cellular genetic profiles.

Systems Bioscience, Inc. (Mountain View, CA) offers a stem cell characterization kit that includes materials to qualify the pluripotent potential of cells using fluorescent-tagged antibodies. Similar kits are also available from many of the larger life science companies. Applied Stem Cell, Inc. (Menlo Park, CA) will use similar methods to characterize cells for researchers and reports both qualitative and quantitative results.

Therapeutics Market

One of the most promising commercial aspects of iPS cells is their potential for therapeutic uses. As a "blank-slate" material for the generation of virtually any human cell type, the use of these cells could be applied to a wide variety of clinical strategies. Though iPS cell therapies are currently only in preclinical stages, the global market for these products appears imminent.

In 2012, the US District Court ruled that an individual's stem cells are essentially drugs once cultured and therefore fall under the regulatory domain of the US FDA. This precedent bestows the authority to regulate clinical stem cell research and trials for all treatments in which cells are manipulated in more than a minimal fashion [112]. Since generating mature adult cells requires extensive in vitro and in vivo culturing of cells [27, 28] companies must first demonstrate safety and efficacy of iPS cells, iPS cell-derived adult cells, and many associated products and services before the full potential therapeutic uses of this technology can be realized. Further, the success of making cells suitable for engraftment is variable between cell types and fully dependent upon generating pure populations of cells at a specific maturation level [29]. Examples of stem cell therapies currently in clinical trials are presented in another section "Clinical Applications."

Reprogramming processes that incorporate retroviral methods have proven inadequate for producing clinically relevant iPS cell populations. These strategies rely on random and stable integration of reprogramming factors into the genome, which have been shown to cause variation even within a patient's own cells and appear to affect the differentiation potential of generated iPS cell lines [143]. New methods are being developed to address the issue of variation in cell products and to remove viral integration schemes from the reprogramming process completely.

Beyond the importance of iPS cells and their derivatives being free of oncogenic transgene elements or viruses, it is vital that final cell products should be free of any undifferentiated stem cells, as these cells are in their essence extremely oncogenic. For this reason, there is a substantial opportunity for the development of methods that generate pure populations of cells. In order to generate clinically relevant cells, reprogramming processes and all subsequent cell manipulations are required to be fully characterized for their suitability and safety in a variety of clinical paradigms.

Logistics and Adjunct Technologies

While research and therapeutic uses of stem cells are being pursued with great vigor by industry, there are a host of other accompanying products and services that are required for successful translation and delivery of stem cell products and therapies to consumers. Logistically, stem cells are alive and therefore require specialized storage and transport systems that will ensure quality products suitable for their final use. Clinical products are tightly regulated from creation to consumption and this requires oversight at every stage in these processes. Thus, this poses significant challenges in a global marketplace.

Consumer Stem Cell Banking Services

While the short-term therapeutics market is limited by previously described development issues, there are growing commercial opportunities in providing acquisition and storage of adult tissue suitable for reprogramming. While cord blood banking services have been popular for a few decades, increasingly services are becoming available to store and propagate tissue suitable for iPS cell derivations.

Recently, Lonza Group (Basel, Switzerland) made available a range of iPS cell services from tissue acquisition to differentiation of client cells into adult cell types. These services are performed under current Good Manufacturing Procedure conditions, therefore making Lonza able to offer cell products to cell therapy clients in support of ongoing clinical trials. This is an important first step in preparing for potential iPS cell-based therapeutics and represents a new market sector entirely.

HLA-Haplotype Banking of iPS Cell Lines

Though iPS cell technologies allow for more personalized medicine approaches through creation of patient-specific cell lines, significant benefits could be made through the use of human leukocyte antigen (HLA) haplotype banking of iPS cells [144]. The HLA system mediates histocompatibility in humans through the use of cell-surface antigens.

This approach would afford many of the immunological benefits of autologous cell therapies by matching donor and patient HLA haplotypes to reduce the potential for rejection of cell grafts. It would also allow for the creation of large clinical iPS cell banks that could be prequalified for use in specific clinical paradigms. The commercial potential of developing such cell banks could also be applied to both drug discovery and clinical applications.

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Part III

Tissue Stem Cells

Spermatogonial Stem Cells in Adult Mice and Men

Courtney Sachs and Marco Seandel

Abbreviations

SSCs Spermatogonial stem cells

Introduction

Spermatogonial stem cells (SSCs) are the resident stem cells in the testes of adult males and are responsible for maintaining lifelong spermatogenesis in mammals, yet represent only a tiny fraction of adult germ cells (e.g., about 0.03 % in mice) [1]. In humans, SSCs seem to be similarly scarce but only indirect estimates have been made, and these are based in part on ethically problematic experiments performed on prisoners who were dosed with radioisotopes in the 1960s [2]. Given the apparent paucity of SSCs, it should come as no surprise that, as yet, we are unable to definitively identify the authentic stem cell population within the testis. Nonetheless, remarkable technology developed by Ralph Brinster and others has enabled the discovery of critical molecular and functional features of SSCs, not only in mice but also in other species, making SSC biology a preeminent model for long-term self-renewing adult stem cells. In addition to maintaining genomic and epigenomic integrity for future generations, SSCs have the unusual property among other adult stem cell types of undergoing spontaneous programming in vitro to produce a pluripotent phenotype, a process that is poorly understood despite a number of recent controversial studies, particularly in humans [3–6]. The goal of this chapter is to present recent discoveries that pertain to the characterization and function of normal adult SSCs in mice and humans and also to address the current understanding of reprogramming of adult male germ cells.

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Spermatogonial Stem Cells in Rodents

According to the classical view, known as the A_s model, mammalian SSCs are characterized by morphological criteria obtained from whole mount preparations of testicular seminiferous tubules. This model, initially proposed by Clermont and Bustos-Obregon [7], defines rodent SSCs as isolated A_{single} (A_s) spermatogonia. These A_s spermatogonia are located on the basement membrane of the seminiferous tubules and are part of a larger subcategory of undifferentiated spermatogonia, A_{undiff}, which are recognized by their apparent lack of condensed heterochromatin in the nucleus. A_s spermatogonia either self-renew, dividing into two new SSCs, or begin to differentiate, forming A_{paired} (A_{pr}) spermatogonia which remain connected by intercellular cytoplasmic bridges [8]. A_{pr} spermatogonia continue on the path of differentiation to form longer chains of 4–32 cells, which are referred to as $A_{aligned}$ (A_{al}) spermatogonia. These A_{al} spermatogonia continue to differentiate, ultimately giving rise to diploid spermatocytes.

More recently, functional and molecular features have essentially supplanted the classic morphological descriptions of putative SSCs. The minimum requirement for stem cell functionality is the ability to maintain the stem cell population while producing differentiating progeny. This functionality can only be definitively assessed by means of transplantation, which was first published as an assay in 1994 by Ralph Brinster and others [9, 10]. The transplantation assay demonstrates that donor SSCs, when injected into the seminiferous tubules of infertile mice, have the capacity to migrate to the proper microenvironment along the basement membrane and carry out long-term self-renewal and spermatogenesis. It was also shown that donor spermatozoa could generate normal offspring and were, thus, fully functional.

It had been widely assumed, in accordance with Clermont's earlier model, that A_s spermatogonia, exclusively, are the true SSCs. However, due to the fact that there

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is no universally accepted A_s-specific marker and that SSCs can only be definitively identified in retrospect using the aforementioned functional transplantation assay, the smallest population that has been proven to have stem cell properties includes all undifferentiated spermatogonia (A_s, A_{pr}, and A_{al}). Furthermore, it is equally unclear whether the stem cell population is limited to an even smaller subset of undifferentiated spermatogonia than the A_s spermatogonia.

Recent studies have presented convincing data suggesting that Clermont's original model is likely flawed. Using an in vivo lineage tracing strategy, Nakagawa et al. described two functional populations of SSCs in the mouse testis; these were referred to as "actual stem cells," which are self-renewing, and "potential stem cells," which have the ability to self-renew but only do so under stress [11]. A recent study by this same group showed that the putative stem cell pool, as defined by the A_s model, is heterogeneous and that the actual stem cell population is contained within a subpopulation of A_s spermatogonia [12]. Other studies have cast doubt on the schema of self-renewal and differentiation suggested by the Clermont model, according to which differentiation is linear and nonreversible, and have shown that the commitment of spermatogonia to the differentiation pathway is indeed reversible [12, 13]. The extent to which this phenomenon is generally applicable to SSCs in other mammals, including humans, is not currently clear.

In addition to the above studies, the ability to characterize SSCs based on molecular markers that are present on the cell surface has greatly accelerated the field. In 1999, Shinohara et al. showed that α_6-integrin and β_1-integrin were expressed on the surface of SSCs [14]. Later, in 2003, Kubota et al. identified Thy1 (CD90) on mouse SSCs. Kubota showed that 95 % of the SSCs in the adult mouse testes are contained in the Thy1$^+$ cell fraction [15]. Kanatsu-Shinohara previously found that mouse SSCs express CD9, though the CD9$^+$ testis cell fraction was found to be enriched only 6.9-fold for SSCs [16]. In a more recent study, Kanatsu-Shinohara showed that SSCs are most concentrated in CD9$^+$EPCAM$^{-/low}$ population [17]. GPR125 was also shown to be a marker for undifferentiated spermatogonia in the mouse [3]. Purification of SSCs has also been facilitated by the use of negative selection against molecules such as α_V-integrin [18].

While cell surface markers are particularly useful for isolation of live SSCs, other signature genes have been identified, many of which are nuclear. These include, but are not limited to, PLZF, LIN28, NANOS2, and OCT4, which are all expressed by undifferentiated spermatogonia, but not specifically by A_s spermatogonia [19–24]. Conversely, KIT expression is absent in undifferentiated spermatogonia and marks the transition to differentiating type A spermatogonia [25]. In a recent paper, however, Oatley et al. showed that ID4 is expressed exclusively in A_s spermatogonia [26].

Spermatogonial Stem Cells in Humans

According to studies beginning with Clermont and Heller, primate spermatogonia were characterized morphologically as A_{dark} (A_d) and A_{pale} (A_p) spermatogonia, based on the distinct levels of chromatin condensation in the nuclei and the consequent intensity of the staining with hematoxylin [27, 28]. Both A_d and A_p spermatogonia were considered undifferentiated and it was suggested that the A_d spermatogonia are the reserve stem cells and the A_p spermatogonia are the actively renewing stem cells [27–30]. In this model of spermatogenesis, the A_p spermatogonia divide to form either new A_p spermatogonia or differentiated type B spermatogonia. The type B spermatogonia continue to divide, differentiating to form primary spermatocytes and spermatids. Other models of human SSC population dynamics suggest that the A_p spermatogonia, which undergo regular divisions, are actually transit-amplifying progenitors, whereas the A_d spermatogonia are the true SSCs [31, 32]. It has also been proposed that the A_d and A_p nuclear phenotypes may represent spermatogonia at distinct stages of the cell cycle as opposed to spermatogonia with differing stem cell fates [33]. Due to the difficulty of culturing human SSCs and the paucity of available assays, however, the true identity of the human SSC remains unknown, though it is most likely true that the human SSCs exist as a smaller subpopulation of the A_d or A_p spermatogonia [5].

In the last decade, however, progress has been made to define human SSCs using the same approaches as were used with rodent models. Izadyar et al. showed that putative SSCs in the adult human testis are phenotypically characterized as SSEA-4$^+$, CD49f$^+$, CD90$^+$, GPR125$^+$, and c-Kit$^{neg/low}$ [34]. The same study also found that about one-third of repopulating spermatogonia express OCT4 and NANOG, signifying the existence of populations of spermatogonia in the adult human testes with at least some characteristics of pluripotent cells.

In a 2010 study, the Dym group used human testicular material from deceased organ donors and confirmed that human spermatogonia express THY1, GFRα1, ITGα6 (although ITGα6 is also expressed in Sertoli cells), and PLZF, all of which are also markers of rodent SSCs [35]. Localized expression of GPR125 was observed in 1–2 spermatogonia per seminiferous tubule cross-section, and they proposed that GPR125 might be a marker of SSCs. In a more recent study, von Kopylow et al. shed substantial light on the original morphology-based model proposed by Clermont [36]. It was found that the gene expression profile of A_p and A_d spermatogonia differed in regard to expression of KIT, Ki-67, and DMRT1, while many putative SSC markers were common to A_p and A_d spermatogonia. Specifically, they found that KIT, Ki-67, and DMRT1 were restricted to

subtypes which lacked nuclear rarefaction zones, i.e., types A_p and B spermatogonia only. A_d spermatogonia, however, were marked by high levels of exosome component 10 (EXOSC10) in the nuclear vacuole, which may reflect differential nuclear RNA metabolism in the A_d spermatogonial population; this feature was linked to the cell's immature state. Thus, as additional molecular correlates of stemness in the human testis are validated, it is likely that the utility of morphological assessments will continue to decline.

While several groups reported that OCT4 expression is not conserved in human spermatogonia [6, 35], Bhartiya et al. suggested that the reason for the discrepancy between findings in rodents and humans may be that the antibodies and primer sets used were derived from the overlapping domain between OCT4A and OCT4B rather than from an exon specific to OCT4A [37]. A novel population of 5–10 μm cells was found to express nuclear OCT4A and also other pluripotent markers such as NANOG and TERT, suggesting that these cells may represent a distinct population of cells with pluripotent features in the testis. Given the numerous pitfalls associated with accurate and valid measurement of pluripotency genes and of possible markers of human SSCs, it remains to be seen whether genes such as OCT4, NANOG, and SOX2 are meaningfully expressed, either at the level of mRNA or protein in human SSCs. Such questions become particularly relevant when addressing the reprogramming of adult germ cells, as discussed in the final section of this chapter.

Microanatomy of the Spermatogonial Stem Cell Niche

A stem cell niche is the specialized microenvironment that supports self-renewal and survival of the stem cell population. Stem cell niches are formed by contributions from surrounding support cells, which provide extrinsic stimuli to regulate self-renewal and differentiation both through secreted growth factors and extracellular matrix support. Spermatogenesis occurs within the seminiferous tubules of the testis, which are surrounded by the basement membrane (Fig. 1). The developing germ cells and Sertoli cells together form the seminiferous epithelium [38]. Tight junctions formed between the Sertoli cells create both a basal compartment, which houses all undifferentiated spermatogonia, and an adluminal compartment. Peritubular myoid cells line the outside of the basement membrane and provide structural support for the tubules. The interstitial region between the tubules consists predominantly of Leydig cells, which secrete testosterone, along with the vascular network, and also tissue macrophages. Each of the cell types mentioned, in addition to vascular contributions, have been implicated as contributors to the SSC niche [39–44].

The Sertoli cell is the only somatic cell type within the seminiferous tubule; in addition to critical roles in fostering the latter stages of spermatogenesis, it is generally accepted

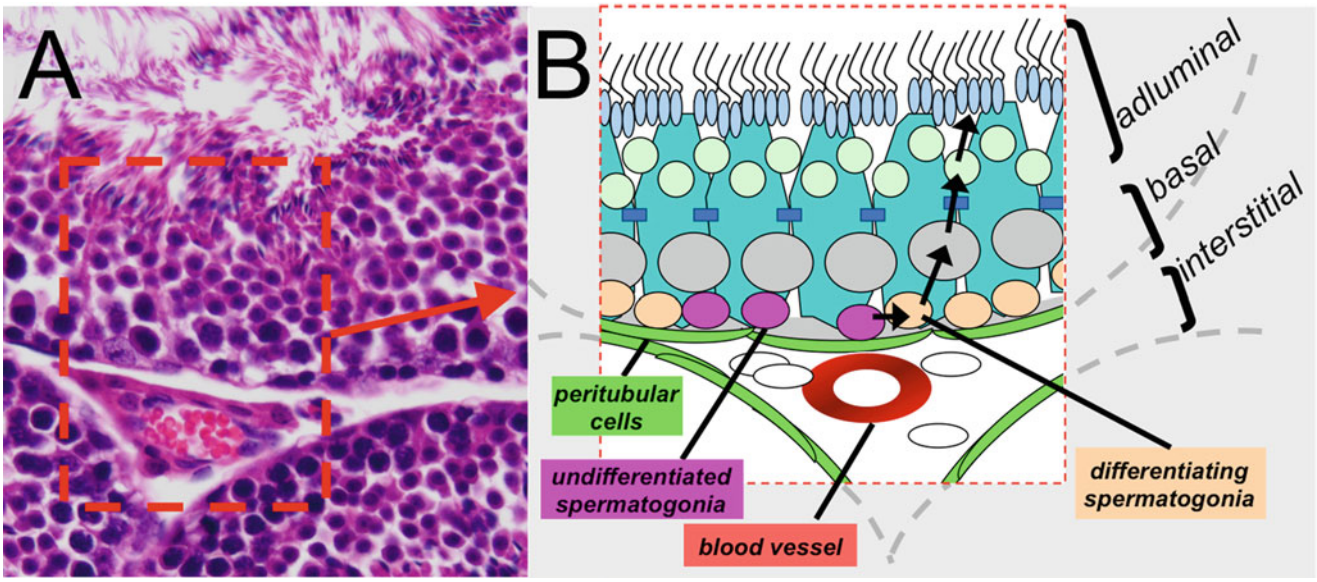

Fig. 1 Structure of the mouse seminiferous tubule and SSC niche. (**a**) Hematoxylin- and eosin-stained section of adult mouse testis. *Red*, *dashed line* shows area that is illustrated in (**b**). (**b**) Cartoon showing undifferentiated spermatogonia, including stem cells, are nurtured from within the seminiferous tubule by signals produced by Sertoli cells (*turquoise*) and also from the outside of the tubule by other somatic cell types, such as peritubular myoid cells (*green*) and others. Additional cell types of note that are present in the interstitial region include endothelial cells, macrophages, and Leydig cells

that the Sertoli cell is the predominant participant in the SSC niche. Oatley et al. recently provided more direct evidence that the Sertoli cells regulate the SSC niche, showing that increasing the number of Sertoli cells in the testes of mice concomitantly increases the number of niches accessible for colonization by SSCs posttransplantation [45].

While the Sertoli cell is critical, somatic cell populations in the interstitial tissue likely contribute to the niche as well [39, 40]. Chiarini et al. showed that undifferentiated spermatogonia accumulate in areas of the seminiferous tubule where the basement membrane is more closely associated with the interstitial tissue. Additionally, Yoshida et al. (2007) implicated the vascular network of the testes in regulation of spermatogonia by showing that during the process of differentiation, undifferentiated spermatogonia migrate away from areas of the tubule that are associated with the interstitial vasculature [46]. The functional roles of vascular-derived instructions in SSC self-renewal have yet to be elucidated.

Extrinsic Factors Regulating Fate Decisions

Substantial progress has been made in identifying extrinsic stimuli that control the decision of SSCs to self-renew rather than differentiate and in using this knowledge to establish culture conditions that support the long-term propagation of SSCs in vitro [47–49]. The first, and arguably the most important, extrinsic regulator of SSC self-renewal and propagation to be found was glial cell line-derived neurotrophic factor (GDNF) [50]. Produced by Sertoli cells, GDNF is a member of the transforming growth factor beta (TGFβ) superfamily. Meng et al. (2000) were among the first to recognize the importance of GDNF signaling in the maintenance of undifferentiated spermatogonia. It was shown that spermatogenesis is disrupted in GDNF-deficient mice, while overexpression of GDNF in transgenic mice results in the accumulation of undifferentiated spermatogonia [50]. These findings ultimately enabled the successful creation of an in vitro culture system that could sustain SSCs long term. In 2004, Kubota et al. found that the addition of recombinant GDNF to serum-free medium did indeed promote the long-term expansion of mouse SSCs [49]. A recent study has shown that GDNF is required not only for the initial establishment of the stem spermatogonial pool but also for the maintenance of the SSC population in the normal adult testis [51]. GDNF was found to promote self-renewal over differentiation of replicating stem spermatogonia in the normal mature testis. GDNF is also known to signal via the GFRα1/RET co-receptor through activation of Src family kinases, Ras, and PI3K-Akt pathways and subsequently induces expression of target genes in SSCs [52–55].

In addition to GDNF, other growth factors that enhance SSC self-renewal have been identified. Kubota et al. (2004) found that while fibroblast growth factor 2 (FGF2) alone does not support SSC expansion, it does increase the rate of proliferation when added in conjunction with GDNF [49]. Kanatsu-Shinohara et al. (2005) also found that inclusion of either FGF2 or EGF in serum-free medium along with GDNF supports long-term expansion of SSCs [56]. Leukemia inhibitory factor (LIF) supports SSC growth in vitro and, thus, may also play a role in the regulation of SSC fate decisions in vivo, although it is not strictly required in vitro [57]. Of note, the Shinohara group recently demonstrated that activation of MAP2K1 downstream of FGF2 drives expression of ETV5 and BCL6B in SSCs [58].

Two recent studies, using gene expression profiling, found that Csf1r, the receptor for Colony Stimulating Factor 1 (CSF1), is highly expressed in undifferentiated spermatogonia isolated from mouse testes [42, 59]. The ligand, CSF1, was thus implicated as a potential extrinsic factor in the regulation of SSC proliferation. When added to cultures of undifferentiated spermatogonia, which were also supplemented with GDNF and FGF2, CSF1 did not enhance proliferative activity but did increase SSC content. These data indicate that CSF1 exposure alters the balance of SSC self-renewal versus differentiation and demonstrate that CSF1 influences SSC self-renewal without affecting proliferation of non-stem spermatogonia. Because CSF1 alone (i.e., without GDNF) did not support cluster formations, it was speculated that CSF1 likely acts in collaboration with or through GDNF. CSF1 expression was observed in both Leydig cells and select myoid cells, suggesting that these cells, too, contribute to the SSC niche [42, 59].

The Wnt family of proteins, which comprises secreted glycoproteins, is another group of cell-extrinsic signals that have been implicated in SSC maintenance in vitro [60, 61]. Yeh et al. (2011) showed that Wnt5a, in particular, supports SSC maintenance and enhances survival of stem spermatogonia in vitro, while Wnt3a may target progenitors [60]. Because the effects of Wnt5a were eliminated by the inhibition of a β-catenin-independent signaling pathway and also because germ cells with active β-catenin signaling lacked SSC activity, these data suggest that Wnt5a supports SSC self-renewal independently of β-catenin. Interestingly, it was also shown that Wnt5a is expressed by Sertoli cells and that SSCs express the cognate receptors. In contrast, Golestaneh et al. found that Wnt3a induces cell proliferation of spermatogonia [61]. It was suggested that Wnt3a acts through the β-catenin-dependent pathways. Unfortunately, direct comparison of these studies is difficult due to substantial methodological differences.

Intrinsic Molecular Mechanisms Regulating Spermatogonial Stem Cell Maintenance

In the SSC system, germ cell-intrinsic factors have essential roles in the maintenance of stem cells and, thus, contribute to the niche in a cell-autonomous manner. Because GDNF is

generally regarded as the most important extrinsic factor in the regulation of SSC self-renewal, the study of cell-intrinsic mechanisms involved in SSC maintenance have focused on those pathways that are regulated by GDNF. To date, numerous genes have been found to intrinsically regulate SSC maintenance. These include POU3F1, ETV5, BCL6B, LHX1, and NANOS2 [53, 62–66]. Wu et al. recently demonstrated that POU3F1 is an intrinsic regulator of GDNF-induced survival and self-renewal of mouse SSCs [63, 64]. The Brinster group showed that siRNA silencing of POU3F1 induces apoptosis in cultured $THY1^+$ spermatogonia and, in transplantation assays, greatly reduces that number of colonies formed in the testes of recipient mice [63, 64]. These studies strongly suggest that POU3F1 is an integral intrinsic regulator of SSC survival and likely acts as a suppressor of apoptosis-related genes.

ETV5 is another gene that has been strongly implicated as an upstream regulator of SSC fate in the GDNF-signaling cascade [62, 63]. Wu et al. (2011) demonstrated that ETV5 knockdown and GDNF withdrawal both dramatically reduced the expression of BCL6B, LHX1, Brachyury, and CXCR4. These data provide evidence to the fact that ETV5 is an upstream of effector of all four genes and is itself regulated via GDNF activation [63]. Loss of BCL6B, a transcriptional repressor, has been shown to upregulate genes associated with apoptosis [63]. LHX1 knockdown by siRNA impairs SSC maintenance in vitro [53]. NANOS2, a zinc finger RNA-binding protein, has an expression pattern consistent with undifferentiated spermatogonia, including A_s, A_{pr}, and some A_{al} [65]. While NANOS2 was initially thought to be unaffected via GDNF, a recent paper demonstrated that the GDNF signaling pathway induces NANOS2 expression [62, 66]. Disruption of NANOS2 results in rapid depletion of undifferentiated spermatogonia, while overexpression results in accumulation of undifferentiated spermatogonia and reduction in the number of differentiating spermatogonia [65].

In parallel to GDNF-activated signaling pathways, additional cell-intrinsic factors have been identified in the self-renewal and survival of the SSC population. One of these factors is promyelocytic leukemia zinc finger protein (PLZF), a transcriptional repressor [19, 20]. It was previously shown that male mice lacking PLZF expression undergo progressive germ cell loss and testis atrophy, strongly suggesting that PLZF is a cell-intrinsic factor that is necessary for the maintenance of germ cell lineage [19, 20]. Hobbs et al. (2010) then showed that $PLZF^{-/-}$ spermatogonial progenitor cells can be maintained in long-term culture [18]. Similarly, Wu et al. (2011) found that PLZF silencing did not affect the ability of SSCs to self-renew in vitro [63]. However, PLZF promotes in vivo SSC self-renewal indirectly by repressing mTORC1 activity, which inhibits normal spermatogonial progenitor cell response to GDNF [18].

FOXO1, another transcription factor, was recently found to be essential to both SSC homeostasis and spermatogenesis [67]. As a specific marker of a subcategory of spermatogonia with stem cell potential in addition to mouse gonocytes, it was revealed that FOXO1 is closely associated with the "stemness" of the spermatogonia. This group also showed that FOXO1 is an important effector of PI3K-Akt signaling in SSCs, thus revealing novel FOXO-dependent mechanisms that affect SSC fate decisions [67]. Thus, a plethora of signals are emerging as regulators of SSCs under normal physiologic conditions.

Loss of Lineage Commitment: Culture-Induced Acquisition of Pluripotency

As opposed to SSC self-renewal which can be demonstrated in vivo or in vitro, reprogramming of adult germ cells into a pluripotent state is generally considered a culture-induced phenomenon, wherein a unipotent germ cell converts into an ES-like state (Fig. 2). In contrast, reprogramming in vivo either in adult mice or in men is an extremely rare event (<1 in ~11,000 in wild-type laboratory mice and <1 in ~16,000 in humans) [68, 69]. The basis for studying reprogramming of SSCs in vitro rests upon (1) the availability of technology to derive and maintain SSC lines in vitro which we regard as germ lineage-committed, non-pluripotent cells and (2) the identification and functional validation of cells that have actually undergone reprogramming to a pluripotent state, concomitant with the loss of most germ cell features. Multiple studies in mice have shown that the resultant pluripotent cells are highly similar but not identical to ES cells with respect to gene expression, function, and epigenetic features [3, 70–73].

The reprogramming of spermatogonia in vitro is akin to induced pluripotency in which a different type of stable precursor (e.g., fibroblasts) is reprogrammed into a pluripotent state, with an unambiguous distinction between the precursors (e.g., fibroblasts or spermatogonia) and the resultant pluripotent cell type [74]. However, such an unambiguous distinction requires that the precursors be clearly defined, most critically, by functional assays for long-term self-renewal both in vitro and in vivo. Unfortunately, these stringent criteria are not met in many cases.

Following the seminal observations by Shinohara et al. (2004) that SSCs derived from neonatal mice could reprogram in vitro after long-term culture, the same group demonstrated that even after single cell cloning of SSCs, such potency was retained [70]. In 2007, we showed, using GPR125 to track germ cells, that even adult SSC lines in long-term culture retain the ability to reprogram spontaneously [3]. As per standard criteria for pluripotency, the reprogrammed cells derived from adult SSC lines were shown not only to form teratomas in immunocompromised mice but also to contribute to chimeric tissues upon blastocyst injection, even though gene expression was not identical to that of ES cells. Guan et al. (2006) demonstrated pluripotent cells

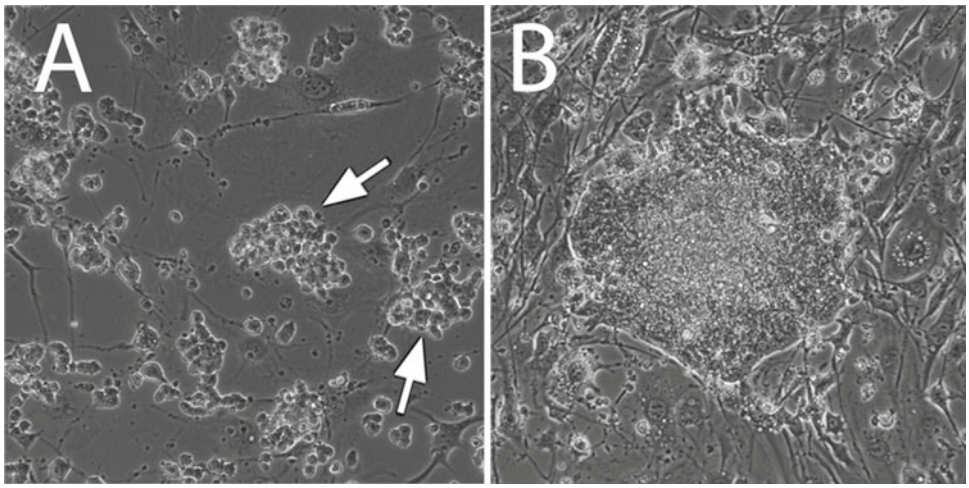

Fig. 2 Reprogramming of adults mouse SSCs in culture. (**a**) Cultures of SSCs exhibit variably sized grape-like clusters (*arrows*) of cells that are tightly associated with each other but loosely attached to underlying feeder cells. (**b**) Spontaneous reprogramming of SSCs yields embryonic stem-cell line colonies with sharp, refractile borders that can be maintained as such if transferred to culture conditions designed from mouse embryonic stem cells. Reprogrammed cells rapidly differentiate when maintained in suboptimal conditions

could be derived from the adult testis but the precursor population was less defined in that study due to the absence of a long-term SSC culture phase [75]. Subsequently, the Scholer group showed, using OCT4-GFP reporter cells, that the culture-induced reprogramming of adult SSCs was highly dependent upon plating density [72].

While the origination of pluripotent stem cells from long-term cultures of cells with testis-repopulating activity strongly argues that SSCs are the substrate for conversion, it has not been clearly demonstrated whether all spermatogonia are similarly potent or alternatively whether only a subset give rise to pluripotent colonies. Izadyar (2008) presented data that the $OCT4^{+}/KIT^{+}$ fraction of spermatogonia were enriched for cells that could be reprogrammed which is interesting, because KIT expression has been considered marker for commitment to differentiation of adult spermatogonia [72]. Intriguingly, Morimoto et al. (2012) recently found that whereas freshly isolated $CD9^{+}$ testis cells (enriched for SSCs) could produce ES-like colonies upon transfection of the Yamanaka factors (Oct4, Klf4, Sox2, and Myc), cultured SSCs could not, suggesting that in vitro propagation of cells has a negative influence on reprogramming [76].

The first evidence of culture-based reprogramming of human spermatogonia came from the Skutella group who found that testicular cells expressing germ cell markers rapidly upregulated OCT4 during the first week in culture [6]. Subsequently, colonies of putative pluripotent cells were formed continuously during the following weeks in culture. Upon differentiation, the pluripotent cells were able to form functional tissues in vitro and limited teratomas in immunocompromised mice. Despite substantial increases in expression of pluripotency genes, the levels were nonetheless significantly lower than those observed in human ES cells. Subsequently, the Scholer group questioned these findings and concluded that the testis-derived cells thought to be pluripotent were actually more closely related to fibroblasts [77, 78]. An additional caveat is that the Conrad et al. study lacked a long-term self-renewal phase of SSCs in culture prior to reprogramming, without which it is difficult to be sure of the identity of the precursors to the cells that underwent reprogramming.

Following the study by Conrad et al., several studies have found evidence for the ability of normal human testicular cells to undergo apparent reprogramming, although the cell of origin and mechanism are not entirely clear [4, 5, 79, 80]. However, no study to date has demonstrated reprogramming of validated human SSCs from long-term, self-renewing cultures that have been maintained for longer than several months. Kossack et al. (2009) observed appearance of ES-like colonies within several weeks of culture of testicular cells and found not only expression of OCT4 and SOX2 but also the ability of stem cells to differentiate robustly in vitro, but no teratomas were formed in vivo [4]. Subsequently, the van Pelt group also showed in vitro differentiation into all three germ layers but not teratoma formation by ES-like cells derived from testicular cell cultures that had been maintained up to 8 weeks but not thereafter [79, 80]. Since teratoma formation is one of the few assays for pluripotency available for human cells in vivo, the observed reprogramming may have been incomplete or inadvertently produced an intermediate cellular state. Subsequently, the same group concluded that similarly derived ES-like cells were not, in fact, pluripotent due to the absence of spontaneous tri-lineage differentiation. In contrast, Golestaneh et al. (2009) discovered that ES-like cells appear after only 4 days of culture of testicular cells from organ donors; within 4 weeks, lines of pluripotent stem

cells were obtained that could form teratomas in vivo [5]. Unfortunately, none of the aforementioned studies was able to unequivocally identify the precursor for the reprogrammed cells, which would require a combination of single cell cloning and subsequent functional characterization of both the putative SSCs (using germ cell transplantation assays) and their ES-like progeny (through formation of teratomas).

Conclusions

The rapid progress of the SSC field beyond morphological criteria and into a phase of functional and molecular studies has ushered in a new era. With the ability to rigorously define this cell type, various groups are moving forward with strategies to address urgent clinical problems, such as treatment-related infertility, using SSCs. Of course, such approaches will require that the level of data produced from the aforementioned rodent studies are at least matched, where possible, using human tissue. At the same time, it is urgent to understand the mechanisms behind reprogramming not only for safety-related reasons in SSC-based cell therapy but also if reprogrammed germ cells are ever to be used for disease modeling or other translational purposes.

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Characterization of the Hematopoietic Stem Cell Niche: Cellular and Molecular Analysis

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Introduction

Multipotential/unipotential stem cells are tissue stem cells found in almost all tissues of the adult and fetus. It is assumed that the steady state of a tissue with high turnover rate (blood, epidermis, intestine) results from the adequate balance at the stem cell level of self-renewal and commitment.

Adult tissue stem cells are rare cells difficult to identify by phenotype. After more than 20 years of research it is now possible, at least in the mouse, to purify blood-forming hematopoietic stem cells (HSCs) almost to purity using antibodies against membrane antigens and cell cycle markers (review in [1]). However, even in this case, it remains impossible to define within a stem cell population which cell will behave as a bona fide HSC capable of repopulating lethally irradiated primary and secondary recipients. This may be due to our incomplete understanding of the genetic networks operative in HSCs, leading us to omit some essential markers for HSC sorting. However, it may also be related to the fact that "stemness" is a state reached at certain time points by a given immature hematopoietic cell oscillating between different states due to gene network noise (reviews in [2, 3]). This latter hypothesis may explain why HSCs express variable level of lineage markers, in particular transcription factors, a characteristic known as lineage priming (review in [4]).

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Adult stem cells are usually studied as native cells directly collected from their tissue of origin. However, some of the stem cell types may be culture-amplified while maintaining their regenerative potential. Such is particularly the case for connective tissue-forming mesenchymal stem cells (MSCs).

Historically, the concept of niche has been devised as the cellular and molecular components associated to an HSC to insure appropriate HSC functioning [5]. It has been subsequently applied to most types of tissue stem cells including germinal cells (reviews in [1, 6]). Study of stem cell niches is difficult since tissue stem cells are not easily identified or visualized. However, study is made easier for the niches of the germinal stem cells in Drosophila due to the specific spatial organization of the testes and ovaries where differentiation proceeds along the anterior–posterior axis of the organ with germinal stem cells at one end, the tip, in contact to specialized cap or hub cells constituting the niches. Moreover, analyses can be performed in Drosophila with a wide range of genetic modifications affecting stem or niche cells. Studies in these flies have shown that a candidate stem cell niche depleted of its stem cell was able to take up and maintain a newly introduced stem cell. A model was established whereby germ stem cells remaining in contact with microenvironmental/stromal cells of the niche kept their stemness property, whereas stem cells that lost this contact became determined to germ cell progenitors. Studies in mammals of intestinal, epidermal, and neural stem cells have largely benefited from the Drosophila germ stem cell model since the corresponding tissues are strictly organized with stem cells localized in well-defined areas. Whatever the tissue stem cell type, the self-renewal capacity might result from either a "lineage mechanism" (asymmetrical division) or a "population mechanism" (symmetrical division of a stem cell giving two daughter stem cells associated to symmetrical division of another stem cell giving two committed progenitors). The amplification of the stem cell pool requires a population mechanism, while for stem cell maintenance a lineage mechanism is sufficient. Since tissue stem cells have to be amplified during development or in conditions of stress, both

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mechanisms may be at play depending on developmental stage or tissue injury.

Definitive hematopoiesis is known to proceed in mammals by distinct developmental steps: emergence in the ventral aorta from the hematogenic endothelium, then migration to and amplification in the fetal liver and finally migration to and maintenance in the bone marrow (review in [7]). Emergence of HSCs from the aortic ventral wall occurs by E10.5 in mice and 3.5 gestational week (GW) in humans. In mice, HSCs with definitive properties of adult tissue stem cells are first detected in the aortic wall and later in development or after explant culture in presumptive gonads and mesonephros region, hence the name for this primary hematopoietic site of aorta-gonads-mesonephros (AGM). Hematopoiesis at this site is short-lived (from E10.5 to E12 in mice, a few days up to 5.5 GW in humans), contrarily to that of the fetal liver where hematopoiesis is detected from E9 in mice and 5 GW in humans up to gestation end in the two species. Bone marrow hematopoiesis that takes place in bone in both species after birth lasts over the entire life span.

There are two other sites of primary definitive hematopoiesis, the spleen and the placenta. The red pulp of the spleen serves primarily as the site of filtration of effete red cells that have reached their term of 120 days of life (in humans). However, it may also be a site of migration and subsequent proliferation of HSCs in stressed mice and diseased humans with bone marrow failure or inefficient hematopoiesis as observed in myelofibrosis (extramedullary hematopoiesis). Recent studies, such as that in [8], indicate that the placenta constitutes another developmental site of primary hematopoiesis throughout human gestation.

In this chapter we will focus on bone marrow HSC niches due to the wealth of data generated over more than 4 decades, allowing us to outline the makeup of the niches. We will then indicate the data progressively unraveled concerning fetal liver and AGM, before giving full attention to the molecular mechanisms underlying the HSC: niche cell cross talk.

The Bone Marrow HSC Niche

In the bone marrow of mammals the hematopoietic logettes filled with differentiating hematopoietic cells are overwhelming, making the identification of highly motile HSCs difficult. Study of HSC niches has therefore relied on several complementary approaches: cocultures of HSCs and stromal cells, in vivo monitoring of cell subsets containing phenotypically defined HSCs after transplantation in mice, and study of transgenic mice with potential niche cell defects.

In vitro studies were first to be implemented (see reviews in [9, 10]). In 1976, Dexter et al. established a long-term culture system where mouse bone marrow cells were seeded on plastic recipients in a medium containing a rich mixture of cytokines and extracellular matrix (ECM) molecules provided by fetal calf and horse sera [11]. In such cultures an adherent layer was generated in approximately 2 weeks. This layer made of macrophages and stromal cells was required for the maintenance for several months (depending on the mouse strain) of HSCs with self-renewing capacity. Remarkably, in the following year, 1977, the same team demonstrated that stromal cells from normal mice were able to maintain HSCs from mice with intrinsic HSC Kit receptor defect, whereas stromal cells from mice mutated for the Kit ligand (Kitl) were unable to sustain HSC production from normal mice. These data evidenced that HSC functioning required other cooperating cell populations, mainly stromal cells, non-hematopoietic in origin, since HSC-derived macrophages were shown to produce inhibitors limiting the growth of stromal cells and to act as scavengers to eliminate spent cells such as enucleated cells in erythroblast islands. These studies provided with an easily expanded system that made it possible to study in vitro the relationship between HSCs and stromal cells and validated the former hypotheses of "hematopoiesis supportive microenvironment" (review in [12]) presumed upon results from in vivo studies of mice transplanted with HSCs.

This culture system was adapted to humans in the 1980s. Moreover, it was refined by allowing to culture cell subsets enriched by phenotype in HSCs and hematopoietic progenitors onto immortalized stromal lines fully supportive of HSCs, or supportive of myeloid or lymphoid progenitors only, or devoid of supportive capacity. Lines of stromal cells can be readily generated in the mouse, either by subsequent passaging and/or by transfer of immortalization genes such as viral T SV-40 or E6/E7 papilloma antigens. Many of the supportive lines still exert their supportive ability when seeded with human HSCs (xenogeneic systems). However, human lines with similar properties are very difficult to obtain: viral antigen transfection induces crises; telomerase integration may lead, probably by associated mutations, to malignant transformation after a number of passages.

Many observations have indicated the mesenchymal nature of the stromal cells, in particular their motility and capacity to synthesize and assemble a proteoglycan-rich ECM [3]. Connective tissue-forming mesenchymal cells include specialized cell types such as osteoblasts, chondrocytes, adipocytes, and smooth muscle cells. Distinct stromal cell populations were shown to present many of the markers of these specialized types such as fat-laden vesicles characteristic of adipocytes, and collagen I, alkaline phosphatase, bone sialoprotein, and osteocalcin characteristic of osteoblasts (review in [13]). We have described the vascular smooth muscle differentiation of human and murine stromal cells and lines acquiring with time in culture vascular smooth muscle cytoskeletal markers, up to the most specific h-caldesmon and smooth muscle myosin heavy chains [14, 15].

The future lies probably in the development of 3D-cultures using diverse scaffolds pegged or not with cytokines or other mediators and substrates of precise elasticity, a biophysical parameter known to affect the self-renewal capacity and the differentiation potential of stem cells.

Even if in vitro systems are amenable to fine-tuned analysis of the different interacting populations, they can at best be regarded as surrogate niches [16]. In vivo observations are mandatory to validate the in vitro results and experiments in animal models are required to provide the crucial information at the cell-to-cell level (review in [9, 10]). In 1979 "adventitial reticular cells" located in the wall of sinusoids and expressing alkaline phosphatase were described in mouse and rat bone marrow [17]. In 1989, the team of Guilio Gabbiani described in human bone marrow peri-sinusoidal "myoid" cells expressing the vascular smooth muscle marker alpha-SM actin [18]. We confirmed the presence of such cells not only in adult bone marrow where they extend cytoplasmic processes deep into the marrow space, making contact with numerous hematopoietic cells, but also in fetal bone marrow where they are present before HSC colonization in "primary logettes" and increase in size and number after colonization [14, 19]. These data suggested that cells associated to the vasculature or to bone might act as niches for different types of hematopoietic cells.

More recent studies have allowed to precise the nature of the HSC niche. One approach has been to monitor the dynamics of labeled HSCs within bone marrow following transplantation. This strategy has been made possible due to major progress in HSC identification and to the possibility to visualize cell trafficking. Nilsson et al. in 2001 sacrificed nonirradiated mice at regular intervals after injection of labeled hematopoietic precursors and examined serial bone marrow biopsies [20]. They found that candidate HSCs significantly distributed to the endosteal region (within 12 cells of the bone surface), 15 h after transplantation. Other investigators, by examination of the calvarium of living mice using a combination of high resolution confocal microscopy and two-photon video imaging, found similar distribution to the endosteal region of candidate HSCs, at earlier time points (5 h after transplantation) and only when the mice had been lethally irradiated or were Kit mutants, situations in which there was real engraftment after several weeks [21]. No direct contact with osteoblasts was observed and the distinction between osteoblastic and vascular niche was not feasible, osteoblasts being perivascular.

A final approach to study the HSC bone marrow niches has been to evaluate the HSC compartment in animals with potential niche defects. In 2003, two teams published simultaneously studies made in mice with increased osteoblastic pool due either to the conditional inactivation of the bone morphogenetic protein receptor 1A (Bmp1a) or to the constitutive activation of the parathormone receptor under the control of collagen I promoter (Col1-CaPPR mice) [22, 23]. In both cases there was a modest, but significant, increase in the number of bone marrow HSCs with competitive repopulation capacity, while the progenitor compartment was not affected. Moreover, normal HSCs injected in Bmpr1a mutant mice increased over a 3-month period, which was not observed when HSCs from mutant mice were administered to normal mice, showing that the HSC increase in the mutant mice was non-stem cell autonomous. In Bmpr1a mutant mice, localized ectopic formation of trabecular bone was observed; in these areas there was an increase in the number of spindle-shaped N-cadherin$^+$ osteoblasts lining the bone surface to which putative HSCs were attached. In the Col1-CaPPR mice, there was a noticeable increase in the Jag1$^+$/osteopontin$^+$ osteoblasts found in metaphyses. These data indicated that some of the bone marrow HSC niches consisted in specific populations of bone-lining osteoblasts. The specificity of the niche-forming osteoblastic cell subset was confirmed in a later study showing that reduction of trabecular bone osteoblasts in biglycan-deficient mice did not lead to decrease in HSCs or to defect in any hematopoietic lineages [24]. Other works further specified the phenotype of osteoblasts of HSC niches as expressing activated leukocyte cell-adhesion molecule and angiopoietin-1 [25, 26]; this latter angiogenic factor enhanced the ability of bone marrow Tie-2$^+$ HSCs to become quiescent.

Other data from genetically modified mice indicated that, beside osteoblasts, vascular cells might also provide a niche for bone marrow HSCs. In thrombopoietin receptor-deficient mice treated by the S-phase-specific cytotoxic drug 5-fluorouracyl and by antibodies neutralizing vascular-endothelial cadherin or Cxcl12 chemokine, the central vascular region of long bones was depleted of hematopoietic cells contrarily to the endosteal region, which indicated the distinctive role of these factors in the two regions [27]. Another study confirmed the role of Cxcl12-Cxcr4 ligand–receptor interaction, showing that induced deletion of Cxcr4 in adult mice led to severe decrease in HSCs, and that cells expressing high amounts of Cxcl12 were associated with candidate HSCs; many of these cells were peri-sinusoidal [28]. The demonstration that vascular cells, either endothelial or perivascular, constitute bone marrow HSC niches has been recently provided by the team of Sean Morrison [29]. Using Kitl knock-in mice, these investigators showed that Kitl was expressed in bone marrow by peri-sinusoidal cells and a few cells surrounding venules and arterioles, but not by bone-lining cells. Candidate HSCs were located adjacent to or in immediate vicinity of the vascular cells. Kitl conditional deletion from hematopoietic cells or osteoblasts did not affect the HSC compartment, contrarily to its deletion from Tie2$^+$ endothelial cells or leptin receptor$^+$ perivascular cells that led to decrease in HSCs with additive effect when Kitl was deleted in both populations (leading to almost complete loss of HSCs and significant reduction of marrow cellularity).

Taken together, these data indicate that diverse types of differentiated cells, non-hematopoietic in origin, may constitute the niches of HSCs in the bone marrow. The diversity of in vivo niches and the rarity of stromal lines fully supportive of HSCs, suggested that the niches might derive from another type of stem cells also present in the bone marrow.

In the 1990s, following the pioneering work of Alexander Friedenstein, several investigators from the orthopedic field described a clonogenic precursor for osteoblasts, chondrocytes, and adipocytes called MSCs (review in [30]). It was soon shown that this precursor gave rise to hematopoietic supportive stromal cells capable of differentiating into vascular smooth muscle cells, as did stromal cells grown in long-term marrow cultures. Enumeration of MSCs in vitro, done in most cases by counting the number of colony-forming units fibroblasts (CFU-fs), indicated their very low frequency (0.1–0.01 % of the bone marrow mononuclear cells in humans). Cogent demonstration of MSC self-renewal was provided by showing that one bone marrow human CFU-f colony consisting in CD146$^+$/CD90$^+$ cells was still containing after ectopic in vivo amplification in the immune-deficient mouse a very small fraction of CD146$^+$/CD90$^+$ cells capable of yielding, upon secondary plating, one or two CFU-fs [31]. These data clearly indicate that MSCs are bona fide stem cells since they are clonogenic, multipotential and self-renewing.

Lineage priming is another property of stem cells shared by MSCs [32]. We have recently performed a meta-analysis of transcription factors expressed in a number of tissues, including hematopoietic and mesenchymal cells, and in HSCs and MSCs. We have found that a few transcription factors were prevalent in each tissue type (results for mesenchymal tissues and hematopoietic cells are presented in Fig. 1a, b). Half of the transcription factors prevalent in mesenchymal and hematopoietic cells were also present in MSCs (Fig. 1c) and in HSCs (Fig. 1d), respectively. Two patterns of differentiation can therefore be described. In one case (lineage-priming model), transcription factors are present early in stem cells and are increased in differentiating cells, while the other ("blank slate" model) transcription factors are detected at substantial level only in differentiating cells. Lineage priming in MSCs might be related to the known plasticity of cells of the mesenchymal lineage, i.e., their capacity to adapt to changing microenvironments (review in [30, 33]).

Culture-amplified MSCs are known for their hematopoietic supportive capacity. Experiments in vivo have confirmed this supportive activity. In 2006, Muguruma et al. reported observations made after intra-bone injection of human labeled culture-amplified BM MSCs in immune-deficient mice [34]. Ten weeks after transplantation, 60 % of the labeled cells were alpha-SM actin$^+$ and located in the vicinity of sinusoids, while 30 % were alkaline phosphatase$^+$ and located in the endosteal region. Transplantation of human cord blood cells after that of MSCs revealed the frequent interaction of MSC progenies and candidate HSCs. In 2007, the team of Paolo Bianco reported results obtained after subcutaneous injection of human CD146$^+$ bone marrow CFU-fs in immune-deficient mice. Four weeks after transplantation, the few human cells that retained the expression of CD146 were located on the abluminal side of mouse-derived endothelial cells forming incipient sinusoids [31]. By week 8, foci of hematopoietic cells were clearly associated to the CD146$^+$ peri-sinusoidal cells. Remarkably, implantation of a single CFU-f gave identical results. Finally in 2010, different teams working in collaboration described one of the murine equivalents to human MSCs as nestin$^+$ cells constituting a very rare population of perivascular cells frequently associated with putative HSCs [35]. Remarkably, in vivo depletion of nestin$^+$ MSCs led to significant decrease in HSCs and to severe impairment of HSC homing to bone marrow. Nestin$^+$ cells were often associated with nerve fibers, which may be due to the fact these cells are probably neuro-ectodermal in origin, contrarily to leptin receptor$^+$ cells that may be derived from the mesoderm [29]. Indeed, it has been shown in mice that MSCs can derive from either of these germ layers (review in [30]).

Taken together, the data suggest a unifying model for bone marrow HSC niche-forming cells, i.e., that they are MSCs or their direct progeny (Fig. 2). Such view should reconcile the opposite tenets of osteoblastic vs. vascular niche since an MSC migrating in the marrow parenchyma may give rise to either lineage, depending on its location at a given time point. In that respect bone marrow HSC niches might differ from niches of other types of stem cells where niche cells are specialized and differentiated (review in [1]).

Fig. 1 (continued) keratinocytes (Epi, GSE7216), brain cortex (Neural, GSE7307), skeletal muscle (Muscle, GSE6798), liver (Hepato, GSE7307), HSCs (GSE17054), and skeletal stem cells (GSE6460). Results were normalized before comparison. For technical details, see [67]. (**a**) Transcription factors prevalent in mesenchyme. (**b**) Transcription factors prevalent in hematopoiesis. (**c**) Levels of transcription factors prevalent in mesenchyme in differentiated mesenchymal tissues vs. stem cells (MSCs). On the *left hand side* are factors expressed both in stem and differentiated tissues (in decreasing order). On the *right hand side* are factors expressed only in differentiated tissues (in increasing order). The *left hand side* set corresponds to factors fitting with a lineage-priming model of differentiation. The *right hand side* set corresponds to factors fitting with a "blank slate" model of differentiation. Lineage priming is exemplified in MSCs by the expression of the master transcription factors SOX9 and RUNX2. (**d**) Comparison of levels of transcription factors prevalent in hematopoiesis in hematopoietic differentiating cells vs. stem cells (HSCs). Lineage priming is exemplified in HSCs by the expression of the master transcription factors IKZF1 and TAL1

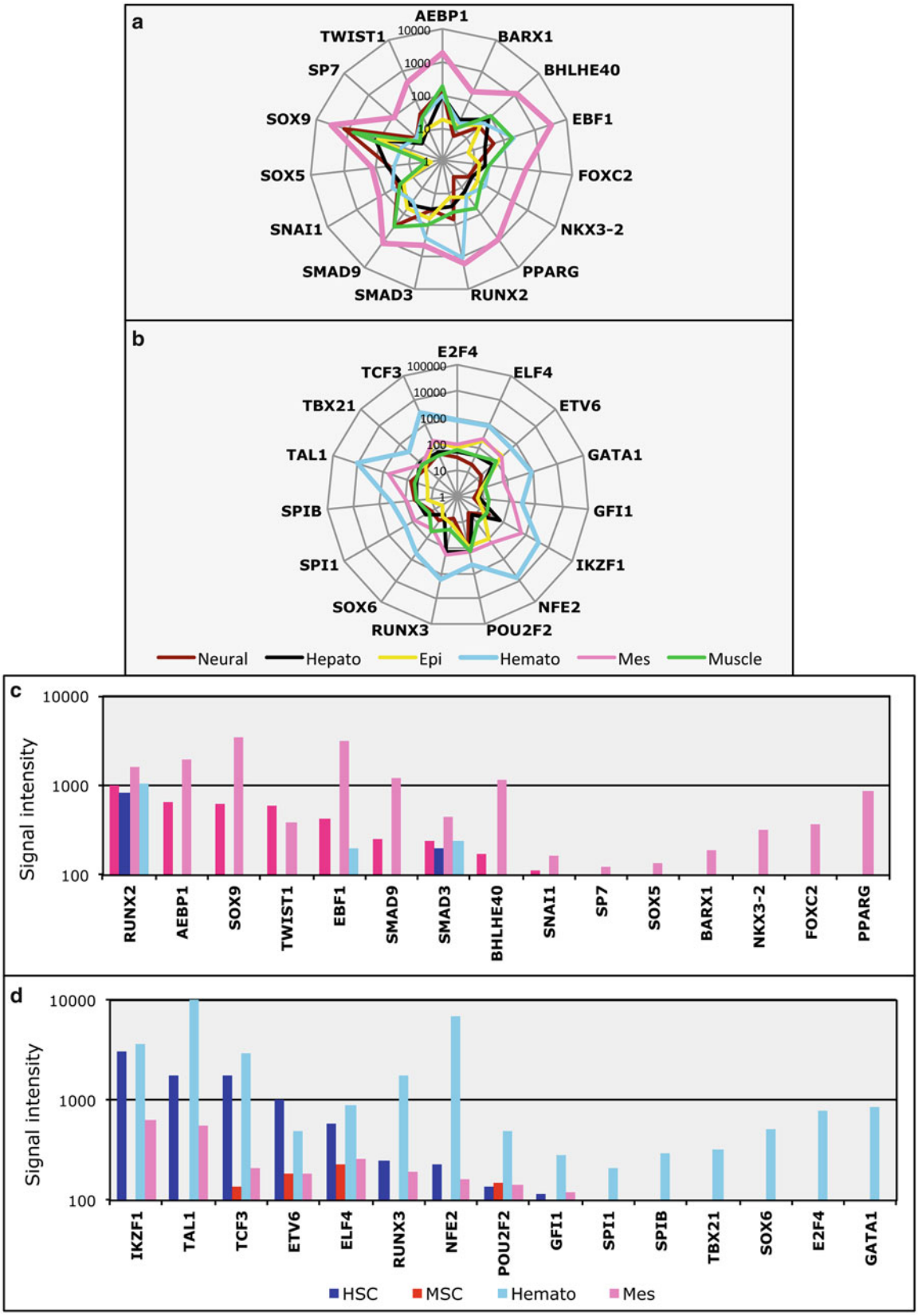

Fig. 1 Transcription factors in human mesenchymal and hematopoietic cells. We used Affymetrix micro-arrays to estimate the levels of transcription factors (SignalLogRatio) in the SiPaGene/BioRetis (http://www.bioretis.de) database for mesenchymal tissues (Mes: bone, cartilage, and fat), hematopoietic cells (Hemato: $CD45^+$, $CD11b^+$, and $CD235a^+$ cells), and MSCs. In addition we used GEO datasets for

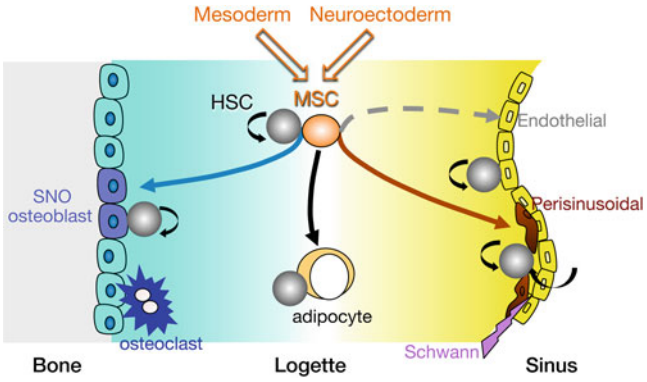

Fig. 2 A model for HSC niches in BM. MSCs, which can be of neuroectodermal or mesodermal origin, can serve as HSC niche on their own or may give rise to spindle-shaped,N-cadherin$^+$ osteoblasts (SNO) or to peri-sinusoidal cells or endothelial cells, thus forming the osteoblastic or vascular niche where sheltered HSCs preserve their self-renewal and commitment potential (see references in the text). On the contrary, the differentiation of MSCs into adipocytes results in adipocytic bone marrow, which is poor in HSCs and hematopoietic progenitors [68]. Two additional, non-MSC-derived niche components, were recently described, neural Schwann cells [69] and osteoclasts [70]. Neural Schwann cells are found in both endosteal and more central bone marrow regions, while osteoclasts line the bone trabeculae. Whether these cells occupy the same niches as osteoblasts or vascular cells is not clear. However, it is known that nerve terminations are found in the vicinity of arterioles both in rodents and in humans [71, 72] where they are known to regulate the bone marrow blood flow and HSC migration into blood [73]

Depending on their location in the bone mesenchyme (i.e., their microenvironment) MSCs might differentiate into a specific subset of osteoblasts, into perivascular pericyte-like cells and even into endothelial cells. Available literature suggest a common origin for pericytes and vascular smooth muscle cells [36], and some data point out to a possible endothelial differentiation of bone marrow MSCs [37], which is compatible with the known derivation of vascular progenitors from embryonic stem cells [38].

Potential Niches in Other Hematopoietic Sites

The HSC niches in fetal liver and embryonic aorta are yet poorly understood. The role of the stromal cells constituting these niches have to be, at least partly, different from that of the bone marrow niche(s) since fetal liver is the site where HSCs amplify and aorta at the site where adult-type HSCs emerge, most probably from the hematogenic endothelium. These niches contrast therefore with niches in the bone marrow where it has been shown that some of the niche components participate in the maintenance of the HSC quiescent state [25].

As mentioned earlier in this chapter, stromal lines represent an exceptional tool to investigate the role and the cellular complexity of the HSC microenvironments. Most of these stromal lines, characterized by their ability to support or not HSCs, differentiate along the vascular smooth muscle cell pathway, many lines being arrested at a distinct differentiation stages along this pathway [39]. However, some of the lines still retain the capability to give rise to other mesenchymal lineages. For example, UG26.1B6, one of the most HSC-supportive AGM stromal lines [40] efficiently gives rise to osteoblasts when cultured in osteogenic conditions [41] and AFT024 [42] and BMC9 stromal lines, generated from fetal liver and bone marrow, respectively, are multipotential with the ability to differentiate into vascular smooth muscle cells, osteoblasts, chondrocytes, and adipocytes [43]. With the aim to further characterize the HSC microenvironment in the human placenta, Robin et al. have recently established a panel of stromal lines at different developmental stages of human gestation [8]. These lines express markers of mesenchymal cells and most of them exhibit an osteogenic, adipogenic, and endothelial-like differentiation potential.

Studies have been implemented to trace the origins of MSCs in early development. By grafting quail or mouse embryonic aorta into chick recipients, Minasi et al. have shown the existence of vessel-associated multipotential progenitor cells in the vertebrate embryo [44]. In vivo, these cells had the ability to integrate the host vasculature and to give rise to several other mesenchymal derivatives such as bone and cartilage. In another study, fate-mapping analysis revealed that mouse mesenchymal progenitors, with the ability to differentiate in vitro into osteoblasts, chondrocytes ,and adipocytes, first appeared in the AGM region in E11 mouse embryo [45]. Whereas osteogenic and chondrogenic progenitors were found both in the dorsal aorta with its surrounding mesenchyme and the urogenital ridges, adipogenic progenitors were exclusively detected in the urogenital ridges. Except for chondrogenic potential in the somites and limb buds (attributed to already differentiated prechondrogenic mass), none of the other examined tissues (yolk sac, head, heart, liver, and vitelline and umbilical arteries) harbored any mesenchymal differentiation potential. Other studies identified bona fide MSCs in human AGM and fetal liver [46, 47]. In these latter studies, coculture experiments with cord blood CD34$^+$ cells indicated that MSCs were able to support hematopoiesis in vitro.

Taken together, these data suggest that MSCs are also constituents of HSC niches during development. As for bone marrow MSCs, they may serve as HSC niches on their own or because they give rise to an adequate progeny, which is probably vascular (pericyte-like or endothelial) at these sites. As noted for other stem cell niches [1] and in an earlier study on CFU-fs made by Wolf [48], the stromal niche constituents appear to be present before their occupancy by HSCs. MSCs appear in the aorta at mid-gestation, circulate in the fetal blood, and colonize the fetal liver and bone where they amplify and will further constitute a supportive microenvironment for

HSCs. These results highlight the intimate relationships between MSCs and HSCs during development as well as in the adult.

A final point concerns the HSC niche in the spleen. A recent study has indicated that candidate HSCs in mice treated with mobilizing agents were found associated to sinusoids [49].

Major Extrinsic Signaling Pathways Implicated in HSC Regulation

Stromal cells regulate HSC functions through the production of specific cytokines (including Kitl, Flt3, thrombopoietin [Tpo], interleukins IL-3 and IL-6), chemokines (such as Cxcl12), angiopoietic factors and molecules of the ECM (reviews in [10, 50]). In the present part, we will focus on a few extrinsic signaling pathways that have been shown recently to control the maintenance of HSCs in the adult bone marrow; we will also review the recent advances concerning the molecular characterization of the AGM hematopoietic microenvironment (Fig. 3).

BMP-4 is a member of the bone morphogenetic protein family and is required for the establishment of the blood lineages from the ventral mesoderm at early embryonic stages. This morphogen has been shown to play a critical role in the regulation of the bone marrow HSC niche. In mice, BMP-4 expression has been reported in osteoblasts, endothelial, and perivascular cells [51] and, as already underlined, the BMP signaling pathway has been shown to control the size of the BM HSC niche and the numbers of HSCs [23]. Using hypomorphic BMP-4 mice, Goldman et al. reported that the frequency and absolute numbers of HSCs were reduced in mutant mice as compared to controls. Furthermore, transplantation experiments suggested that the ability of HSCs to self-renew is affected when HSCs are placed in a microenvironment deficient for BMP-4. The role of BMP-4 as an important extrinsic regulator of HSCs had been previously demonstrated using in vitro culture systems. Human cord blood HSCs exposed to low concentration of BMP-4 (5 ng/mL) differentiate, whereas at higher concentrations (25 ng/mL) BMP-4 maintains ex vivo HSCs with repopulating activity [52]. These results suggest a dose-dependent effect of BMP-4 on adult HSCs. Whereas human cord blood and, at lower levels, bone marrow HSC candidates express mRNAs encoding the BMP receptors Bmpr1a/Alk3 and Bmpr1b/Alk6 and the downstream signaling molecules Smad1, 4, and 5 [52], studies in mice revealed that bone marrow HSCs cells do not express BMP receptors [22]. Thus, BMP-4 may control HSC activity through direct and indirect mechanisms in humans and mice, respectively.

The Notch pathway is also a major extrinsic signaling that regulates HSCs. In 2003, as already mentioned, it was shown, using transgenic mice with constitutive activity for the parathyroid hormone receptor, that osteoblasts are critical components of HSC niches in the bone marrow and that the

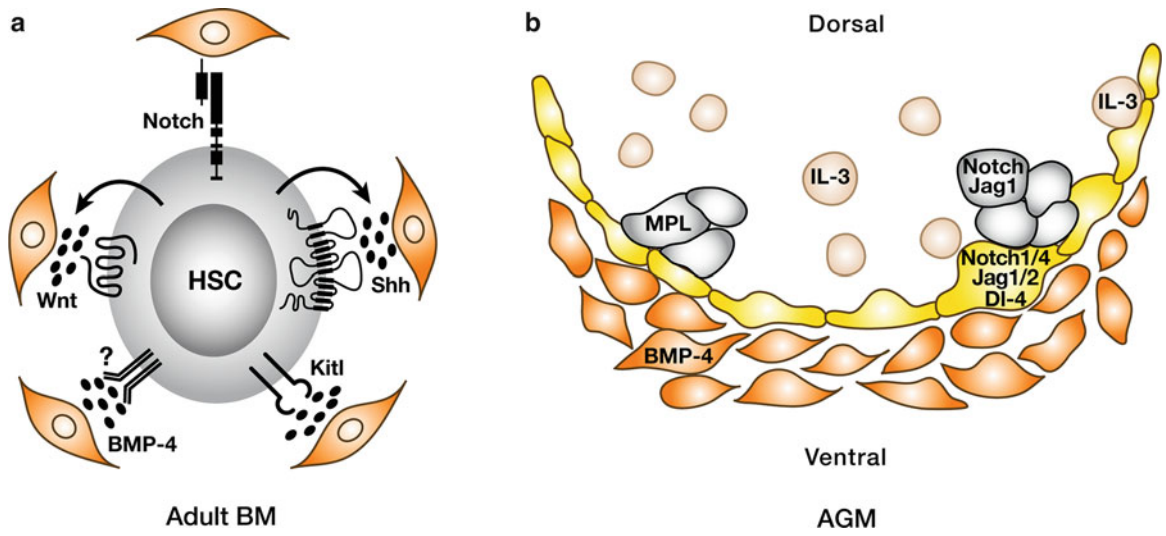

Fig. 3 Schematic representation of some of the extrinsic signaling pathways occurring in the bone marrow HSC niche and in the AGM hematopoietic microenvironment. (**a**) Bone marrow stromal cells regulate HSCs through the production of specific cytokines (such as Kitl and BMP-4), chemokines and morphogens (Wnt, Notch, and Shh). How an HSC integrates these complex extrinsic signals to proliferate, survive, self-renew, or differentiate is a critical issue in the field of stem cell biology and regenerative medicine. (**b**) During ontogeny, the first adult-type HSCs autonomously emerge in the AGM region. Hematopoietic intra-aortic clusters (in *gray*) most likely derive from hematogenic endothelial cells (in *yellow*) in the ventral side of the aorta. BMP-4, which is expressed in stromal cells (in *orange*) underneath the endothelial lining of the aorta, and the Notch pathway, activated in aortic endothelial cells and hematopoietic clusters, have been shown to regulate AGM HSCs. IL-3-expressing cells found in the circulation (in *beige*) and sometimes closely attached to the endothelium and the Mpl/Tpo pathway participate also in the regulation of HSCs

Notch/Jagged1 pathway plays a critical role in the cross talk between HSCs and osteoblasts [22]. A role for Notch as an important extrinsic regulator of HSCs came also from ex vivo studies. The constitutive activation of Notch1 signaling in presumptive HSCs led to the generation of immortalized cell lines with the ability to produce myeloid and lymphoid cells in vitro and in vivo [53]. Using transgenic Notch reporter line, it has been shown that the Notch pathway, preferentially active in bone marrow HSCs, is down-regulated when HSCs differentiate [54]. Inhibition of Notch signaling increases the differentiation of HSCs in vitro and in vivo and thus Notch activity is essential to maintain the pool of HSCs.

A number of studies in mice and humans revealed that the Wnt signaling pathway regulates the activity of HSCs. Wnt molecules may be directly produced by stromal cells and also by HSCs themselves, suggesting that Wnt proteins could play a role on HSCs through either paracrine or autocrine mechanisms. In 2003, in vitro studies elegantly demonstrated that the Wnt pathway is critical for regulation of bone marrow HSC self-renewal [55]. Presumptive HSCs transduced with a retroviral vector allowing the constitutive expression of an activated form of β-catenin (a critical molecule in the Wnt canonical pathway) were shown to expand in vitro while retaining an immature phenotype and to reconstitute efficiently the hematopoietic system of irradiated recipients. Thus, activation of the Wnt pathway promotes the proliferation of HSCs without inducing their differentiation. Using reporter mouse lines, the authors showed also that the Wnt signaling pathway is active in HSCs and induces the expression of HoxB4 and Notch1, two other important regulators of HSCs. Interestingly, both the Wnt and Notch pathways appear to be active simultaneously in the majority of HSCs and are integrated at the functional level. When cultured in the presence of Wnt3a and Kitl, HSCs deficient for Notch signaling showed accelerated differentiation as compared to controls [54]. These results reveal that the Wnt pathway does not properly control the maintenance of bone marrow HSCs in the context of Notch inhibition.

As compared to the bone marrow HSC niche, very little is known about the molecular identity of the AGM hematopoietic microenvironment. In vertebrate embryos, the hematopoietic activity in the AGM is characterized by the emergence of hematopoietic clusters closely attached to the ventral part of the aortic endothelium. A number of studies have shown that hematopoietic stem/progenitor cells most likely derive from specialized hematogenic endothelial cells lining the aortic floor (review in [56]). In a recent work it has been reported that hematopoietic progenitors are present in mouse aortic clusters located both in the dorsal and ventral parts of the artery, while it is only in the ventral part that HSC activity is detected [57]. Altogether, these results reveal the heterogeneity of the intra-aortic hematopoietic clusters and the role for mesenchymal stromal cells underneath the aorta in the emergence and/or regulation of AGM HSCs. Studies in the human, mouse, chick, and zebrafish embryos have shown that BMP-4 is expressed in the underlying mesenchyme of the aorta, suggesting that the expression pattern of BMP-4 in the AGM is strictly controlled during vertebrate evolution (review in [58]). Functional analysis revealed that loss of BMP signaling in the zebrafish prevents the emergence of HSCs in the aorta and that inhibition of the BMP pathway abolishes the activity of AGM HSCs in the mouse [59, 60]. Using in vitro long-term cultures, it has been shown that BMP-4 also increases the growth/survival of AGM HSCs [61]. Furthermore, bioinformatics and molecular studies revealed that the BMP signaling controls the activity of the transcription factor Runx1, which is essential for the establishment of definitive hematopoiesis, and together with Runx1 is integrated in a specific transcriptional network [62]. Interestingly, BMP-4 in combination with Hedgehog molecules appears to play a critical role in the dorsal–ventral polarization of the AGM hematopoietic activity [60]. Notch is also an important regulator of AGM hematopoiesis since it plays a role both in the specification of arterial vessels and in the hematopoietic development (review in [63]).

Although hematopoietic cytokines are known to play a critical role in the cross talk between HSCs and stromal cells in the bone marrow, very few of them have been shown as implicated in the AGM hematopoietic microenvironment. Using Runx1$^{+/-}$ mouse embryos as a model, interleukin-3 (IL-3) was identified as a strong positive regulator of embryonic HSCs. As shown by in vivo transplantation, IL-3, but not other hematopoietic cytokines such as Kitl, was able to rescue the defective HSC potential of Runx1$^{+/-}$ AGM [64]. IL-3, not only plays a role on AGM HSCs but also on placental HSCs, and increases the number of HSCs with repopulating activity. Interestingly, transplantation experiments indicated a dramatic decrease in the numbers of HSCs in IL-3-deficient AGM and placenta as compared to controls. This suggests that the level of IL-3 as found in wild-type animals is absolutely required for optimal production of HSCs in the embryo. In the embryo, IL-3 expressing cells were found in the circulation and in the developing stomach, which is close to the AGM region. More recently, the thrombopoietin receptor Mpl, a well-known regulator of megakaryopoiesis and HSCs in the adult, has been shown to regulate HSCs both in the AGM and the fetal liver in the mouse [65]. An elegant strategy based on refined HSC localization in the AGM and on comparison of gene expression profiles have led to the identification of novel regulators of AGM HSCs [66]. One of them, p57Kip2 (Cdkn1c), expressed in HSCs, was also detected in the underlying mesenchyme of the dorsal aorta at E11. This result suggests that p57Kip2 may regulate AGM HSCs through HSC autonomous and/or nonautonomous mechanisms. The same study also revealed the presence of insulin-like growth factor-2 and its receptors in the AGM and a positive role for IGF-2 in the survival/proliferation of immature AGM hematopoietic progenitors.

Conclusion

How an HSC integrates the signals emanating from its microenvironment to either differentiate, proliferate, survive, or self-renew is still a major issue in the field of stem cell biology and regenerative medicine. The present intensive investigations on the cellular and molecular characterization of HSC niches in the adult and during ontogeny are of considerable interest since they increase our knowledge on the molecular mechanisms implicated in the emergence, amplification, and maintenance of HSCs, which should improve the ex vivo HSC manipulation for clinical scenarios.

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Glandular Stem Cells (GSCs): Stem Cells in Glandular Organs

Ming Jiang, Karin Williams, and Simon W. Hayward

Introduction

A gland is an organ that synthesizes a substance for release; examples include prostate fluid, breast milk, mucus, and hormones, either inside the body or to its outer surface [1]. Glands can be divided into two groups: exocrine glands, secreting their products through a duct or directly onto the apical surface, and endocrine glands, which are glands that secrete their products through the basal lamina and lack a duct system. Glandular stem cells (GSCs) are defined as multipotent stem cells including progenitor (adult stem) cells in glandular organs. GSCs can be obtained from exocrine glands such as pancreas or salivary glands using well-established cell culture methods [2–4]. The resulting cell populations are characterized by a high proliferative capacity and an unusually high plasticity. Cells from pancreas have the capacity to differentiate into many lineages including into oocyte-like cells [3]. The preparation methods for GSCs can be applied to cells from many vertebrates, including fishes and birds. Since the cells are excellently cryopreservable, this finding has been utilized to establish a new stem cell bank for preserving living cells of rare and wild animals.

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Embryonic stem cells (ESC) are totipotent, having the plasticity to give rise to all of the cell types required for development and adult function. However, in the adult, stem cells have a more restricted repertoire with various levels of potency. Accepted definitions of adult stem cells (ASC) include the ability to continue to self-renewal in adulthood and multipotentiality (the ability to give rise to daughter cells with more than one phenotype). Asymmetric cell division, where the stem cell gives rise to a clone of itself as well as a daughter cell of a different cell fate, is a defining characteristic of stem cells [5, 6]. Stem cells are often described as normally having low proliferative activity with the ability to multiply in response to appropriate stimuli, such as in wound healing and inflammation. This potential to repopulate a tissue was thought to be restricted to the organ in which the stem cell resides. However data have accumulated suggesting that the plasticity of ASC may be far wider, perhaps encompassing the repertoire of an entire embryonic germ layer.

Current perceptions of stem cells focus upon the plasticity of the differentiated phenotype and on mechanisms which might induce changes from different levels of differentiation. Traditional models of differentiation suggest that there are certain irreversible points of commitment between the totipotent ESC and the fully differentiated cells in functional adult tissue. In contrast to this idea is the concept of a continuum of differentiation with cells exhibiting less propensity to exhibit stem cell-like behavior as they express more specific characteristic markers [7]. On a molecular basis the "developmental commitment" can be considered as being encoded in combinations of transcription factors [8]. This represents the resurrection of the idea of combinatorial gene control proposed more than 30 years ago [9]. Commitment to a specific phenotype is considered reversible allowing the possibility that individual cells can revert to a less-committed phenotype or alter to a different committed phenotype by changing the combination of transcription factors that they express. The caveat to this is that as differentiation precedes the degree of difficulty involved in reversing or reprogramming, the process seems to increase. Induced pluripotent

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stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an ESC-like state by being forced to express genes and factors important for maintaining the defining properties of ESC [10–12]. It is not known if iPSCs and ESCs differ in clinically significant ways. These cells would seem to meet the defining criteria for pluripotent stem cells; however it is not clear that in the face of continued expression of factors involved in promoting "stemness," these cells ever differentiate in a fully normal manner.

This chapter will examine the broad topic of ASC in glandular organs rather than the role of stem cells in a particular organ. We will address the questions of what GSCs are and whether a given organ such as the gland is served by a truly organ-specific stem cell population. We will focus on GSCs in epithelial tissues and will discuss the interactions of these cells with their local microenvironment. We will also discuss the phenomena of metaplasia and transdifferentiation as they apply to our comprehension of stem cell biology.

Models of Glandular Stem Cell Biology

Stem cell-based models of development and proliferation/differentiation have been proposed in a number of organs. These have generally suggested the presence of a stem cell compartment with the potential to undergo many rounds of division. Divisions of stem cells are proposed to give rise to daughter cells which are either stem cells or which join a population of transient-amplifying cells capable of undergoing clonal expansion finally giving rise to a cell population which is fully differentiated but has limited potential for proliferative activity [13, 14]. Such a stem cell and transit-amplifying cell model was proposed for the prostate by Isaacs [15]. The prostate is potentially a good model in which to study stem cell biology as it is dependent upon androgenic stimulation for its development and for maintenance of its glandular structure in adulthood. If androgens are removed, the prostate will regress; if they are reintroduced, it will regrow. The stem cell and transit-amplifying cell models provide a simple basis from which to determine how the growth and maintenance of organs could come about (summarized in Fig. 1). However these models do not address the question of where stem cells are located, how this location affects glandular structures, and what the phenotypic characteristics of stem cells might be.

An in vivo translational tissue recombination-xenografting mouse model has been used to functionally remodel human prostatic glandular tissues. Adult tissue regeneration or remodeling is believed to initiate from multipotent stem and progenitor cells. Two human adult nontumorigenic prostatic epithelial cell lines NHPrE1 and BHPrE1 were established in our laboratory [16]. NHPrE1 cells were designated as putative progenitor cells, showing high expression levels of stem cell-associated proteins CD133, CD44, OCT4, and PTEN as

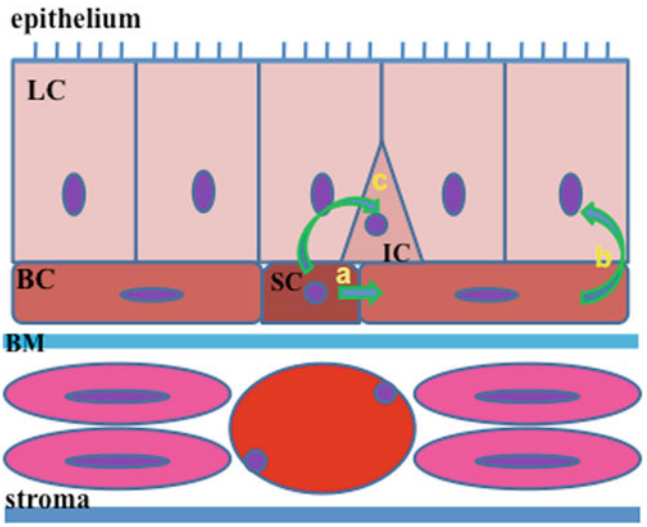

Fig. 1 A regeneration model representing possible cell lineage differentiation in the prostate, as an example of glandular organ development. This figure illustrates a lineage model of human prostatic glandular regeneration such as might occur within a tissue recombinant. In this model androgen-independent glandular stem cells (SC), including stem and/or progenitor cells, are able to initiate the growth of prostate tissue and retain glandular rudiments following castration which can then undergo expansion to repopulate the gland in response to androgenic stimulation. Stem/progenitor cells can potentially rise to either basal cells (BC) (indicated by *a*) which can subsequently differentiate into luminal cells (LC) (indicated by *b*). Alternatively SC may give rise to an intermediate cell phenotype (IC) as indicated by *c*. In case of the prostate, an androgen-dependent gland, androgen-independent and androgen-dependent populations seem to exist. Following androgen depletion it is possible that either the intermediate cells or the stem cells may be preserved to support the regrowth of the glandular epithelium following the reintroduction of androgens. *LC* luminal cell, *BC* basal cell, *SC* stem cell, and *BM* basal membrane

detected by immunofluorescence staining and Western blotting. BHPrE1 were defined as intermediate or transit-amplifying (TA) cells that expressed cell cycle regulation-related biomarkers p63, p53, p21/WAF1, and Rb. A tissue recombination-xenografting mouse model was utilized to functionally compare regeneration of human prostatic glandular tissues in vivo. In a tissue recombination model utilizing urogenital sinus mesenchyme (UGM) as a glandular inducer, both NHPrE1 and BHPrE1 cells were able to regenerate human-pattern benign secretory ductal-acinar architecture in vivo, containing intact basal and luminal epithelial layers with neuroendocrine cells. Appropriate cytokeratin profiles were seen in the epithelial tissue layers. Prostate differentiation-associated proteins, such as androgen receptor (AR), prostate-specific antigen (PSA), NKX3.1, and 15-lipoxygenase-2 (15-LOX-2), were appropriately expressed in the remodeled epithelia. This remodeling was found to be more efficient when initiated from putative progenitor (NHPrE1) rather than intermediate (BHPrE1) cells. Both NHPrE1 and BHPrE1 cell lines provide important data on progenitor and intermediate cell phenotypes and represent significant new tools for elucidation of molecular mechanisms of signal transduction in

human prostatic regeneration, pathogenesis, and carcinogenesis (the cell origin of cancer). These cell lines are useful models for investigating molecular and cell biological mechanisms related to the genesis of benign and malignant human prostate diseases and their relations to metabolic syndromes, inflammation, and interactions between epithelium and stroma (microenvironment).

The location of stem cell populations in glandular tissues has been most closely studied in the gastrointestinal (GI) tract. Stem cells of the gut epithelium give rise, via asymmetric cell mitosis, to daughter cells with more than one phenotype. These phenotypes include a range of distinctive cell types, including the Paneth, goblet, and endocrine cells. The perpetually self-renewing gut tissue consisting of proliferating, differentiating, migrating, and dying cells is not directly the progeny of the stem cells but rather of stem cell daughters. The stem cell daughters retain proliferative potential giving rise to a clonally expanding population; however the proliferative potential of each daughter is finite unlike the potential of the stem cell. Commitment of daughter cells to a specific secretory cell-type fate in the small intestine apparently occurs in a cell-type determined manner at different points between a stem and a differentiated cell. For example, cells become committed to the endocrine lineage at an early point, but do not commit to one of the nine or so different endocrine cell types until a later point [17].

Gut stem cells all reside within the epithelial units that are known in the stomach as glands and in the intestine as crypts. Both of these units consist of areas in which the stem cells and their proliferating daughters reside: migratory zones in which cells are both migrating and differentiating and a fully differentiated zone in which mature and dying cells are located. Work by Nomura and coworkers has identified the existence of at least three to four stem cells located within the isthmus region of the developing/maturing stomach glands and maintenance of one or more stem cells in the mature units [18]. This elegant study examined cell lineage by use of a lacZ-carrying X chromosome. Chimeric (mixed blue and white) glands were observed at early time points indicating the presence of more than one progenitor cell. Two progenitor cells would produce 50 % mixed 25 % homochromatic ratios. The ratios noted by Nomura were however closer to 75 % indicating that at least three stem cells are involved. Glands are therefore initially seeded by more than one stem cell [18], but mature glands generally are derived from only one stem cell [18, 19], possibly as a result of stem cell competition in which a stem cell must compete for a favored position within the gland [20] or due to symmetrical/asymmetrical cell divisions removing stem cells from the population. As compared to the stomach, the intestinal crypts progress to monoclonality in a similar but accelerated fashion [21–27]. While crypts are generally accepted to be monoclonal, it is thought that there are four to six functional stem cells per crypt [28]. This apparent discrepancy arises in part because if the stem cells present within the gland are themselves clonal (i.e., are daughters of a single stem cell produced by symmetrical division). Such cells cannot be differentiated using the present techniques of following X-chromosome inactivation patterns, chimeric lineage analysis, or retroviral integration.

Repopulation experiments have failed to answer the question of "how many stem cells are present?" as immature first-to-third-generation stem cell daughters (which are of course themselves monoclonal) may be able to repopulate the entire crypt in times of crisis by reprogramming back into stem cells [29–32]. Targeted ablation studies using transgenic mice carrying reporter genes have confirmed that certain cell lineages of the gut epithelium share a multipotent progenitor-intermediate [17]. However, Bjerknes has found that 80–90 % of the long-lived clones in the small intestine are unipotent with the remainder being multipotent [28]. This finding does not undermine the existence of true stem cells but does indicate the problems that arise when the stem cells and the long-lived but finite first-to-third-generation daughters cannot be readily distinguished from each other.

Multiple stem cells are also found within a single niche in the tracheal epithelium and submucosal glands [33]. Lineage analysis using recombinant retroviruses has demonstrated the existence of both unipotent and multipotent progenitors in submucosal glands [33]. Borthwick and coworkers have further localized the stem cells to specific regions of the glands and trachea [34].

Studies in the urogenital tract using chimeric BALB/c/C3H mice indicate that glandular organs such as the prostate and seminal vesicles, as well as nonglandular structures such as the epididymis, bladder, ureters, and kidney, were not clonal [35]. This finding suggests that these structures were derived from more than one progenitor cell. In contrast, in the same study, uterine glands were never found to be chimeric. The authors suggest that this indicates a monoclonal origin for uterine glands. However it is entirely possible that these glands are in fact originally polyclonal but undergo sorting procedures similar to the stem cell selection described for intestinal crypts.

The submandibular salivary gland is generated from a diverse array of progenitor cells that contribute to the functional and architectural complexity. It has been implied in many reports in the literature ([36] and references within) that small cell undifferentiated carcinomas of the salivary gland arise from a ductal stem cell with a multidirectional capacity. This putative stem cell is thought to reside within the intercalated ducts.

Stem cells can be isolated and identified based on a distinctive profiling of cell-surface biomarkers, transcription factors, and enzymes including ABCG2, ALDH1, CD44, FOXA2, and SOX2 [37]. There has been a long search for specific cellular markers which will identify stem cells within a tissue. Cytoskeletal proteins such as cytokeratins have been

utilized in an attempt to identify specific cell phenotypes and lineages in proliferative and nonproliferative compartments in glands such as the breast and prostate [38–44]. These studies suggest that there may be specific profiles of cellular marker expression which define the position of a cell within the differentiation process and suggesting that expression of certain combinations of markers is restricted to early or stem cell populations. Other studies suggest that putative stem cells can be identified either by the absence of a specific marker (e.g., $p27^{Kip1}$ in the prostate [45, 46]) or the overexpression of certain specific markers (e.g., pp32 in the intestine and prostate [47, 48]). Such markers of stem cell phenotypes are covered more fully in other chapters.

Although the concept that cancers arise from "stem cells" or "germ cells" was first proposed about 150 years ago, there is a concept in the field of cancer research that rare cancer stem cells (CSCs) or tumor-initiating cells (TICs) exhibit some common characteristics of normal stem cells [49]. A CSC is defined as a cell that has the ability to self-renew and to differentiate into all subpopulations of cells that compose a tumor, maintain tumor homeostasis, and mediate tumor metastasis [50, 51]. CSCs also have the ability to give rise to tumor recurrences if they survive treatment [52].

Interactions Between Epithelium and Stroma in Glandular Organs

Glandular organs are generally composed of an epithelial parenchyma surrounded by stroma. For a long time the stroma was considered to be a supporting matrix which aided in organ function. For example, the fibromuscular prostatic stroma provides the force needed for ejaculation, while smooth muscle in the gut provides the peristaltic action needed to move food. It is now clear that the stroma plays an active role in both development, where mesenchyme directs epithelial differentiation and, in the adult, where the differentiated state of the epithelial cells is maintained by continuous interactions with the adjacent stromal cells [53]. This continuous crosstalk between tissues in an organ also regulates functions such as proliferation and apoptosis [54–57].

Following gastrulation the mammalian embryo is composed of cells representing the three germ layers which will give rise to all of the tissues of the body. In a simplistic representation the external surface is covered with a layer of ectoderm which will give rise to the skin, as well as the sweat, mammary, and preputial glands. The endoderm will give rise to the gastrointestinal tract and those structures, such as the liver, pancreas, prostate, and bladder, which are derived from it. The mesodermal layer, which occupies the space between these surfaces, will give rise to all of the mesenchymal tissues, including the muscles and connective tissues. As a result of mesenchymal to epithelial transitions, mesoderm also gives rise to epithelial structures including the urogenital tract derivatives of the Wolffian and Müllerian ducts (the ureters, epididymis, ductus deferens and seminal vesicles, and the Fallopian tubes and uterus, respectively).

A series of tissue recombination experiments have established that epithelial tissues from a range of sources can respond to inductive mesenchyma by changing their pattern of differentiation. These experiments involve the separation of epithelial and mesenchymal cells from different organs and their heterotypic recombination, as shown schematically in Fig. 2. Recombined tissues are then grown as sub-renal capsule implants in either syngeneic or immunodeficient rodent hosts, or alternatively in the case of embryonic birds grafted in ovo, and subsequently examined to determine the nature of the resulting recombinant tissue.

Two classes of mesenchymal and stromal interaction with epithelium have been recognized. These are permissive effects and instructive effects [58]. A *permissive* effect supports a previously determined developmental program already specified by the epithelium, for example, supporting differentiation of an adult tissue. An *instructive* effect elicits a new program of markers in the epithelium specified by the mesenchyme, as found in the process of organogenesis. The first effect can be illustrated in heterospecific but homotypic intestinal recombination experiments, as summarized in Table 1. In these experiments 14-day fetal rat intestinal mesenchyme and endoderm were recombined with endoderm and mesenchyme from five-and-a-half-day-old embryonic chick intestines. The resultant recombinants were developed as intracoelomic grafts in ovo and examined in terms of endodermal expression of brush border enzymes. Recombinants of rat mesenchyme with chick endoderm expressed sucrase but not lactase while the opposite recombination produced the opposite enzyme patterns. This pattern of expression would be expected of the source endoderm since at this stage of development chick intestine expresses sucrase but not lactase while rats have the opposite expression [59]. Thus the mesenchyme in these experiments supports the differentiation of the gut endoderm and allows the expression of markers characteristic of the species from which the epithelial tissue was derived.

Instructive interactions of mesenchyme with the epithelium have been demonstrated in the development of the gut, using the chicken and the Japanese quail as model systems as summarized in Table 2. Mesenchyma from the stomach and small intestine of these birds exert instructive influences on the morphogenesis of both allantoic epithelium and on epithelium from various levels of the digestive tract [60]. So when stomach mesenchyme is recombined with allantoic epithelium, the morphology and expression of markers by the epithelium changes to that of stomach epithelium. Similar experiments have demonstrated glandular induction in ectoderm. For example, embryonic mammary mesenchyme can induce mammary gland differentiation from skin [61], and rabbit corneal epithelium likewise has been demonstrated to

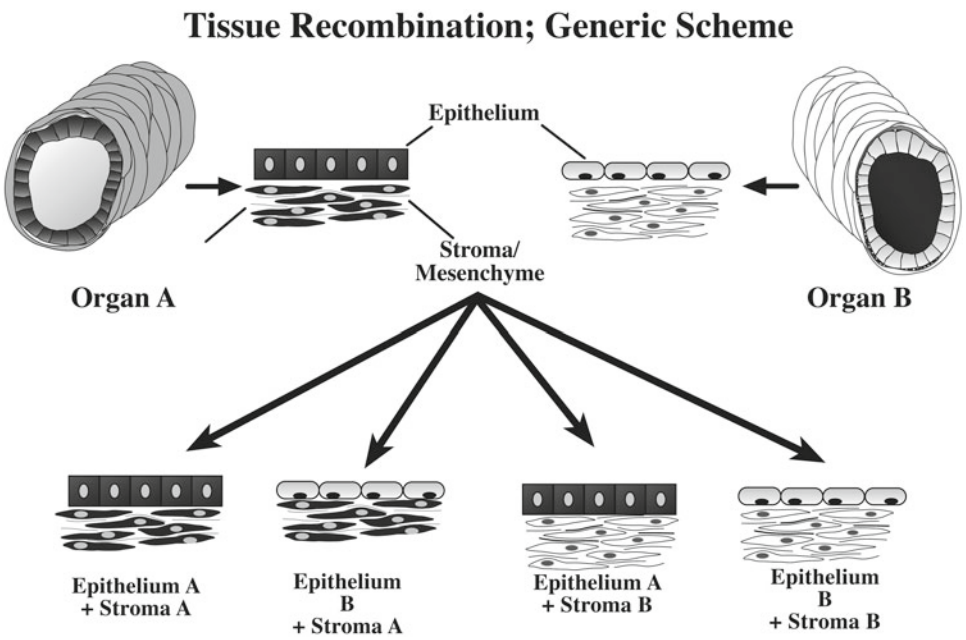

Fig. 2 Schematic representation of tissue recombination. Stromal and epithelial tissues are separated from each other and isolated. The tissues are then recombined in either homotypic (stromal and epithelial cells from the same organ) or heterotypic (stromal and epithelial cells from different organs) combinations

Table 1 Illustration of a permissive mesenchymal effect on epithelium in a heterospecific but homotypic recombination experiment

Epithelium source	Mesenchyme source	Marker expression pattern
Fetal rat intestine	Embryonic chick intestine	Lactase (rat-specific marker)
Embryonic chick intestine	Fetal rat intestine	Sucrase (chick-specific marker)

The mesenchyme supports expression of a set of markers already specified by the epithelium (Data from Kedinger et al. [59])

Table 2 Illustration of an instructive mesenchymal effect on epithelium in a heterospecific and heterotypic recombination experiment

Source mesenchyme	Source epithelium	Resultant epithelium
Chick stomach	Quail allantois	Quail stomach
Quail intestine	Chick allantois	Chick intestine

The mesenchyme changes the expression of the markers expressed by the epithelium to those of the epithelial type which would be associated with the mesenchyme (Data from Haffen et al. [58])

produce sweat glands or pilosebaceous units when combined with embryonic dermis [62].

Experiments performed with epithelial and mesenchymal tissues from the endodermal hindgut and Wolffian gut derivatives have shed more light on the role of the germ layer of origin in determining epithelial cell fate. UGM will produce prostatic morphogenesis in the epithelium of the urogenital sinus, vagina, adult prostate, and the embryonic urinary bladder [63–67]. Stromal-epithelial interactions were once thought to irreversibly determine the developmental fate of the epithelium. However, recombinations of adult bladder epithelium with UGM demonstrated that adult tissue could, in some instances, be made to redifferentiate along another pathway [68, 69]. The adult bladder epithelium gives rise to secretory prostatic structure as a result of proliferation and reorganization of the adult bladder basal cells. This process resembles closely the sequence occurring in normal prostatic development. The ductal-acinar structures formed in this experiment resemble prostatic epithelium in terms of histology, histochemistry, expression of androgen receptors, androgen dependency for DNA synthesis, and production of PSAs [68, 69]. More recent studies have confirmed that the adult human bladder urothelium can also be induced to generate prostatic structures by rat UGM [70]. In similar experiments it was demonstrated that bladder epithelium can also respond to rectal mesenchyme giving rise to glandular structures with characteristic intestinal histology and secretions [71]. This study also documents that a subpopulation of epithelial cells take on a glandular appearance. As in recombinants involving UGM, significant areas of transitional differentiation were still seen in recombinants composed of bladder epithelium and rectal mesenchyme.

Instructive interactions have also been demonstrated using tissue recombinants to foster the development of tissues from embryonic stem (ES) cells. Risbridger's group was able to show the development of prostatic tissue using human ES cells [72]. Similar studies using mouse ES cells

Table 3 Role of glandular epithelial germ layer in response to inductive mesenchyme

Epithelium	Germ layer origin	Mesenchyme	Resultant tissue
Ductus deferens	Mesoderm	Urogenital sinus mesenchyme or seminal vesicle mesenchyme	Seminal vesicle
Ureter			
Seminal vesicle			
Bladder	Endoderm		Prostate
Prostate			
Urethra			

The response of adult urogenital tract epithelia to inductive mesenchyme is limited by the germ layer of origin of the epithelium. Mesodermally derived epithelia give rise to seminal vesicle in response to either urogenital sinus or seminal vesicle mesenchyme. In contrast endodermally derived epithelia respond to the same inductive influences by generating prostatic tissue

demonstrated the ability of bladder mesenchyme to specifically induce bladder differentiation [73, 74].

Descriptions of bladder epithelium + UGM and bladder epithelium + rectal mesenchyme recombinants strongly suggest that the process which is occurring is not one of transdifferentiation (the change of mature adult cells from one phenotype to another) but rather one of induction of a new phenotype. Thus the bladder epithelium does not uniformly respond to the inductive effects of the mesenchyme by changing its pattern of differentiation, but rather a subpopulation of cells within the urothelial layer responds to the inductive mesenchyme and gives rise to new prostatic or intestinal glandular structures. However studies using CMFDA cell tracking have suggested that direct transdifferentiation of urothelial cells into prostatic epithelium under the influence of UGM can occur [75]. These two mechanisms, transdifferentiation and induction, of stem cell populations are (at least conceptually) not mutually exclusive, and the contribution of each phenomenon to the historic observations is presently unclear. In both the bladder and the prostate proliferative rates are naturally normally extremely low. In contrast the rectum which is a part of the gastrointestinal tract has a much higher rate of cell turnover. Thus stem cell populations must have a capacity to engage in a wide range of proliferative activity with that activity being dependent upon the organ in which the cell is finally located. Regulation of stem cell proliferation in this context is thus likely to be controlled by interactions with the local stromal microenvironment.

Like UGM the mesenchyme of the newborn rat's seminal vesicle (SVM) is a powerful inducer of glandular differentiation. In tissue recombination experiments, SVM induces seminal vesicle differentiation in the epithelial tissues derived from the seminal vesicle, ureter, and ductus deferens [76–78]. Like the seminal vesicle, ureter and ductus deferens are both derived from the Wolffian duct, which is, in turn, a mesodermally derived epithelial tissue.

Thus epithelial tissues from all three germ layers can be induced to give rise to new glandular tissues by exposure to appropriate mesenchyma. All of the experiments described above demonstrate the ability of mesenchymal cells to induce gland formation within a germ layer of origin. A further series of experiments in the urogenital tract examined the ability of mesenchymal cells to induce changes across germ layer boundaries. Tissue recombinants prepared using UGM with mesodermally derived epithelium gave rise to glandular structures with seminal vesicle morphology and secretions [79, 80]. In contrast SVM recombined with endodermally derived epithelium gave rise to glandular tissues with prostatic phenotype and secretory activity [81]. This series of experiments is summarized in Table 3. The epithelial tissues of the bladder, urethra, and ureter all have the same transitional phenotype in vivo and are essentially indistinguishable in terms of appearance and expression of differentiated markers. Yet, in these experiments they give rise to tissues reflecting their embryonic germ layer of origin. The induction of glandular morphology by UGM in human ES cells gave rise specifically to a prostatic and not to seminal vesicle phenotype [72]. This might suggest that the differentiation pathway followed that seen in normal development with endodermal structures (of which the prostatic epithelium is one) likely becoming fixed and not undergoing the epithelial to mesenchymal transformation required to generate a trilaminar embryo, and subsequent mesenchymal to epithelial transformation needed to generate a mesodermal epithelium such as that of the seminal vesicle.

Tissue recombination experiments suggest that there is a subpopulation of cells within embryonic and adult epithelial tissues which can respond to inductive mesenchyma by giving rise to new tissue types. These experiments thus demonstrate either that putative stem cells have the potential to produce a wider variety of daughter cells than are found in their tissue of origin or, alternatively, that tissues contain stem cells for more than one tissue type. On the basis of the experiments described above, this second option would suggest that the bladder, for example, contains a range of stem cell types including prostatic and rectal stem cells, in addition to its native "bladder stem cell" population. Such an explanation is unappealing both intellectually and biologically. In particular findings described in the introduction to this chapter would suggest that developmental plasticity of stem cell populations

is a reasonable explanation of this ability to repopulate multiple organs. These tissue recombination data would further suggest that changes in stromal environment are sufficient to change the developmental program executed by stem cells within the confines of their germ layer of origin.

Metaplasia and Transdifferentiation

Metaplastic changes, where cells of one tissue type take on characteristics of another, have been recognized by pathologists for many years [82]. Metaplasia demonstrates that adult epithelia retain an ability to take on a new differentiated phenotype. This may be due to true transdifferentiation (the conversion of one differentiated cell type to another) or may represent a proliferative response of stem cells implying that stem cells have wider specificity than the cells of the organs from which they are derived [8, 83]. Although these phenomena might be rare in nature, we can imagine the possibility of deliberately reprogramming cells from one tissue type to another by manipulating the expression of transcription factors, which could generate new therapies in organogenesis and regenerative medicine [84, 85].

The phenomena of metaplasia and transdifferentiation represent changes in the differentiation of epithelial tissues. Metaplasia represents the presence in a tissue of an epithelial cell phenotype not normally found in the location being examined. Many examples of metaplasia are recognized by pathologists. In many cases these are protective responses. For example, many epithelial tissues will take on a squamous phenotype following injury or persistent insult, covering and sealing the wounded surface. In other cases metaplasia may result from a chemical or hormonal insult. In the prostate, estrogen exposure results in the formation of squamous metaplasia [57, 86–89]. In human prostate cancer patients, treatment with synthetic estrogens gives rise to squamous metaplasia, a phenomenon now rarely seen in countries where this treatment regimen has been discontinued. In utero exposure to female sex hormones, notable diethylstilbestrol, also results in squamous differentiation [90]. Metaplasia can be a benign protective response to a particular insult. However metaplasia is often seen as a premalignant condition because the insult which gave rise to a metaplastic response can persist and induce malignant transformation. Thus, for example, cigarette smoke induces stratified squamous metaplasia in the trachea and bronchi. Continued smoking leads to malignant transformation to squamous carcinoma in the respiratory tract. It is noteworthy that all of the clinically observed metaplastic changes are apparently restricted to the repertoire of the germ layer from which the epithelium is originally derived.

Metaplasia can be a result of proliferation of a stem cell population to give rise to daughter cells with an inappropriate or abnormal phenotype. Metaplasia includes the more restrictive class of cellular changes known as transdifferentiation. Unlike other forms of metaplasia, in which tissues can result from proliferation of stem or transitional/amplifying cell populations, transdifferentiation is defined as "an irreversible switch of one type of already differentiated cell to another type of normal differentiated cell" [8, 91]. It should be noted that many papers use the word transdifferentiation without due regard for its strictly defined meaning. While there are limited documented examples, transdifferentiation occurs during development [92] and in some specialized laboratory situations such as in cell culture [93]. However in normal and benign adult disease, transdifferentiation is not common. In malignant disease epithelial to mesenchymal transitions (EMTs) are well documented. However it can be argued that these fall outside of the definition of transdifferentiation because they do not result in the formation of a normal differentiated cell. In the context of the present communication, transdifferentiation is apparently not relevant to stem cell biology since, in contrast to other forms of metaplasia, it involves only previously differentiated adult cells.

Conclusions

In this brief overview we have attempted to highlight some of the available data suggesting a high degree of plasticity in adult glandular epithelial stem and progenitor cell populations with the interaction of surrounding stromal microenvironment. We note that glandular epithelial tissues are capable of changing their pattern of differentiation under both naturally occurring and experimental conditions. These data suggest that the glandular epithelial tissues contain a cell population, GSC, which is capable of generating epithelial tissue characteristic of multiple organs. Thus we would support the contention that the idea of organ-specific stem cells may no longer be viable. Rather available data suggest that GSCs are capable of repopulating many of the organs of their germ layer of origin with relative ease. It is certainly clear that signals from inductive mesenchyma can elicit this response although at present specific molecular pathways that result in the induction of any given gland are unknown.

Recent data would also suggest a cellular plasticity that allows cells to repopulate tissues even beyond their germ layer of origin. However the mechanism by which this phenomenon may occur is presently unclear. Current models of differentiation suggest that fully differentiated and fully plastic states are defined by expression of specific transcription factors by cells [7, 83]. Such models predict that cells have a capacity to move between these states. It is further suggested that the degree of difficulty involved in moving between differentiated and plastic states is a function of the

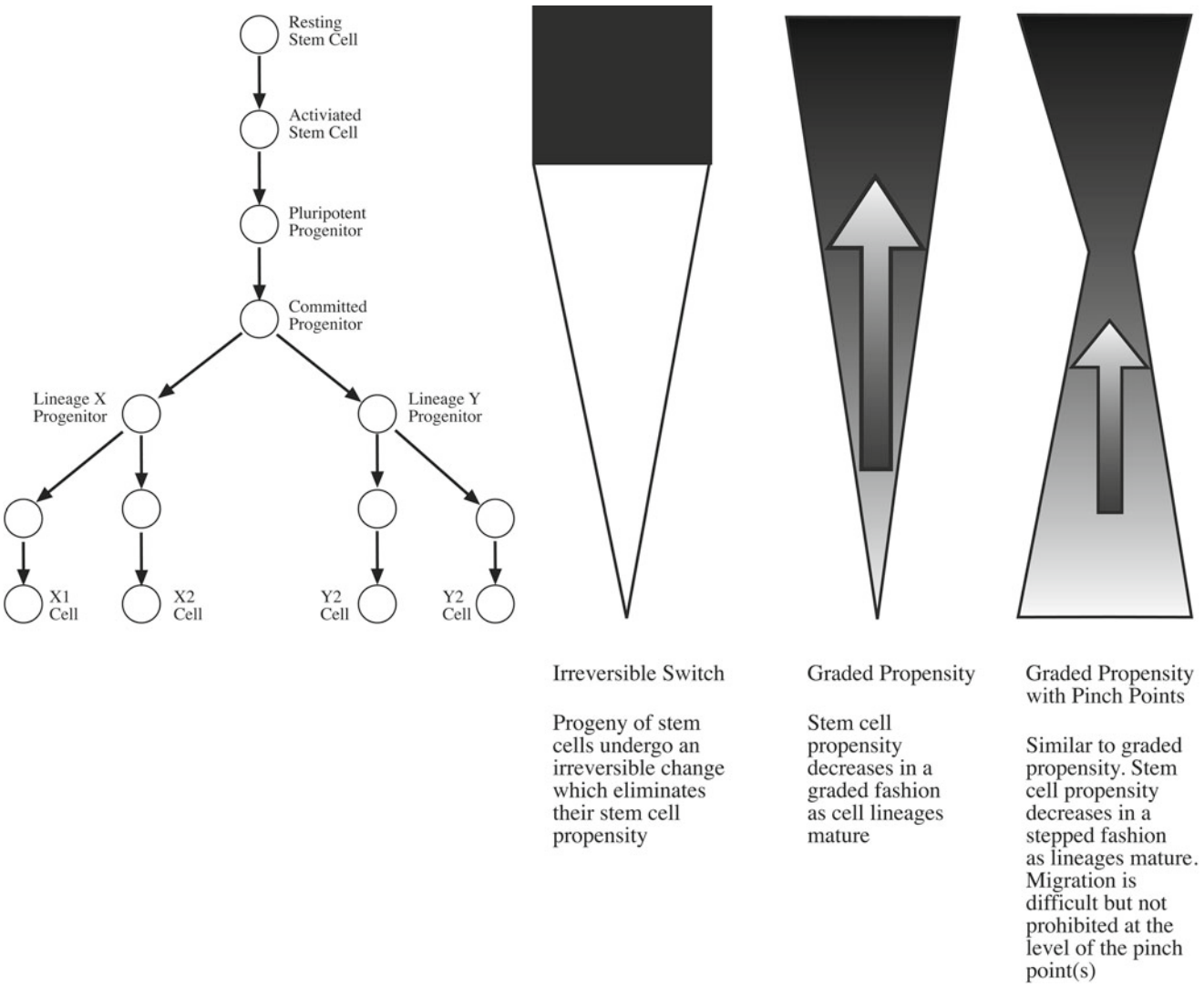

Fig. 3 Concepts of the propensity of cells to migrate between totipotent and committed cell types. Traditional views of cell maturation suggest that at some point on the differentiation pathway, cells undergo an irreversible switch and are no longer able to dedifferentiate. More contemporary views suggest that there is a gradation between totipotent embryonic stem cells and fully differentiated adult cells which can be traversed in either direction. Increased commitment is associated with expression of tissue-specific transcription factors. The energetic and biological costs associated with transformations from differentiated to totipotent cell types apparently make this sort of event unlikely. We propose that this graded propensity model contains discreet pinch point(s). Movement from below the pinch point to above is more restricted than other forms of change from a committed to stem cell phenotype. An example of such a pinch point might be a change across a germ layer boundary (Redrawn and modified from Blau et al. [7])

distance moved (see Fig. 3). Data from tissue recombination experiments suggest that exposure to inductive mesenchyma is insufficient to force transdifferentiation of committed epithelial cells but is sufficient to stimulate a stem cell population to generate daughter populations different from the tissue of origin. The process of transdifferentiation, while it is well documented, is apparently not common supporting the idea that moving from one differentiated state to another, while possible, is difficult. Data from tissue recombinants and from naturally occurring metaplastic responses further suggest that crossing the germ layer boundary is a significantly more difficult step than moving differentiation patterns within a germ layer. Thus the mechanisms required to shift between germ layers are apparently different and perhaps more fundamental than reprogramming within a layer. Thus the germ layer might represent a "pinch points" in the continuum from totipotent to committed cells.

Thus we return to the question "what is a glandular stem cell?" The data presented would suggest that GSCs occurring in adult glandular organs are simply the least differentiated and most plastic epithelial cell type found in adult glands, suggestive of progenitor cell properties. Adult glandular epithelial stem and progenitor cells exist towards one end of a continuum between totipotent ESC and differenti-

ated adult epithelium. They can easily repopulate a range of glandular organs within the confines of a given germ layer.

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Human Mammary Epithelial Stem/ Progenitor Cells

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Abbreviations

ALDH	Aldehyde dehydrogenase
α-SMA	Alpha smooth muscle actin
CALLA	Common acute lymphoblastic leukemia antigen
CK	Cytokeratin
EGF	Epidermal growth factor
EMA	Epithelial membrane antigen
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ESA	Epithelial-specific antigen
HIM	Human-in-mouse
HMEC	Human mammary epithelial cell
MaSC	Mammary stem cell
ME	Myoepithelial
MRUs	Mammary repopulating units
MUC1	Mucin-1
PR	Progesterone receptor
TDLU	Terminal ductal lobular unit
TEB	Terminal end bud
vHMEC	Variant human mammary epithelial cell

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Human Mammary Development and Architecture

The human breast epithelium is organized into 5–10 lobes that radiate outward from the nipple. Each lobe contains a branching epithelial network of ducts leading to the functional structures of the breast, the terminal ductal lobular units (TDLUs), which are comprised of numerous alveolar sac-like structures. The bilayered epithelium consists of two main lineages: luminal and basal/myoepithelial (ME) cells. The luminal lineage contains polarized cells that line the lumens of the ducts and alveolar luminal cells that comprise the inner layer of the lobular component of the tissue and perform the secretory functions of the gland during lactation. Cells within the basal/ME lineage are basally positioned in the tissue, between the luminal epithelial layer and the basement membrane that separates the epithelium from the stromal compartment. In response to hormonal cues, fully differentiated myoepithelial cells have contractile functions that serve to squeeze secretions from the alveolar cells and move them along the ductal tree to the nipple.

During prenatal development in both mouse and human tissues, the mammary epithelium develops from the surface ectoderm when cues derived from the underlying primordial mesenchyme specify the formation of a mammary bud-like structure that subsequently invades into the mesenchymal tissue [1]. In humans, both male and female fetuses sprout rudimentary ducts from the primary mammary bud that further develop into a variety of structures, ranging from simple blunt-ended ducts to well-developed lobular structures that reflect the influence of maternal hormones in the perinatal period [2]. Following birth and the loss of circulating maternal hormones, the tissue involutes to simple ductal structures that remain until puberty in both males and females in humans. In females, the hormones produced during puberty allow for further development of the tissue, signaling for ducts to elongate and branch and TDLUs to form [1, 3]. The TDLUs continue to develop and mature through each successive menstrual

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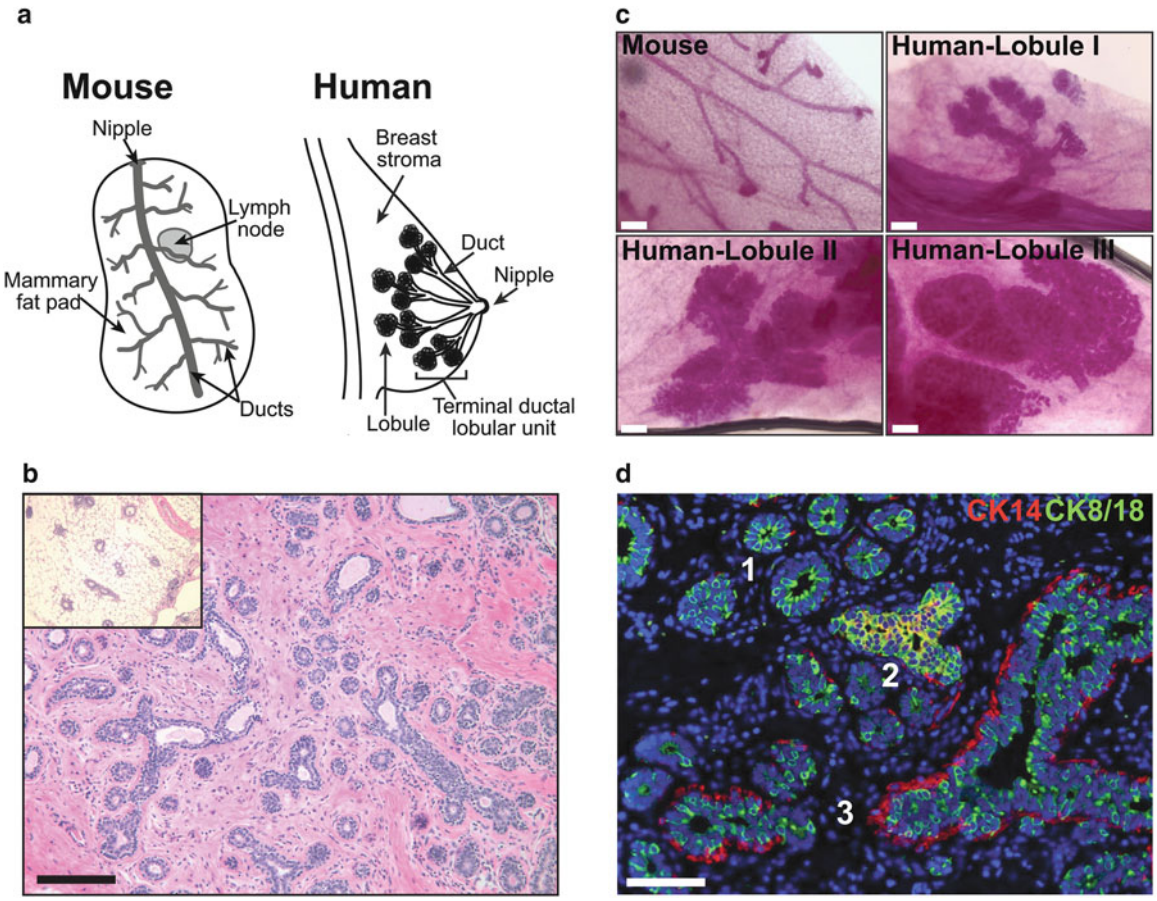

Fig. 1 Distinctions between mouse and human mammary tissue. (**a**) Schematic representations of mouse and human mammary architecture. (**b**) Hematoxylin- and eosin-stained sections of mouse (inset) and human breast tissue, illustrating the differences between the adipose-rich mouse and the collagen-/fibroblast-rich human stromal compartments (bar = 200 μm). (**c**) Whole mounts of tissue from nonpregnant mouse and human mammary glands stained with carmine alum dye. Lobules are much more complex and varied in human mammary tissue (bar = 200 μm). (**d**) Lobular and ductal structures in human mammary tissue show much more heterogeneity in expression of lineage-specific keratins than the mouse. Some regions show little expression of CK14 (1), are double positive for CKs 14 and 8/18 (2), or show restricted expression of basal/ME CK14 and luminal CK8/18 (3; bar = 100 μm)

cycle, and full differentiation of the epithelium occurs during pregnancy and lactation as the TDLUs expand and dilate to support the production of milk [1, 3].

The mouse is the primary model system for the study of stem/progenitor cell activity during mammary development, adult homeostasis, and the proliferation and differentiation needed during pregnancy and lactation. While there is much similarity in the general features of the mouse and human epithelium (both are bilayered branching ductal/alveolar structures), there are also key differences that may point to altered stem/progenitor cell activity and regulation [4]. In contrast to the radial arrangement of multiple lobes of branched epithelium in humans, the mouse mammary gland is composed of a single, simple ductal tree that lacks TDLUs (Fig. 1a). In the absence of pregnancy, only strain-specific rudimentary alveolar budding occurs; pregnancy is required for full lobular-alveolar development in mice [4]. The stromal support network for the epithelial tissue is also quite distinct in mice and humans (Fig. 1b). In contrast to the adipose-rich stromal compartment of the mouse mammary gland, the human breast epithelium is embedded in a collagen-rich stroma, where two fibroblastic compartments can be observed: the intralobular stroma immediately surrounding individual lobules and the denser interlobular stroma found between lobules. Additional cell types such as adipocytes, endothelial cells, and hematopoietic cells also provide support to the epithelium [1, 5].

There are also likely differences between mice and humans in the hormonal regulation of stem and progenitor activity. The breast epithelium is regulated by the actions of estrogen, progesterone, and prolactin, which oscillate throughout the menstrual cycle and play important roles during pregnancy and lactation. During mammary development in the mouse, estrogen and progesterone primarily regulate ductal elongation and branching while prolactin plays an essential role in alveolar development during pregnancy; these hormones may play analogous roles in human breast tissue, but it is much more difficult to study and has not been

well elaborated [3]. For tissue homeostasis, the estrous cycle of the mouse differs significantly from the menstrual cycle in humans. Estrogen levels are highest during the estrus phase in the mouse, which corresponds to peak proliferation within the mammary gland. In contrast, peak proliferation within the human mammary gland correlates with the luteal phase of the menstrual cycle, in which progesterone and estrogen are both active [3]. Unlike mice, human prolactin acts at both the endocrine and autocrine/paracrine levels, as human mammary epithelial cells (HMECs) produce prolactin endogenously, although the regulation of this secretion is not well understood [6]. The proliferative cells within mouse and human mammary tissue, as well as potential stem/progenitor cells identified in mouse tissue, are hormone receptor negative [3, 7], thus pointing to the importance of both paracrine signaling within the gland and the differences in hormonal regulation of mammary proliferation in mouse and human tissue for stem/progenitor cell regulation.

Lastly, the lobule architecture of human breast tissues is much more complex, is highly variable from woman to woman, and reflects factors such as pregnancy (parity status). Four major types of lobules, proposed to represent a developmental continuum, have been described from whole mount analysis of human breast tissues from parous and nulliparous women (Fig. 1c). Type I lobules are thought to be the least differentiated and consist of an average of 11 immature alveolar buds. These structures are then thought to further develop with each successive menstrual cycle and pregnancy into larger and more mature lobules, Types II and III, consisting of an average of 47 and 80 alveolar ductules, respectively. Type IV lobules would represent the fully differentiated lactating lobule [8, 9]. Nulliparous women, where the overall breast tissue is less differentiated, harbor primarily the least mature lobules (Type I) in contrast to parous women, whose breast tissue contained a higher proportion of well-developed Type III lobules [8, 9]. Comparison of noncancer-associated breast reduction mammoplasty specimens to tissues from women prone to hereditary breast cancers (carriers of BRCA1 mutations), as well as tissues from women with a diagnosed breast cancer, indicated that there was an enrichment of Type I lobules in the cancer-associated or BRCA1 carrier tissues [9]. Given that Type I lobules are thought to be the least differentiated, this supports the idea that stem/progenitor cells, presumed to be enriched in Type I lobules, may be the likely targets for breast cancer development.

Factors that affect TDLU biology, such as parity, lifetime exposure to hormones, and changes in the stromal microenvironment in human breast tissues, are known etiological factors associated with increased risk of breast cancer development. TDLUs have been proposed to serve as the anatomical origin for the majority of human breast cancers [10]; thus, it is important to understand how the differences in hormonal cycling, stromal microenvironment, and anatomical development between mice and humans contribute to regulation of stem/progenitor activity in TDLUs in human tissue and how this becomes disrupted under pathological conditions.

Mammary Lineage Markers

Immunohistochemical studies of prenatal, pubertal, and both normal and cancerous adult tissues have been instructive for identifying markers of the luminal and basal/ME lineages in the breast. Keratins are some of the most common markers used to distinguish the lineages; in general, luminal lineage cells are characterized by expression of simple cytokeratins (CKs) 7, 8, 18, and 19, while basal/ME lineage cells are characterized by expression of stratified epithelial CKs 5, 14, and 17 [11, 12]. During human mammary development the primary mammary bud is initially CK14 and CK19 negative, but during the secondary bud stage, cells show reactivity to both CK14 and CK19 as well as other basal/ME lineage markers such as p63, α-6-integrin (CD49f), CK17, and α-1-integrin (CD29) [13]. Nearer to birth, CK14 and CK19 expression becomes more lineage restricted, and the basal/ME layer begins to show expression of terminal differentiation markers such as α-smooth muscle actin (α-SMA) [12]. Hormone receptors for estrogen (ER) and progesterone (PR) are important markers of the luminal lineage in the adult tissue, but their expression in the developing breast has not been extensively studied. From the small number of samples examined, expression appears to be low until puberty; in general, in adult tissues PR expression seems to be higher than ER [14]. Other markers that have been used to characterize the luminal lineage include epithelial cell adhesion molecule (EpCAM, also known as epithelial-specific antigen, ESA), CD24, and mucin-1 (MUC1, also known as epithelial membrane antigen, EMA). Common acute lymphoblastic leukemia antigen (CALLA, also known as CD10) and THY1 are used to characterize the basal/ME lineage.

While in general, the lineage markers are expressed similarly in mouse and human tissues, there are some critical differences. CD24, which marks luminal lineage cells exclusively in humans, is more of a pan-epithelial marker in the mouse tissue [15]. In addition, CK8/18/19 and CK5/14 have strict lineage restrictions in the mouse tissue to luminal and basal/ME cells, respectively. However, in human TDLUs and ducts, there is much more variability, with expression of CK14 seen in luminally positioned cells, variable expression of CK14 in basal/ME cells in lobules versus ducts, and luminal epithelial cells that are CK19 negative, suggesting there is potentially more complexity to the cell types contained within the two lineages in human tissue [16, 17] (Fig. 1d). Further supporting this complexity, there is also evidence that there are specific markers that can potentially differentiate luminal and basal/ME cells in the different structural regions of the breast tissue (i.e., in ducts and lobules), such

as CK6a and CK17 showing preferential expression in luminal and basal/ME cells of ducts and BCA-225 and WT1 marking the same lineages in lobules [16].

Defining the Mammary Hierarchy

Mammary stem and progenitor cells clearly play roles in both the prenatal and postnatal development and maintenance of the human breast, as well as in the regeneration of the tissue through repeated pregnancies. The simplest form of the epithelial hierarchy of the mammary gland is proposed to consist of a stem cell at its apex that gives rise to a bi-potent progenitor cell that then in turn gives rise to luminal and basal/ME lineage-committed progenitors that differentiate to form the functional cells of the luminal epithelial and myoepithelial bilayer (Fig. 2a). In the mouse, alveolar progenitors, which are induced during pregnancy, have also been described to maintain alveolar development during successive cycles of pregnancy and lactation [18]. Whether such cells exist in human tissues during pregnancy or whether these cells exist in nonpregnant human tissue is unclear. However, since TDLUs undergo maturation and development throughout the life of women, it is likely that there is a role for alveolar progenitor cells in regulating this process.

For mouse epithelium, the presence and functional activity of potential stem and progenitor cells has been elucidated through the use of two methods: (1) the mammary fat pad transplantation assay, pioneered by DeOme et al. [19], where tissue fragments or dissociated cells can be implanted in a secondary host to regenerate a mammary tree, and (2) lineage-tracing experiments with genetically engineered mouse strains where markers such as green fluorescent protein or LacZ are expressed from lineage-specified promoters [18, 20]. In addition, mammary tissue can be readily obtained from multiple embryonic and postnatal developmental stages for study. During embryonic development, mouse mammary multipotent stem cells are enriched within the mammary bud and expand dramatically between embryonic days 13.5 and 18.5 to participate in the establishment of luminal and basal/ME lineages [21]. Following birth, postnatal multipotent or bi-potent mammary stem cells (MaSCs) were proposed to be localized within the specialized terminal end bud (TEB) structures that mediate ductal elongation through the fat pad [15]. However, recent evidence from lineage-tracing experiments indicates that bi-potent progenitor cells do not contribute to the maintenance of the gland after the initial postnatal period; instead, lineage-restricted progenitor cells are responsible for the bulk of adult tissue homeostasis [20]. This suggests that bi-potent progenitor activity ascribed to

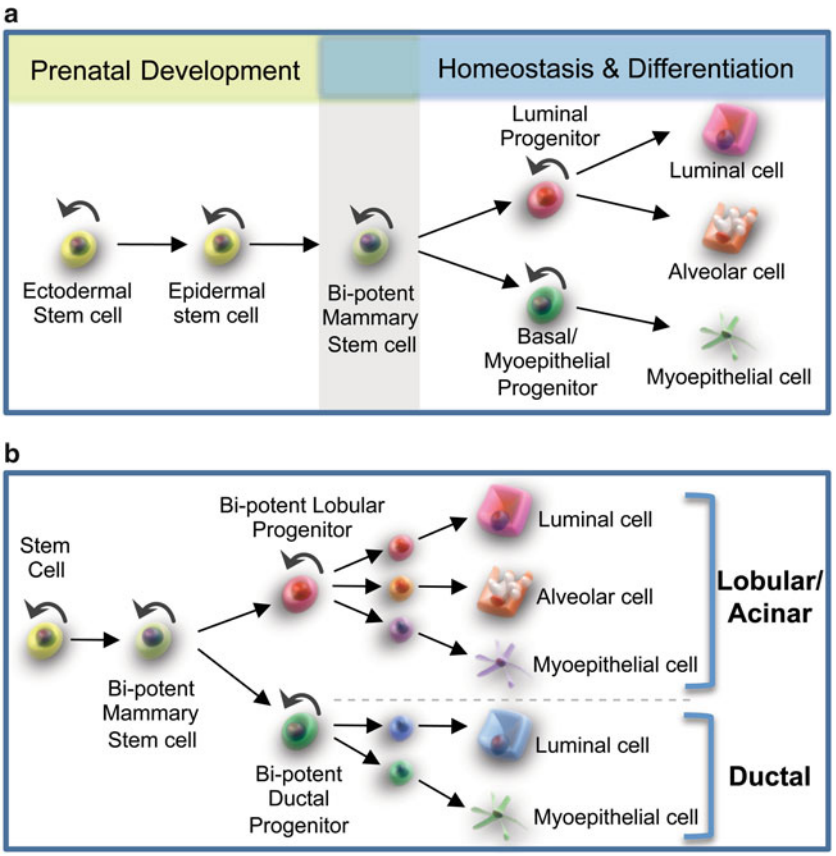

Fig. 2 Human mammary epithelial hierarchy. (**a**) The mammary epithelium has developmental origins in surface ectoderm and epidermal tissue and, in its simplest expression, is thought to be maintained by an epithelial hierarchy whereby a bi-potent progenitor cell gives rise to luminal and basal/myoepithelial lineage-committed progenitors to maintain the differentiated cells in the adult breast tissue. (**b**) The complete human mammary epithelial hierarchy has not been fully defined, but likely contains additional levels of complexity, such as structurally oriented progenitors (lobular and ductal) and additional intermediates that have yet to be identified

adult-derived tissues may be a result of transplantation challenge or in vitro culture. Despite their potentially limited relevance to adult homeostasis in situ, transplantation assays have indeed indicated the presence of adult tissue-derived multipotent and bi-potent progenitor cells that can generate mammary outgrowths from a single cell in the mouse [22–24]. Bi-potent progenitors have also been identified that specify morphological differentiation in the mouse, i.e., alveolar-limited and duct-limited bi-potent progenitors, suggesting that the mammary epithelial hierarchy should also encompass structural differentiation capacity in addition to luminal and basal/ME differentiation [25] (Fig. 2b).

In contrast to the wealth of tools to study the contribution of stem/progenitor cells in mouse mammary development, for early human mammary development, researchers are restricted to drawing inferences from whole mount or immunohistochemical staining of archival tissue, of which there is limited material. In adult tissue, X-linked marker inactivation patterns have suggested the presence of multiple stem cells contributing to lobule formation throughout the human breast [26], but to demonstrate stem/progenitor activity involved in adult tissue homeostasis in humans, in situ lineage-tracing and orthotopic transplantation experiments are not feasible. Thus, studies of stem/progenitor activity have been based on analysis of sorted populations of cells to identify in vitro bi-potent progenitor activity through colony formation assays in 2D and 3D culture and, in limited uses, modified in vivo transplantation assays such as the human-in-mouse (HIM) orthotopic transplantation model and kidney capsule implantation, both of which rely on human mammary fibroblasts to provide engraftment support [27, 28]. The starting material for all of these studies is tissue derived from breast reduction mammoplasty surgery, where epithelial cells can be isolated from either organoids (small clusters of epithelial tissue) or single-cell suspensions through serial digestion steps with the enzymes collagenase, hyaluronidase, and trypsin; single-cell suspensions can be further separated into enriched populations of cells through either immunomagnetic bead or fluorescence-activated cell sorting (FACS) techniques. The major caveat for human tissue-based studies is that, aside from than ages of the patients from which they are derived (so that experiments can be conducted with largely premenopausal tissue, <50 years old), there is often little other information known about the tissue. Thus, experiments cannot be controlled for parity status, menstrual cycle phase, or hormonal birth control use—factors known to contribute to changes in the behavior and state of the tissue. In contrast to inbred mouse strains, there is also tremendous genetic heterogeneity between patient samples, leading to considerable patient-to-patient variability in the behavior of cells that can complicate interpretations of results.

Stem/Progenitor Populations: Bi-potent Differentiation

With the gold-standard technique of mammary fat pad transplantation and the use of limiting dilution analysis coupled with multi-protein flow cytometry to sort freshly dissociated tissue, populations of mouse mammary cells can be assessed for their ability to reconstitute a functional mammary tree. Sorted populations that form extensive outgrowths from low numbers of injected cells are described as containing mammary repopulating units (MRUs) or MaSCs. In the mouse, MaSCs are contained within populations defined by the expression profiles of $Lin^{-}Sca1^{lo}CD24^{med}CD49f^{high}$ or $Lin^{-}CD29^{hi}CD24^{+}$; the marker profiles of these populations are consistent with them being associated more closely with basal/ME differentiation states [23, 24] (Fig. 3a). For human epithelial outgrowths, given the limitations described above, stem/progenitor activity has most often been assessed in vitro as "bi-potent progenitor activity," the ability to differentiate into cells of both lineages, most frequently defined by expression of the luminal (CK8/18/19) and basal/ME (CK5/14) keratins (Fig. 3b). Through these types of analyses, populations of cells that contain differentiated luminal, luminal progenitor, and MaSC-like cells have been prospectively identified from human tissues; a recent comparison of these populations to those established from the mouse indicates significant transcriptomal overlap between these populations in both species [29].

Several groups have used immunomagnetic beads or FACS to isolate human epithelial populations to assess bi-potent progenitor activity. The most commonly used markers are the luminal markers EpCAM or EMA/MUC1 and the basal markers CD10, THY1, and CD49f [16, 28, 30–35]. Bi-potent differentiation has been defined most often as (1) the ability to form colonies showing luminal and basal differentiation in 2D culture conditions, usually on collagen-coated plates in serum-free growth conditions, and (2) the ability to form branched TDLU-like structures in Matrigel outgrowth assays (Fig. 3b). A few groups have also tried to assess bi-potent differentiation in vivo through use of a humanized mouse model system or kidney capsule implantation [28, 35–37]. Colonies formed in 2D with bi-potent differentiation primarily have two morphologies: either a ring of "myoepithelial"-like cells (staining positively for myoepithelial markers such as CK14) surrounding a core of luminal cells (staining positively for luminal markers such as CK18) or a colony with a mixed population of cells that show single-positive and double-positive staining for both lineage markers (Fig. 3b). In addition to flow cytometry for surface markers, alternative methods of assessing stemlike behavior such as the ability to grow in nonadherent mammosphere

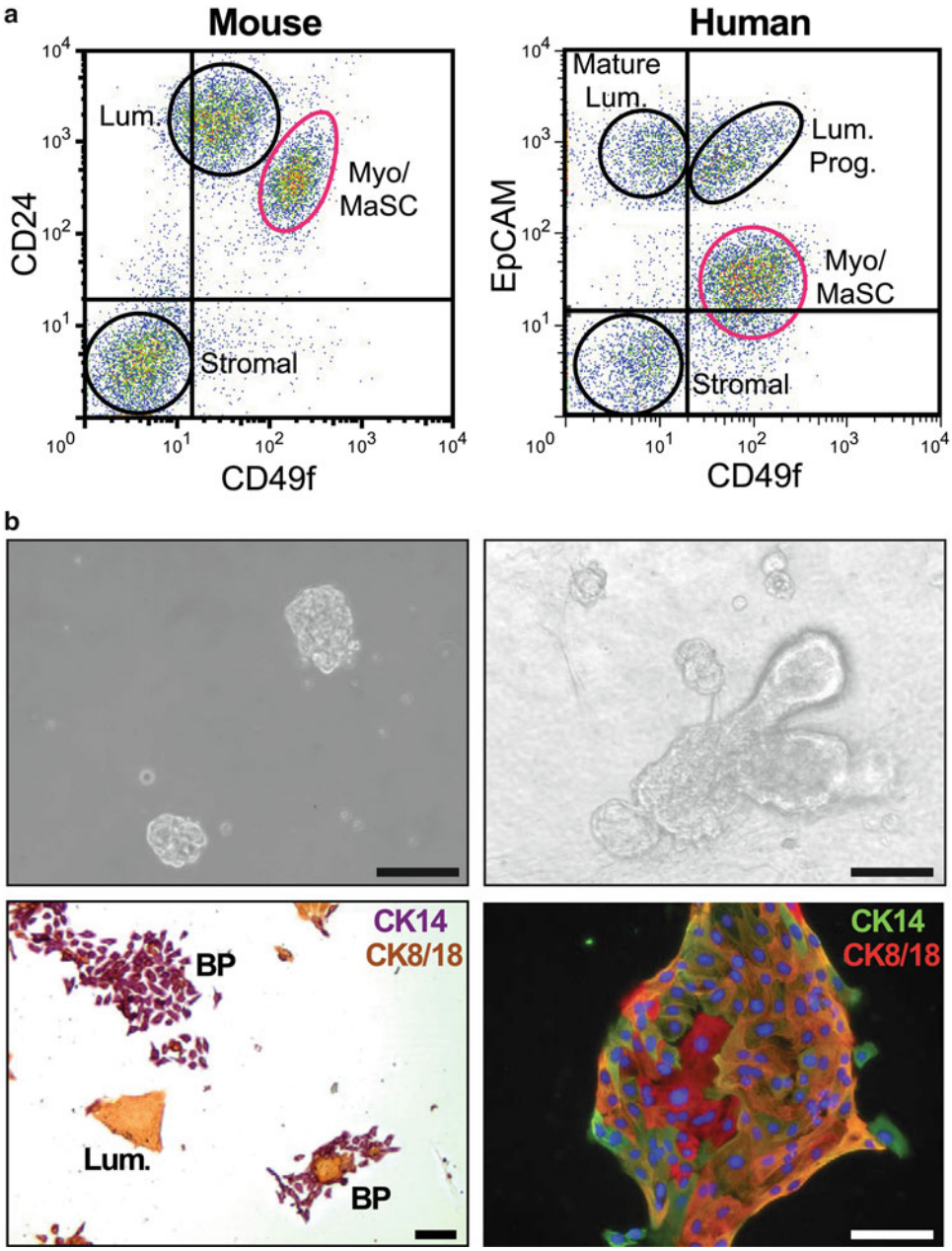

Fig. 3 Identifying progenitor activity in human breast epithelial cells. (**a**) Flow cytometry is the most common technique used to isolate different populations of mammary epithelial cells. For both mouse and human cells, MaSC/bi-potent progenitor activity has been demonstrated most frequently from the basal/myoepithelial population: $CD24^{+}CD49f^{hi}$ (mouse) or $EpCAM^{lo/-}CD49f^{+}$ (human). (**b**) For HMECs, bi-potent progenitor activity has been ascribed to cells that can generate mammospheres in nonadherent culture conditions (*top*, *left*), branching and budding structures in Matrigel 3D colony formation assays (*top*, *right*), and/or differentiate into cells expressing markers of both lineages in 2D culture (*bottom*, *right,* and *left*). Colonies demonstrating bi-potent differentiation (BP) have two characteristic morphologies (*bottom*, *left*), presenting either with a mixed population of $CK14^{+}$, $CK8/18^{+}$, and double-positive cells or with a core of $CK8/18^{+}$ cells ringed by $CK14^{+}$ cells (bar = 100 μm)

culture, Hoechst dye-effluxing side-population cells, long-term lipophilic dye-retaining cells, or the expression of aldehyde dehydrogenase (ALDH1), a marker associated with stem cells in hematopoietic and neural stem cell populations, have also enriched for cells with bi-potent progenitor activity [33, 36–38], though it is still somewhat controversial as to which types of cells are being marked with these methods.

In general, populations containing cells with bi-potent progenitor activity have been described as having marker profiles that suggest basal/ME differentiation ($EpCAM^{lo}CD49f^{+}$, $EpCAM^{+}MUC^{-}$, $EpCAM^{-}CD49f^{+}$, $EpCAM^{+}CD10^{+/-}MUC^{-}$, and $EpCAM^{+}CD49f^{+}CD10^{+}THY1^{+}$), consistent with the mouse mammary literature. However, other groups have found that cells within populations that can be described as

having a luminal progenitor phenotype ($EpCAM^{hi}CD49f^{+}$) can also have bi-potent progenitor activity [16], consistent with early reports that had suggested that cells with a luminal differentiation state can differentiate into myoepithelial-like cells in culture [39, 40]. Additionally, at non-limiting dilution conditions, populations of cells that have both luminal ($EpCAM^{+}CD10^{-}$) and basal/ME traits ($CD10^{+}$) were both shown to have bi-potent differentiation capacity in vivo in a humanized mouse model, suggesting that cells within both lineages could have bi-potent differentiation capacity [41]. The confusion in the field likely stems from both an overly simplistic view of the mammary hierarchy and differences in experimental conditions. As the two most commonly used markers, EpCAM and CD49f, are also used to identify populations of mature luminal and basal/ME cells; no unique set of cell surface markers can currently discriminate progenitor cells from more differentiated cells contained within sorted populations. Thus, it is unclear which cells are actually being assessed with current sorting strategies.

Genetic manipulation of mice has allowed for much investigation into pathways involved in mammary development [15]. Given the limited tools available, there has been much less investigation into the signaling pathways that may be involved in differentiation state transitions within the human mammary hierarchy. One approach has been to use the mammosphere assay, concurrent with ALDH1 expression, as a readout of MaSC maintenance and expansion in the presence of signaling pathway modulation. Through the use of this approach, it has been shown that a hedgehog-Gli1-Bmi1 signaling axis, HER2 overexpression, and an Akt/Wnt signaling axis (mediated by knockdown of PTEN) can promote expansion of both primary and serially passaged mammospheres, as well as $ALDH1^{+}$ cells [42–44]. BRCA1 has also been implicated in progenitor cell maintenance. Knockdown of BRCA1 in primary breast epithelial cells led to an expansion of undifferentiated and $ALDH1^{+}$ cells, and studies of prophylactic mastectomy tissues from BRCA1 mutation carriers indicate increased basal differentiation in luminal cells both in vitro and in vivo, suggesting BRCA1 may regulate differentiation of luminal progenitor cells [45, 46]. This regulation may occur in part through inhibiting the expression of SLUG, knockdown of which has been shown to increase luminal differentiation in primary breast epithelial cells and cultures [46]. Notch proteins have also been implicated in regulating the bi-potent progenitor to luminal progenitor transition through analysis of cultured cells derived from primary tissues. Cells from the $EpCAM^{lo}CD49f^{+}$ ($THY1^{+}CD10^{+}$) bi-potent progenitor population could be induced to form colonies with reduced luminal differentiation and increased basal/ME differentiation in the presence of downregulated Notch3 signaling [34]. Similarly, it was shown that in $CD10^{+}$ populations, Notch can downregulate ÄNp63, which was shown to be critical to maintain basal/ME identity and promote luminal differentiation, further implicating Notch signaling in control of luminal differentiation state [47]. Lastly, organoid cultures indicate that HER1/epidermal growth factor (EGF) receptor ligands differentially regulate differentiation. Amphiregulin, along with fibroblast growth factor receptor ligands, contributes to normal maintenance of ductal structures, but EGF specifically expands cells with basal/ME differentiation, potentially by expanding cells with bi-potent differentiation and then directing them to differentiate along the basal/ME lineage [48]. While our understanding of pathways controlling the human mammary hierarchy is still incomplete, these results, along with transcriptomal comparisons of isolated mouse and human progenitor populations, suggest that there is considerable overlap between the two species**.**

Cultured Cell Lines and Their Relationship to the Mammary Hierarchy

It has long been evident that cultured HMECs of the luminal and basal/ME lineages have differing requirements in vitro. Cells expressing luminal keratins show limited growth capacity in culture in general but tend to be supported for a few passages in media containing serum [39, 49, 50]. In contrast to the limited growth of luminal lineage epithelial cells in culture, much greater expansion can be seen for basal/ME lineage cells, which are the predominant cells that grow out in the most commonly used serum-free defined media formulations [51]. Given the limited growth of luminal epithelial cells in culture, there still does not exist a robust manner in which to study luminally differentiated cells in vitro. This may have severe limitations on our understanding of the potential bi-potent differentiation capacity of cells within this lineage, since the majority of bi-potent progenitor activity for human cells is assessed in vitro using culture media that supports basal/ME cell expansion.

HMECs cultured for an extended period in vitro under serum-free conditions undergo a characteristic growth curve where, after a period of proliferative growth for several passages, cells enter a stasis period for about 2 weeks, which then leads to the formation and expansion of colonies of small refractile cells termed variant HMECs (vHMECs) [52]. These cells show expression of markers consistent with a relatively undifferentiated basal-like cell, in that they highly express basal CK14 but show limited expression of luminal keratins such as CK8/CK18 and show no expression of more differentiated markers of basal/ME or luminal differentiation such as α-SMA, CK19, or ER [49, 51]. vHMECs exhibit significant differences in gene expression profiles, lineage markers, and chromatin methylation states compared to primary HMECs and are characterized by methylation of p16 [52–54]. It has been proposed that vHMECs may represent

MaSCs [51], and indeed, cells that go on to become vHMECs appear to be contained within the population that has been characterized as containing MaSC [41]. In addition, vHMECs were demonstrated to have metaplastic abilities when cultured in an in vitro epidermal differentiation assay, in that they were able to form stratified epidermal-like tissues, suggesting that the epigenetic changes that accompany the formation of vHMECs leads to a partial loss of mammary specification and a gain of the ability to access more primitive differentiation states, such as those of the epidermal precursors to mammary tissues during development [41]. Additional studies are necessary to determine whether epidermal progenitor cells exist in an uncommitted state within adult human breast tissues or whether the acquisition of epidermal progenitor-like features may be due to genetic and/or epigenetic events that take place during formation of vHMECs in long-term culture.

The Mammary Hierarchy and Breast Cancer Heterogeneity

The definition of the mammary hierarchy in human breast tissue, while incomplete, provides a framework for understanding human breast tumor heterogeneity. Ductal carcinomas are broadly categorized into two types, ER+ and ER− tumors, but can be further subdivided molecularly and histologically into subtypes with different prognostic outcomes and therapeutic sensitivities [11, 55]. Molecular classification of tumors has shown that ER+ and ER− tumors generally retain expression of markers of the two major differentiation states of normal human breast tissue: ER+ tumors, which encompass the molecular subtypes luminal A and B, express hormone receptors and genes characteristic of luminal epithelial cells (e.g., CK8/18, CK19, CD24, MUC1, GATA3, EpCAM); in contrast, ER− tumors, which encompass the molecular subtype basal-like, retain characteristics of epithelial cells that lack estrogen-responsive genes and express markers characteristic of basal/ME cells (e.g., SMA, CD49f, p63, CK14, EGFR, CD44). ER− status is also found in rarer types of cancers, such as medullary, adenoid cystic, metaplastic carcinomas, and the recently described molecular classification of claudin-low type tumors, where the tumor cells not only lack ER-responsive and luminal genes but also exhibit features of alternate cell types not found in normal breast epithelium [56, 57].

Given the reflection of the normal breast lineages in tumor subtypes, two main hypotheses have been put forward to explain breast tumor heterogeneity: (1) that ER+ and ER− tumors arise from transformation of different lineage-committed progenitors (namely, luminal and basal/ME progenitors) or (2) that specific genetic and epigenetic events arising in a common cell of origin drive tumor differentiation to generate tumor subtypes [58]. Though it is likely that specific genetic mutations and epigenetic changes also play a role in tumor differentiation, recent evidence suggests that different cells of origin also likely contribute to the formation of tumor subtypes. Molecular gene expression profiling of isolated human breast populations ($EpCAM^+CD49f^-$ differentiated luminal, $EpCAM^+CD49f^+$ luminal progenitor, and $EpCAM^{-/lo}CD49f^+$ basal/myoepithelial/MaSC) and comparison of this data to the gene expression profiles of the intrinsic breast cancer subtypes indicate that, as expected, differentiated luminal cells match most closely with the most differentiated tumor types, luminal A and B [35]. In contrast to the hypothesis that basal progenitors would give rise to basal-like breast cancers, it was the luminal progenitor gene expression signature that was most enriched in expression data from basal-like breast cancers [35]. This may be reflective of the fact that tumors with basal differentiation (expression of CK5/6 and 14) for the most part do still show expression of luminal epithelial keratins such as CK8/18, and the vast majority of human breast tumors express the differentiated luminal keratin CK19 [11, 17].

The luminal progenitor origin of basal-like breast cancers is also supported by recent data from mouse and human experimental systems that suggests that the cells of origin in BRCA1-associated breast cancer, known to be associated with basal-like breast tumors, are luminal progenitor cells and that these cells may have impaired differentiation due to the loss of BRCA1 [35, 46, 59]. Adding to this, for nonmutant BRCA1 tissue, transformation of $EpCAM^+CD10^-$ luminal epithelial cells gave rise to both ER+ and ER− tumors that had characteristics of both luminal and basal-like differentiation, and ER− tumors were enriched from transformed cells derived from the luminal progenitor $EpCAM^+CD10^-CD49f^+$ population [41]. In contrast, transformation of the $CD10^+$ basal/myoepithelial population gave rise to tumors that were ER− and had squamous differentiation as well as decreased CK19 expression; molecular profiling of these tumors indicated that their gene expression profile matched most closely with rare subsets of ER− tumors, such as claudin-low and metaplastic breast cancers [41]. Given that CD10+ cells are enriched in the population proposed to contain the human MaSC ($EpCAM^{-/lo}CD49f^+$), this suggests that rare subsets of human breast cancers may emerge from transformation of potential MaSCs. In contrast, mounting evidence indicates that the vast majority of human breast cancers of luminal and basal-like molecular subtypes arise from transformation of cells within the luminal lineage. A deeper understanding of the regulation of the mammary epithelial hierarchy and the relationship of cells within the hierarchy to the cells of origin for tumors will generate new avenues of investigation to develop improved diagnostic and treatment options for patients, especially for basal-like breast cancers that typically have an aggressive clinical course.

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Mammary Epithelial Stem Cells

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Abbreviations

AR	Amphiregulin
DLLC	Differentiating large light cells
ER	Estrogen receptor
MMTV	Mouse mammary tumor virus promoter
MRU	Mammary repopulating unit
PI-MEC	Parity-identified mammary epithelial cells
PR	Progesterone receptor
SLC	Small light cells
TDLU	Terminal ductal lobule unit
ULLC	Undifferentiated large light cells
WAP	Whey acidic protein promoter

Experimental Evidence Supporting the Existence of Mammary Stem/Progenitor Cells

The experiments that originally demonstrated the existence of stem cells in the mammary gland were based on the pioneering studies of DeOme and his students, Les Faulkin and Charles Daniel. They developed and optimized serial transplantation of normal mammary gland into the cleared mammary fat pad of syngeneic mice [1, 2]. They demonstrated that the normal mammary gland contains cells that will grow and fill the fat pad with a normal ductal mammary tree and respond to hormones with a normal differentiation program [3]. The progeny of the transplanted cells could be serially transplanted into the appropriate recipients for multiple times; however, unlike preneoplastic or neoplastic cells, the normal cells always senesced after multiple serial transplants, generally 5–8 transplant generations [3]. This was interpreted as indicating mammary stem cells possessed a finite proliferative activity (i.e., life span). This finite life span is a fundamental difference between normal and preneoplastic/neoplastic mammary cells. Cells with an indefinite in vivo life span (i.e., immortalized) have been identified in numerous mammary model systems, including MMTV-induced alveolar hyperplasias [4], chemical carcinogen-induced ductal and alveolar hyperplasias [5, 6], hormonally induced alveolar hyperplasia, spontaneously immortalized ductal hyperplasias [7, 8], and cells containing specific genetic alterations (i.e., p53 deletion, polyoma mT antigen) [9, 10]. These immortalized populations can be non-tumorigenic, weakly tumorigenic, or highly tumorigenic [10–12]. One might speculate that the ability to proliferate over 8–12 serial transplant generations before exhibiting a decrease and loss of proliferation activity would indicate an increase of stem cell number or activity as a consequence of some treatment. As of the end of 2011, this assay has not yet been applied in any stem cell study.

Subsequent studies demonstrated that stem cells were located along the entire mammary ductal tree and represented in all the different developmental states of the mammary gland. Host age and reproductive history had little influence on the frequency of stem cells as measured by percent successful takes and life span assay [13, 14]. Mammary cells taken from 26-month-old virgin mice had the same transplant potential as cells taken from 3-week-old mice. Both cell populations senesced after five transplant generations. Similarly, mammary cells in 12-month-old multiparous mice had the same serial transplant potential as cells from 3-week-old virgin mice [13]. Finally, continuous hormone stimulation did not induce additional loss of ductal growth potential. These

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results have important implications for understanding the role of mammary stem cells in normal mammary development because they emphasize that the mammary stem cell is a relatively quiescent cell that is only activated under conditions of gland repopulation (i.e., fetal growth stage, pubertal growth phase). Under other conditions, such as pregnancy, it is likely that ductal and alveolar progenitor cells form the bulk of the increased mammary epithelial cell population [15] (see discussion in next section).

These early studies emphasized that stem cell life span is intricately linked to proliferation activity. For example, life span was correlated with the interval of serial transplantation. Thus, transplanting at 12-month intervals instead of 3-month intervals prolonged the ultimate life span of normal cells [13, 16]. Similarly, transplanting from the periphery of the ductal outgrowth (i.e., such cells would have undergone more cell divisions) resulted in earlier senescence than transplanting cells from the center (i.e., the original transplant site) of the outgrowth. In summary, these early studies suggested the presence of a mammary cell that could repopulate the mammary gland and undergo a normal and complete morphogenetic program (i.e., a stem cell). Such cells are spaced throughout the mammary tree, are quiescent, and have a finite life span. A commonly stated assumption that normal mammary stem cells are an ideal target for oncogenic transformation because they, like cancer cells, share a long life span (i.e., replicative potential) is not supported by the transplantation results. At least for the mammary gland, the evidence to date suggests that mammary stem cells have a finite life span.

Morphologic Evidence of Stem Cells Among Mammary Epithelium

Distinguishing mammary cells was first based on their ultrastructural appearance [14]. Undifferentiated (pale) cells were found which exhibited the expected behavior of stem cells in mammary explants induced in vitro to differentiate toward secretory cell fates. It was discovered that mouse mammary explants, like mammary epithelium in situ, contained pale- or light-staining cells and that it was only these cells that entered mitosis when mammary explants were cultured.

Light cells were analyzed in mouse and rat mammary glands in the electron microscope utilizing their ultrastructural features to distinguish them from other mammary epithelial cells (Fig. 1) [17]. Both small light cells (SLC) and undifferentiated large light cells (ULLC) (Fig. 1) were observed with condensed mitotic chromosomes indicative of their replicative competence in mouse mammary explants, pregnant and lactating mouse mammary glands, and rat mammary gland from 17 stages of development beginning with nulliparity through pregnancy, lactation, and involution [17–20]. Partially differentiated ULLC or differentiating large light cells (DLLC) were observed in rapidly proliferating mammary epithelium during pregnancy and probably represent transient-amplifying epithelial cells committed to a secretory fate. Using all of the above features, a more detailed description of the epithelial subtypes that comprise the mammary epithelium was established.

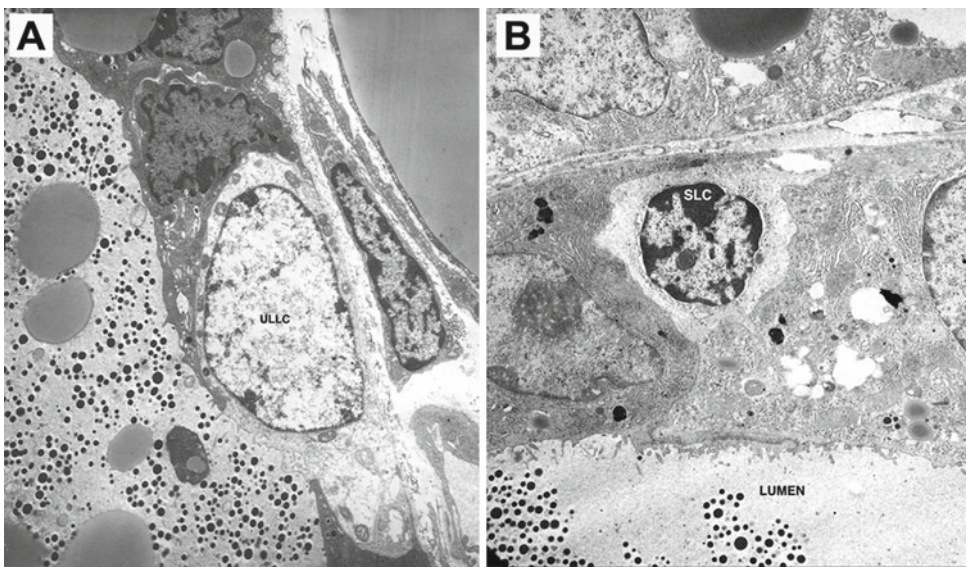

Fig. 1 Electron micrographs taken of a secretory acinus in a fully lactating female mouse showing (**a**) large (ULLC) light cell juxtaposed (but undifferentiated) to differentiated secretory dark epithelial cells and (**b**) a small light (SLC) cell depicted in a secretory acinus. SLC are found exclusively located near the basement membrane and never are found in contact with the lumen (shown here at the *bottom* of the figure characterized by the presence of microvilli on the surface of the secretory cells and the presence of dark casein micelles)

Evaluation of the 17 stages of mammary gland development showed that the population density (number of cells/mm^2) of SLC among mammary epithelium did not change from puberty through post-lactation involution. The proportion of SLC in the epithelial population remained unchanged. This means that although the number of mammary epithelial cells increased by 27-fold during pregnancy in the mouse, the percent of SLC in the population did not change [21, 22]. Therefore SLC increase and decrease in absolute number at the same relative rate as the expanding epithelial cell population, suggesting that they have a capacity for self-renewal. In contrast, ULLC numbers were much more variable, perhaps indicative of their transitional nature.

Absence of SLC and ULLC in Growth Senescent Mammary Tissues

Mammary epithelial cells bearing the morphological characteristics of undifferentiated stem cells (i.e., SLC and ULLC) likewise disappear from senescent populations simultaneous with growth cessation [23]. In premalignant mammary epithelial populations, which exhibit indefinitely prolonged growth potential, both of these cell types (SLC and ULLC) are maintained.

A study of human breast epithelium demonstrated the presence of mammary epithelial cells possessing the ability to regenerate elaborate branching structures resembling mammary terminal ductal lobular units both by morphology and marker expression, in vivo and in vitro [24]. The experimental approach was based upon ultrastructural studies in the mouse mammary gland, which described SLC and ULLC as putative epithelial stem cells. SLC and ULLC do not commonly contact the duct or lobule lumen [25]. Indeed suprabasal breast epithelial cells were found with these properties and demonstrated that these cells possessed stem cell properties. This discovery lends strong experimental support for the conclusion that the undifferentiated SLC and ULLC represent a multipotent epithelial cell population in the mouse and that a similar epithelial subset exists in the human breast.

Mammary Stem/Progenitor Cell Hierarchy

Evidence for lobule-limited and duct-limited pluripotent mammary epithelial cell activities has been established for both rats and mice by transplantation of limiting dilutions of dispersed mammary epithelial cells into hosts that were subsequently impregnated and/or treated with hormone combinations to produce alveologenesis [15, 22, 26, 27]. Studies with retroviral-marked clonal mammary populations demonstrated that both of these lineage-limited activities were present within clonal populations through repeated transplant generations indicating their derivation from a single pluripotent antecedent [22, 28]. In addition, serial passage of the retroviral-marked mammary epithelial clones in pregnant hosts showed that the capacity of individual outgrowths to produce lobulogenesis or ductal elongation was independently lost during the acquisition of growth senescence among individual transplants [28]. With the development of the WAP-Cre model used in combination with the Rosa26LacZ reporter mice (R26R), evidence for a LacZ-marked lobule-limited progenitor observable in parous mouse mammary epithelium surfaced [29]. These LacZ-positive, parity-identified mammary cells (PI-MEC) were found to be pluripotent, self-renewing, and capable of maintaining their lobule-limited progenitor activities following serial transplantation in epithelium-free mammary fat pads when the hosts were subsequently impregnated (Fig. 2) [30]. During pregnancy in these hosts, the PI-MEC proliferated and gave rise to luminal progeny that were PR- or ERα-positive and luminal progeny that were bereft of these steroid receptors. Further, in the developing secretory acini, they contributed not only secretory progeny but also myoepithelial cells. Further study indicated that these cells were present in the mammary tissue of nulliparous females and that they could be detected in explant cultures after treatment of the fragments with growth factors that do not induce lactogenic differentiation [31]. Additional evidence demonstrates that PI-MEC are found to be virtually 100 % present in the CD49f^{hi} population [32]. This population was shown earlier to possess essentially all of the mammary repopulating activity [33]. Subsequent transplantation of CD49f^{hi}-positive PI-MEC and the CD49f^{lo} epithelial cells into epithelium-divested mammary fat pads indicated that all the repopulating activity was associated with the PI-MEC fraction [32].

Functional Assays for Monitoring Mammary Stem and Progenitor Cells (Limitations)

The accepted standard of functional mammary stem cell assays remains the repopulation of a cleared mammary fat pad and subsequent secondary transplantation of any ensuing mammary outgrowth first reported in 1959 [1]. The main deterrent to these experiments is that they are expensive, time-consuming, and not amenable to high-throughput assays. While these assays work well for the detection of murine stem cells and other rodent sources, no equivalent model has yet been established for the study of human mammary stem cells. Human tissue nonresponsiveness and the murine host mammary stroma are the main causes for experimental failure [34–37].

One alternative method for testing engraftment capacity of human mammary stem/progenitor cells involves the injection of human mammary fibroblasts into the cleared murine fat pad prior to the transplantation of the human mammary epithelial cells [38, 39]. This assay allows for the establishment

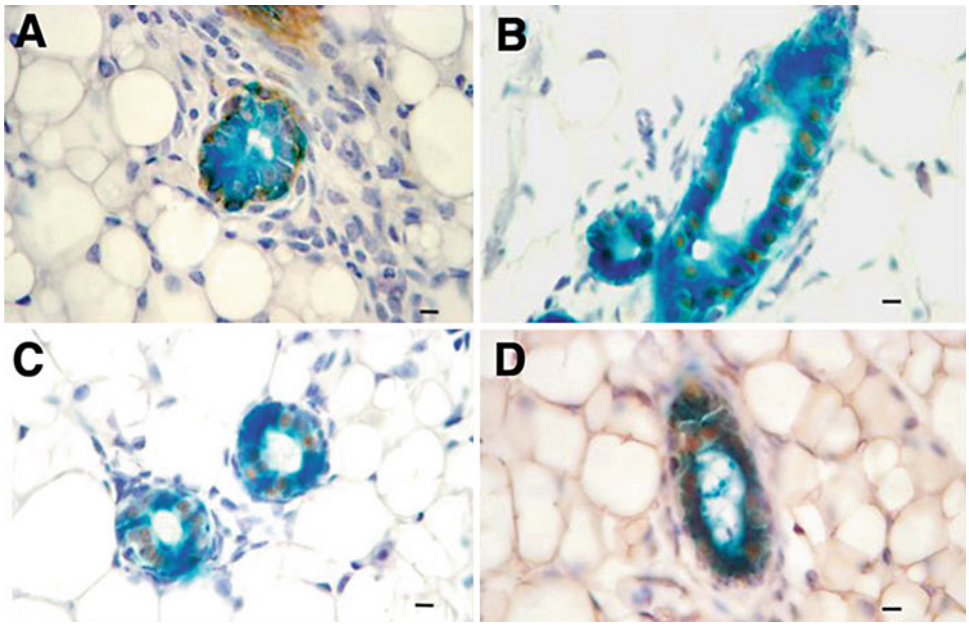

Fig. 2 (**a**–**d**) Parity-identified multipotent mammary epithelial cells (PI-MEC) marked by lacZ expression (*blue*) are capable of producing both myoepithelial (**a**) progeny characterized by the simultaneous expression of lacZ and smooth muscle actin (*brown*) and luminal epithelial progeny during lobulogenesis (**b**–**d**). Luminal epithelial progeny may be positive for progesterone receptor (*brown* nuclear stain in **b** and **c**) or estrogen receptor (*brown* nuclear stain in **d**) or luminal progeny negative for either progesterone or estrogen receptor. This evidence indicates that PI-MEC represent the lobule-limited multipotent epithelial progenitor cells in the mouse. Scale bars = 15 mm

of human mammary stroma or "humanization" creating a basement structure allowing for the engraftment and expansion of the human mammary epithelial cells. In this system the epithelial cells are able to expand and differentiate into histologically normal-appearing human mammary structures comprised of luminal and myoepithelial cells.

A second human into mouse implantation model has been investigated. In this model human mammary epithelial cells are mixed with irradiated fibroblast, embedded within collagen gels, and implanted into highly vascularized areas such as underneath the kidney capsule [40]. After 4 weeks the histologically sectioned tissue resembles normal human breast tissue with both luminal and myoepithelial cells. These outgrowths have fully differentiated luminal cells that express ER and PR and form functional secretory epithelial cells that synthesize milk proteins if the host becomes pregnant.

Phenotypic Analysis of Mammary Stem and Progenitor Cells

The most common technique used to identify and isolate mammary stem and progenitor cells is based on cell surface markers using magnetic and/or fluorescent sorting methods. By sorting for combinations of cell surface markers, researchers have been able to establish a rough idea of what markers are expressed by different classes of mammary progenitor cells.

Mouse

The cell surface markers used currently to establish mammary progenitor populations include CD14, CD24 (a pan-epithelial marker used to discriminate against stromal cells), β1-integrin (CD29), α6-integrin (CD49f), β3-integrin (CD61), and Sca-1. Cells bearing these markers can be sorted based on the intensity of the fluorescent activity that correlates to the expression levels of each of the markers. The populations are sorted into high, med, and low populations (e.g., $CD24^{med}Sca\text{-}1^{low}CD29^{high}CD49f^{high}$). A $CD24^{med}Sca\text{-}1^{low}CD29^{high}CD49f^{high}$ cell is referred to as a mammary repopulating unit (MRU). It is estimated that 1 MRU can be isolated from every 60–90 mammary epithelial cells [33, 41]. The MRU designation is based on its ability to form a mammary colony in vitro; its in vivo regenerative capacity is yet to be determined. Based on the expression levels of CD24, CD29, and CD49f, it is believed that MRUs occupy basal positions in the mammary epithelium. These cells express basal keratin 5 further evidence of the basal position in situ [42].

These markers have been useful but only to a limited extent. Recently the Cre-Lox recombination system was used in the mouse, and the results indicate that many mammary cell types, as characterized by keratin expression, contribute progeny to outgrowths generated by injection of dispersed cells [43]. In addition, it has been shown in human breast cancer cell lines that the markers for tumor-initiating cells

and for luminal non-tumor-initiating cells do not indicate the exclusivity of these markers to tumorigenesis per se [44].

There are conflicting reports regarding the importance of these surface markers and their relevance to the prospective isolation of populations of epithelial cells enriched for their ability to produce competent mammary epithelial reconstitution in transplanted mammary fat pads. Two groups have claimed that $CD49f^{hi}/CD24^{pos}$ or $CD29^{hi}/CD24^{pos}$ cells constitute populations highly enriched for mammary stem cell activities competent for regeneration of a complete and functional mammary gland and capable of self-renewal [33, 41]. Reports from another group indicate that the bulk of in vivo reconstituting activity resides in the $CD24^{lo}$ population, and practically none is associated with $CD24^{hi}$ in cells isolated from mammary tissue using this single-cell surface marker [45]. Removal of CD24 from the genome has little to no effect on mammary gland development or function in the mouse [46].

Human

In vitro and in situ studies indicate that the mammary stem cells reside in the intralobular ducts of the human mammary gland and not the terminal ductal lobule units (TDLUs) [47]. The markers used to isolate human mammary stem cells include epithelial cell adhesion molecule (EpCAM; also known as epithelial cell antigen (ESA) and CD326), CD49f, and luminal-specific glyco-mucin protein MUC1 [40, 47–50]. CD49f is expressed at higher levels on basal epithelial cells and lower levels on luminal cells, while EpCAM is expressed at higher levels on luminal cells and lower levels on basal cells. Human MRUs have an $EpCAM^{lo}CD49f^{high}MUC1$ phenotype indicating a basal position similar to those of the mouse [48, 51].

The lack of a species specific in vivo model has hampered the characterization of the human mammary stem cells as all of these results are based on in vitro experiment or transplantation studies utilizing immune-deficient mice.

Functional Assays for Monitoring Mammary Stem and Progenitor Cells (Limitations)

The early serial transplantation studies did not provide precise data on stem cell frequency as the experiments utilized fragments of mammary cells instead of cell suspensions. One study provided an upper estimate of stem cell frequency in different portions of the mammary fat pad [14]. This study calculated the total number of mammary epithelial cells in a mammary fat pad and then divided the fat pad into 80–100 fragments for transplantation. Using this approach, the authors calculated the upper frequency of stem cells in virgin duct and end buds from 6-week-old virgin mice as 1/7,200 and 1/2,200, respectively. Studies done with semi-purified cell suspensions prepared by enzymatic digestion and using a limiting dilution approach provided more definite results, although the frequency was very dependent upon the procedure used for preparation of the cells. For example, using cells prepared from 10- to 12-week-old virgin BALB/c female mice a 3-h digestion with collagenase-hyaluronidase yielded a repopulating frequency of 1/2,200 when cells were injected in PBS solution. However, when cells were digested overnight and followed by a short exposure to trypsin and implanted with Matrigel (in a 1:1 volume ratio), the repopulating frequency was 1/250. These results were evaluated using Poisson distribution statistics, which required five dilutions, thus imposing very stringent criteria. Using other less demanding approaches can only provide estimates, which are less reliable. The improved protocol was developed by Moraes et al. [52]. In their study, they provided estimates of repopulating frequency of 1:100 for cells taken from FVB strain normal adult virgin mammary gland. These studies have implications for any study on mammary stem cells [53]. Recent approaches using sorted cell populations estimate stem cell frequency at least an order of magnitude greater than the above studies. One has to consider the factors that might contribute to the underestimate of stem cell frequency in studies using flow-cytometry-guided cell sorting. What is the impact of cell damage and cell loss on the interpretation of the results?

Is there a fundamental difference between implanting a cell suspension and a fragment of mammary cells? Surprisingly, this question has not been addressed in any recent study that focuses on the identification of mammary stem cells. In the older published literature, there is limited data and discussion of the events occurring within 72 h after implantation of a mammary fragment. An early study demonstrated that transplanted fragments of normal ductal tissue dissociate into small aggregates within 24 h after transplantation [54]. By 72 h, ductal tubular organization is established with an intact basement membrane and significant mitotic activity. A similar pattern of histogenesis is observed upon the transplantation of hyperplastic alveolar nodules [55]. It is unknown (although highly likely) if this initial cell dispersion and reaggregation represents the interaction of different subsets of mammary cells. If this early histogenic activity is critical for subsequent cell proliferation, how does one interpret the results where cell suspensions of sorted populations representing one subset of mammary cells are cited as evidence for the existence of the "stem" cell? The current assays do not distinguish between engraftment capability and stemness.

Dispersed Cell Implantation Compared to Fragment: Clonal or Combinatorial

It has been shown, both directly by retroviral-tagging in serially transplanted MMTV-infected mammary outgrowths and more recently by implantation of "visually confirmed" single

cells, that an entire functional mammary gland may be developed from the progeny of a single cell [22]. On the other hand, considerable evidence exists that transplantation of dispersed mammary epithelial cells comprised of unsorted heterogeneously marked epithelial cells produces complete outgrowths that are frequently (in some cases invariably) mixtures of the progeny derived from the variously marked donor cells [28, 30, 32, 56, 57]. In the absence of ERα expression, duct elongation and development fails both in pubertal and in parous females [56]. The amphiregulin null (AR^{null}) mouse mammary gland phenocopies this deficiency indicating that AR is a major duct-specific growth signal mediated through ERα-positive mammary epithelial cells, Despite this, both $ER\alpha^{null}$ and AR^{null} mammary epithelial cells are capable of contributing progeny to all mammary epithelial subtypes when dispersed and mixed with wild-type mammary epithelium before injection into cleared mammary fat pads [56, 57]. The evidence from progesterone receptor (PR) null models reveals that alveologenesis cannot proceed in the absence of paracrine signals from PR^+ epithelial cells [58]; nevertheless dispersed PR^{null} cells marked by LacZ expression contribute alveolar progeny when mixed together with wild-type epithelial cells in pregnant hosts. This clearly demonstrates that a complete mammary epithelial outgrowth cannot be formed without $ER\alpha^+$ and PR^+ epithelial cell subtypes. These findings argue that a single mammary cell injected into an empty mammary fat pad must at a minimum divide asymmetrically (and remain a stem/progenitor cell) to produce an $ER\alpha^+$ daughter and later again to produce cap cell progeny in order to begin ductal growth and still later to produce a PR^+ cell to support side branching and, subsequently, alveologenesis. The clear existence of lineage-limited, pluripotent duct and lobule progenitors within the nulliparous mouse's mammary epithelium raises the strong probability that these cells might combine to produce mammary outgrowths comprising both ductal and lobular development when inoculated in dispersed cell populations. PI-MEC (i.e., lobule-limited stem/progenitor cells) produce PR^+ and $ER\alpha^+$ as well as progeny negative for these receptors when contributing to mammary outgrowths in pregnant host [30]. Similar findings were obtained when duct-limited outgrowths were tested for the presence of these steroid nuclear receptors. These results indicate that each of these lineage-limited stem/progenitors is capable of producing cell progeny shown above to be indispensable for complete mammary development. Thus the lines between the primary antecedent and the downstream stem/progenitors become blurred regarding their relative importance in producing complete mammary outgrowths in transplanted fat pads. Serial transplantation of clonal populations by fragment implantation into subsequently impregnated hosts showed that the capacity of any given fragment to produce alveologenesis and/or duct elongation was lost independently during the onset of growth senescence [28]. Earlier, serially transplanted growth senescent duct fragments were shown to be able to generate lobulo-alveolar growth upon impregnation of the transplant host [59]. More recently, it has been shown that fragment versus dispersed cell implantation demonstrates that no change in the ability to produce regenerated glandular structures (hence no change in stem cell function) results from either age or reproductive longevity [60]. The conclusion drawn from these observations postulates that either each lineage-limited stem/progenitor activity decays independently from the other during outgrowth development or that the primary mammary stem cell loses the capacity to produce one or the other lineage-limited downstream stem/progenitor during its own self-renewal during its expansion in the previous generation.

To summarize, both dispersed cell and fragment implantation led to mammary epithelial outgrowths comprised of progeny produced by independently self-renewing stem/progenitor populations. These facts do not in any way dispute the existence of a primary mammary stem cell antecedent. However, they do indicate the persistence of multiple pluripotent stem/progenitor cell activities within the mammary epithelial population that is capable of independently contributing diverse epithelial progeny during mammary gland growth and regeneration. The current understanding of the mouse mammary stem/progenitor cell hierarchy is summarized in Fig. 3.

Influence of the Mammary Microenvironment over Stem Cells

To highlight the influence of diverse mammary epithelial cell types in bringing about the successful regeneration, near-limiting dilutions of dispersed mammary epithelial cells were comingled with testicular cells isolated from adult WAP-Cre/Rosa26R mice [61]. The resulting mixtures were inoculated into cleared fat pads, and mammary ductal morphogenesis was allowed to proceed. Subsequently, a fraction of the transplant hosts were maintained as virgins, and the rest were mated and permitted to complete a full pregnancy, lactation, and involution cycle. Only male cells possess the WAP-Cre and Rosa26 LacZ reporter gene. Thus, LacZ-positive cells among the regenerated mammary epithelium indicate the presence of testicular cell progeny. The mammary nature of these LacZ-positive cells was confirmed by staining for mammary-specific markers for milk protein synthesis, cytokeratins K5/K14, and smooth muscle actin. FISH analysis confirmed that these cells were male and indicated the absence of fusion between male and female cells. LacZ-positive cells were found in all second-generation transplants from the male/female chimeric outgrowths, indicating their capacity for self-renewal. These experiments demonstrate

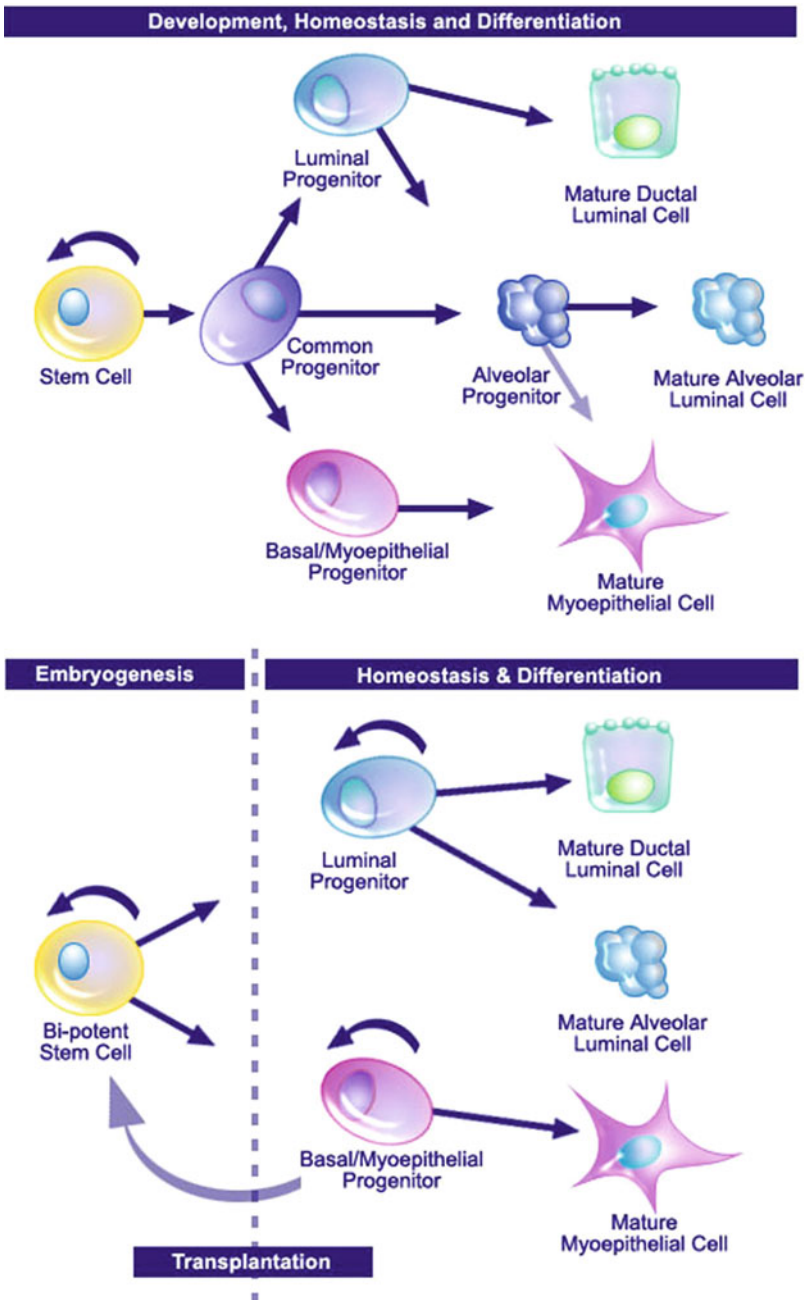

Fig. 3 Schematic illustration depicting classical hierarchy of mammary stem and progenitor cells where one stem cell results in two lineage-restricted progenitors (*upper panel*). The current understanding of the mouse mammary stem cell hierarchy where bi-potent progenitors participate in embryonic development or during regeneration following transplantation of basal progenitors while unipotent progenitors maintain luminal and basal lineages in adult mice

the overarching importance of the signals provided by mammary epithelial cells for the development of microenvironment(s) capable of sustaining stem cell activity and differentiation. Experiments have also demonstrated that neural stem cells and lineage-negative bone marrow cells isolated from WAP-Cre/Rosa26 LacZ reporter mice responded in the same manner as the testicular cells in this mammary niche assay (Fig. 4) [62, 63]. Not only is the normal mammary microenvironment able to direct stem cells derived from non-mammary tissues but also directs tumor-derived cells, mouse mammary tumor, or human testicular carcinoma to adopt a normal mammary phenotype [64, 65]. In both cases differentiation of the tumor-derived cells required the presence of ERα$^+$ and PR$^+$ cells in the surrounding environment. Without the cues provided by these cells, tumors formed.

In the human breast, little transplantation biology is available due to technical difficulties in establishing mammary outgrowths in vivo. Recently, progress has been made in this area through humanization of the mouse mammary fat pad with human-derived stromal cells [38]. The results of successful implantations of normal human organoids indicate that independent ductal, lobular and acinar structures may be generated within humanized mouse mammary fat pads by

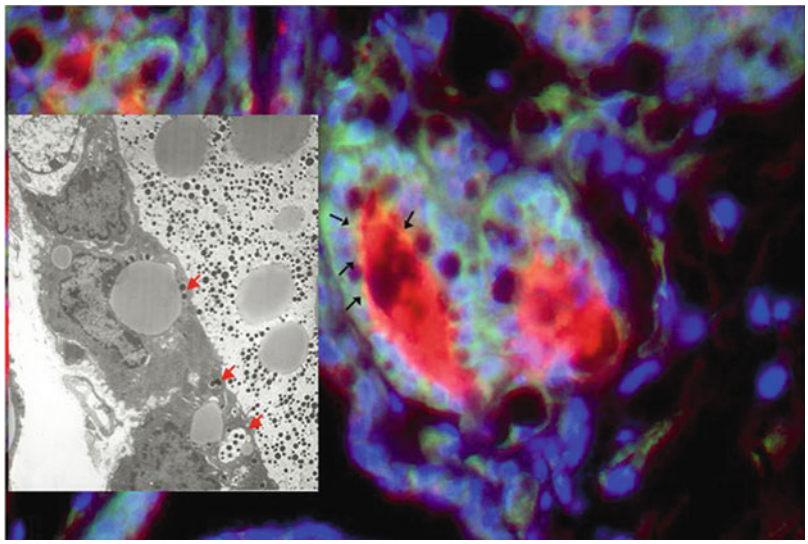

Fig. 4 A neural stem cell/mammary epithelial cell chimeric outgrowth from a lactating host is shown. Casein protein expression is indicated by the red fluorescence and beta-galactosidase by the green fluorescence, *Yellow* indicates the overlapping of the two stains. In the *inset*, an electron micrograph of a lactating acinus is depicted. The *arrows* indicate the presence of casein micelles in the secretory cells at their luminal surface. The *black arrows* in the fluorograph show that casein and beta-galactosidase staining is present (*yellow*) at the luminal surface of the beta-galactosidase-positive (neural-derived) cells

human mammary epithelial cells. This result and those demonstrating the association of bi-potency with individual mammary epithelial cells (of the mouse mammary fat pad with human-derived stromal cells suggests that a similar stem/progenitor cell hierarchy exists in human breast epithelium) [38, 39].

Future Directions and Challenges for Mammary Stem Cell Biology

The foregoing discussion supports the concept that the tissue microenvironment can affect the cellular repertoire of an adult stem cell. This influence in the murine mammary gland appears to be manifest in signals emanating from the epithelial cells as well as the stromal elements of the mammary fat pad. Several questions remain to be answered. For example, what is the role if any of mammary stem/progenitor cells in this process? Does the mammary fat pad selectively support the reprogramming in conjunction with the mammary epithelial cells or can any fat pad in the female mouse demonstrate this activity? Both testes and neural tissues develop from ectodermal precursors, will cells developing from mesoderm or endoderm precursors respond similarly when mixed with mammary epithelial cells in the context of the mammary fat pad? In fact cells derived from mesoderm tissue demonstrate this capacity [63]. Finally, what are the cellular, genetic and molecular components that define the mammary epithelial-specific stem cell niche and how can these factors be utilized for developing new paradigms for stem cell control and cancer therapy?

Preliminary experiments have shed a small amount of light on the questions mentioned above. First, enriching or depleting the mammary epithelial cells for cells expressing the currently accepted cell surface markers for mammary stem/progenitor cells (CD49f, CD29, or CD24) did not affect the efficiency of reprogramming non-mammary cells [33, 41, 45]. Testing mammary epithelial cell populations from various gene knockout models has thus far not revealed any particular gene product that is essential for reprogramming. However recent findings have delineated at least one essential epithelial cell characteristic necessary for the process of reprogramming.

Serial transplantation of the mammary epithelium inevitably leads to growth senescence, which has clearly been linked to the number of mitotic events required for stem cell activity to reach the outermost periphery of the regenerated gland. Studies designed to determine whether growth senescent mammary epithelial cell populations that are unable to support in vivo mammary epithelial regeneration by themselves may be able to reprogram non-mammary stem/progenitor cells have begun. Thus far, those growth-deficient mammary populations that have been tested were able to reprogram non-mammary stem cells and in the process were able to generate full mammary outgrowths in cleared mammary fat pads. These findings have strong implications for recruitment of transformed cells to growth-deficient niches and neoplasia. In addition, these studies have led to the examination of the response of cancer cells in this experimental model, as cancer cells show considerable plasticity when placed in developing tissue environments [66, 67]. Present work demonstrates that signals from the mammary microenvironment in the context of the regenerating gland are capable of redirecting the repertoire of adult somatic stem cells from at least three non-mammary tissues. Further efforts to extend these initial findings will elucidate at least some of the mechanisms involved.

Although untested, another possibility for the appearance of growth senescence might be due to failure of the microenvironment (niche) to provide the signals appropriate for stem

cell self-renewal. This deficiency would by necessity involve the epithelial cell population surrounding the stem cell proper since transplantation always occurs into young mammary fat pad stroma. This possibility is easily tested in current model systems where mammary cells carry the β-gal marker. A corollary to this possibility would be that signals emanating from the transformed progeny surrounding the self-renewing premalignant/tumorigenic cell rather than a property intrinsic to the premalignant/tumorigenic cell are responsible for the infinite replicative lifetime of an immortalized mammary population. This latter situation would require that asymmetric divisions from the self-renewing tumorigenic cell generate these supporting "niche" cells.

Our challenge is not to sort out from this mixture the primal mammary stem cell but instead to comprehend the interaction among these components that allows the long-term maintenance of mammary stem cell activity. We want to emphasize that focusing our primary deliberations upon the primordial mammary stem cell deflects our attention from the important issue of extending our understanding of how stem/progenitor cells and their progeny interact to maintain mammary homeostasis and how this may be disturbed during neoplastic transformation.

Acknowledgment Figure 3 was illustrated by Eve E. Kingsley Booth.

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Gastric Stem Cell Biology: Proliferation Kinetics, Differentiation Hierarchies, and Role in Carcinogenesis

Sherif M. Karam

Introduction

The glandular epithelium of the stomach has unique morphological, physiological, and molecular properties which have attracted many research interests. Over the years, much information has been discovered regarding the role of this epithelium in the production of mucins, pepsinogens, and hydrochloric acid which are involved in the pathogenesis/therapy of peptic ulcer disease [1–3]. There has also been an increasing interest in the perpetual renewal of this epithelium which implies the presence of stem cells [4].

The gastric epithelial stem cells produce different cell lineages secreting the various components of the gastric juice in addition to different hormones. Pioneering experiments using DNA labeling by radioactive nucleotides and the radioautography technique demonstrated that these stem cells are characterized by a high proliferative capability and are anchored in a specific location along the epithelium [5]. During the last few decades, our understanding of these proliferative "stem" cells and the interrelationships of their cellular hierarchies has been gradually increasing especially with the development of genetically manipulated animal models and the technology of genetic lineage tracing (the values of these techniques are reviewed in Robinson et al. [6]; Kawaguchi et al. [7]). Thus, the renewal concepts of the gastric epithelium have become fundamental to understanding its structure and function in health and disease.

During development, the gastric epithelium starts as a single layer of proliferative endodermal stem cells which gradually undergoes gene expression modifications associated with massive morphological changes due to regional specifications [8]. Eventually, enlargement and compartmentalization of the primitive stomach become associated with a remarkable increase in the epithelial surface area, proliferation/differentiation of endodermal stem cells leading to production of various cell lineages. It is generally believed that some of these endodermal stem cells are maintained throughout life to ensure the perpetual renewal of the gastric epithelium. This chapter starts with an update on the gastric stem cells and a summary of the main morpho-dynamic features of the various cell lineages along the body (corpus) and pyloric antral regions of the stomach. Then, based on studies conducted in mice and humans, evidence in support of a role for stem/progenitor cells in the origin of gastric cancer will be presented.

Structural Organization of the Gastric Epithelium

For many years, it has been recognized that the mammalian gastric epithelium is organized to form numerous glandular structures comprising two main types: mucus-producing glands in the pyloric region and acid-producing glands in the corpus region [9]. These glands open into the luminal surface via foveolae (pits) which are shorter in the corpus than in the antrum (Figs. 1 and 2). The pit-gland units of the corpus are called "zymogenic units," and those of the pylorus are called "mucous units" [10].

The zymogenic units consist of a structurally and functionally heterogeneous population of cells that include several major cell types (Fig. 1) [11]. (1) The mucus-secreting *pit cells*, or surface mucous cells, are found in the pit region and on the luminal surface which are characterized by a group of dense mucous granules packed in an organelle-free apical area called ectoplasm. (2) The acid-secreting *parietal cells* are scattered throughout the pit-gland units and are characterized by an intracellular canalicular system, cytoplasmic tubulovesicular elements, long numerous microvilli lining canalicular/apical membranes, and large numerous

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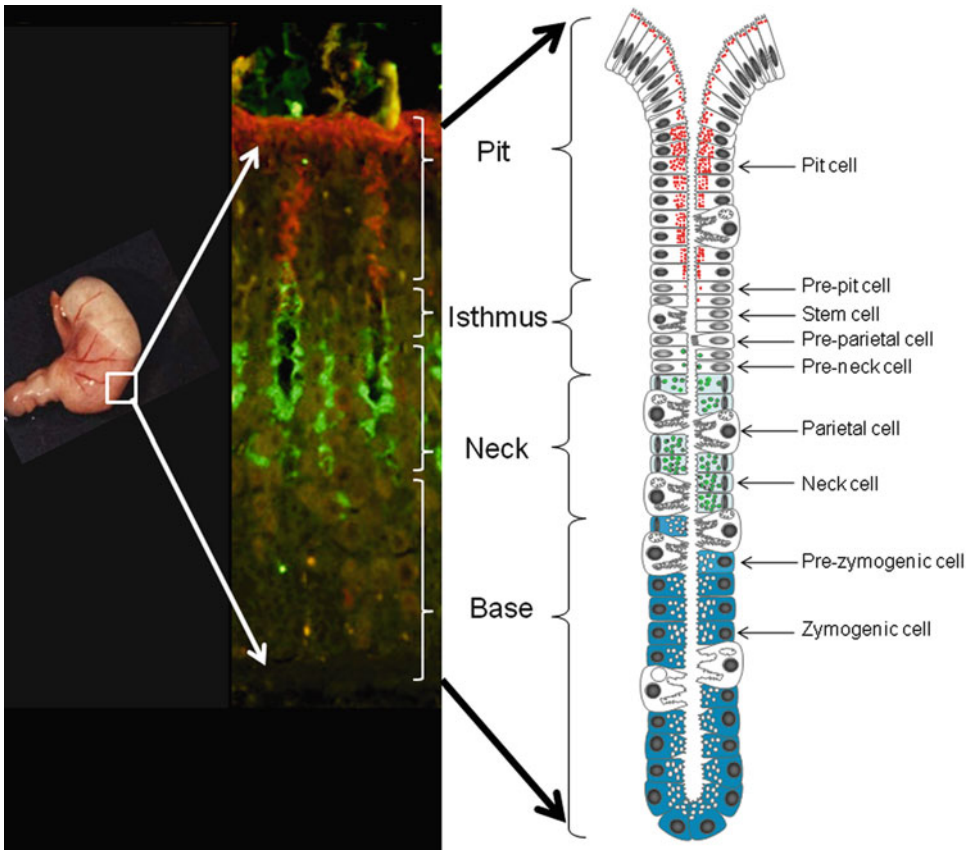

Fig. 1 The corpus region of the mouse stomach seen on the *left* is presented in a paraffin tissue section probed with two lectins: *Ulex europaeus* 1 agglutinin and *Grifforia simplifolica* II specific for mucus-secreting pit cells (*red*) and neck cells (*green*). Note the area in between pit and neck cells which shows little or no fluorescence labeling. This area represents the isthmus. The diagram on the *right* demonstrates an epithelial zymogenic unit as it appears in the corpus region of the mouse stomach. The unit is made of four regions: pit, isthmus, neck, and base. Pit cells are found in the pit, neck cells in the neck, and zymogenic cells in the base. Parietal cells are scattered in the four unit regions. The isthmus is characterized by small cells that include the undifferentiated (granule-free) stem cells and their descendant partially committed progenitor cells (pre-pit, pre-neck, and preparietal cells) which give rise to the three main cell lineages secreting mucus, acid, and pepsinogen. Members of the enteroendocrine and caveolated (tuft) cell lineages are not presented in this diagram

mitochondria. (3) The mucus-secreting *neck cells* are interspersed between parietal cells in the neck region and are characterized by numerous cored secretory granules throughout the cytoplasm. (4) The pepsinogen-secreting *zymogenic cells* are located in the base region of the glands and are characterized by a basal stack of rough ER cisternae and apical zymogen granules with a homogeneously pale content. (5) *Pre-zymogenic cells* are located in the upper segment of the base region between neck and zymogenic cells and are characterized by Golgi apparatus producing prosecretory vesicles and secretory granules whose contents appear to be intermediate between those of neck cells and zymogenic cells. (6) The peptide-secreting *enteroendocrine cells* are scattered throughout the pit-gland units and include several subtypes based on the shape/size of their secretory granules and their peptide content. (7) The villin-rich *caveolated cells* are also scattered throughout the pit-gland units and are characterized by a microvillus tuft protruding into the glandular lumen and long narrow spiral caveolae that open between the microvilli.

In the pyloric antrum, the mucous pit-gland units (Fig. 2) consist of a monolayer of cells which are less heterogeneous than those of the zymogenic units (Fig. 1). In addition to various enteroendocrine cells and a few caveolated cells, these mucous units include two major mucus-secreting cell types (Fig. 2) [10]. (1) The *pit cells*, or surface mucous cells, are found in the pit region and on the luminal surface. They are characterized by a group of dense mucous granules. (2) The *gland cells*, or gland mucous cells, are found in the neck and base regions of the pyloric antral glands. These cells are characterized by numerous cored granules which appear similar to those of mucous neck cells of the zymogenic units.

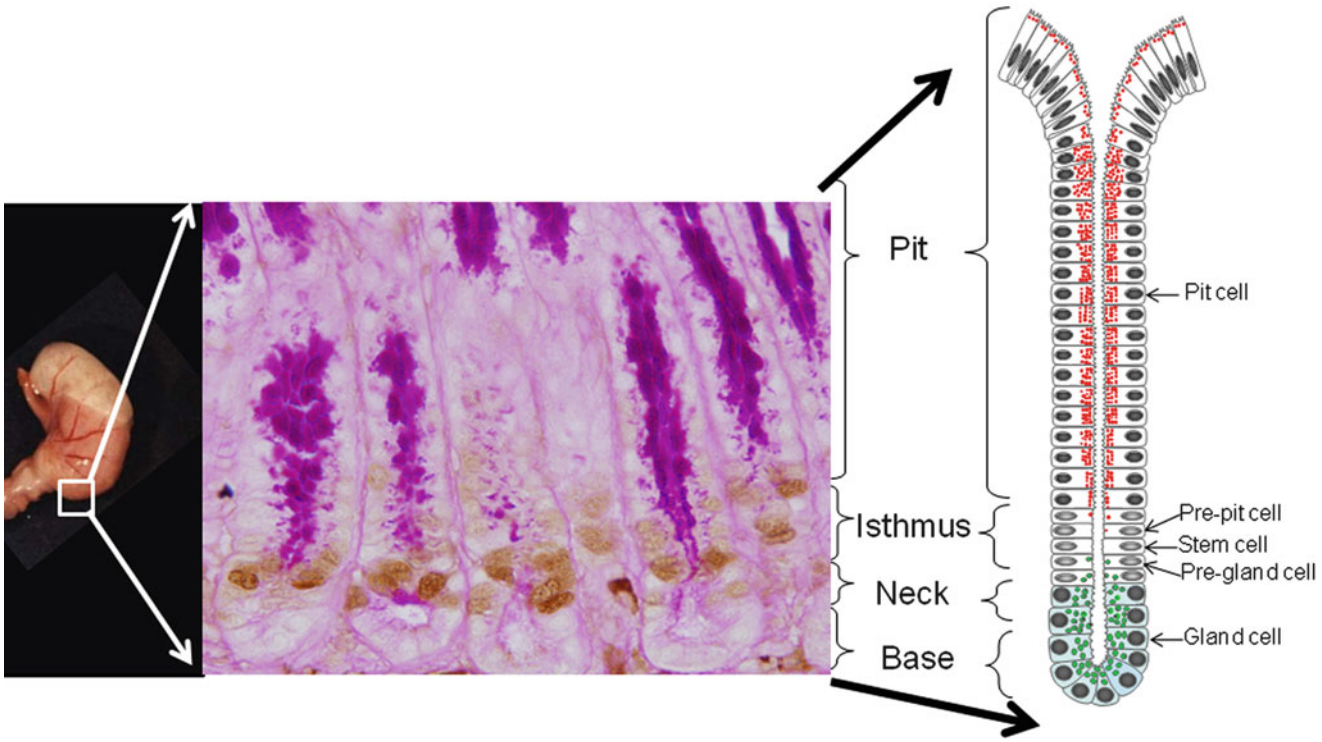

Fig. 2 The pyloric antral region of the mouse stomach seen on the *left* is processed for immunohistochemistry using an antibody specific for bromodeoxyuridine (BrdU). The mouse was injected with BrdU 1 h before sacrifice. The tissue section is counter stained with period acid Schiff (PAS). Note the area (isthmus) where brown nuclei (BrdU-positive) are located. They are closer to the bottom of epithelial units than the luminal surface. Note also that pit cells in the area above the level of BrdU-labeled cells are PAS positive. Some of the dividing BrdU-positive cells of the isthmus contain little PAS staining due to presence of mucous granules (pre-pit cells). The diagram on the *right* demonstrates an epithelial mucous unit as it appears in the pyloric region of the mouse stomach. The unit is made of four regions: pit, isthmus, neck, and base. Pit cells are found in the pit and gland cells in the neck/base. The isthmus is characterized by small cells that include the undifferentiated (granule-free) stem cells and their descendants which are partially committed progenitor cells (pre-pit and pre-gland cells). They give rise to the two main mucous cell lineages populating the unit. For simplicity, members of the enteroendocrine and caveolated (tuft) cell lineages as well as the recently discovered villin-rich and Lgr5-positive cell types are not presented in this diagram

Identification of Gastric Stem Cells

In the zymogenic unit, while the cellular components of the pit, neck, and base regions are well characterized, the cells found in the narrow isthmus region appear to be small and devoid of prominent signs of differentiation. In 1953, Stevens and Leblond [12] were the first to provide a clue regarding these isthmal cells which were capable of incorporating radioactive nucleotides soon after injection into mice. It was therefore concluded that these are the mitotic cells of the epithelium and proposed to be the source of dying mucous cells seen at the luminal surface of the stomach. This continuous process of cell production, migration, and death was considered to be the normal physiological cell renewal.

The presence of these frequently dividing cells was confirmed in high-resolution radioautographs and electron micrographs. These dividing cells were found to be undifferentiated and exhibit embryonic cell-like features: high nucleus-to-cytoplasm ratio, a nucleus with much diffuse chromatin and large reticulated nucleoli, and cytoplasm containing a few small organelles but many free ribosomes [11, 13]. In a time course 3H-thymidine labeling experiment, these cells were labeled during their mitosis and followed afterwards during their differentiation-associated bidirectional migration toward the luminal surface and the base region of the zymogenic unit [14]. Therefore, the gastric stem cells were initially defined by two major criteria. First, morphologically, they are undifferentiated with embryonic cell-like features. Second, functionally, they have a high capacity to proliferate so as to ensure their own renewal while producing lineage precursors which differentiate while migrating to form transit cells committed to become mature cells.

The presence of few "undifferentiated" cells among the isthmal cells has been reported not only in the stomach of the mouse [15] but also in rats [13] and rabbits [16]. In humans, when normal mucosal biopsies of the body region of the stomach were examined systematically with electron microscopy,

such undifferentiated granule-free cells were not identified [17]. Therefore, it seems that the common granule-free stem cell of the mouse stomach had no counterpart in humans. The least differentiated cells encountered in the body region of the human stomach carried a few small (210 nm) secretory granules and were referred to as "mini-granule cell." The available evidence suggested that either these rare poorly differentiated cells or one of the other partially committed progenitors act as the stem cells of the human epithelial unit [17].

In the pyloric antrum, while the pit-gland units were smaller and less heterogeneous than those of the body region, the renewal process appears to be more complicated. *First*, using 3H-thymidine radioautography combined with electron microscopic analysis, undifferentiated granule-free cells with embryonic cell-like features were identified in the pit-gland junction (isthmus region). Because these cells are also highly proliferative and capable of bidirectional migration as demonstrated by pulse-chase experiment, it was concluded that they were the stem cells of the pyloric antral units [18]. *Second*, using the powerful tool of genetic lineage tracing, Samuelson and coworkers identified a rare cell type expressing villin, which could be found in the isthmus and base regions of some epithelial units of the mouse pyloric antrum [19]. These rare villin-positive cells were quiescent or postmitotic. However, their division could be stimulated by treatment with interferon gamma, and within a few weeks, they became capable of populating some epithelial units in the pyloric antrum. The villin-positive subpopulation of progenitor cells was identified in epithelial units mainly close to the lesser curvature next to the nonglandular epithelium of the stomach fundus. Since this junctional epithelium is known to be a common site for cancer, it was proposed that these villin-expressing cells not only act as stem cells but might also play a role in gastric carcinogenesis [19].

Third, more recently and also by using the in vivo lineage-tracing technique, Hans Clevers and coworkers were able to identify another stem cell population located at the bottom of the gastric units (not in the isthmus) of the pyloric antrum. They were characterized by the expression of a Wnt target gene referred to as leucine-rich G protein-coupled receptor or Lgr5 [20]. These cells were not quiescent, but capable of mitosis as demonstrated by 3H-thymidine incorporation and expression of markers of proliferating cells. Lineage tracing showed that Lgr5-positive cells were capable of generating the whole epithelial lining of the units. However, the morphological features of these cells were not like the undifferentiated granule-free cells. Electron micrographs of Lgr5-positive cells showed a considerable amount of rough endoplasmic reticulum in the basal cytoplasm which indicates their involvement in protein synthesis. Indeed, the Golgi apparatus of these cells appeared active and associated with some secretory granules. These features question the necessity of the undifferentiation nature of adult stem cell populations. So, in the pyloric antrum, some stem cells may not have to be undifferentiated.

When the pyloric antral Lgr5-positive stem cells and their immediate daughter cells were isolated by fluorescence-activated cell sorter and processed for a microarray analysis, they appeared to express some other Wnt target genes, such as Cd44, Sox9, Sord, Prss23, Cldn2, and Sp5. Therefore, in the pyloric antral glands, it seems that the canonical Wnt signaling pathway plays a very important role in control of stem cell dynamics. Furthermore, some of the Lgr5-positive cells (designated as Lgr5 "low" cells and considered as immediate descendants of Lgr5-positive cells) highly expressed enteroendocrine-specific cell markers such as chromogranin, somatostatin, and gastrin [20]. This finding may be taken as evidence to place pre-enteroendocrine cells before other progenitors (pre-pit and pre-gland cells) in the differentiation hierarchy of gastric stem cell in the pyloric antrum.

Immediate Descendants of Gastric Stem Cells

In mice, the presence of undifferentiated granule-free cells was confirmed in serial sections of the isthmus region of the zymogenic units. In addition, these serial sections revealed the presence of seven other types of cells with different signs of early commitment [11, 14]. All seven cell types cited below have embryonic cell-like features similar to those of the granule-free cells, but in addition each cell type has a feature indicating early commitment. (1) *Pre-pit cell precursors* are characterized by Golgi apparatus producing prosecretory vesicles similar to those of the pit cells, but no secretory granules. (2) *Pre-neck cell precursors* have Golgi apparatus producing prosecretory vesicles similar to those of the neck cells, but no secretory granules. (3) *Pre-pit cells* are characterized by a Golgi apparatus producing prosecretory vesicles similar to those of pre-pit cell precursors and pit cells. They also have dense secretory granules similar to those of pit cells but are fewer and smaller. (4) *Pre-neck cells* are characterized by a Golgi apparatus producing prosecretory vesicles similar to those of pre-neck cell precursors and neck cells. They also have cored secretory granules similar to those of neck cells but are fewer and smaller. (5) *Preparietal cells* are characterized by parietal cell-like features, i.e., long apical microvilli and an incipient intracellular canaliculus; they include three subtypes: one carrying a few secretory granules similar to those of pre-pit cells, the second with cored granules similar to those of pre-neck cells, and the third devoid of any granules. (6) *Pre-enteroendocrine cells* are characterized by a few endocrine-type secretory granules similar to but smaller and fewer than those of enteroendocrine cells. (7) *Pre-caveolated cells* are characterized by few caveolae and microvilli similar to those of caveolated cells.

In the pyloric antrum, the isthmus region of the mucous pit-gland units includes proliferative granule-free cells with morphological features similar to those of the zymogenic units. Descendants of these undifferentiated cells include six different types of progenitors. *Mottled-granule cells* are uncommitted precursors which are characterized by embryonic cell-like features like those of the granule-free cells, but in addition they carry a few small mottled granules in their apical cytoplasm [18]. *Mixed-granule cells* are descendants of the mottled granules and are characterized by a mixture of small dense granules and large cored granules. When mixed-granule cells divide, they give rise to (3) *pre-pit cells* characterized by a few dense mucous granules and (4) *pre-gland cells* carrying a few cored mucous granules. In addition, *pre-enteroendocrine cells* and *pre-caveolated cells* originate directly from the undifferentiated granule-free cells [18].

Dynamic Features of Gastric Stem Cells

In both the corpus and pyloric antral regions, 3H-thymidine radioautography has been utilized to study the dynamism of the proliferating cells of the pit-gland units and to follow their differentiation/migration pathways with time. Thus, radioautographs represent the source of valuable data on the labeling indices of various cell types at different time intervals.

In the corpus region of the mouse stomach, mitotic cells are found in the isthmus region of the zymogenic units where granule-free cells are the most proliferative. Pre-pit and pre-neck cells and their precursors are mitotic, whereas preparietal cells do not divide. In 3H-thymidine pulse-chase experiments, the shift in radiolabeling that occurs with time from pre-pit to pit cells has explained the similarities in their morphological features and indicated that they both constitute a continuum or one cell lineage that migrates upwards to the free surface [21]. The shift in the labeling from pre-neck to neck cells has also confirmed the morphological similarities in their features and indicated that they belong to one cell lineage. Because of the morphological features of pre-zymogenic cells and the fact that they acquire 3H-thymidine labeling after neck cells and before the zymogenic cells, it appears that these pre-zymogenic cells represent a transition during the transformation of neck cells into zymogenic cells [22]. Thus, pre-neck, neck, pre-zymogenic, and zymogenic cells all constitute one lineage that migrates toward the bottom of the zymogenic unit (Fig. 1). Also, the shift in labeling from preparietal to parietal cells indicates that they constitute a third cell lineage but with a bipolar mode of migration toward either the pit orifice or gland bottom [17].

Similar to parietal cells, the enteroendocrine and caveolated cells develop in the isthmus and undergo bipolar migration [23]. The turnover time of the different gastric epithelial cell types is determined by continuous infusion of a low dose of tritiated thymidine into mice, which are then sacrificed at different time intervals. From the cumulative increase in the labeling indices of each cell type, the rate of cellular turnover and the turnover time was estimated.

In the pyloric antrum, of the mouse stomach, cell proliferation occurs in the isthmus region of the mucous units where granule-free cells divide and give rise to mottled-granule cells. The turnover time of granule-free cells is about 1 day [18]. The uncommitted mottled-granule cells are relatively numerous and represent 39 % of the isthmal cells. Mottled-granule cells act like transit cells which undergo clonal expansion and divide 4 times before giving rise to the mixed-granule cells which are also proliferative. They give rise to pre-pit cells and pre-gland cells. The turnover time for each of the mottled- and mixed-granule cells is about 1 day [18]. Pre-pit and pre-gland cells are also proliferative. Following 3H-thymidine pulse-chase experiments, the shift in radiolabeling that occurs with time from pre-pit to pit cells has confirmed the similarities in their morphological features and indicated that they both constitute a continuum or one lineage that migrates upwards to the free surface [10]. The shift in the labeling from pre-gland to gland mucous cells has also confirmed the morphological similarities in their features and indicated that they belong to one lineage [24].

The powerful technology of mouse aggregation chimeras and transgene expression have provided new insights regarding the clonality and number of the multipotent stem cells in each epithelial unit and their capacity to encode spatial memory or retain a positional address along the cephalocaudal axis of the gut (reviewed in Gordon et al. [25]). In the stomach, the stem cell hierarchy is established during development of the pit-gland units. This process involves a selection among several multipotential stem cells so that ultimately only one survives to supply descendants to the fully formed units. Using genetic mosaic analysis, and based on the expression pattern of an X-linked *LacZ* transgene that female mice inactivate randomly during development, the clonality of zymogenic and mucous pit-gland units appear to be different. Most of the mucous pit-gland units of the pyloric antrum appear to be monoclonal [26]. However, in the human stomach, histochemical mutation analysis clearly showed that the zymogenic pit-gland unit contains multiple stem cells [27]. In another study, using chemical mutagenesis, Bjerknes and Cheng provided additional evidence supporting the view that most zymogenic pit-gland units arise from a single multipotent stem cell. However, some gastric epithelial units carried mutant cells of only one cell lineage. Hence, some units might not contain a multipotential stem cell, but maintain a long-lived committed progenitor that replenishes a single cell lineage [28].

In humans, while the organization of the gastric epithelial pit-gland units and the allocation/features of different mature cell types are more-or-less similar to those in mice, the situation is a bit different in the stem/progenitor cell zone. It was

not possible to find in humans an undifferentiated cell similar to those of granule-free cells of the mouse zymogenic pit-gland units [17]. However, McDonald et al. [27] followed spontaneous mutations in the mitochondrial cytochrome c oxidase gene and demonstrated its propagation in all cell lineages. Hence, they are derived from multipotent stem cells, probably the mini-granule poorly differentiated cells which are identified by electron microscopy [17].

Cell Lineages in the Gastric Epithelium

Serial sections of the zymogenic pit-gland unit reveal that it is made of a monolayer of about 200 cells [11]. Whereas the pyloric antral pit-gland unit is lined by a monolayer of about 250 cells [12]. The cells populating these two different types of epithelial units are involved in the production of mucins, acid, pepsinogen, hormones. These cells originate from multipotent stem cells which give rise to seven different cell lineages (Figs. 1 and 2).

Corpus Pit Cell Lineage

Members of this cell lineage are known for their secretion of mucus and involvement in gastric mucosal protection [29]. They are also capable of adapting to injury by forming cytoplasmic extensions to cover denuded basal lamina, a phenomenon called restitution [30].

Pre-pit Cell Precursors. In the isthmus, about 67 % of the progeny of the stem cells produced daily become pre-pit cell precursors. They are characterized by a small Golgi apparatus that produces prosecretory vesicles at the trans-face. These vesicles vary in density but contain uniformly fine particulate material. These cells are partially committed and have two different progenies. The majority (99 %) become pre-pit cells and only 1 % become preparietal cells with pre-pit cell-like secretory granules. The development of a pre-pit cell precursor into a pre-pit cell is manifested by the maturation of the trans-Golgi vesicles into dense secretory granules. In the case of the preparietal cell, there is also an elongation of the apical microvilli [14].

Pre-pit Cells. Pre-pit cells are located in the upper portion of the isthmus and are characterized by a few 200-nm-wide, dense secretory granules. An average of 10 pre-pit cells are present in each isthmus. Radioautography has revealed that they have two sources of origin. About 57 % come from the differentiation of pre-pit cell precursors, the remaining 43 % from their own mitosis. After a pulse of 3H-thymidine, 25 % of pre-pit cells become labeled. With time, label increases to reach 33 % at 6 h, then gradually decreases to 1 % at 4 days, and completely disappears thereafter. In continuous 3H-thymidine labeling experiments, almost all pre-pit cells become labeled by 2 days. Both single injection and continuous labeling experiments confirm the short turnover time of pre-pit cells (2.5 days). The fate of pre-pit cells is to become pit cells. This occurs as the activity of the cell increases, and an increasing number of larger and larger secretory granules are produced and accumulate at the apex before exocytosis [14].

Pit Cells. These cells are located in the pit region and are characterized by a dense apical group of mucous granules. In the mouse, there are 37 pit cells per unit; their Golgi apparatus produces mucus in the form of a uniformly fine electron dense particulate content packed in prosecretory vesicles which eventually form secretory granules. The granule contents are homogeneously dense except on the free surface where they may acquire a core [11, 14]. Two lectins, *Ulex europaeus* type 1 agglutinin and cholera toxin B subunit, can be used as markers for pit cells in adult and developing mice [31, 32]. The pit cells migrate outward along the pit wall to reach the gastric luminal surface in a few days [21]. During pit cell migration, the apical group of granules enlarges due to increase in number and size of newly produced secretory granules, from 250 to 400 nm. In addition, cells gradually elongate with tapering of their basal cytoplasm, nucleoli become condensed, the amount of ribosomes diminishes, and the mitochondria decrease in size. Pit cells close to the pit-isthmus border retain some ability to divide. Thus, pit cells are not only developed from maturation of pre-pit cells, but some are also produced by their own mitosis. Even though a pit region may include a few large parietal cells, the migration of pit cells along the pit wall occurs in a fairly regular pipeline manner. It takes about 60 h for a pit cell to reach the surface. At the luminal surface, the transit time is only 12 h. The overall turnover time of pit cells averages 3 days [21].

Cells of the pit lineage express some member of the trefoil family of peptides (TFFs) which are implicated in various biological processes: protection, restitution, repair, and cell proliferation/differentiation program [33]. In the zymogenic unit, TFF1 is synthesized and packaged with mucus within the same secretory granules of pit cells and their committed/uncommitted progenitors [34, 35]. Since TFF1 knockout mice exhibit a reduction in the number of parietal cells at the expense of pit cells, it has been proposed that TFF1 plays an important role in the commitment program of the progenitors of pit cell lineage [34]. Sonic and Indian hedgehog expression analyses, respectively, show decreasing and increasing gradients from the corpus region of the stomach toward the pyloric antrum [36]. This differential expression pattern of hedgehog may explain some of the morpho-dynamic differences between mucus-secreting pit cell lineages in the corpus vs. antrum [10, 21].

Zymogenic Cell Lineage

Pre-neck Cell Precursors. About 24 % of the stem cells produced daily become pre-neck cell precursors. These precursors are characterized by prosecretory vesicles at the trans-face of their Golgi apparatus containing dense irregular material with light periphery. They are partially committed precursors and the fate of 98 % of them is to become pre-neck cells; the remaining 2 % become preparietal cells with cored secretory granules similar to those of pre-neck cells [14].

Pre-neck Cells. Pre-neck cells are located in the lower portion of the isthmus; they average 1.8 cells per isthmus. They are characterized by a few 400-nm-wide secretory granules which appear dense with a light core. They are mitotically active (11 % become labeled after a radio-thymidine pulse) and their division yields new pre-neck cells and cells committed to develop into neck cells. The turnover time of pre-neck cells is about 3 days [14].

Neck Cells. These cells, also called "mucous neck cells," are located in the neck region and are characterized by their Golgi apparatus producing dense irregular material packed in the center of prosecretory vesicles and light material packed at the periphery of the same vesicles. These vesicles develop to form numerous dense mucous granules which usually contain a light core made of pepsinogen [37]. These cored granules are scattered throughout the cytoplasm in comparison to the apical granules of pit cells. The *Griffonia simplicifolica* II lectin can be used as a marker for neck cells [31, 32] in both adult and developing mice. These mucus/pepsinogen-secreting neck cells have been well characterized as an entity separate from zymogenic and pit cells by Wattel and Geuze [38]. The mouse stomach contains 13 neck cells per gland. Neck cells close to the isthmus have fewer and smaller granules (430 nm wide) than those close to the base (700 nm). After their production in the isthmus from transformation of pre-neck cells or in the high neck segment from their own mitosis, neck cells migrate inward while completing their differentiation toward the mucous phenotype. Neck cells are not end cells, i.e., their fate is not to degenerate and die. They spend from 7 to 14 days in the neck region. Then, at the neck-base border, their phenotype gradually changes from mucous to serous [22].

Pre-zymogenic Cells. In the upper segment of the base region of the pit-gland unit of the mouse, there is a group of cells producing secretory granules which are intermediate between those of neck cells and those of zymogenic cells. These granules contain two different components: electron dense mucus and light pepsinogen. Cells with similar criteria are also described in guinea pigs [37], rats [39], and humans [17]. In the mouse stomach, there are 5 pre-zymogenic cells per unit. These cells can be identified by neck cell-specific lectins and pepsinogen-specific antibodies, markers for both neck and zymogenic cells [32, 40]. In the mouse, these cells are classified into subtypes I, II, and III, according to whether the dense mucous component is, respectively, more abundant than, about equal to, or less abundant than the light pepsinogenic component. Moreover, prosecretory vesicles at the Golgi trans-face of each of these subtypes exhibit differences parallel to those occurring in the granules. The size of the secretory granules showed an increase from 760 to 830 to 930 nm, respectively, in subtypes I, II, and III. In the basal cytoplasm, rough ER cisternae are more abundant in subtype III than in subtypes I and II. The existence of further intermediates between these subtypes indicates that they transform into one another (I → II → III) and thus gradually change their phenotype to become more and more pepsinogenic. The gradual decrease in their mucus production has led to the production of granules which are entirely pepsinogenic [22].

Zymogenic Cells. These pepsinogen-secreting cells are characterized by spherical zymogen granules with homogeneously light pepsinogenic content. These cells have been extensively studied by Samloff [41] and Hersey [42]. The mouse stomach contains 67 zymogenic cells per unit; they are typical serous cells characterized by a basal stack of rough ER cisternae and apical zymogen granules [11]. Antibodies against pepsinogen and intrinsic factor are utilized as markers specific for mouse zymogenic cells [32, 40, 43]. As zymogenic cells migrate inward, their phenotype specificity increases, as suggested by the measurement of zymogen granules, 780-nm-wide in the high base vs. 1,070-nm-wide in the low base. The production of larger and larger granules is in line with the increase in the amount of rough ER cisternae and also with the enlargement of the nucleolus. Zymogenic cells are end cells which eventually acquire signs of degeneration and finally die at the gland bottom after a long turnover time of ~194 days [22].

Differentiation of the zymogenic cell lineage is associated with the expression of the transcription factor Mist1 [44] and regulated by mesenchymal connective tissue cells via bone morphogenic protein 4 [45] and members of the parietal cell lineage probably via Sonic hedgehog [46].

Parietal Cell Lineage

This cell lineage is responsible for acid secretion and hence has attracted many scientists and pharmaceutical companies interested in defining the molecular events and mechanisms involved in this secretory process.

Preparietal Cell Precursors. Little is known about these precursors (P2 in Fig. 2). They are defined in developing

transgenic animal model in which the precursors of acid-secreting cell lineage have been amplified. They are characterized by embryonic cell-like features, in addition to having numerous apical microvilli with little glycocalyx [32, 40].

Preparietal Cells. Preparietal cells are characterized by having long apical microvilli and incipient canaliculi. Preparietal cells do not undergo mitosis at any stage of their development [4]. Based on the presence or absence of some secretory granules, preparietal cells are divided into three variants: (1) preparietal cells with no secretory granules which directly develop from preparietal cell precursors, (2) preparietal cells with a few small dense granules similar to the granules of pre-pit cells which develop from pre-pit cell precursors, and (3) preparietal cells carrying a few cored granules similar to those in pre-neck cells which develop from pre-neck cell precursors. Development of preparietal cells into parietal cells occurs in three stages. *First*, an increase in the surface area of the apical plasma membrane forms long numerous microvilli. *Second*, a few small H,K-ATPase-containing tubules and vesicles appear in the cytoplasm, and the apical membrane invaginates to form an incipient canaliculus at one side of the nucleus. *Third*, an additional canaliculus appears on the other side of the nucleus, and the number and size of mitochondria gradually increase. Finally, expansion of the canaliculi and overall increase in cell size leads to formation of a fully mature parietal cell [4, 16, 47]. The formation of a preparietal cell takes about 1 day, and their maturation into a parietal cell requires at least two more days [4].

Parietal Cells. These cells have been extensively investigated due to their role in pathogenesis and therapy of acid-related diseases [3, 48]. In the mouse, there are 26 parietal cells per unit; they are scattered throughout the pit-gland units and are characterized by an intracellular canalicular system, cytoplasmic tubulovesicular elements, long numerous microvilli lining canalicular/apical membranes, and large numerous mitochondria. Antibodies against the alpha- and beta-subunits of the H,K-ATPase [16, 49, 50], the cytoskeletal protein ezrin [51], and the Lewis x blood group antigen, Galb1,4(Fuca1,3) GlcNacb1 [32, 52], as well as the lectin *Dolichos biflorus* agglutinin [31, 32], are all molecular markers for parietal cells in developing and adult mice. Parietal cells are produced in the isthmus and migrate bidirectionally along the pit-gland axis. This migration pathway has been visualized by radioautography. Radiolabeled cells are first seen in the isthmus. With time, they appear in the pit in an outward direction and also in the neck in an inward direction until they reach the blind end of the unit. In situ hybridization studies and biochemical analysis have demonstrated that the synthetic/secretory activity of parietal cells vary along the pit-gland axis [47]. Young parietal cells in the isthmus and neck are more active than old parietal cells in the pit and base regions. The estimated turnover time of parietal cells is about 54 days [4]. Ablation of parietal cells in genetically manipulated animal models [32, 40, 53–56] has been associated with a block in the terminal differentiation of zymogenic cells. Thus, in addition to the fact that parietal and zymogenic cell lineages have a common source of origin, it seems that the former produces some regulatory factors necessary for the terminal differentiation of the latter. DNA profiling of isolated parietal cells revealed the identity of various genes that could be responsible for this regulatory function of parietal cells [57].

Several factors are involved in the regulation of parietal cell dynamism. *Gastrin* is a peptide hormone secreted by the pyloric antral G cells and is a well-known acid secretagogue [58]. It also plays an important role in the homeostasis of the gastric epithelium and parietal cell lineage. Gastrin knockout mice are not only achlorhydric but also deficient in parietal cells [59, 60]. Gastrin also stimulates the expression of some genes involved in the regulation of parietal cell dynamism: *Sonic hedgehog* and the epidermal growth factor receptor ligands *amphiregulin* and *heparin-binding epidermal growth factor-like growth factor* [61]. Sonic hedgehog is associated with the tubulovesicles and the proton pump of gastric parietal cells and secreted with histamine stimulation [62]. However, when acid secretion is inhibited by omeprazole and hypergastrinemia develops, Sonic hedgehog is upregulated in parietal cells [63]. In addition, knockout mice deficient in Sonic hedgehog of parietal cells develop pit cell hyperplasia, hypochlorhydria, and hypergastrinemia [64]. *Bone morphogenic proteins 2 and 4* are also involved in the regulation of parietal cell renewal. Blocking of these parietal cells' bone morphogenic proteins in noggin transgenic mice induces a reduction in the number of parietal cells and alteration in the zymogenic cell lineage [45].

Pyloric Pit Cell Lineage

Pyloric Pre-pit Cells. Pre-pit cells of the pyloric epithelial units represent about 17 % of all isthmal cells and are usually located near the pit border. Both the morphological features and dynamic behavior of these cells are quite similar to those of the pre-pit cells in the isthmus region of the oxyntic pit-gland units [10, 14].

Pyloric Pit Cells. These cells are located in the pit region and represent about 180 cells per pit-gland unit. The mode of migration and structural features of these cells are similar to those in the oxyntic epithelium [14]. They are also fast migrants and have a turnover time of about 3 days [10].

In addition to TFF1 which is biosynthesized and secreted by pre-pit and pit cells in the pyloric antrum, TFF3 is expressed in a subpopulation of pit cells near the isthmus region of mucous units, suggesting an important role in the differentiation/migration program of this cell lineage [65].

Pyloric Gland Cell Lineage

Pyloric Pre-gland Cells. These poorly differentiated cells are characterized by having a few small (280 nm) cored granules. The pre-gland cells represent about 28 % of the isthmal cells and predominate near the neck border. These cells duplicate before their differentiation-associated migration to cross the neck border and become gland cells [24].

Pyloric Gland Cells. These mucus-secreting gland cells are located in the neck and base regions of the mucous pit-gland unit and represent about 37 cells per unit. Along the neck-base axis, gland cells exhibit more and larger cored granules toward the base. The granule size varies from 380 nm in the neck to 580 nm in the base. In addition, with the inward migration of gland cells, the amount of ribosomes diminishes, the rough ER cisternae become numerous, and the Golgi apparatus increases in size [24]. The isthmus of each mucous unit produces about 12 gland cells per day by differentiation of pre-gland cells. Gland cells also retain some mitotic activity which gradually diminishes toward the gland bottom. Thus, a total of 29 gland cells are added daily to the gland cell population. Gland cells migrate inward to the gland bottom in a gradually decreasing rate. The average time spent by a gland cell in the neck region is about 10 h and in the base region, about 200 h. This pattern of gland cell renewal is known as "cascade" pattern of cell renewal. Therefore, the overall turnover time of gland cells is highly variable, from 1 to 60 days [24].

Enteroendocrine Cell Lineages

The peptide- or polypeptide-secreting enteroendocrine cell lineages are scattered throughout the gastrointestinal epithelium. The enteroendocrine cells have been characterized and extensively studied [66]. These cells include several subtypes which vary based on the shape/size of their secretory granules and their peptide content. In the mouse, quantification shows that there are 13 enteroendocrine cells per zymogenic unit comprising several types. The identification of enteroendocrine cell types depends on the size, shape, electron density, and immunocytochemical specificity of their secretory granules [67]. They are named by different letters: D, EC, ECL, G, P, PP, and X/A-like cells, respectively, secreting somatostatin, serotonin, histamine, gastrin, bombsin-like peptide, pancreatic polypeptide, and ghrelin. Gastric enteroendocrine cells are scattered along the four unit regions but are mainly found in the base [11]. In mice, they represent about 7 % of all cells in the corpus pit-gland unit [11] and 3 % in the pyloric antral unit [10]. Interestingly, the percentage of enteroendocrine cells keeps decreasing toward the small intestine (0.5 %; [68]) and the colon (0.4 %; [69]).

After a long debate about the neuronal vs. epithelial origin of these cells, it has been established that enteroendocrine cells share a common stem cell with other epithelial cell lineages [23, 68, 70, 71]. Thus, enteroendocrine cells represent several cell lineages which originate from the common epithelial stem cells.

Pre-enteroendocrine Cells. These are immature cells producing a few small endocrine-like secretory granules. They have been described in the isthmus region of the oxyntic glands of the stomach [23], in the crypt base of the small intestinal epithelium [68], and in the crypt base of the descending colon [69]. Pre-enteroendocrine cells are occasionally seen undergoing mitosis in the stomach [23] and intestine [68]. Thus, they originate mainly by differentiation of the stem cells as well as by their own mitoses. Radioautographic labeling of these immature forms of enteroendocrine cells has revealed that in the stomach body, they mature in the isthmus and then migrate bidirectionally to reach the pit and base regions after about 16 days [23]. In the small intestine, they migrate outward and reach the crypt top by 1–2 days where they produce more and more granules. After 1–2 more days, they reach the villi where they are transformed into mature enteroendocrine cells [68]. In the colon, it takes at least 1 day for a pre-enteroendocrine cell to be formed; it differentiates into enteroendocrine cell and reaches the mid-crypt by 7 days. Pre-enteroendocrine cells are, thus, left behind by the more rapidly migrating pre-vacuolated and pre-goblet cells [69].

Enteroendocrine Cells. These are the mature forms of the endocrine cells which are located throughout the gastric pit-gland units, the small intestinal villi, and the colonic crypts [23, 68, 69]. They are characterized by a large group of granules in the infranuclear cytoplasm and may have bundles of cytoplasmic filaments which appear relatively few due to increase in cell size. With time, enteroendocrine cells migrate in inward or outward directions. The overall turnover time of enteroendocrine cells is estimated at about 60 days in the stomach corpus [23], 4 days in the small intestine [68, 70], and 23 days in the descending colon [69].

Caveolated Cell Lineage

It was Nabeyama and Leblond who found and described mature members of this lineage throughout the gastrointestinal epithelium [72]. These caveolated cells are characterized by: a microvillous tuft protruding into the glandular lumen, long filamentous rootlets, and long narrow convoluted caveoli that open between the microvilli. These cells can also be identified by immunohistochemistry using antibodies specific to villin and fimbrin [73]. In the gastric epithelial units, caveolated cells are scattered mostly in the pit, isthmus, and neck regions [11].

Pre-caveolated Cells. These immature cells are described in the isthmus regions of the epithelial units of the corpus mucosa [23] and the crypt base regions of the descending colon [69]. They are very rare and appear plump with narrow apices and few caveolae. Pre-caveolated cells originate by differentiation of the epithelial stem cells [23, 69]. Their maturation is followed by bidirectional migration in the gastric units and by their outward migration in the descending colonic crypts.

Caveolated Cells. These mature cells are characterized by a plump body with narrow apex projecting microvilli into the luminal surface. The cytoplasm exhibits prominent lysosomes and numerous caveolae separated by bundles of filaments extending from the core of the microvilli deep to the sides of the nucleus [23]. The long axis of the ovoid nucleus tends to be parallel to the basement membrane. In the mid-crypt regions of the descending colon, caveolated cells exhibit a basal cytoplasmic process which becomes longer in the crypt top but short at the luminal surface [69]. In the stomach, the little data available have shown that caveolated cells follow a bidirectional mode of migration similar to that of enteroendocrine and parietal cells [23]. In the descending colon, it is estimated to take about 1 day for a caveolated cell to be produced; they migrate outward and spend about 4 days in the crypt base and 0.5 day in each of the middle and upper thirds of the crypt. The overall turnover time of caveolated cells is about 8 days in the colon [69].

Role of Gastric Stem/Progenitor Cells in the Origin of Cancer

A fundamental question in cancer research is the nature of the cells which are capable of initiating and sustaining neoplasia. Nowell [74] initially proposed that cancer is monoclonal and originates from a single stem cell as a result of several genetic alterations. This hypothesis of the stem cell origin of cancer is supported by several studies [75–77]. Our analysis of three different genetically engineered mouse models and various human stomach tissues supports this hypothesis of the stem or progenitor cell origin of cancer.

1. Amplification and Eventual Invasiveness of Gastric Stem/Progenitor Cells in TFF1 Knockout Mice

   TFF1 is synthesized and packaged in the same mucous granules of the gastric pit and pre-pit cells [33, 35]. TFF1 is thus secreted with mucus and both function in enhancing gastric mucosal protection and regeneration [33]. In addition, several experimental studies suggested that TFF1 acts as a tumor suppressor that may be involved in development and/or progression of gastric cancer [78–80]. Lack of TFF1 in a knockout mouse model was associated with a fivefold increase of mitotic figures in the pyloric antrum and an elongation of the pit regions of the mucosa, and finally the pit-gland units lost their tubular appearance and adenoma developed in all deficient mice. In many of these TFF1 knockout mice, the cells of the adenoma acquired malignant changes and a localized carcinoma in situ developed [81–83].

   This remarkable role of TFF1 as a tumor suppressor is strongly supported by screening of different types of human gastric cancer which revealed an apparent down-regulation of TFF1 expression due to either allelic loss at the TFF1 gene locus, TFF1 promoter methylation, or TFF1 gene single-point mutations [80, 84]. Thus, the TFF1 knockout mouse provides an excellent model to look at the alterations that are associated with precancerous lesions and to understand the development of gastric adenocarcinoma [82, 83].

   Systematic cell lineage analysis in the oxyntic mucosa of the TFF1 knockout mice and their control littermates starting from birth up to more than 1 year of age demonstrated that TFF1 is localized initially in the Golgi saccules, prosecretory granules, and secretion granules of pre-pit cells [34]. In addition, with age, TFF1 knockout mice demonstrated a sequence of events starting with a gradual increase in the length of the gastric pits associated with a decrease in the number of acid-secreting parietal cells. This was attributed to a change in the commitment program of pre-pit cells [34]. In the pyloric antrum, the situation was more pronounced where nodular lesions and even carcinoma in situ in the basal portion of the mucosa were observed at around 6 months of age [81]. In the 12-month-old knockout mice, some amplified glandular cells find their way through a gap in the muscularis mucosae and invade the submucosa [83]. These invading cells grow in the connective tissue of the submucosa and maintain some capacity to differentiate. This is in support of the concept of autocrine control of gastric stem cells and their capacity to differentiate outside their niche; so they are the source of instructions for their own commitment program [57]. Thus, the TFF1 knockout mouse model recapitulates the classical chronological scheme of multistep carcinogenesis including the initiation (due to the TFF1 deficiency), promotion, and progression of the cancer cells [83].

   Collectively, analysis of the TFF1 knockout mice at different age groups supports the hypothesis of the stem cell origin of gastric cancer [75, 76, 85–88]. In the pyloric antrum of the TFF1-deficient mice, the cells which are responsible for the formation of early mucosal thickening, the carcinoma in situ, as well as the submucosal invasion with cyst-like structures are mainly epithelial progenitors. The fact that gastric progenitors including the undifferentiated granule-free stem cells are amplified in early stages of gastric tumorigenesis and formed the invasive cells in

gastric adenocarcinomas raises a potential biological role of stem cells in the tumorigenesis cascade. Therefore, this mouse model could be taken as an evidence for the stem/progenitor cell origin of gastric cancer.

2. Preparietal Cell Proliferation, Transdifferentiation, and Carcinoid Formation in T Antigen Transgenic Mice

A lineage progenitor has typically been thought to be committed to the production of a mature cell type that performs a specific function. Thus, a preparietal cell gives rise to a parietal cell, not an enteroendocrine cell [4]. A recent analysis of a transgenic mouse model of gastric cancer has provided some evidence for more plasticity for progenitor cell commitment and differentiation than previously considered possible. In these mice, the transcriptional regulatory elements of the H,K-ATPase beta-subunit gene were used to deliver the product of Simian virus 40 large T antigen gene to preparietal cells. This forced expression of an oncoprotein in preparietal cells induced their proliferation from day 1 of postnatal life [32, 47] and led to a massive (50- to 70-fold) expansion in their population by 1–2 months of age [55]. However, differentiation of preparietal cells to mature parietal cells and neck cells to zymogenic cells was blocked [55].

When these mice became 3–6 months old, preparietal cell hyperplasia became associated with progressive mucosal thickening and glandular cyst formation. Areas of dysplasia were also developed. They were characterized by nuclear heterogeneity, loss of polarity, and stratification of glandular epithelial cells. In 10-month-old transgenic mice, areas with typical features of carcinoma in situ developed. These areas were characterized by complete loss of glandular architecture. Invasive epithelial cells formed loose trabeculae or ribbons. The cells had large nuclear-to-cytoplasmic ratio and much condensed heterochromatin. By 1 year of age, invasive gastric cancer developed with local and distal (hepatic) metastases [89].

In this mouse model, immunohistochemical characterization of the gastric epithelial cells that form the invasive carcinoma revealed an interesting result. The transition from preparietal cell hyperplasia to neoplasia is marked by increased expression of neuroendocrine cell markers (chromogranin A and DOPA decarboxylase) and loss of preparietal cell marker (H,K-ATPase). So, it seemed as if preparietal cell had switched their phenotype from H,K-ATPase synthesizing cells to enteroendocrine-type synthesizing chromogranin A and DOPA decarboxylase. Electron microscopic examination of these focal neoplastic areas demonstrated the transdifferentiation of preparietal cells into enteroendocrine cells [89]. These findings may provide a possible explanation for the cellular origin of neuroendocrine cancer in the stomach which appears to be more common than generally thought [90].

3. Hyperplasia and *Helicobacter pylori* Modulation of Gastric Stem/Progenitor Cells in Diphtheria Toxin Transgenic Mice

H. pylori is a Gram-negative bacterium which colonizes the stomachs of more than half of the world's population. These *H. pylori*-positive individuals may remain asymptomatic throughout their life [91]. On the other hand, some *H. pylori*-infected individuals may develop pathological changes leading to chronic atrophic gastritis [92] which is a preneoplastic condition characterized by loss of acid-producing parietal cells [93]. In these individuals, *H. pylori* is found in the protective mucous layer of the stomach or closely attached to the cell membranes of the lining epithelium [94]. Attachment of *H. pylori* is mediated via adhesin molecules which have affinity to NeuAcα2,3Galβ1,4-containing glycans (sialyl-Lewis x) on the apical plasma membranes of mucous cells [95].

When a transgenic mouse model of parietal cell ablation was generated by using the attenuated diphtheria toxin (DT) A fragment (tox176) and the lineage-specific promoter Atp4b [55], gastric epithelial stem/progenitor cell proliferation was stimulated leading to a progressive amplification of normally rare progenitors expressing NeuAcα2,3Galβ1,4 glycan [4, 96]. Parietal cell loss and amplification of glycan-positive progenitors are features of humans with chronic atrophic gastritis [96].

Inoculating the stomachs of these DT-transgenic mice with *H. pylori* strains recovered from patients with or without chronic atrophic gastritis was associated with the growth and attachment of the bacteria to the amplified dividing and nondividing epithelial progenitors expressing glycans specific to adhesins of *H. pylori* [97]. Scanning confocal microscopy, combined with multilabel immunohistochemistry and electron microscopy, confirmed that a subset of gastric epithelial progenitors provided not only a surface for attachment of *H. pylori* but also a habitat which supported formation of intracellular communities of *H. pylori*. The development of these intracellular bacterial communities in adult mammalian epithelial progenitors provides a new view of how *H. pylori* persists in some of its hosts as well as an opportunity to consider how the biological features of these progenitors may not only support but also be influenced by intracellular bacterial communities.

To test the consequences of *H. pylori* invasion on gastric epithelial progenitors, an in vitro assay was developed by using a mouse gastric epithelial progenitor (mGEP) cell line expressing the *H. pylori*-specific glycans [98]. Incubating mGEP cells with *H. pylori* strains isolated either from chronic atrophic gastritis or from cancer patients showed that, while the former strain is adhesive to the progenitor cell membranes, the latter is invasive and capable of forming intracellular communities [99].

Therefore, this intimate relationship between *H. pylori* and gastric epithelial progenitors as demonstrated both in vivo and in vitro is associated with changes in gene expression leading to carcinogenesis and provides another strong piece of evidence for the hypothesis of adult stem/progenitor cell origin of cancer.

Comparative studies of the gastritis and cancer strains of *H. pylori* support this concept. The cancer strain induces higher levels of expression of ornithine decarboxylase and antizyme inhibitor (Azin1) in cultured mGEPs and upregulates these transcripts in GEPs recovered by navigated laser capture microdissection from the stomachs of colonized gnotobiotic transgenic DT mice. Thus, regulation of polyamine availability by intracellular *H. pylori* could affect the proliferative status of GEPs. Intriguingly, ornithine decarboxylase exhibits increased expression in gastric adenocarcinoma compared with tissue without metaplasia [100]. Additional factors likely affect the outcome of this intimate association between *H. pylori* and gastric epithelial stem cells. Compared with the gastritis-associated *H. pylori* strain, infection of mGEP with the cancer-associated strain results in lower levels of expression of the elements of ephrin receptor signaling pathways known to control proliferation of gastrointestinal stem cells [101] as well as several tumor suppressors, including Kangai1. Downregulation of Kangai correlates with poor prognosis of human gastric cancer [102].

Thus, results of TFF1-, SV40-, and DT-mouse models together with the increasing data supporting the stem cell origin of cancer strongly suggest that some members of the isthmal progenitor cells are involved in epithelial tumorigenesis and may have an early diagnostic, therapeutic, and/or prognostic clinical value.

4. Amplification of Gastric Stem/Progenitor Cells During Chronic Atrophic Gastritis

Morphological analysis of the pit-gland units of the normal adult human stomach in both the corpus and pyloric regions revealed no undifferentiated granule-free (stem) cells which were previously identified in the corresponding regions of the mouse stomach. The least differentiated cells in human gastric units included a few small mucous granules [17, 103]. These cells were found at the junction between pit cells and mucus-secreting neck (or gland mucous) cells in the corpus (or antrum). Because of their morphological features, central location, and mitotic activity, these cells are qualified to be the stem cells of the gastric epithelium.

Systematic examination of gastric mucosal tissues obtained from informed patients undergoing endoscopy (for recurrent upper gastrointestinal symptoms) and from patients undergoing gastrectomy (for adenocarcinoma) supported the multistep process of gastric carcinogenesis [103]. Morphological analysis of the gastric units of these tissues revealed an early amplification of the actively proliferative gastric stem/progenitor cells which carry a few scattered mucous granules. This progenitor cell hyperplasia coincides with atrophic gastritis and loss of the mature gland mucous cells. Therefore, it seems that gastric stem/progenitor cells are the target of oncogenic hit which occurs during progression of gastritis into metaplasia and finally adenocarcinoma.

5. Upregulation and Dislocation of the Stem Cell Transcription Factor Oct4 During Multistep Gastric Carcinogenesis

Oct4 is a transcription factor that belongs to the POU family of proteins and binds octamer DNA motifs in the promoters of several genes to regulate the pluripotency of embryonic stem cells [104]. Oct4 is also expressed in some adult stem cells and is involved in their regulation [105]. The self-renewal transcription factor Oct4 could also be involved in the development of some tumors [105–107]. Expression of Oct4 in the adult human stomach was demonstrated by Western blot analysis of histologically normal gastric mucosal biopsies obtained from the pyloric antrum. The expression of this transcription factor in the gastric epithelium in a region that corresponds to the proliferative cell zone (isthmus) of the antral pit-gland was confirmed using immunohistochemistry [108]. However, these cells also produce mucous granules. So, they are not undifferentiated, but are the committed progenitors of pit and gland (pre-pit and pre-gland) cells which are capable of self-renewal and differentiation. The level of Oct4 expression does not seem to differ with or without *Helicobacter pylori* infection in these microscopically normal biopsies [108].

Immunohistochemical analysis of a series of tissue sections assembled in the form of an array representing the multistep process of gastric carcinogenesis revealed that the expression of Oct4 is upregulated early during development of cancer. There is a significant increase in the expression of Oct4 in mucosal tissues with evidence of severe gastritis, metaplastic/dysplastic transformation, and gastric cancer. Quantification showed an increasing trend from 2 % labeling area in control to 6 % and 16 % in gastritis and cancer tissues, respectively, suggesting a role for Oct4 in the early stages of cancer development. Detailed analysis of pyloric antral mucosal cancer tissues revealed not only an increase in Oct4 expression but also a change in its subcellular localization (from nuclear to cytoplasmic). This observation was confirmed when some tissue samples were processed for subcellular fractionation and Western blotting using the same antibodies. This alteration in the subcellular distribution of Oct4 could be due to inhibition of cytoplasm-to-nucleus translocation during carcinogenesis. These findings will be helpful in designing new modalities for early detection and/or therapy of gastric cancer.

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Progenitors of Islet Cells

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Abbreviations

CAII	Carbonic anhydrase-II
DE	Definitive endoderm
DTR	Diphtheria toxin receptor
EMT	Epithelial-to-mesenchymal transition
ES cells	Embryonic stem cells
FZD3	Frizzled-3
GPCR50	G-protein-coupled receptor 50
hiPS	Human-induced pluripotent stem
IDE	Inducer of definitive endoderm
MMP16	Matrix metalloproteinase 16
TGF	Transforming growth factor

Introduction

Insulin-dependent diabetes results from the loss of insulin-producing cells, also known as beta cells, in the pancreatic islets of Langerhans. The loss of beta cells leads to uncontrolled and persistently high blood glucose levels that require clinical management. Noteworthy, even the daily control of glycemia by insulin injections by a patient cannot prevent chronic episodes of hyperglycemia. On the long term, these can lead to several serious complications including renal failure, atherosclerosis, limb amputation, and blindness. Patients on insulin therapy can also suffer from dangerous hypoglycemic episodes. A lot of hope has been raised for diabetes patients and their physicians that one day it will be possible to restore a functional beta cell mass even after complete loss of the original one. This might be obtained either by transplanting beta cells from exogenous sources like cadaveric organ donors or stem cells or by stimulating the generation of new beta cells from endogenous stem/progenitor cells. Proof of concept has already been obtained that transplanting islet beta cells from cadaveric organ donors can restore glucose control in diabetes patients [1, 2]. However, insufficient numbers of beta cells can be obtained from available donor organs, making this therapeutic approach inefficient at present. A lot of research efforts have been done, and are still being done, to find unlimited sources of transplantable beta cells. A breakthrough was obtained in generating pancreatic endodermal progenitor cells from human embryonic stem (ES) cells [3] and in deriving beta cells thereof [4, 5]. Further efforts are focusing on improving the efficiency of in vitro derivation of beta cells from ES cells and on finding safe ways to implant these and to prevent rejection. We will further discuss this breakthrough at the end of this chapter. First, we will address the state of the art regarding the elusive adult stem/progenitor cells in the pancreas.

Adult Stem/Progenitor Cells in the Pancreas

The possibility of beta cell "neogenesis," i.e., that new beta cells could regenerate in the adult pancreas even after complete loss of the original ones, has always fascinated researchers. Every possible hypothesis has been raised in this context, with every cell type in the pancreas being considered in specific models as a possible progenitor/precursor of the beta cell.

Exocrine Acinar Cells

Exocrine acinar cells are the most abundant cell type in the pancreas and therefore represent an interesting target for cell reprogramming in order to generate new beta cells. They are notorious for their pronounced plasticity in de- and transdifferentiation or senescence whenever their environment is

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changing [6]. Rodent acinar cells have the capacity to transdifferentiate in vitro into functional endocrine beta cells in the presence of selected growth factors or culture supplements like epidermal growth factor and leukemia inhibitory factor or nicotinamide [7, 8]. The acinar origin was ascertained by lineage-tracing experiments [8, 9]. The newly formed beta cells were able to normalize the glycemia after transplantation into diabetic animals [9]. Further, it was shown that acinar cells first dedifferentiate during culture whereby they acquire ductal and pancreatic embryonic progenitor characteristics like co-expression of Ptf1a and Pdx1, activation of the Notch pathway, and expression of progenitor markers like Hnf1b, Cpa1, and Sox9 [6, 10]. In vivo, acinar cells were found to transdifferentiate into beta cells following forced expression of transcription factors like Pdx1, Ngn3, and MafA [11]. It is rather unlikely that this transdifferentiation may ever take place under physiological or most pathological conditions. Genetic lineage tracing of acinar cells revealed no conversion into endocrine cells during adult life, partial pancreatectomy, pancreatitis, partial duct ligation, or transforming growth factor alpha (TGF alpha) stimulation [12–14]. However, the transdifferentiation capacity of acinar cells might prove useful for the generation of larger numbers of transplantable beta cells in vitro. An important question remains whether human acinar cells possess a similar capacity. Tracing technology has been applied to follow human acinar-to-ductal transdifferentiation in vitro [14]. Within 1 week of culture, virtually all surviving acinar cells had adopted a ductal phenotype. In future, it will be important to establish a protocol to efficiently induce transdifferentiation of acinar cells to beta cells.

Duct Cells

The duct cells are the cells lining the transport route of the acinar exocrine enzymes to the duodenum. They have also long been considered as potential precursor cells of the beta cell. This was initially based on histomorphological observations of extra-islet insulin-positive cells residing in the vicinity of the duct epithelium and therefore regarded as being recently "budded" off from that epithelium. However, recent genetic lineage studies showed that duct cells contributed to the formation of endocrine and exocrine cells during embryogenesis but no longer after birth. Even in conditions of severe tissue damage, including the experimental model of duct ligation, duct cells did not give rise to new beta cells. These conclusions were found in four independent studies that made use of three different duct-specific tracers, namely, the genes for Hnf1b, Sox9, and mucin [15–18]. They are, however, at stake with a previous study in which a ductal derivation was reported of postnatal beta cells and acinar cells [19]. Also in the duct ligation conditions, duct cells seemingly contribute to the expanding beta cell mass in this study. Human carbonic anhydrase-II gene was used as a (postnatal) duct-specific tracer. Previous studies reported that this transgene may be inappropriately expressed in mouse tissues [20] which might explain the discrepancy of the CAII tracing study with the more recent genetic lineage-tracing studies that made use of the mouse genes for Hnf1b, Sox9, and mucin, respectively. Terminal duct cells or centroacinar cells have also been suggested to generate new beta cells after injury. However, lineage tracing revealed that these cells, marked by Hes1 expression, do not behave as adult stem cells in the normal or injured pancreas [21]. The least we can say is that the ductal origin of beta cells in the adult pancreas is highly controversial, if not unproven.

Alpha Cells

In transgenic mice with near-total beta cell ablation, the Ins-DTR mice, genetic lineage tracing revealed that a certain degree of beta cell regeneration occurs as a result of alpha cell transdifferentiation [22]. In these mice, the diphtheria toxin receptor (DTR) was placed under transcriptional control of the insulin promoter. After diphtheria toxin administration, more than 99 % of the beta cells were ablated. The mice received exogenous insulin for survival during 5 months. From the sixth month on, the mice survived without the need for exogenous insulin. The beta cell mass recovered up to 44 % compared to animals shortly after beta cell ablation. This corresponds on average to a recovery of 10 % of normal beta cell mass, which represents the lowest amount of beta cells needed for a near-normal basal glycemia. In this study the regeneration of beta cells was rather slow and was insufficient to restore normal glycemia. On the other hand, a rapid transdifferentiation of alpha cells to beta cells was described in a model in which partial duct ligation and alloxan-mediated ablation of the beta cells were combined [23]. Within 2 weeks, large islets appeared in which most of the beta cells were neogenic. Indirect evidence was shown for alpha-to-beta cell conversion. Unfortunately, no lineage tracing was performed to prove this point.

The remarkable transdifferentiation potential of alpha cells was also described in a study of Collombat et al. in which the transcription factor Pax4 was specifically overexpressed in mature alpha cells [24]. A vast expansion of the beta cell mass was described which was attributed to a continuous neoformation of beta cells and a concomitant decrease in the amount of alpha cells.

Considerable challenges still need to be addressed before this knowledge could be translated into therapeutic applications. Determining the underlying mechanisms and showing the transdifferentiation potential of human pancreatic alpha cells will be important. Given the relatively low numbers of

alpha cells in the pancreas, a particularly robust method to induce alpha-to-beta cell transdifferentiation needs to be developed in order to obtain potential clinical benefit.

Delta Cells

Islet cells expressing somatostatin, the hormone characteristic for delta cells, were reported to differentiate into beta cells [25, 26]. In both studies, the mice were given a single dose of streptozotocin, to eliminate the beta cells. In the study of Guz et al., insulin treatment was given to restore normoglycemia. Double-positive cells for Pdx-1 and somatostatin appeared with or without insulin expression [25, 27]. They suggest a transition from somatostatin-expressing cells into insulin-expressing cells.

Beta Cells

In vivo, the beta cell mass continues to expand at a relatively slow pace during adult life [28, 29]. Dynamic changes in the beta cell mass occur in response to altered metabolic demands, e.g., during pregnancy or obesity. With genetic lineage tracing, Dor et al. demonstrated that during physiological development in adult mice, new beta cells are formed by proliferation and not by neogenesis from progenitor cells. Even in the regenerating pancreas, after partial pancreatectomy [30] or partial genetic beta cell ablation [31], after treatment with exendin-4 [32] or overexpression of TGF alpha [12], the beta cell mass was expanded primarily due to proliferation of preexisting beta cells.

Expansion of human islet beta cells represents an attractive strategy as a source for beta cell therapy. Therefore, culturing and proliferation of isolated islets has been studied frequently. Inducing proliferation of pancreatic beta cells in vitro proved to be challenging and is often associated with loss of function. In 2004, Gershengorn et al. introduced the concept of epithelial-to-mesenchymal transition (EMT) of beta cells. The process of EMT is important for morphogenesis during embryonic development, and it is also associated with pathological conditions such as fibrosis and cancer. According to this study of Gershengorn et al., isolated adult human islets lose their epithelial characteristics, gain a mesenchymal phenotype, and become highly proliferative. By serum deprivation, these cells could be induced to form hormone-positive islet cells [33]. This result was confirmed by culturing trypsinized islet cells that underwent some degree of dedifferentiation and started to proliferate. Treatment with betacellulin induced redifferentiation by restoring expression of multiple beta cell genes [34]. This concept of EMT of pancreatic beta cells was challenged by several lineage-tracing studies in mice [35–38]. They used genetic lineage-tracing experiments based on the Cre/lox system to label beta cells and their progeny in vitro. Fibroblast-like cells could be derived from mouse islets and these could be induced to redifferentiate into low hormone-expressing islet-like cells, comparable with the human studies. Although the tracing experiments revealed that mouse beta cells could adopt a mesenchymal morphology, the beta cells did not significantly contribute to the proliferating fibroblast-like cell population. It was argued that these "precursor" cells most likely originate from expansion of mesenchymal cells that are present in the pancreatic islet preparations. The abovementioned lineage-tracing studies that put forward the idea that beta cells are not precursor cells were all performed in mice. In 2007, also a human study revoked the beta cell EMT concept and attributed the human islet-derived precursor cells to mesenchymal stromal cells present in the pancreatic islet preparations [39].

The first direct evidence for dedifferentiation and significant proliferation of human pancreatic beta cells in vitro came from the study of Russ et al. They used a lentiviral Cre/lox system to indelibly label human beta cells in culture [40]. Furthermore, they show the very limited replication of dedifferentiated mouse beta cells, thus emphasizing the species difference between mouse and human beta cell proliferation. Further work of the group of Efrat revealed that human beta cells undergo true EMT. They start to express several mesenchymal markers (e.g., N-cadherin, vimentin) and mesenchymal stem cell markers (e.g., CD90, CD105) [41]. The expanded dedifferentiated human beta cells could be induced to redifferentiate into glucose responsive beta-like cells [42]. Downregulation of HES1 in the expanded cells additionally promoted the redifferentiation process [43]. The latter further differentiated after transplantation into hyperglycemic immunodeficient mice and were capable of restoring euglycemia. These results offer hope for overcoming the donor shortage for beta cell therapy for diabetes, although extra work is still needed to determine the extent of differentiation of the obtained beta-like cells and to circumvent the need of viral vectors for HES1 inhibition, before possibly going to the clinic.

Adult Stem Cells

A few studies reported that cells from enzymatically dissociated adult mouse pancreas showed in vitro clonogenic potential (e.g., [44]) or at least the potential of serial passaging [45]. More recently the former group extended their results to human cells and reported that clonogenic multipotential stem cells can be isolated from adult islets and that these express insulin [46]. With lineage tracing, they indicate that these cells originate from the pancreatic endoderm. It is still unclear what is the significance of such cells for islet beta cell regeneration in vivo or whether these putative adult stem

cells represent a tissue culture artefact. Moreover, it cannot be excluded that such insulin-expressing cells with strong proliferative capacity were derived from dedifferentiated beta cells (see above) rather than representing true stem cells. The problem in identifying stem/progenitor cells in pancreas tissue is the lack of stem cell markers. Most putative stem cell markers have turned out to be also expressed by mature differentiated cells. For example, the CD133 stem cell marker is also expressed by all duct cells in the pancreas [47]. Nestin was taken as a pancreatic stem cell marker [48] but later turned out to be expressed also by all pancreatic stellate cells, a mesenchymal cell type located in the pancreatic interstitium [49].

There was a lot of excitement when it turned out that following partial duct ligation, a manipulation of the pancreas which causes severe damage to the exocrine acinar cells, Ngn3 expression significantly increased and Ngn3-expressing cells were regularly observed in the vicinity of ducts [50]. Ngn3 is a transcription factor that is expressed by the embryonic precursors of all islet cell types and that was initially thought to be no longer expressed postnatally in the pancreas. Furthermore, Ngn3-expressing cells isolated from duct-ligated pancreas could differentiate in vitro into hormone-expressing islet cells. It was hypothesized that duct ligation awakened a population of dormant stem cells located in ducts which, via an intermediate stage expressing Ngn3, generated new beta cells in the pancreas. Later it was found that a low level of Ngn3 expression is maintained in mature islet beta cells and is required for their function [51]. Under conditions like duct ligation, Ngn3 expression in islet cells can increase [17]. In the latter study, genetic lineage tracing of Sox9-expressing duct cells revealed that indeed Ngn3 became expressed by some duct cells but that these Ngn3-positive cells did not contribute to the beta cell mass, thus making the dormant progenitor hypothesis controversial.

In conclusion, many interesting hypotheses on the mechanism of beta cell regeneration have been proposed, some of which have been dismissed by others, like the ductal neogenesis hypothesis, whereas others remain to be confirmed, like the insulin-expressing stem cell hypothesis (Fig. 1). The controversy is fuelled both by the attractiveness of the prospect to regenerate beta cells in diabetics and by the lack of decisive evidence. Progress in this field is also hampered by the frequent lack of reproducibility, which may in part be due to the technical sophistication of the experimental models.

If anything, what has become clear is that several cell types in the adult pancreas, including acinar cells, alpha cells, and beta cells themselves, have pronounced plasticity in differentiation and proliferation (Fig. 1). It remains to be demonstrated whether this plasticity could be used to restore a functional beta cell mass in diabetic patients.

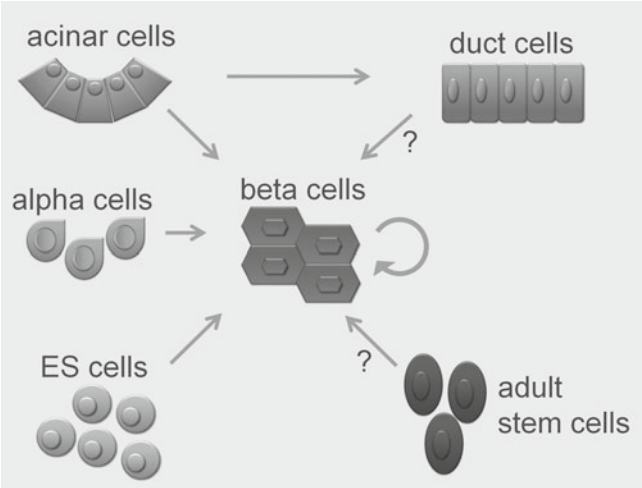

Fig. 1 Potential cellular sources for beta cell neogenesis. Acinar cells have been demonstrated to de/transdifferentiate to a duct-like phenotype and to transdifferentiate with or without this intermediate phenotype to beta cells. Also the conversion of alpha cells and pluripotent ES cells to beta cells has been clearly demonstrated. Another option is to induce proliferation of beta cells themselves in vitro by inducing EMT. The hypothesis of duct cell to beta cell transdifferentiation and the presence of adult pancreatic stem cells still remain highly controversial

Breakthrough on Pluripotent Stem Cell Differentiation

Whereas the study of adult tissue stem cells and beta cell regeneration in the pancreas remains controversial and inconclusive, directed differentiation studies of pluripotent stem cells have reached an important breakthrough and seem to come closer to clinical application. The success of this method primarily relies on the implementation of basic knowledge from embryonic pancreas development onto pluripotent stem cells in culture. Interestingly, pancreas progenitors generated in vitro from human ES cells give rise to functional beta cells upon implantation in animals and long-term follow-up. Despite these recent advances, the path to clinical application is still tortuous and requires more investigations in order to prove the safety of the technique and the functionality of pluripotent cell-derived beta cells in human settings. Although integration of the findings from mouse and human stem cell models can be beneficial for a clear understanding of the system and its improvement, we will limit this section mainly to the data from human pluripotent stem cells.

Induction of Pancreatic Progenitors In Vitro

Pluripotent stem cells that are in the pipeline for beta cell therapy in the future include human embryonic stem (hES) and human-induced pluripotent stem (hiPS) cells. They are

both characterized by an unlimited division while maintaining the undifferentiated state and the potential to generate derivatives of the three developmental germ layers (ectoderm, mesoderm, and endoderm) when exposed to specific culture conditions. Whereas the very early studies suggested the possible expression of pancreatic genes by mouse and human ES cell progenies [52–54], the differentiation of insulin-producing cells was inefficient and inconsistent, a condition in part attributed to the stochastic models used. Because the pancreas normally develops from the embryonic definitive endoderm (DE), efforts were made afterwards to enrich this cell lineage first during stem cell differentiation in vitro. In 2005, D'Amour et al. showed for the first time that hES cells, similarly to their mouse counterpart, also respond to high levels of TGFb signaling by preferentially differentiating into endoderm cells [3, 55]. These findings were concordant with the endoderm-inducing function of this pathway previously described in lower vertebrates such as zebra fish and frog [56, 57]. Activin A is mainly used in vitro as the leading TGFb ligand and was found to efficiently induce definitive endoderm from numerous hES and hiPS cell lines, especially when combined either with Wnt3a, BMP4, FGF2, VEGF, or with small molecules [58–66]. Although several other protocols for definitive endoderm induction that made no use of activin A have been reported [67, 68], it is nowadays common knowledge that TGFb stimulation via Smad2/3 is crucial and required in this process.

In a pioneer study by D'Amour et al. [69], several stages of pancreas differentiation were mimicked in vitro by the sequential exposure of DE cells to particular combinations of growth factors related to embryonic pancreas development. These treatments allowed for the generation of primitive gut tube, posterior foregut, pancreas endoderm, and endocrine progenitor equivalents; however, the insulin-producing cells obtained with this strategy were not responsive to glucose stimulation. Further refinement of the protocol by several other investigators elucidated the pathways that are fundamental for the differentiation of pancreas progenitors from hES- or hiPS-derived DE cells. These pathways function similarly as during embryonic development in vivo and essentially include a combination of hedgehog inhibition, BMP inhibition, retinoid signaling, and FGF activity [5, 61, 63–65, 70, 71]. Activation of the Wnt pathway or TGFb inhibition was also tested in two studies that generated large proportions of pancreatic progenitors expressing the transcription factor PDX1 [63, 65]. This would indicate that the major sequences of signaling cues required for pancreatic fate commitment from DE cells have been successfully unraveled and implemented in vitro to selectively enrich cultures in pancreatic cells at the expense of hepatic cells, for example (Fig. 2).

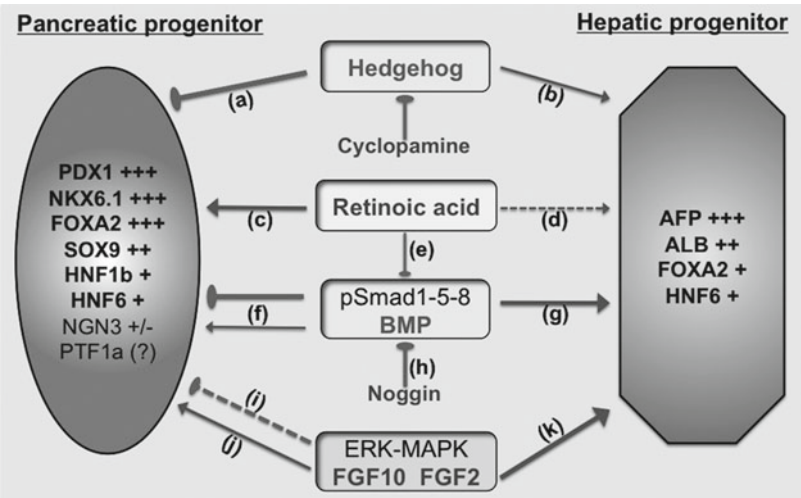

Fig. 2 Four main pathways regulate the early stages of pancreas differentiation from definitive endoderm and control the acquisition of pancreatic versus hepatic fate. Hedgehog signaling is well characterized as a potent inhibitor of pancreatic initiation (**a**) that is expressed in the hepatic domain (**b**) under the influence of FGF signaling. It functions in the establishment of organ domains. This pathway can be blocked by treatment with the alkaloid cyclopamine. Retinoid acid is expressed in the developing pancreas and participates in the induction of PDX1 expression (**c**). It may also contribute to hepatic gene expression to a certain degree (**d**). An inhibitory effect of retinoic acid on Smad1-5-8 phosphorylation (**e**) was demonstrated on hES-derived definitive endoderm seeded at low density, which contrasts with the early studies indicating activation of BMP signaling by retinoic acid. BMP is well known as an inhibitor of early pancreas development (**f**) in contrast to its requirement for hepatic initiation (**g**). This potent inhibitory effect at early stages is later reversed, and pancreatic progenitors require BMP signaling (**f**). The inhibition of BMP signaling in the pancreatic domain is under the control of noggin (**h**). As for BMP pathway, FGF signaling via ERK/MAPK also controls early pancreas induction with high concentration being inhibitory (**i**) whereas low concentrations are required (**j**). In later stages, FGF stimulates the proliferation of pancreas progenitors (**j**). On the other hand, this pathway represents an amplification signal (**h**) for the ALB+AFP+ hepatoblasts. Not displayed on this scheme are the Wnt and TGFb pathways that were recently shown to contribute to pancreatic differentiation from stem cells. The markers displayed in the progenitors recall the current status of protein detection from differentiated hES cells (Adapted from Mfopou et al. [73])

To this end, several embryonic or induced stem cell lines can nowadays be efficiently differentiated towards definitive endoderm and further towards pancreatic progenitors without the need for genetic manipulations, but only by modifications of the molecular microenvironment in which the cells are maintained [72, 73]. Nevertheless, subtle differences exist between cell line propensities for the generation of a particular cell type, and the molecular basis for this is just beginning to be deciphered [65, 74, 75].

Identity of Pancreatic Progenitors Induced In Vitro

In the developing embryo, Pdx1 expression marks the domain of the gut endoderm that will give rise to the pancreas. In both mouse and human, the absence of this transcription factor results in pancreas agenesis [76–78]. For this reason, PDX1 expression has for long been considered as a landmark for pancreatic differentiation from pluripotent stem cells, and many investigators have based their claims on the expression of this transcription factor only. However, during mouse embryonic development, Pdx1 is broadly expressed in the posterior foregut endoderm from which the posterior stomach and duodenum are also derived [79]. Furthermore, the specified pancreas progenitor in vivo is currently defined by the co-expression of a cluster of transcription factors including Pdx1, Nkx6.1, Ptf1a, Sox9, Foxa2, and Hnf6 (reviewed in [80, 81]). Whereas the expression of each of these factors at the pancreas endoderm stage of pluripotent stem cell differentiation has been reported by many authors, their co-expression remains more questionable, and the expression of all of them in the same cell is even more problematic. In the last few years, efforts were made to show double immunoreactivity for PDX1 and one of the other transcription factors (SOX9, FOXA2, HNF6, NKX6.1) [5, 58, 64, 69, 70, 82, 83]. However, the lack of quantitative data casts doubt about the validity of some claims, with the possibility that only a small fraction of the PDX1+ population may express the additional marker. The model used (flat culture or 3D structures) may significantly influence the differentiation of cells co-expressing PDX1 and NKX6.1, for example [84]. Considering the paucity of good commercial antibodies against some of these transcription factors, it is presently technically challenging to demonstrate the differentiation of fully specified pancreatic progenitor based on triple labeling for PDX1, NKX6.1, and PTF1a. Taken together, the current studies provide sufficient evidence that posterior foregut equivalents can be efficiently differentiated from pluripotent stem cells and characterized by PDX1 expression, but the formal demonstration of specific pancreas progenitor (PDX1+ NKX6.1+ PTF1a+) derivation is not yet a common achievement for all investigators. This is presently only indirectly assumed [82].

Selection of PDX1+Pancreatic Progenitors Based on Surface Markers

Despite the initial high enrichment in definitive endoderm cells by treatment of pluripotent stem cells with activin A, it is not possible to generate pure cultures of PDX1+ progenitors. Residual undifferentiated cells or cells committed towards mesoderm and ectoderm can still be found at later stages of differentiation and are usually responsible for the development of teratoma upon transplantation [5, 82, 85, 86]. This implies that selection of the desired cell type, namely, the pancreatic cells, would represent an advantage provided that surface markers are identified and specifically expressed by these cells. This assay can theoretically be performed to select for beta cells at the final stage or for pancreatic progenitors at intermediate stages in order to further characterize and differentiate them. Recent studies suggested that few surface markers are expressed by the PDX1+ progenitors or by endocrine cells differentiated from pluripotent stem cells. These include CD24, CD142, Frizzled-3 (FZD3), G-protein-coupled receptor 50 (GPCR50), and matrix metalloproteinase 16 (MMP16) for the PDX1+ progenitors, and CD200 and CD318 for the endocrine cells [82, 87, 88]. While the importance of this selection step was demonstrated by the absence of teratoma formation following implantation of positive populations [82], the excitement brought by these reports was attenuated by a reevaluation of one marker (CD24) in mouse and human ES cell differentiation models, indicating no segregation of its expression in the pancreatic cells despite the analysis with several different antibodies [87, 89]. Additional studies will be required in this field to confirm the usefulness of non-reevaluated markers and to discover new ones that show specific expression in the pancreatic progenitor or endocrine cells.

Only Insulin-Producing Cells, and Not Genuine Beta Cells, Can Be Produced In Vitro

The ultimate achievement in stem cell-based diabetes therapy would be to derive mature and functional beta cells in vitro. The genuine beta cell in the pancreas is characterized not only by the expression of insulin but also by its regulated secretion in the bloodstream in concert with changes in blood glucose levels. These variations are sensed and acted upon by a sophisticated molecular machinery that involves glucose transporters (GLUT2), high-affinity hexokinase (hexokinase IV), metabolic coupling factors (ATP, ADP), potassium-dependent channels, and voltage-dependent calcium channels [90]. It is anticipated that beta cells derived from pluripotent stem cells should harbor all these properties in order to efficiently replace the donor beta cells in the transplanted patients. Insulin expression in progenies of human embryonic or induced pluripotent stem cells has been

demonstrated repeatedly in vitro and taken as a proof of concept that stem cells can generate beta cells. In several studies, insulin-positive cells were also shown to express transcription factors that mark the beta cell such as PDX1, NKX6.1, or MAFA; and C-peptide positivity was used to rule out the possible cellular accumulation of insulin by uptake from the culture medium [4, 61, 63, 65, 69, 71, 83]. Whereas these findings constitute a significant progress as compared to the very early studies in the field, the problem is not yet completely solved considering that the insulin-positive cells differentiated to date are usually poly-hormonal (also expressing glucagon or somatostatin) and fail to release insulin upon glucose stimulation. Therefore, because they lack a functional glucose-sensing machinery, they resemble immature insulin-producing cells, but do not concisely compare to the genuine beta cells [63, 69, 91, 92]. There are nevertheless a few studies in which glucose-stimulated insulin secretion was reported [61], but without any comparison to human beta cells in the same assay. In the future, it should be desirable that standardized assays are developed for the measurement of glucose response in order to allow for data comparison between different laboratories.

PDX1+ Pancreatic Progenitors Generate Functional Endocrine Cells In Vivo

Success in the differentiation of functional beta cells in vitro is still hampered by the inadequate knowledge of the extracellular cues required for the differentiation of endocrine cell types in the developing pancreas. However, several researchers were able to show that PDX1+ pancreatic progenitor cells derived from pluripotent stem cells gave rise to endocrine progenitors and beta cells upon implantation in immune incompetent animals [5, 85, 86, 92, 93]. Worthy to note, although the generation of pancreatic progenitors occurs in about 2 weeks in vitro, which is quite short as compared to the time scale in normal human embryonic development, the in vivo differentiation of endocrine progenitors and beta cells required at least 12 weeks. Therefore, endocrine cells differentiated in this system arise in a developmental period (about 14 weeks) that is comparable to that in embryonic life. They produce insulin and release human C-peptide in the bloodstream of transplanted animals upon treatment with a glucose bolus [5, 82, 85, 94]. In the study by Kroon et al., it was clearly shown that the insulin-producing cells present in the grafts were functional. This was performed by selectively destroying the endogenous beta cells with the beta cell toxin streptozotocin and showing that the mice remained normoglycemic [5]. On the contrary, a significant decrease in glycemia could not be obtained following transplantation to diabetic mice of endocrine cells that were differentiated in vitro beyond the pancreas progenitor stage [85]. All these data have opened a new line of thought, which assumes that pluripotent stem cell-derived pancreas progenitors can be produced in a scalable system and transplanted to diabetic patients [94, 95], thereby bypassing the current limitations in deriving mature beta cells in vitro. In this case, the in vivo microenvironment of the recipient would allow for further differentiation of the grafted progenitors towards functional beta cells as observed in the mouse. For the transplantation of pluripotent stem cell progenies, it is envisioned that encapsulation in specific matrices could protect the cells from immune rejection by the recipient. This issue is presently under investigation [95].

Small Molecule Inducers of Pancreatic Differentiation

In the course of pluripotent stem cell differentiation, several growth and differentiation factors described in mouse embryonic development are used in a sequential manner. These are essentially recombinant proteins that activate specific receptors expressed by the differentiating cells, some of which are used at very high concentrations or are not sufficiently stable. Considering the high cost of these recombinant proteins, one can assume that producing large amounts of beta cells required for each diabetic recipient can turn out to be very expensive. Small molecules can solve this issue because they are affordable and usually more stable in culture. In the past year, several small molecule screening platforms have identified few that recapitulate the functions of particular recombinant proteins. For instance, inducer of definitive endoderm-1 and endoderm-2 (IDE1 and IDE2) was recently shown to activate the TGFb downstream effectors SMAD2/3 in human ES cells, resulting in efficient DE differentiation [67]. The recombinant protein Wnt3a was also replaced in few protocols by small molecule inhibitor of GSK3b such as BIO or CHIR, which also supported activin-induced endoderm differentiation [63, 68, 96]. In the same line, wortmannin and sodium butyrate were supplemented to activin A during DE induction [61, 92]. During the pancreatic and endocrine progenitor stages of pluripotent stem cell differentiation, several small molecules are applied in order to boost PDX1 or NGN3 expression. These include in particular the retinoid agonist retinoic acid, the hedgehog inhibitor cyclopamine, the BMP inhibitor dorsomorphin, the TGFb inhibitor SB431542, the focal adhesion kinase inhibitor, and novel molecules such as (−)indolactam V [63–66, 97–99]. In the future it may be possible to derive pancreatic cells using more small molecules and less recombinant proteins [63]; but current data indicate that pancreatic cells have not yet been successfully generated from a purely "chemical soup."

Concluding Remarks

Despite many years of intensive investigations by many excellent research groups, the search for elusive adult stem cells in the pancreas has not been conclusive (Fig. 1). The major challenge is to prove the existence of adult stem cells in pancreas tissue and to find reliable cellular markers for their identification and isolation for further study.

Important progress in our understanding of beta cell renewal, growth, and regeneration in this organ has been achieved. New knowledge was obtained by implementing lineage-tracing methods that allow us to test various hypotheses on the origin of islet cells. These studies revealed an important plasticity in the adult pancreas with regard to the capacities for cell proliferation, dedifferentiation, and transdifferentiation, not only of exocrine (acinar) cells but also of endocrine (alpha and beta) cells (Fig. 1). At present it is not clear which of these biological phenomena, if any, could be efficiently exploited clinically to restore a functional beta cell mass in diabetic patients. In many instances it is not yet known whether the knowledge obtained from animal models also holds for the human system or how it could be translated to induce beta cell regeneration.

The most comprehensive progress in the past years has been achieved by studies of pluripotent stem cells. By implementing basic knowledge of embryonic pancreas development, important breakthroughs were obtained in guiding expansion and pancreatic differentiation of cultured human pluripotent stem cells. This research avenue seems to come closer to clinical application, for example, by implanting encapsulated hESC-derived pancreatic progenitor cell grafts as a treatment for type I diabetes [94]. The remaining challenge for basic research in this field is to discover the embryonic conditions that control the terminal differentiation of endocrine islet cells.

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Stem Cell Populations Giving Rise to Liver, Biliary Tree, and Pancreas

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Abbreviations

The stem cell or progenitor cell populations are indicated by an acronym which is preceded by a small letter indicating the species: m = murine; r = rat; h = human.

AFP	Alpha-fetoprotein
CD133	Prominin 1
CFTR	Cystic fibrosis transmembrane conductance regulator
CK	Cytokeratin
C-PEP	C-peptide
CS-PG	Chondroitin sulfate proteoglycan
CXCR4	CXC-chemokine receptor 4
CYP450	Cytochrome p450
DS-PG	Dermatan sulfate proteoglycan
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule (CD326)
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXA2	Forkhead box A2
GAG	Glycosaminoglycan
GCG	Glucagon
GFAP	Glial fibrillary acidic protein
HA	Hyaluronan
hBTSC	Human biliary tree stem cell

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HDM	Serum-free, hormonally defined medium
HGF	Hepatocyte growth factor
hHB	Human hepatoblast
hHpSC	Human hepatic stem cell
HNF	Hepatocyte nuclear factor
HP-PG	Heparin proteoglycan
HS-PG	Heparan sulfate proteoglycan
ICAM-1	Intercellular adhesion molecule-1
INS	Insulin
iPS	Induced pluripotent stem
KM	Kubota's Medium
LGR5	Leucine-rich repeat-containing G protein coupled receptor 5
MIXL1	Mix paired-like homeobox gene (expressed in primitive streak in embryos)
MUC6	Mucin 6, oligomeric mucus/gel-forming
NCAM	Neural cell adhesion molecule
NGN3	Neurogenin 3
PBG	Peribiliary gland
PCNA	Proliferating cell nuclear antigen
PDG	Pancreatic duct gland
PDX1	Pancreatic and duodenal homeobox 1
PROX1	Prospero homeobox protein 1
SALL4	Sal-like protein 4
SEM	Scanning electron microscopy
SMAD	Homolog of the *Drosophila* protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein, SMA
SOX	Sry-related HMG box
TEM	Transmission electron microscopy
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial cell growth factor

Introduction

Liver, biliary tree, and pancreas are midgut endodermal organs central to handling glycogen and lipid metabolism, detoxification of xenobiotics, processing of nutrients for optimal utilization, regulation of energy needs, and synthesis of diverse factors ranging from coagulation proteins to carrier proteins (*e.g.*, AFP, albumin, transferrin). The integrity of the body depends heavily on liver, biliary tree, and pancreatic functions, and failure in any of them, especially the liver, results in rapid death. In recent years it has become apparent that these tissues comprise maturational lineages of cells that are in epithelial-mesenchymal cell partnerships. Each lineage tree begins with an epithelial stem cell (*e.g.*, hepatic stem cell) partnered with a mesenchymal stem cell (MSC) (*e.g.*, an angioblast). These give rise to cellular descendants that mature coordinately. The maturational process generates epithelial and mesenchymal cells that change stepwise with respect to their morphology, ploidy, growth potential, biomarkers, gene expression, and other phenotypic traits. More detailed presentation of the literature on the phenotypic traits of the biliary tree [1], pancreas [2], and of the liver [3] have been given in prior publications (Fig. 1). Moreover, the properties of maturational lineages in the biliary tree are not fully known as few studies have been completed. Here we note only a few examples of changes in the intrahepatic lineages to demonstrate the phenotypic gradients in phenotypic traits that can occur (Table 1).

The net sum of the activities of cells at the sequential maturational lineage stages yields the composite tissue. In this review we provide an overview of stem cell populations giving rise to liver, biliary tree, and pancreas. Several recently published reviews present further details [1, 3–5]. For the sake of brevity, we will not discuss studies involving the lineage restriction of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells to a hepatic or pancreatic fate. This topic is covered elsewhere in the book (Chap. 10). In addition, we have focused this review almost entirely on studies of human tissues. Other chapters in the book address closely related endodermal stem and precursor cells for the stomach (Chap. 19), and others provide further information on stem cells on liver or pancreas (Chaps. 20, 22, 30, and 34) (Fig. 2).

Embryonic Development

During early development definitive endoderm derives from stem cells through the effects of a number of pluripotent transcription factors, including goosecoid, MIXL1, SMAD2/3, SOX7, and SOX17 [6]. Endoderm subsequently segregates into foregut (lung, thyroid), midgut (pancreas, biliary tree, and liver), and both foregut and hindgut (intestine), also through the effects of specific mixes of transcription factors. Those dictating the midgut organs include SOX9, SOX17, FOXA1/FOXA2, Onecut2/OC-2, and others [7–10] (Fig. 3). The liver, biliary tree, and pancreas derive from midgut endoderm established at the gastrulation stage of early embryonic development [11]. Among the other organs of endodermal origin, endogenous adult stem cells have been identified in most, including the small and large intestines [12], the stomach [13], and the lungs [14, 15]. The pancreas is distinct in that lineage tracing experiments indicate that there are only very rare stem cells in the postnatal organ [16–18]. Subsequently, we found evidence that pancreatic stem cells are not located within the organ itself but rather in the biliary tree, particularly the hepato-pancreatic common duct. These stem cells give rise to committed progenitors located in pancreatic duct glands (PDGs) [2].

The formation of the liver and pancreas occurs with outgrowths on either side of the duodenum that extend and ramify into a branching biliary tree structure that, at its end, engages the cardiac mesenchyme to form liver [19].

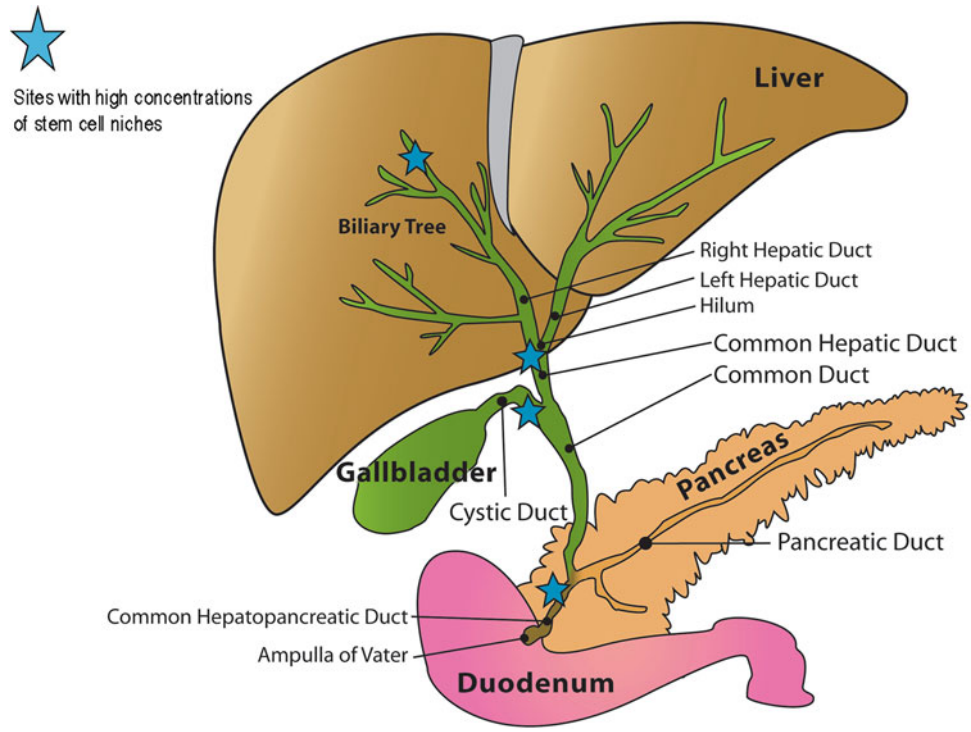

Fig. 1 Schematic of liver, pancreas, and biliary tree. Figure reproduced from Cardinale et al. (2012)

Table 1 Intrahepatic lineage-dependent phenotypic traits in human livers

Maturational lineage stage	Early (stages 1–4; zone 1)	Intermediate (stages 5–6; zone 2)	Late (stages 7–10; zone 3)
Cell size	7–9 μm—stem cells 10–12 μm—hepatoblasts 12–15 μm—committed progenitors 17–18 μm—adult cells	~20–25 μm	~25–35 μm
Ploidy	Diploid	Diploid, and some tetraploid depends on age	Tetraploid or higher
Proliferation	Hyperplastic growth (DNA synthesis with cytokinesis)	Hyperplastic growth and some hypertropic growth (depends on the extent of cytokinesis)	Hypertrophic growth (DNA synthesis with negligible cytokinesis)
Representative genes expressed	*Stem cells*: NCAM, EpCAM, CD44H (no AFP and little to no albumin), CS-PGs[a, d] *Hepatoblasts*: ICAM-1[a], EpCAM, AFP[a], CD44H, constitutive albumin[b], P450A7[a], HS-PGs[a, d] *Hepatocytes*: glycogen synthesis enzymes[a], CX 28[a], HS-PGs[d], partially regulatable albumin[b]	Transferrin[c], TAT[a], fully regulatable albumin[b]	P4503A4[a], glutathione-S-transferase, HP-PGs[d] factors associated with apoptosis[a]

AFP alpha-fetoprotein, *CD44* receptor for hyaluronans, *CS-PG* chondroitin sulfate proteoglycan, *CX* connexins (gap junction proteins), *Cyp450* cytochrome P450s, *HS-PG* heparan sulfate proteoglycan, *ICAM-1* intercellular adhesion molecule-1, *NCAM* neural cell adhesion molecule, *TAT* tyrosine aminotransferase

[a]Levels of expression are due to lineage-dependent activation of transcription

[b]Acquisition of relevant regulatory elements in transcription

[c]Translational mechanism(s)

[d]Posttranscriptional modifications (*e.g.*, in Golgi)

One of the branches connects the gallbladder to the biliary tree via the cystic duct. The branch closest to the duodenum forms the ventral pancreas. On the other side the ducts extend and connect to the dorsal pancreas. The formation of the intestine incorporates a twisting motion that swings the ventral pancreas anlage to the other side where it subsequently merges with the dorsal pancreas anlage to form the complete organ. The liver cannot swing to the

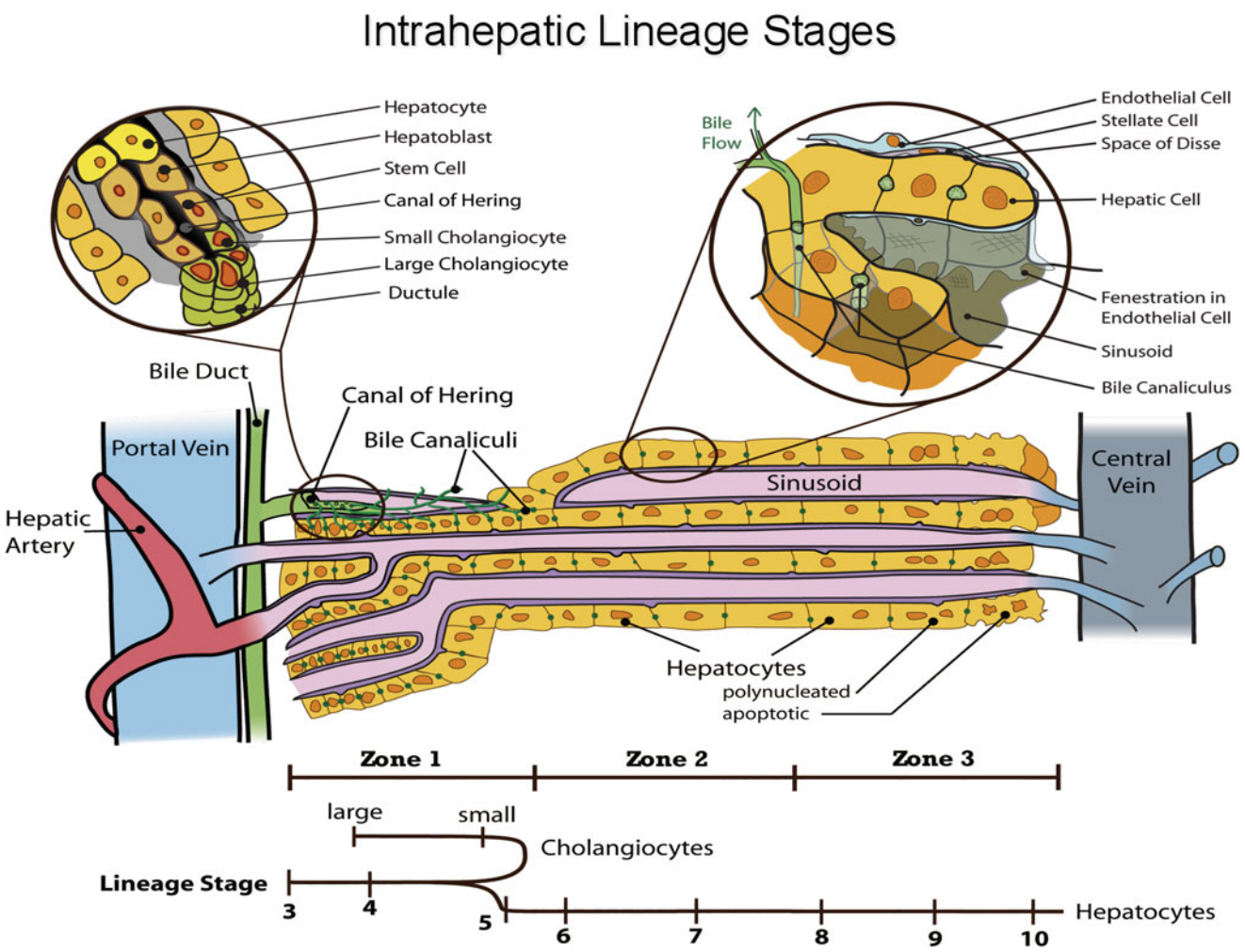

Fig. 2 Schematic of intrahepatic parenchymal lineages in the human liver. Figure is reproduced from Turner et al. (2011)

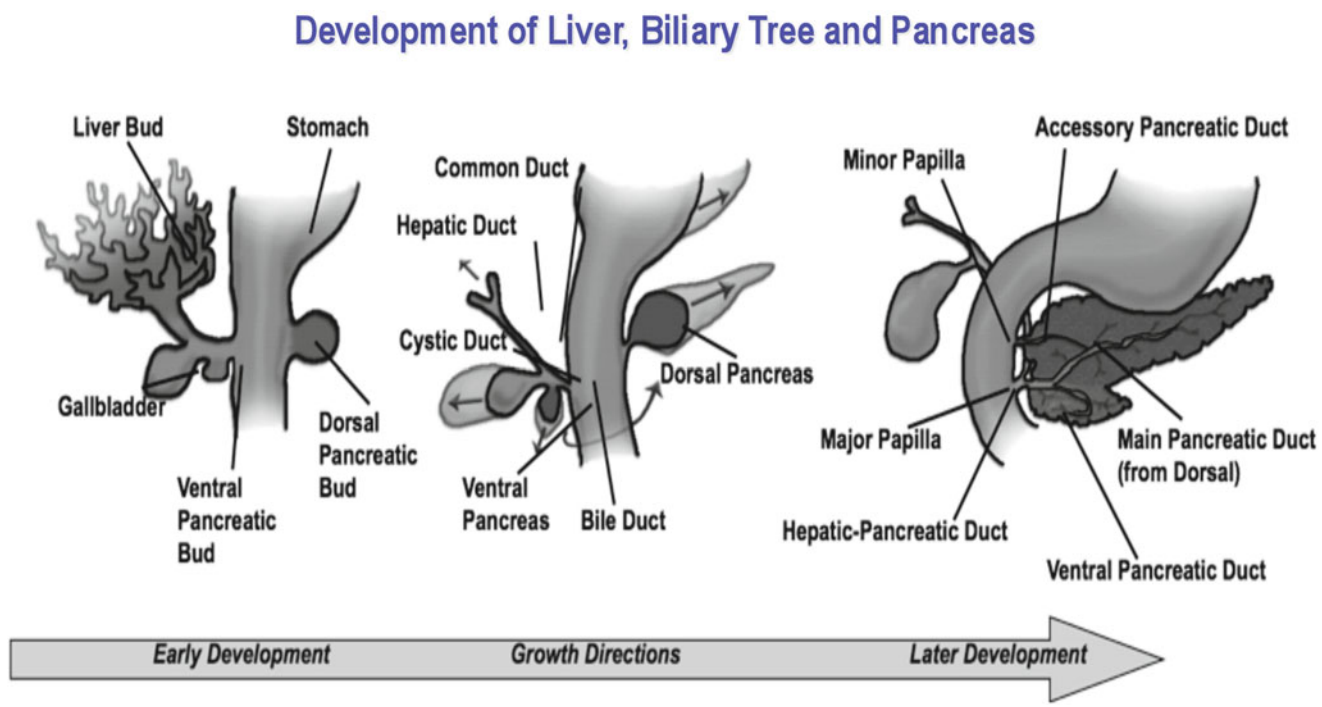

Fig. 3 Development of liver, pancreas, and biliary tree. Reproduced from Cardinale et al. (2012)

opposite side, given its size and its connections into the mesenchyme, connections which are associated with rapid vascularization of the forming tissue. This results in the liver and the ventral pancreas sharing the hepato-pancreatic common duct that connects them to the duodenum, while the dorsal pancreas has a separate connection to the duodenum.

Stem Cell and Progenitor Cell Niches

Stem and progenitor cells reside in discrete locations called niches, each with a unique environment [20] (see also Chaps. 1 and 4) (Fig. 4). The niches for the midgut organs include *peribiliary glands* (PBGs) in the extrahepatic and intrahepatic biliary tree; [2, 21, 22] *the ductal plates* in fetal and neonatal livers; the *canals of Hering*, which are derived from the ductal plates, in pediatric and adult livers; [23–25] and the PDGs [2, 26–28]. These niches form a network that is continuous throughout the biliary tree, with those in the biliary tree anatomically connecting directly to the canals of Hering within the liver and to the PDGs, the reservoirs of committed progenitors, within the pancreas. Many of the cellular components of the niches are known. Stem cell niches comprise epithelial stem cells and their mesenchymal cell partners, angioblasts. Transit amplifying niches or committed progenitor cell niches comprise epithelial transit amplifying cells and committed progenitors and their mesenchymal cell partners, precursors to endothelia, stellate cells, or stromal cells. Paracrine signaling between the epithelial and the mesenchymal cells is essential for viability, proliferative potential, and specilized cell functions. It can be mimicked *in vitro* by use of feeder cells of the relevant mesenchymal type, or by defined mixes of matrix components and soluble signals. To date, the matrix and soluble signals in the stem cell and progenitor cell niches have been only partially defined [29–33]. The known components are listed in Table 2.

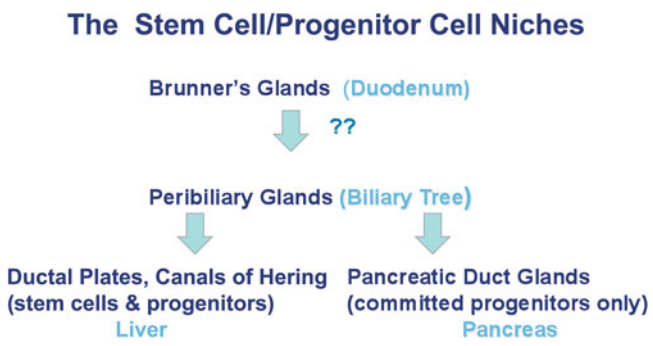

Fig. 4 Network of stem and progenitor cell niches in liver, biliary tree, and pancreas

There are hints, but no proof, that the network of niches begins with the Brunner's glands. These submucosal glands in the duodenum are located between the major papilla, the entranceway to the hepato-pancreatic duct, and the minor papilla, the port connecting the duodenum to the dorsal pancreatic duct. Brunner's glands are not found elsewhere within the intestinal tract. Indeed, they are used to define the transition from the duodenum to the beginning of the small intestine. Future studies should determine their possible relevance to the stem cell and progenitor cell niche network in the biliary tree, liver, and pancreas.

The PBGs occur throughout the biliary tree as *intramural glands*, found within the bile duct walls, and *extramural glands* that are tethered by extensions to the bile ducts [38]. PBGs occur in highest frequencies at the branching points of the biliary tree, with the greatest numbers found in the hepato-pancreatic common duct and the large intrahepatic bile ducts [1] (Fig. 5). Beyond pioneering studies of Nakanuma and associates [38–40], almost nothing is known of the roles of the extramural PBGs.

Each PBG contains a ring of cells at its perimeter and is replete with mucous (PAS-positive material) in its center. The cells in the ring are phenotypically quite homogeneous at some sites (*e.g.*, hepato-pancreatic common duct, large intrahepatic bile ducts) but heterogeneous at other sites (*e.g.*, cystic duct, hilum, common duct). The variations identified thus far implicate maturational lineages for which there are two axes: [22, 41]

- *A radial axis* starting with high numbers of primitive stem cells (characterized by elevated expression of pluripotency genes and other stem cell markers) located in PBGs near the fibromuscular layer in the interior of the bile ducts and ending with mature cells at the lumens of the bile ducts (Figs. 6 and 7)
- *A proximal-to-distal axis* starting with high numbers of primitive stem cells near the duodenum and progressing along the length of the bile ducts to mature cells near the liver or pancreas.

Thus, the radial axis in the biliary tree near the liver results in mature hepatic parenchymal cells. That near the pancreas results in mature cells of pancreatic fate. Radialaxes between liver and pancreas yield cells with mature bile duct markers. The PBGs connect directly into the canals of Hering, the intrahepatic stem cell niches, and at the level of the hepato-pancreatic common duct, the PDGs. The network provides a biological framework for ongoing organogenesis of liver, biliary tree, and pancreas throughout life.

These phenomena parallel the well-described intestinal lineage system. The radial axis of maturation in the intestine progresses from stem cells in the crypts to fully differentiated cells at the tops of the villi. The proximal-to-distal axis follows the length of the intestine and results in distinct

Table 2 Cellular and microenvironment components in precursor cell niches [25, 33–37] (see also Chap. 4)

A. Stem cell niches			
Cells types	Biliary tree and hepatic stem cells	Mesenchymal cell partners: angioblasts	
Markers	NCAM, CD133, LGR5, SOX9, SOX17, PDX1, pluripotency genes that include Nanog, OCT4, SOX2, KLF4, SALL4	CD117, CD133, VEGF-receptor, Von Willebrand factor	
Extracellular matrix components	Type III collagen, hyaluronans, minimally sulfated chondroitin sulfate proteoglycans, a form of laminin that binds to alpha6/beta4 integrin (laminin-5?)		
Soluble factors	Leukemia inhibitory factor (LIF), interleukin 11 (IL-11), and others still being identified		
B. Transit amplifying cell and progenitor cell niches			
Cell types	Hepatoblasts, hepatocytic and biliary committed progenitors, pancreatic committed progenitors	Mesenchymal cell partners: Stellate cell precursors	Endothelial cell precursors
Markers	EpCAM, ICAM-1, SOX9, no expression of pluripotency genes —AFP, albumin, CYP450A7 in hepatoblasts —Glycogen, albumin, CX28 in hepatocytic committed progenitors —Secretin receptor, CFTR in biliary committed progenitors —NGN3, MUC6, amylase, low levels of insulin, and other islet hormones in pancreatic committed progenitors	CD146, alpha-smooth muscle actin (ASMA), low levels of desmin, ICAM-1, but no GFAP and minimal levels of vitamin A	CD133, CD31, VEGF-receptor, Von Willebrand factor
Extracellular matrix components	Hyaluronans, type IV collagen, form(s) of laminin binding to alpha/beta1 integrin, sulfated forms of CS-PGs, and minimally sulfated form of HS-PGs		
Soluble factors	HGF, EGF, bFGF, IL-11, IL-6, and others		

AFP alpha-fetoprotein, *CFTR* cystic fibrosis transmembrane conductance regulator, *CS-PG* chondroitin sulfate proteoglycan, *EGF* epidermal growth factor, *FGF* fibroblast growth factor, *GFAP* glial fibrillary acidic protein, *HGF* hepatocyte growth factor, *HS-PG* heparan sulfate proteoglycan, *ICAM-1* intercellular adhesion molecule-1, *IL* interleukin, *MUC6* Mucin 6, oligomeric mucus/gel-forming, *NGN3* neurogenin 3, *VEGF* vascular endothelial cell growth factor

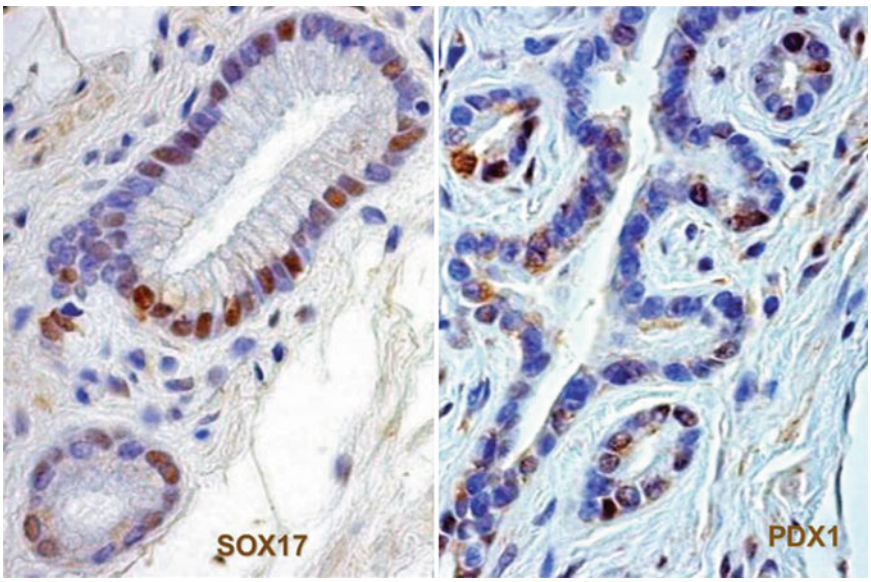

Fig. 5 PBGs in adult human biliary tree tissue stained for SOX17 or PDX1. Figure modified from Cardinale et al. (2011)

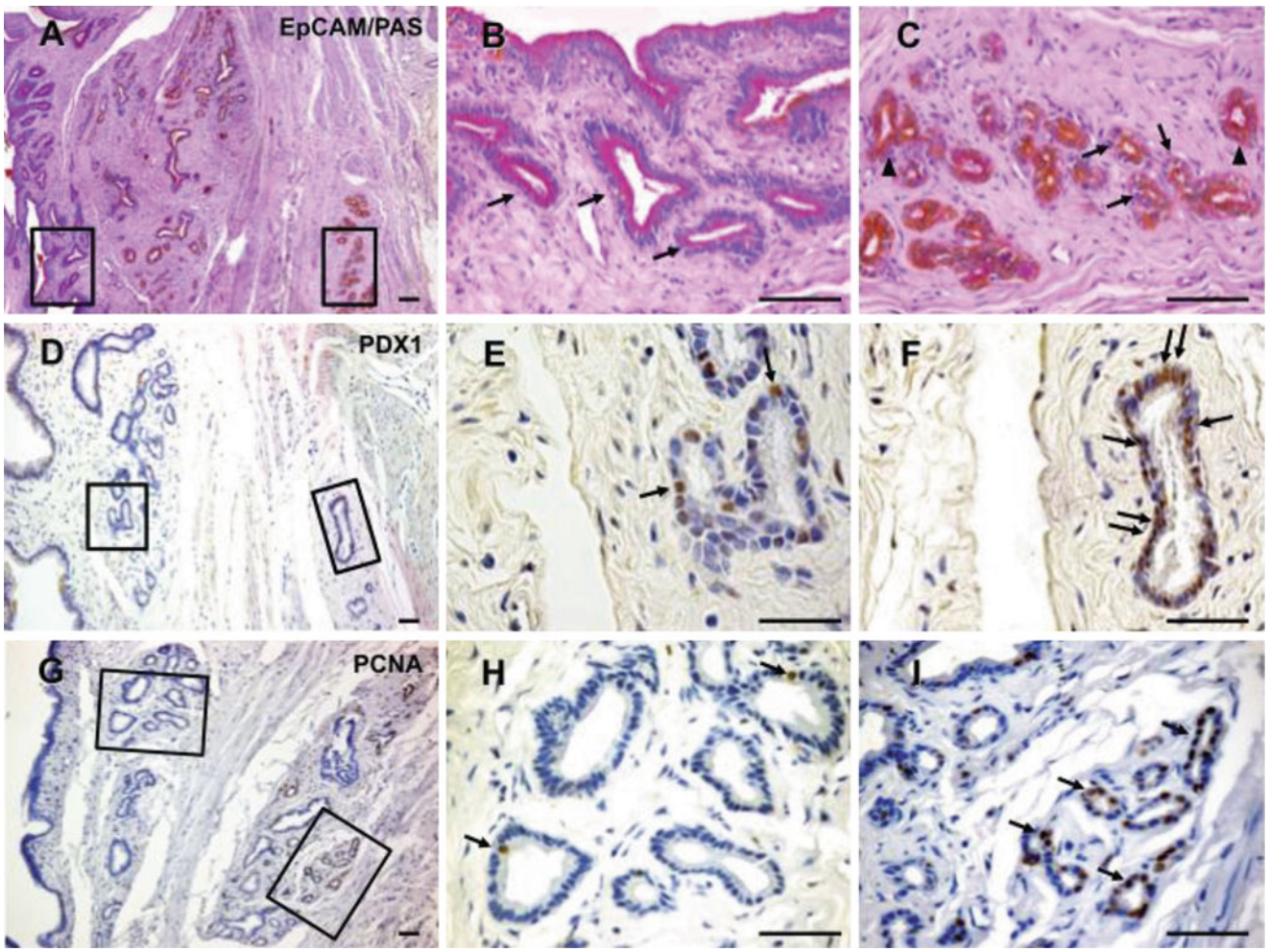

Fig. 6 Radial axis maturational (*blue*) lineage within bile ducts. Gradient in expression of stem cell markers and of cell proliferation in PBGs (*red*). (**a**–**c**) Immunohistochemistry for EpCAMs counterstained with PAS. Glandular elements just beneath the surface epithelium (see magnification in (**b**)) are mostly EpCAMs-negative and PAS-positive (goblet cells); by contrast, acini deeply located near the fibromuscular layer (see magnification in (**c**)) are composed of cells that are or [EpCAM+, PAS+] cells (*arrowhead*). a mix of [EpCAM−, LGR5+] and [EpCAM+, LGR5+] and PAS or negative (*arrows*). (**d**–**f**) Immunohistochemistry for PDX1. PDX1+ cells (*brown*) are mostly situated deeply within duct walls (see magnification in (**f**): *arrows*). PBGs near the surface epithelium are occasionally PDX1+ (*arrow* in (**e**)). (**g**–**i**) Immunohistochemistry for PCNA. Proliferating cells are mostly present in glandular elements located near the fibromuscular layer (*arrows* in (**i**)). Few cells are positive in more superficial acini. Notably, surface epithelial cells are (*brown*) mostly negative for PCNA. Scale Bar = 50 μm. Figure reproduced from Carpino et al. (2012)

mature cells depending on whether they are located in the esophagus, stomach, duodenum, small or large intestine.

The phenotypic changes in cells in the PBGs along the identifiable maturational lineages indicate the existence of multiple subpopulations of stem cells. Populations in PBGs at the start locations (near the fibromuscular layers in the interior of the bile ducts) and those near the duodenum have the highest numbers of stem cells that co-express endodermal transcription factors essential for liver and pancreas formation (*e.g.*, SOX9, SOX17, PDX1). These cells also express genes associated with pluripotency (Nanog, OCT4, SOX2, KLF4, SALL4) and other early lineage stage markers (NCAM, LGR5, CD133) or indicators of proliferation (*e.g.*, Ki67). Furthermore, they do not express detectably markers of mature cells (*e.g.*, insulin, albumin) [22, 41]. The PBGs between those with the most primitive stem cell traits and those with mature markers are characterized by cells with an intermediate phenotype: expression of epithelial cell adhesion molecule (EpCAM); some but not all of the endodermal transcription factors (*e.g.*, PDX1 or SOX17, but not both); less or negligible amounts of the pluripotency genes; fewer, if any, of the other stem cell traits (*e.g.*, LGR5 or CD133); and low but detectable expression of one or more mature cell markers (*e.g.*, albumin or insulin). The extent of expression

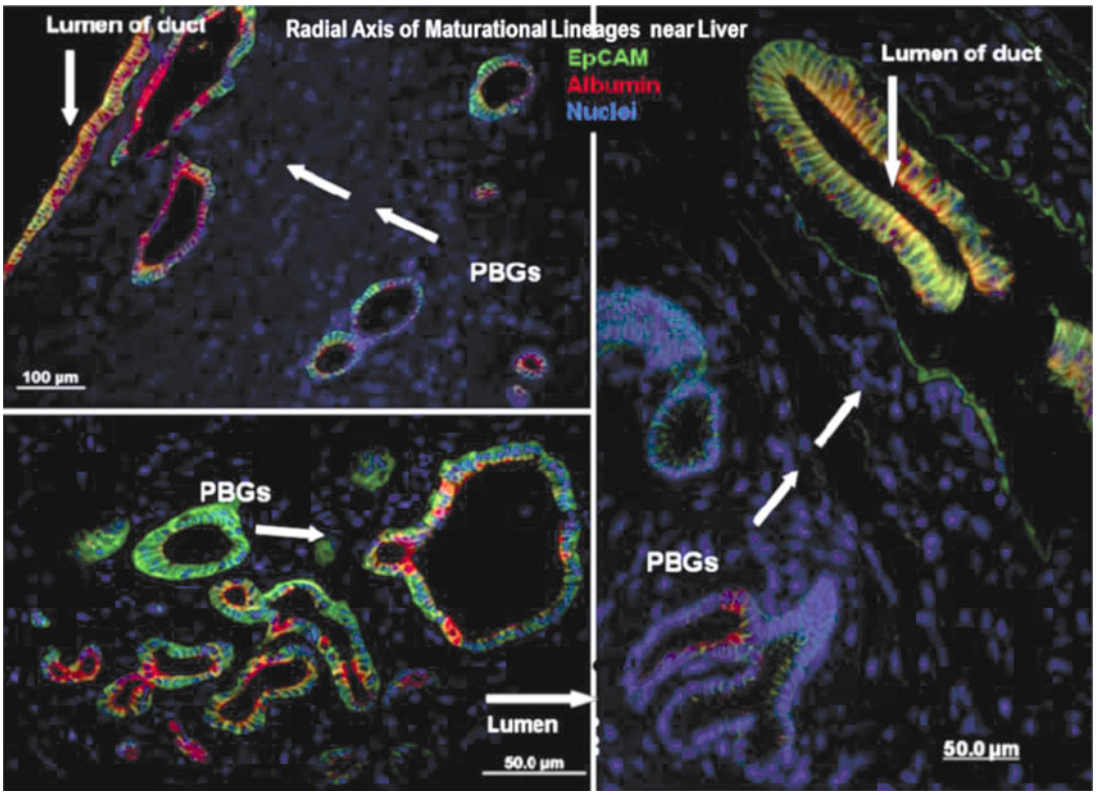

Fig. 7 Radial axis maturational lineage near liver. The markers change from those found in the PBGs near the fibromuscular layer to those found in cells at the lumens of the bile ducts. Here are images tracking two markers, albumin and EpCAM, from the PBGs to the luminal surface of the ducts. Note that some of the cells within the PBGs express EpCAM or albumin, while some do not. With progression towards the surface, there are PBGs in which all of the cells express EpCAM but may be devoid of albumin expression. Finally, the cells at the lumen of the ducts express both EpCAM and albumin. We hypothesize that these findings are evidence for a maturational lineage progressing from the PBGs deep within the walls of the bile duct to the cells at the luminal surface of the ducts and that EpCAM is an intermediate marker and albumin a more mature marker for the cells that are maturing towards a liver fate. This occurs in the portion of the biliary tree closest to the liver. Figure reproduced from a figure in the online supplement of Cardinale et al. (2011)

of the mature lineage markers increases with proximity to the bile duct lumens and to the liver or the pancreas (Figs. 6, 7, and 8).

The *in situ* studies summarized above have been complemented by *in vitro* studies of the biliary tree stem cells, hepatic stem cells, hepatoblasts, and committed progenitors of liver or pancreas. More details on those in the liver are given later in this review. Here, we will summarize observations on biliary tree stem cells (Fig. 9).

These cells can be dispersed into a cell suspension and grown on culture plastic or on certain extracellular matrix components and in *Kubota's Medium*, a serum-free formulation tailored for culture selection and expansion of endodermal stem cells and progenitors. The same medium has proven useful also for angioblasts and their descendants [1, 29, 31, 33, 42] (Fig. 10). Kubota's Medium comprises any rich basal medium with low calcium (~0.3 mM), no copper, selenium (~10^{-10} M), zinc (~10^{-12} M), insulin (~5 µg/mL), transferrin/fe (~5 µg/mL), high-density lipoprotein (~10 µg/mL), and a defined mixture of purified free fatty acids bound to highly purified albumin. Notably, the medium contains no cytokines or growth factors. Mature cells do not survive in Kubota's Medium, only the stem cells and progenitors from both epithelial and mesenchymal cell lineages. Thus, it co-selects for endodermal stem cells and progenitors and their mesenchymal stem/progenitor cell partners, angioblasts and their descendants, precursors of stellate cells or endothelia [29, 33].

We observed two major types of biliary tree stem cell colonies in cultures. Type 1 colonies have cells that undulate ("dancing cells"), are very motile, and initially do not express EpCAM (CD326) but acquire it at the edges (the perimeters) of the colonies, corresponding to slight cellular differentiation.

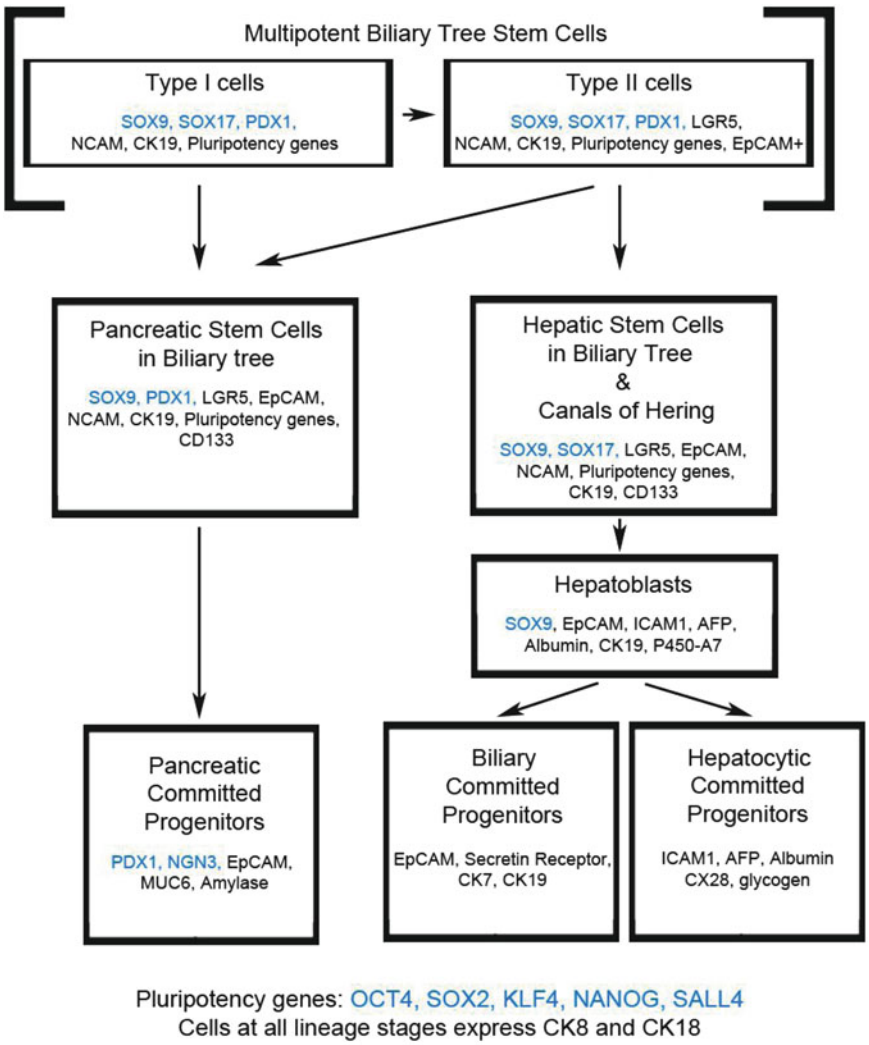

Fig. 8 Major subpopulations of stem cells and progenitors in liver, biliary tree, and pancreas. It is unknown at this time whether the pancreatic stem cells, located within the biliary tree, derive from type I or type II biliary tree stem cells or both. Although the two populations of biliary tree stem cells and pancreatic stem cells are present in highest numbers in the hepato-pancreatic common duct, they are found also in large numbers in the PBGs of the large intrahepatic bile ducts (figure prepared with information from Carpino et al. 2012; Wang et al. 2012)

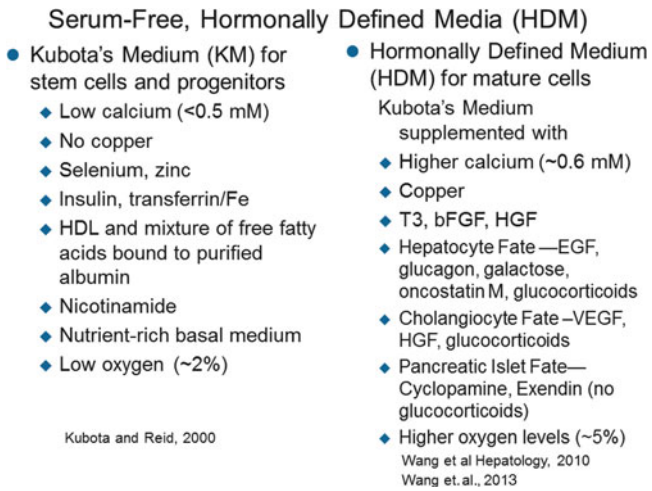

Fig. 9 Serum-free, hormonally defined media (HDM) used for expansion or for differentiation of stem/progenitors. They include Kubota's Medium used for stem/progenitors and HDM tailored to a mature cell type

The type 1 colonies are precursors to type 2 colonies. The latter show uniform expression of EpCAM from the outset and display a carpet-like appearance with cells of uniform morphology. The expansion potential of the cells in culture in Kubota's Medium is considerable: 2–3 cells can grow to colonies of more than 500,000 cells in ~8 weeks [1]. The cells retain a stable stem cell phenotype (i.e., self-renew) throughout months of culture and may be subcultured ("passaging"). Initially cells show a typical division time of about 1–2 days, but within a week, they slow to a division every 2–3 days. At 8 weeks the colonies contain cells in the centers that are morphologically uniform, are small (7–9 μm), and express high levels of stem cell markers. Cells at the edges of the large colonies are slightly larger (~10–12 μm) and have weak expression of EpCAM and expression of markers intermediate in the differentiation pathways, indicating potential loss of stemness and transition to more mature progenitors.

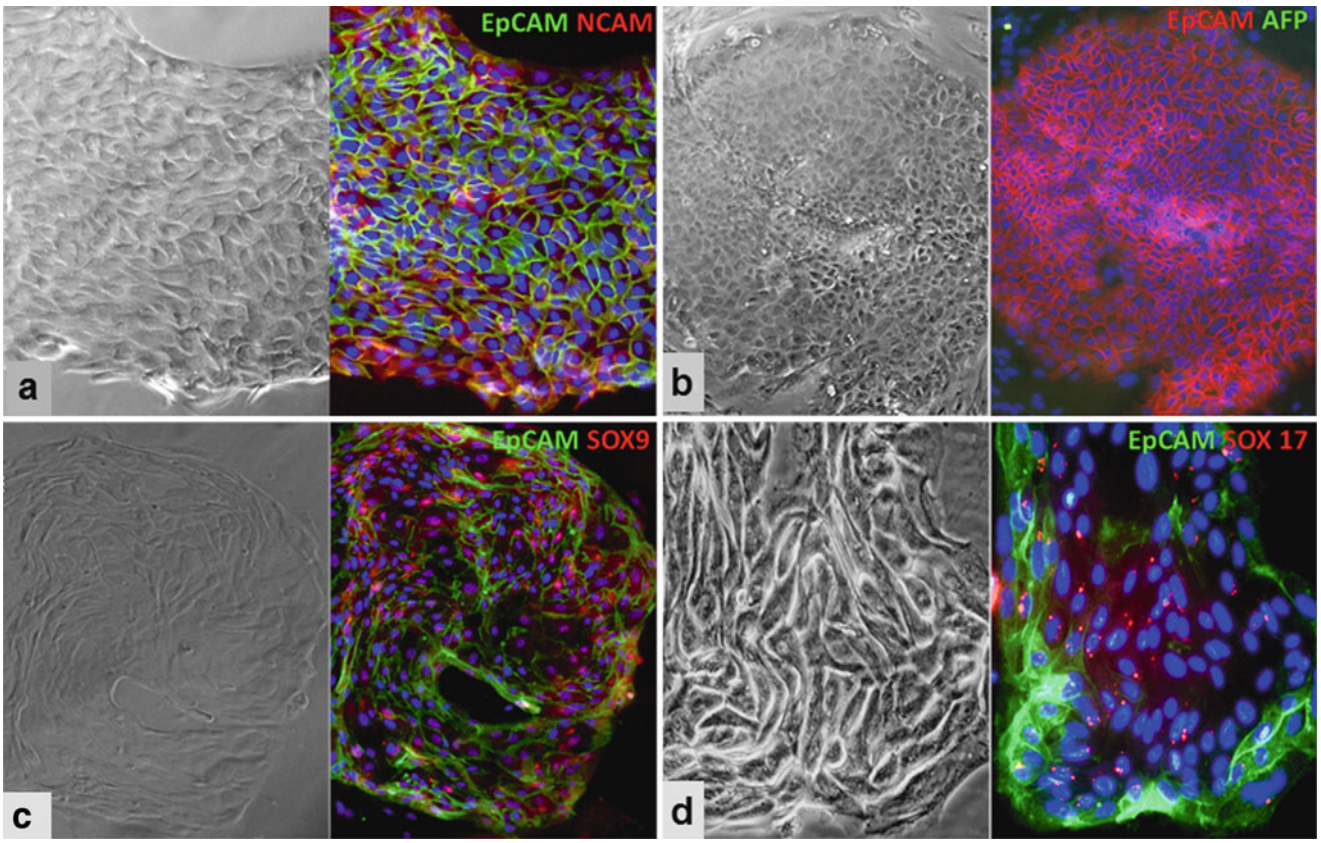

Fig. 10 There are two types of biliary tree stem cell colonies. Type II colonies (**a**, **b**) contain cells those that express EpCAM on every cell from the outset. Type I colonies (**c**, **d**) are those that are EpCAM negative initially but acquire expression of EpCAM at the perimeter of the colonies. Reproduced from Cardinale et al. (2011)

Using three-dimensional (3D) hydrogels and appropriate signaling molecules, the biliary tree stem cells can be induced to differentiate to hepatocytes, cholangiocytes, or pancreatic neo-islets [1]. We have not done studies yet to learn if they can give rise to acinar cells. The differentiation is achieved by embedding the stem cells in specific mixes of extracellular matrix components (hyaluronans and type I collagen for bile ducts, hyaluronans and type IV collagen and laminin for hepatocytes or islets) and providing a serum-free, hormonally defined medium (HDM) tailored for a specific mature cell type. The HDM are prepared by supplementing Kubota's Medium with copper (10^{-12} M), higher calcium (0.6 mM), and bFGF (10 ng/mL) and then adding a unique set of hormones and growth factors for hepatocytes (*HDM-H*, glucagon, galactose, T3, oncostatin M, hepatocyte growth factor (HGF), epidermal growth factor (EGF), glucocorticoids), cholangiocytes (*HDM-C*, HGF, EGF, VEGF, glucocorticoids), or pancreatic islets (*HDM-P*, B27, ascorbic acid, cyclopamine, retinoic acid, HGF, and, after 4 days, replacement of bFGF with Exendin-4). Further optimization of these conditions is underway.

The gene expression profiles of cells in the 3D hydrogels complemented the morphological observations. For example, cells cultured under conditions for hepatocytes produced albumin, transferrin, and P450s. Cells in conditions for cholangiocytes expressed anion exchanger 2 (AE2), cystic fibrosis transmembrane conductance regulator (CFTR), gamma glutamyl transpeptidase (GGT), and secretin receptor. Cells in conditions for pancreatic islets expressed transcription factor PDX1 and the hormones glucagon, somatostatin, and insulin. Specific staining for human C-peptide confirmed *de novo* synthesis of proinsulin, and its secretion was regulated in response to the level of glucose. *In vivo* studies provided further evidence for the multipotency of the human biliary

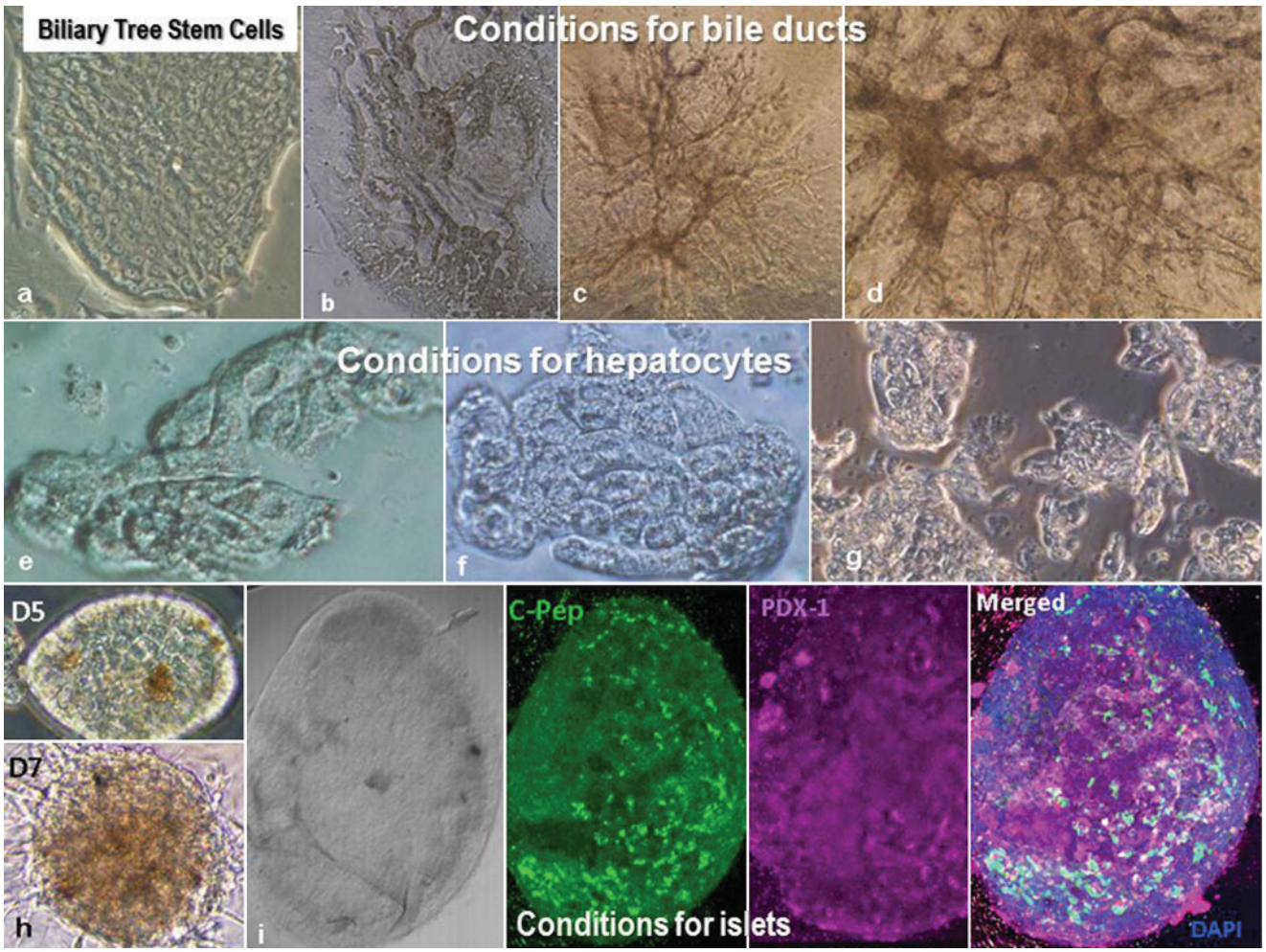

Fig. 11 Self-replication of biliary tree stem cells in Kubota's Medium (**a**). Lineage restriction of the biliary tree stem cells to bile ducts (**b**–**d**) vs. hepatocytes (**e**–**g**) vs. pancreatic islets (**h**–**l**). In (**h**) are shown two images of a neo-islet stained for zinc, a co-factor in insulin synthesis. In (**i**) is a phase contrast image of the hydrogel that was then stained for C-Peptide (**j**), PDX1 (**k**) and a merged image of (**j**, **k**). Figure is modified from a figure in Cardinale et al. (2011)

stem cells for hepatic, biliary tree, and pancreatic fates. Direct injection of the stem cells into the livers of immune-deficient mice generated mature human hepatocytes and cholangiocytes (Fig. 11).

To confirm endocrine pancreatic differentiation, pre-induced neo-islet structures were implanted into mouse fat pads, and the animals were treated with a toxin (streptozotocin) at a dose sufficient to destroy their own pancreatic beta cells, but not human beta cells. Those mice transplanted with the human neo-islets showed significant resistance to hyperglycemia compared to controls that did not receive cell therapy. The presence of functional beta-like cells derived from the biliary tree stem cells produced serum levels of human C-peptide, which was regulated appropriately in response to a glucose challenge [1] (Fig. 12). Further studies have confirmed and expanded upon these initial findings, leading us to conclude that the hepato-pancreatic common duct is the major reservoir of stem cells giving rise to committed progenitors found in PDGs and thence to pancreatic islets throughout life [41]. Ongoing studies are testing whether the maturational lineage involves a migration of cells or, as in the intestine, a type of "conveyer belt" leading to mature cells. See Table 3 for markers occurring at varying stages along the proximal-to-distal axes.

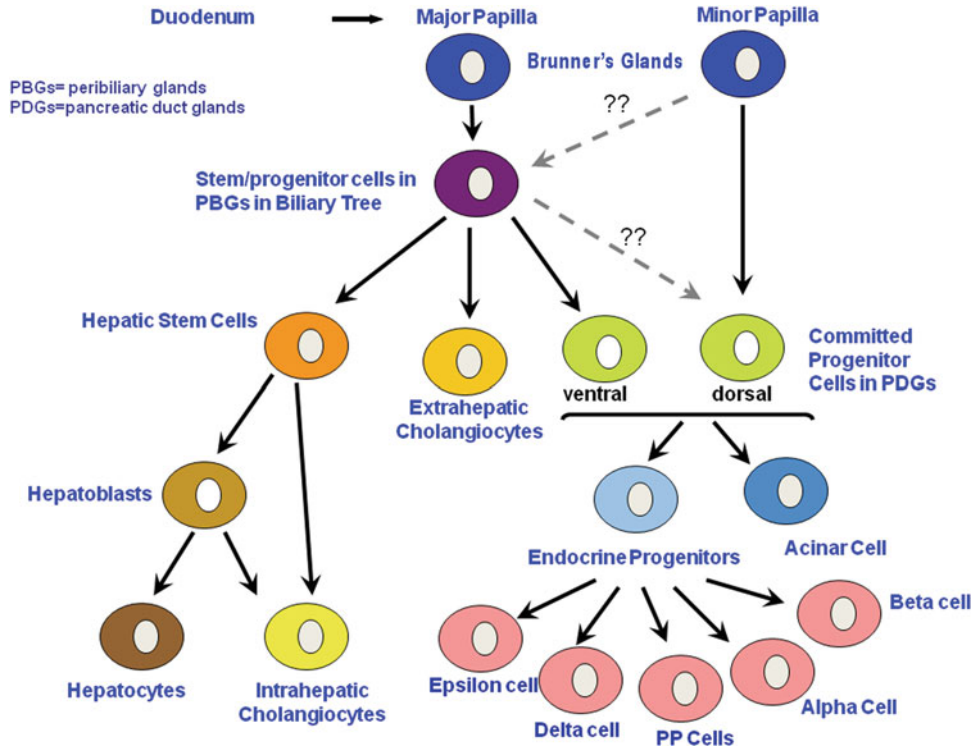

Fig. 12 Schematic of probable lineage pathways for stem cells and progenitors in the biliary tree. Figure is from a review by [1]

Table 3 Comparison of markers of stem/progenitor cells in liver, biliary tree, and pancreas (reproduced from a table in the online supplement of [2]

Proximal-to-distal axis of the maturational lineages						
	Liver		⟷			Pancreas
Cells	Hepatoblasts—adjacent to Canals of Hering [25, 29]	Hepatic stem cells in Canals of Hering [3]	Biliary tree stem cell subpopulations in peribiliary glands (PBGs) [2, 21, 22, 64]			Pancreatic committed progenitors in pancreatic duct glands (PDGs) [2]
Endoderm markers	SOX9	SOX9, SOX17	SOX9, SOX17	[a]SOX 9, SOX17, PDX1	SOX9, PDX1	SOX9, PDX1
Epithelial markers			CK8 and 18, CK19, E-cadherin			
Cell adhesion molecules	αβ1 integrin, ICAM-1 EpCAM	α6β4 integrin [33], NCAM, EpCAM	NCAM, EpCAM	NCAM	NCAM, EpCAM	Integrins [100] EpCAM [101]
			Integrins not yet studied			
Pluripotency genes[b]	None	moderate	Strong OCT4A, SOX2, NANOG, KLF4			None [102, 110]
Other stem cell markers	Weak CXCR4, CD133, SALL4	Strong CXCR4 CD133, LGR5, SALL4 CD117 [31][c]	CXCR4, CD133, SALL4, LGR5			CXCR4 [106], CD133 [104, 107] CD24 [105]
Hedgehog proteins	Weak Indian and Sonic [32]	Strong Indian and Sonic [32]	Indian; Sonic hedgehog expressed			Weak Sonic [102, 104, 111]
Matrix proteins	Laminin, type IV collagen [33]	Laminin-5, type III collagen [33, 62]	Not tested			Fetal islets have collagen IV, V, VI, nidogen, elastin; fetal acinar cells have primarily fibrillar collagens, fibronectin
GAG/PGs	HA, CD44, syndecans, and CS-PGs [33, 66, 67]	HA, CD44, minimally sulfated CS-PGs [33, 103]	HA, CD44; others not yet tested			HA, CD44, fetal islets have syndecans (HS-PG-1 and 3), glypicans; fetal acinar cells have primarily CS-PGs (Wang and Reid, unpublished studies)

(continued)

Table 3 (continued)

Liver traits	Albumin++, AFP+++, P450A7, Glycogen [31]	Albumin+/−, AFP− [31]	None of the mature traits	None
Pancreatic traits	None	None	ISL1, PROX 1, NeuroD, PAX4	NGN3, MAFA, MUC6, Nkx6.1/ NKx6.2 (Nkx6) and Ptf1a [108, 112], GLUT2 [109]
Multidrug resistance genes	MDRI-negative ABCG2-moderate [64]	MDRI-1 moderate, MVR-1 Strong ABCG2 Strong [64] ABCG2 [64]		None

PBGs peribiliary glands, *PDGs* pancreatic duct glands, *HA* hyaluronans, *HS-PGs* heparan sulfate proteoglycans, *CS-PG*$_S$ chondroitin sulfate proteoglycans, *Syndecans* HS-PGs that have transmembrane core proteins, *Glypicans* HS-PGs linked to plasma membrane by PI linkages, *MDRI* multidrug resistance genes

[a]These biliary tree stem cells are the most primitive and found near the fibromuscular layer within the bile ducts; they give rise in the radial axis maturational lineage to EpCAM+ cells

[b]*Pluripotency genes* = OCT4, NANOG, KLF4, SOX2

[c]*CD117* is found in canals of Hering and present on angioblasts that are tightly bound to the hepatic stem cells; it is hypothesized to be found in the PBGs in association with the various stem cell subpopulations. *Hepatoblasts*, transit amplifying cells, give rise to *hepatocytic and biliary committed progenitors* that do *not* express SOX17, pluripotency genes, LGR5, or other markers of stem cells. See also Fig. 8

Hepatic Stem Cells

Those familiar with the myth of Prometheus will recall that the liver possesses a remarkable capacity for regeneration [43]. Yet liver diseases, potentially leading to organ failure due to hepatitis viruses, alcohol consumption, diet and metabolic disorders, and other causes, constitute a major medical burden [44–46] (Fig. 13).

Cell-based therapies and tissue engineering represent possible approaches to address these needs [3, 47–50]. Sourcing of cells for such applications is a significant challenge. In some countries it is possible to obtain fetal tissues. In others neonatal or adult tissues can be used. Given the newly discovered source of stem cell population in the biliary tree, this tissue represents a major potential source of the stem cells for cell therapy and tissue engineering for both liver and pancreas.

Here we will focus specifically on stem/progenitor cells of the liver and biliary tree as they pertain to formation of liver (Fig. 14). The role(s) of stem cells in the normal maintenance of the liver and in regeneration from various insults remains a subject of active research and debate [23, 24, 43, 49, 51–56]. This section of the review focuses more on hepatic stem cells (hHpSCs) and hepatoblasts (hHBs) and what is known of their location and involvement in quiescent vs. regenerative liver tissue.

There are multiple stem cell populations located within the peribiliary glands of the intrahepatic bile ducts in livers of all donor ages [22] and in the ductal plates in fetal and neonatal livers [25, 57, 58]. The ductal plates transition to become canals of Hering in pediatric and adult livers; they consist of small ductules located at each of the portal triads [24, 25, 59, 60]. The canals of Hering give rise to the organ's two specialized epithelial cell types, hepatocytes and cholangiocytes (bile duct cells), via an organized maturational lineage system [3].

Hepatic Stem Cell Isolation and Expansion

We reported several years ago on the isolation of human hepatic stem cells (hHpSCs) from fetal, neonatal, pediatric, and adult human livers by selection with a monoclonal antibody for the surface marker EpCAM [31] (Fig. 15). These cells constitute approximately 1 % (0.5–1.5 %) of the total liver population from early childhood onwards. Unlike mature hepatocytes, they survive extended periods of ischemia, allowing collection even several days after cardiac arrest [61]. The hHpSCs express additional surface markers often found on stem/progenitor cells, such as CD133 (prominin), CD56 (neural cell adhesion molecule, NCAM), and CD44 (the hyaluronan receptor); they also express characteristic endodermal transcription factors SOX9, SOX17, and HES1. They are small (diameter 7–9 μm, which is less than half that of mature parenchymal cells) and express weak or negligible levels of adult liver-specific functions such as albumin, cytochrome P450s, and transferrin. The stem cells display far greater capacity to proliferate in culture than hepatocytes or cholangiocytes and can continue to expand for months with a doubling time of 36–40 h. The colonies that form look remarkably similar to those of embryonic stem (ES) cells or iPS cells [31, 33, 41, 62].

The hHpSCs serve as immediate precursors of hepatoblasts. The hepatoblasts are readily distinguished by the expression of α-fetoprotein and intercellular adhesion molecule-1 (ICAM-1), for which the hHpSCs are negative [25, 31, 33] (Fig. 16). The hepatoblasts, in turn, are precursors of committed unipotent progenitors for hepatocytes and cholangiocytes. When injected into the livers of immune-deficient mice, the hHpSCs give rise to cells expressing characteristic human liver and bile duct proteins, especially after the host's liver has been damaged by treatment such as with carbon tetrachloride.

Whereas there has been limited success to achieve *ex vivo* expansion of hematopoietic stem cells, the stem cell

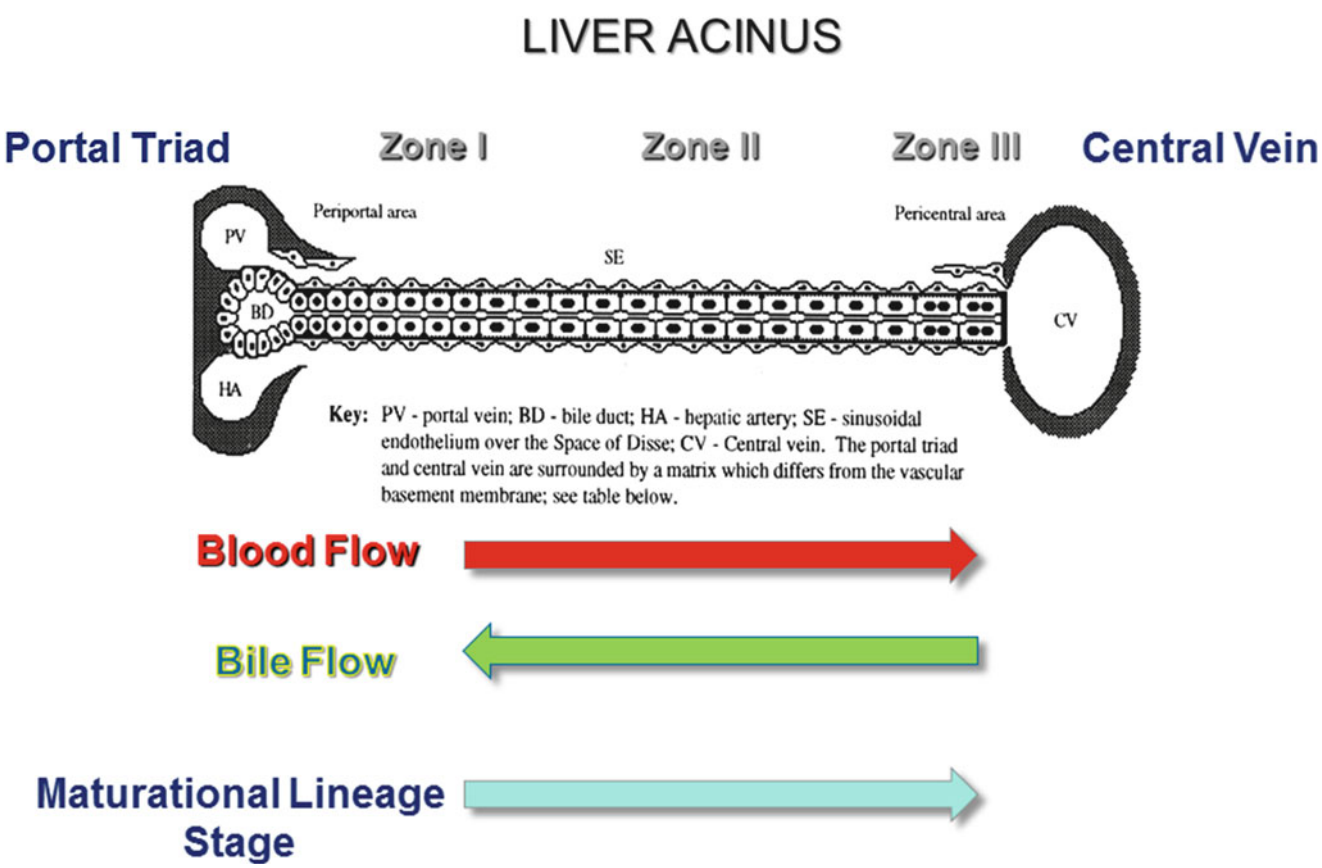

Fig. 13 Schematic of the liver acinus encompassing portal triads to central vein and showing direction of blood flow, bile flow, and maturational lineage. Modified from a figure in Reid et al. (1992)

populations of the hepatic lineages proliferate rapidly and for a sustained period in Kubota's Medium [29] which, as stated previously, contains no additional growth factor or cytokine. Conceivably, pathways important for hepatic stem/progenitor cell survival *in vivo*, such as Hedgehog (Hh) signaling [32], are activated through autocrine loops. The expanded hHpSCs maintain a stable marker phenotype and express the enzyme telomerase, whose mRNA and the protein encoded are localized to the nucleus in the hHpSCs and the hepatoblasts; telomeric enzymatic activity correlated well with both the mRNA and protein and with the protein being found within the nucleus (Fig. 17). However, later lineage stages (committed progenitors to late lineage stage mature cells) have no evidence of synthesis of telomerase but have large amounts of telomerase protein localized cytoplasmically. Telomeric enzymatic activity does not correlate with total telomerase protein levels. We hypothesize that regenerative demands will result in small amounts of the cytoplasmic reserves of telomerase relocating to the nucleus. If we are correct, the enzymatic activity levels should correlate with the amount of telomerase protein in the nucleus [63].

More recently, we have observed that Sal-like protein 4 (SALL4) is strongly expressed in the hBTSCs, hHpSCs, and hHBs but not in committed progenitors of either liver or pancreas [64]. SALL4 is a member of a family of zinc finger transcription factors and a regulator of embryogenesis, organogenesis, and pluripotency. It can elicit reprogramming of somatic cells and is a marker of stem cells. We found it expressed in normal mHBs, normal hHpSCs, hHBs, and hBTSCs, but not in committed hepatocytic or biliary progenitors and not in mature parenchymal cells of liver or biliary tree.

A crucial prerequisite for successful expansion of hHpSCs is to mimic an appropriate microenvironment. When selected for growth *in vitro* on tissue culture plastic and in Kubota's Medium, the hHpSCs grow as colonies with feeders of angioblasts (CD117+, VEGF-receptor+, CD133+, Von Willebrand Factor+); [31, 33, 37] the feeders can be replaced with weakly cross-linked hyaluronans and type III collagen [30, 33, 65]. The cells expand for months under these conditions. By contrast, hHBs survive for only about a week under the same conditions, but they can survive if they are co-cultured with stellate cell precursors (CD146+, alpha-smooth muscle actin+, desmin+, VCAM+, ICAM-1+, GFAP) or feeders of MSCs. The stellate feeder cells (or feeders of MSCs) can be replaced with hyaluronans, type IV collagen, and/or laminin [33, 66, 67]. The medium and matrix conditions

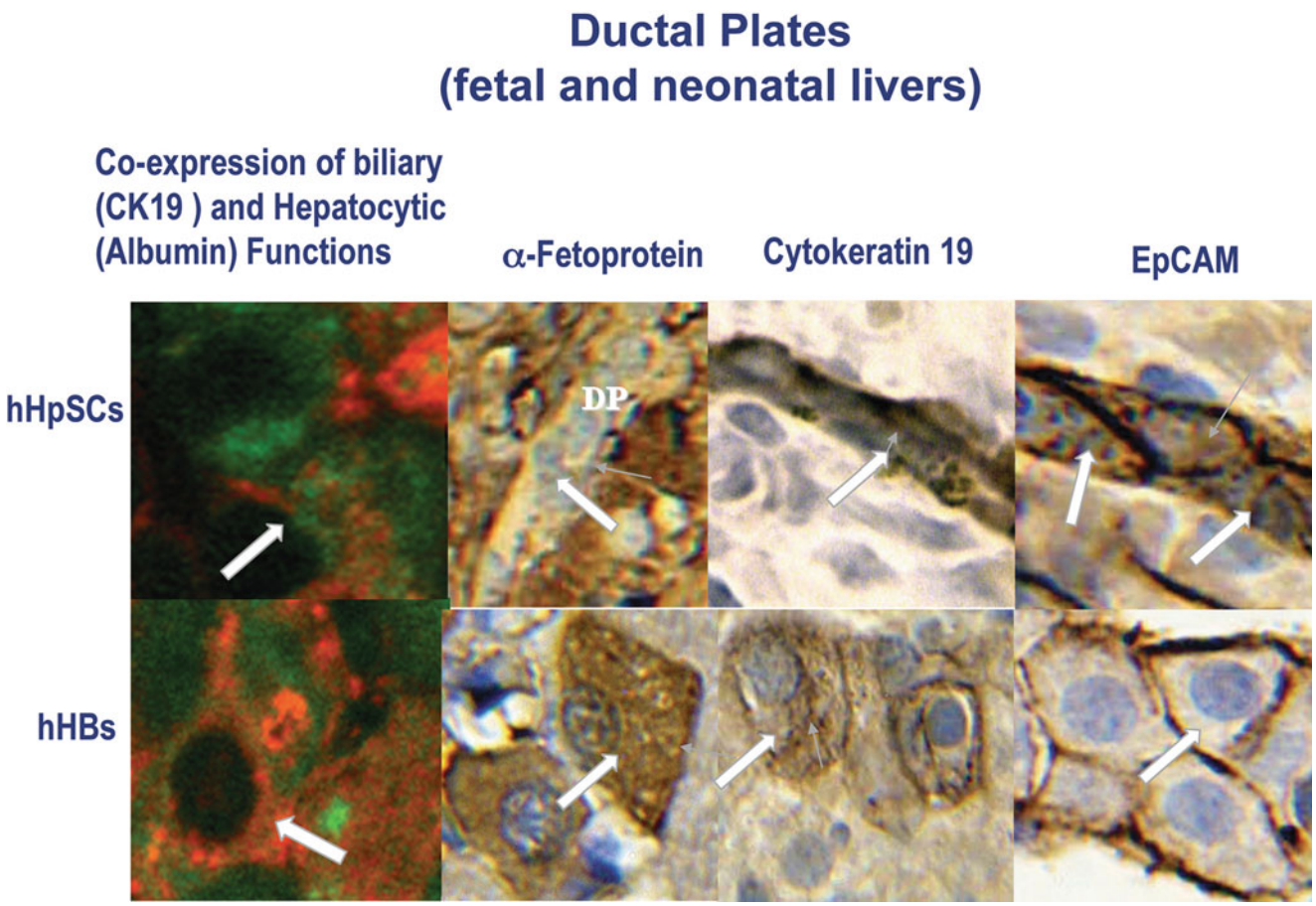

Fig. 14 Ductal plates found in fetal and neonatal livers with the location of hHpSCs and hHBs (see *white arrows*). Both co-express albumin and CK19. The hHBs, but not the hHpSCs, express alpha-fetoprotein (AFP). The expression of CK19 and EpCAM are more intense in hHpSCs. EpCAM in hHBs is confined primarily to the plasma membrane. Published information on the stem cell niches derived from Zhang et al. (2008); figure was first published in a methods review (Wauthier et al. 2008)

described above allow for flow cytometrically purified hHpSCs or hHBs to survive and proliferate in culture and without the need for feeders. Both type III collagen and hyaluronans are constituents of the normal liver stem cell niche [30, 33].

Conversely, 3D cultures in hyaluronans supplemented with other matrix components, and used in combination with serum-free medium supplemented with specific hormones and growth factors (HDM-H or HDM-C), result in differentiation of the cells. The HDM for driving the stem cells to a mature fate consists of Kubota's Medium supplemented with copper (10^{-12} M), calcium (0.6 mM), basic fibroblast growth factor (10 ng/mL), and glucocorticoids (10^{-8} M) and further tailored for hepatocytes with supplementation of glucagon, galactose, triiodothyronine (T3), oncostatin M, EGF, and HGF; an HDM for cholangiocytes contains HGF, vascular epithelial growth factor (VEGF), and EGF [33]. The matrix components used are hyaluronans into which are mixed network collagens (type IV, type VI) and laminin for hepatocytes vs. type I collagen or type I collagen and fibronectin for cholangiocytes.

The hHpSCs also respond to mechanical forces. Initially, it was apparent that hHpSCs grew better on transwells coated with type III collagen rather than hard plastic surfaces with the same coating [30, 62]. A systematic study of hHpSC behavior in 3D cultures using hyaluronan hydrogels of differing stiffness indicated that rigidity of the microenvironment is an important parameter in regulating maintenance of stemness vs. differentiation to more restricted progenitors [68]. This had been studied previously in differentiation of progenitors for bone and other hard tissues, but not for internal organs such as the liver. The optimal expansion of the hHpSCs for clinical applications likely will be achieved in 3D hydrogels containing type III collagen, hyaluronans, and possibly additional matrix components or synthetic mimetics.

The hHpSCs, like human ES cells, grow in tight colonies. Dissociating either type of stem cells has proven to be an

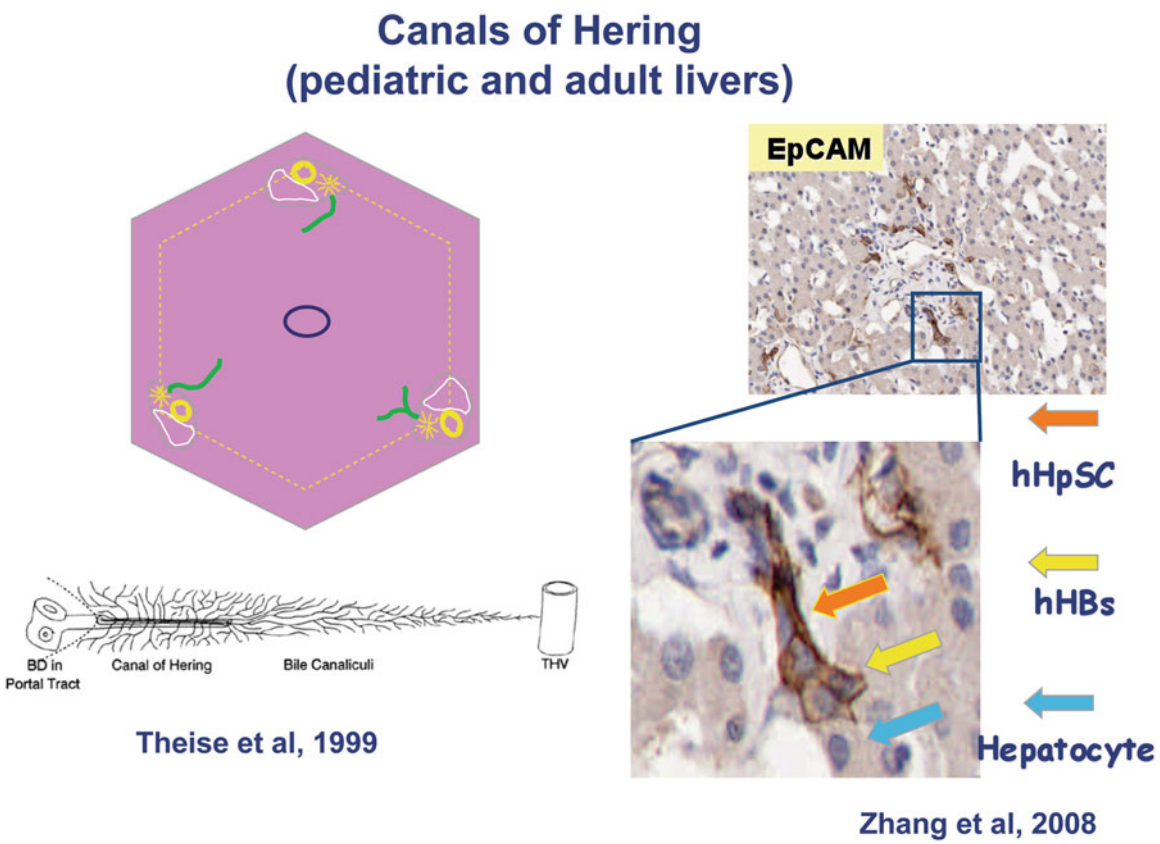

Fig. 15 Canals of Hering in pediatric and adult livers. From Zhang et al. [25] and Theias et al. [24]

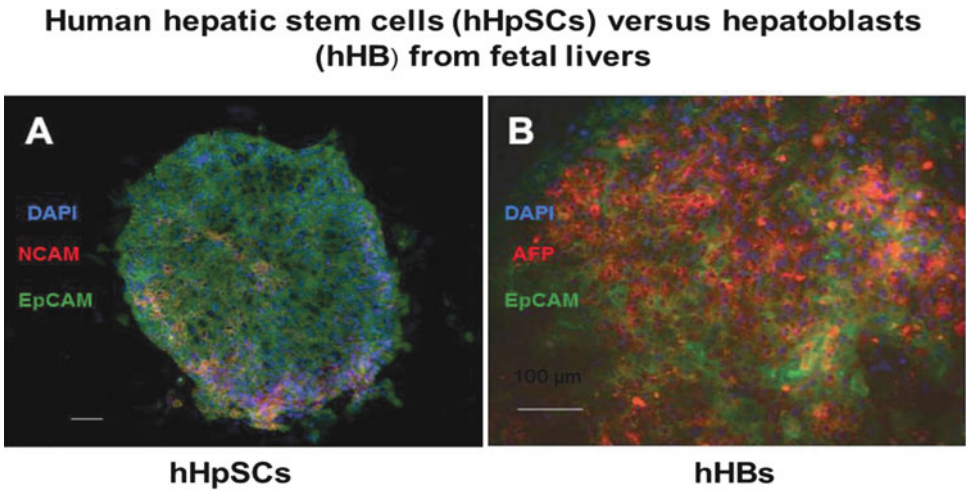

Fig. 16 Colony of hHpSC vs. an hHB colony from human fetal livers. The colonies were stained for EpCAM (*green*) and DAPI (*blue*). The hHpSCs do not express alpha-fetoprotein at all but rather express NCAM (shown in *red*). By contrast, hHBs express alpha-fetoprotein (*red*) and ICAM-1 (not stained). Figure is reproduced from Wang et al. (2010)

important practical problem for their efficient expansion *ex vivo* and for cryopreservation [69]. When treated enzymatically to generate a single cell suspension, both of these stem cell types undergo a high level of cell death. Ding's laboratory screened for chemicals that would enable ES cells to survive enzymatic dissociation and remain pluripotent. They identified two compounds, a 2,4-disubstituted thiazole (Thiazovivin) and a 2,4-disubstituted pyrimidine (Tyrintegin), that met these criteria [70]. They found that Thiazovivin inhibits the Rho-associated kinase (ROCK), a key component of the pathway

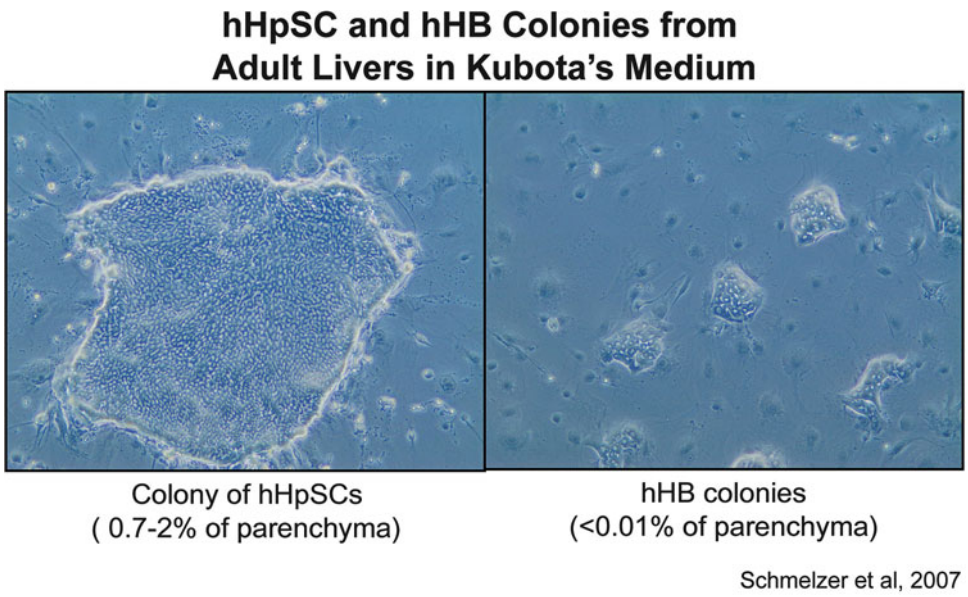

Fig. 17 Phase contrast images of hHpSCs vs. hHBs derived from adult human livers. The hHpSCs form colonies that are similar in appearance to those of ES cell colonies; the cells are relatively uniform in morphology, with high nucleus to cytoplasmic ratio and tightly bound to each other via E-cadherin. The hHB colonies form more cord-like structures interspersed by canaliculi. Image reproduced from Schmelzer et al. (2007). Similar images appear in Wang et al. (2010)

that controls cytoskeleton remodeling, and a likely regulator of cell-ECM and cell-cell interactions. Tyrintegin enhances attachment of dissociated ES cells to ECM and stabilizes E-cadherin. The investigators concluded that ES cell interactions in the normal niche generate signals essential to survival and that small molecules modulating those signals can maintain viability of dissociated cells (Fig. 18).

Likewise, we have observed that hyaluronans, a normal component of most, if not all, stem cell niches, can protect hHpSCs for dissociation and cryopreservation [69]. The addition of hyaluronans was found to protect cell adhesion mechanisms including the hyaluronan receptor, E-cadherin, and certain integrins, markers shared by hepatic and many other stem cell populations [69].

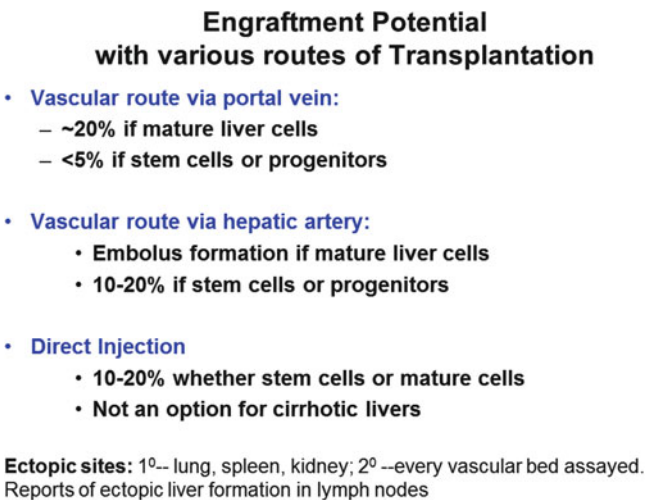

Fig. 18 Summary of engraftment potential of liver cells when transplanted by various routes. Data derive from multiple sources and reviews (Turner et al. 2010; Turner et al. 2012; Puppi et al. 2012)

The Need for Grafting Strategies in Transplantation of Cells from Solid Organs

Transplantation of stem cells into hosts faces challenges applicable to all cell types derived from solid organs. If cells are transplanted via a vascular route, there is inefficient engraftment; the cells disperse to ectopic sites; and emboli may form [65]. Our studies and those conducted by many others have found that mature cells achieve only ~20 % engraftment if injected into the portal vein of the liver [45, 71, 72]. Stem cells are even more challenging, with approximately only 3 % of the cells engrafting if administered via the portal vein (or via the spleen that connects directly to the portal vein). This can be improved to ~20 % engraftment in the liver if stem cells are injected into the hepatic artery [73]. The remaining majority of the cells either die or engraft in ectopic sites, most commonly the lung. Cells that lodge in the vascular beds of ectopic sites can survive for months [74], a finding of unknown significance at this time, but of potential clinical concern.

We have devised grafting strategies for transplantation of hHpSCs embedded into a mix of soluble signals and extracellular matrix biomaterials (hyaluronans, type III collagen, laminin) found in stem cell niches [74]. The hHpSCs maintain a stable stem cell phenotype under the graft conditions. The grafts were transplanted into the livers of immunocom-

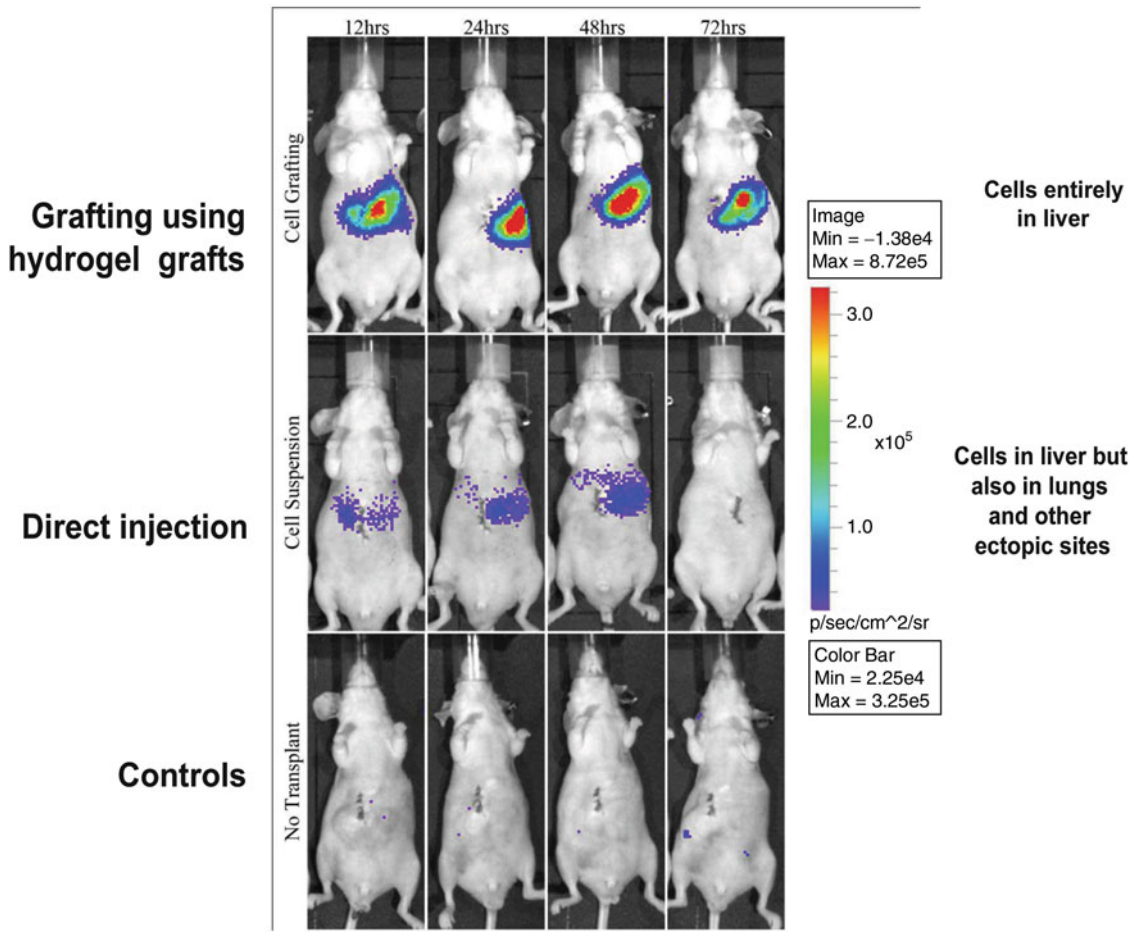

Fig. 19 Grafting strategies dramatically improve engraftment efficiency and minimize ectopic distribution of transplanted cells. Image reproduced from Turner et al. (2012)

promised murine hosts, with and without carbon tetrachloride treatment, to assess the effects of quiescent vs. injured liver conditions. Grafted cells remained localized to the livers, resulting in a larger bolus of engrafted cells in the host livers under quiescent conditions and demonstrated more rapid expansion upon liver injury. We therefore have proposed grafting as a preferred strategy for cell therapies for solid organs such as liver [65, 74] (Fig. 19).

Differentiation

The pharmacology of stem cell differentiation also must encompass both soluble signals (i.e., conventional biologics and/or drugs) and matrix components corresponding to the cells' 3D microenvironments. Cytokines and other soluble factors necessary for liver development and for the maintenance of differentiated hepatocytes have been known for some time [35, 36, 75] (see also Chaps. 4, 22, and 34). However, the specific and efficient directed differentiation of stem or progenitor cells to fully mature hepatocytes and cholangiocytes *ex vivo* has remained a difficult challenge. This, in fact, is a general problem in much of stem cell biology, whether starting with lineage-restricted adult stem cells or pluripotent ES and iPS cells (see Chap. 10).

Biomatrix scaffolds. Approximately 30 years ago, Reid and associates developed a means to provide an environment conducive to maintenance of the differentiated state by presenting cells with ECM components, termed *biomatrices*, prepared by a high-salt extraction procedure [76]. Frozen sections or pulverized liver biomatrices used as cell culture substrata enabled the long-term survival of highly functional hepatocytes, far beyond what could be achieved on plastic or with simple type I collagen gels. Recently, we have established an improved protocol, one involving perfusion strategies and also with high-salt extraction, to prepare decellularized organs. We call the extracts *biomatrix scaffolds*. They are tissue-specific but minimally (if at all) species-specific, and they potently *induce* cell differentiation [41]. The biomatrix scaffolds contain >98 % of the collagens and known collagen-bound matrix components, including most of the fibronectins, laminins, nidogen, entactin, elastin, etc., and essentially all the proteoglycans (PGs). They retain phys-

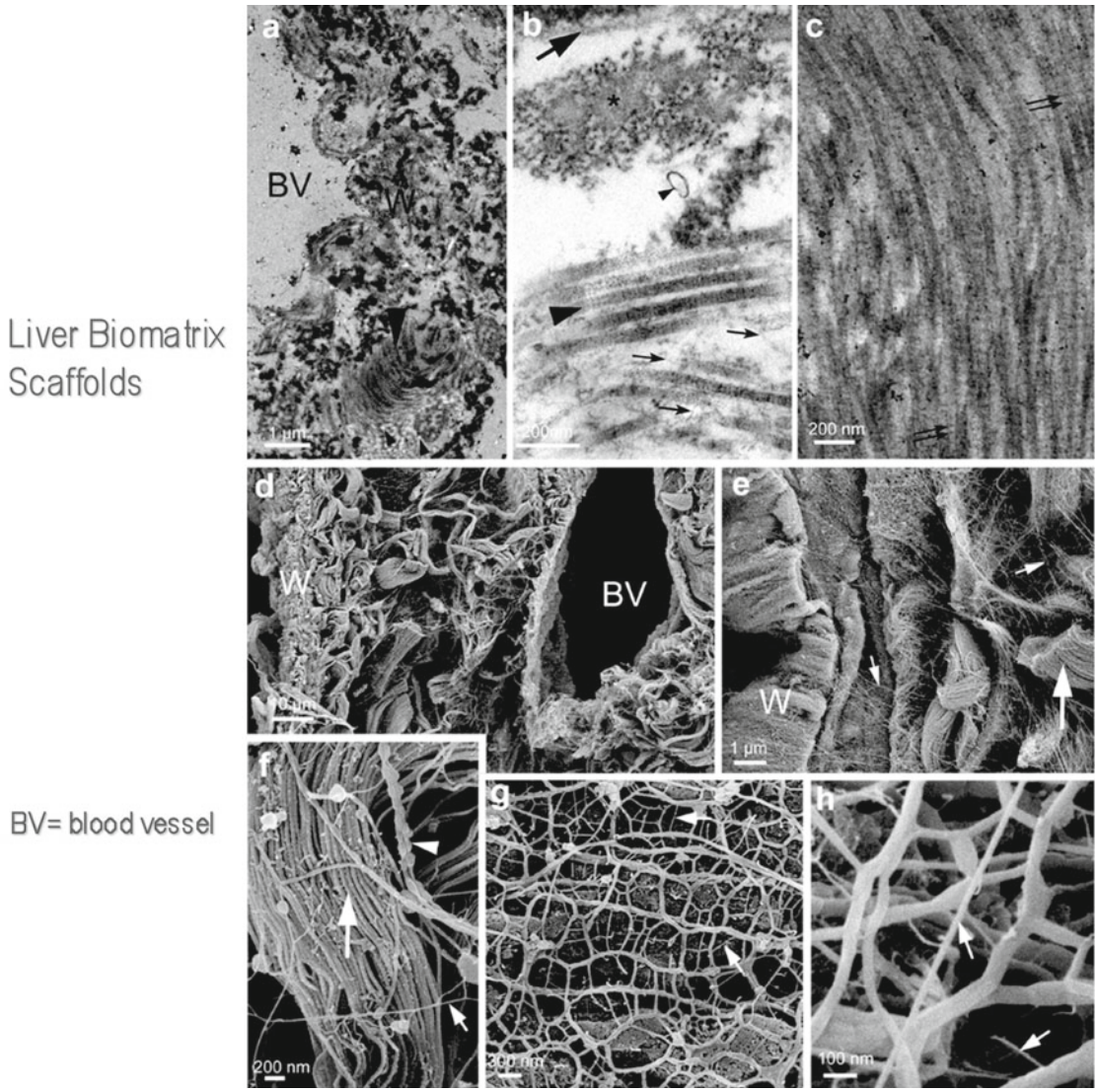

Fig. 20 Transmission and scanning electron microscopy images of rat liver biomatrix scaffolds. (**a**) Low magnification of a blood vessel (BV). probably the portal vein, based on the narrow wall (W) thickness compared to the large diameter of the vessel. The undulations or scalloping of. the vessel (sometimes linked to the internal elastic lamina of an artery) is here probably a partial collapse of the vessel wall. Collagen Type I fibers (*large arrowhead*) are numerous and contains cross-sections of individual fibers that do not take up heavy metal stains (*white dots, small arrowheads*). (A1) Higher magnification of a vessel wall shows basement membrane (*large arrow*), amorphous elastin (*), and associated elastic fibers, a rare membrane vesicle remnant (*small arrowhead*), a collagen Type I banded fiber (*arrowhead*), and small fibrils (*small arrows*). The small fibrils are probably fibrillin (Type VI collagen) that associates closely and helps organize Type I collagen. (**b**) High magnification of Type I collagen with 64 nm banding pattern (*arrows*). (**c**) Low magnification of a vessel with a thin wall (BV) and the wall of a larger vessel (W). (**d**) At higher magnification, the large vessel wall (W) is scalloped, consistent with hepatic artery of a portal triad, see (**a**). Beneath the wall are numerous Type I collagen bundles (*large arrow*) linked by long branching thin, reticular (Type III) collagen fibrils (*small arrows*). (**e**) A large bundle of Type I collagen has characteristic parallel fibers (*large arrow*) associated with a variety of smaller fibers (*arrow*) and nodular or beaded fibers (*arrowhead*). (**f**) 3D meshwork of large/small fibers interlinked in a plane that forms a boundary such as to a liver sinusoid. (F1) Higher magnification of the meshwork showing a variety of fibers (*arrows*): Type III collagen (larger diameter straight), elastic fibers, or Type VI collagen

iological levels of the known matrix-bound cytokines and growth factors found in the tissue. Mature parenchymal cells plated on biomatrix scaffolds in a serum-free HDM remained stable for many weeks and continued to express liver-specific functions equivalent to those of freshly isolated cells.

The hHpSCs seeded onto the liver biomatrix scaffolds in a serum-free defined medium underwent several rounds of cell division, followed by growth arrest and differentiation within approximately a week to mature hepatic parenchymal cells. High levels of specialized hepatocyte and cholangiocyte protein expression and functions could then be maintained for more than 8 weeks [41] (Figs. 20, 21, 22, and 23).

Differentiation of hHpSCs to mature parenchymal cells can be achieved also in 3D hyaluronan hydrogels prepared in

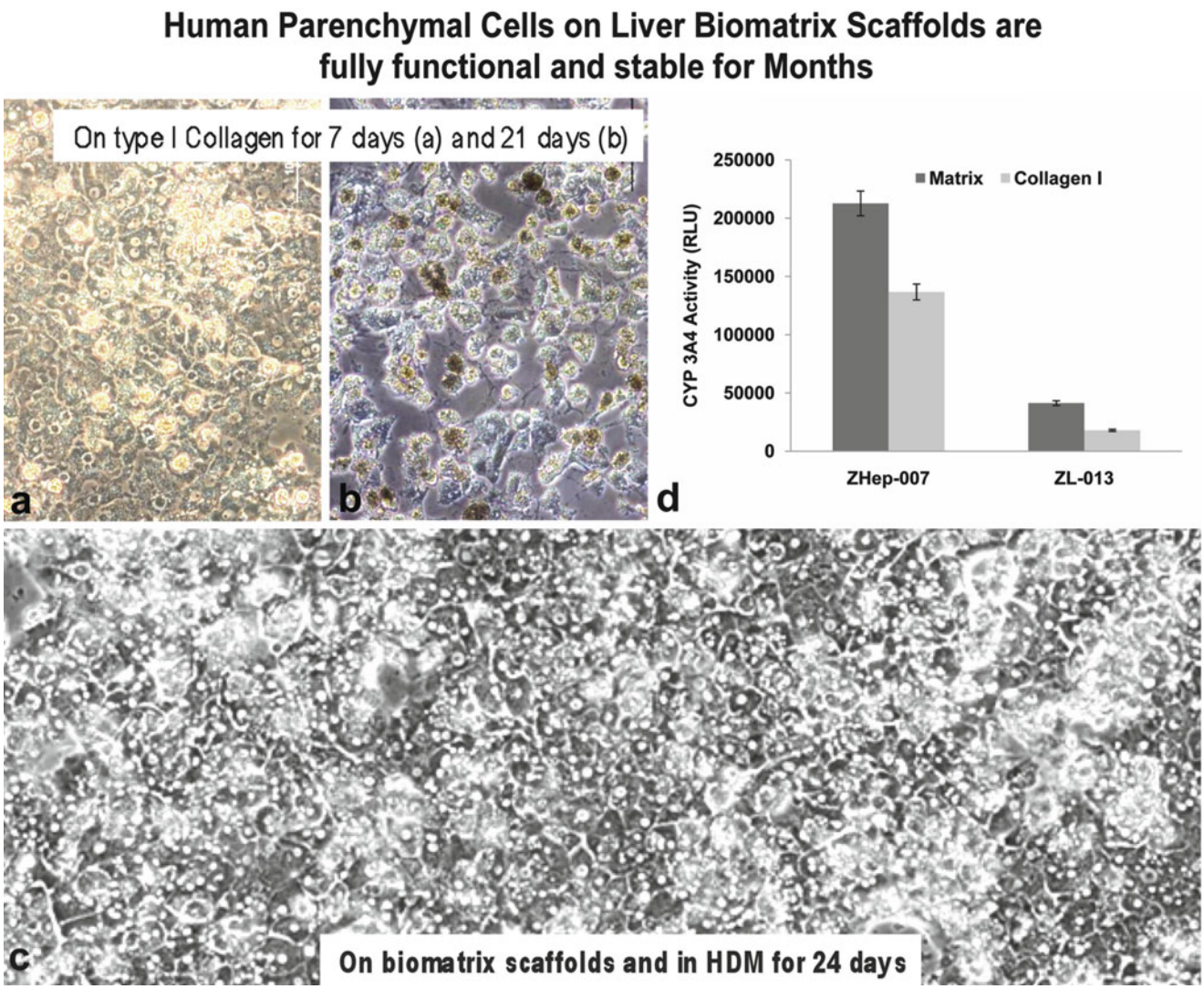

Fig. 21 Cultures of human adult human liver cells, plated in HDM-H and onto either type I collagen gels (**a**, **b**) or onto a frozen section of liver biomatrix scaffolds (**c**). The cultures on collagen gels lasted about 12 days. Those on biomatrix scaffolds lasted more than 8 weeks and with phenotypic traits similar to freshly isolated cells. (**d**) P450 levels in cultures of a "good lot" (ZHep-007) vs. "bad lot" (ZL-013) of frozen human hepatocytes from CellZDirect (RTP, NC) on type I collagen vs. liver biomatrix scaffolds. The "good lots" are ones that will attach on culture plastic; the "bad lots" are those that do not. Reproduced from figure in Wang et al. (2011)

serum-free hormonally defined media (HDM) and supplemented with defined, purified matrix components [41]. As noted above, distinct conditions favor the generation of hepatocytes vs. cholangiocytes. Ultimately, identification of each of the particular tissue-specific matrix molecules necessary for efficient differentiation will be required for mechanistic understanding. It also may be important for clinical translation. The pharmacology of matrix components and their interactions with cytokines and growth factors, the great majority of which bind to the glycosaminoglycan chains of PGs, is a rich, albeit highly complex area that promises to contribute greatly to regenerative medicine [77, 78]. Understanding of the role of complexes of specific growth factors or cytokines bound to defined glycosaminoglycan saccharides [79] in the regulation of cell differentiation and tissue-specific gene expression [80–82] is still in its infancy, but it is likely to become a dominant factor in the maintenance and regulation of stem cells for clinical and non-clinical purposes.

Liver Regeneration

The renowned regenerative capacity of the liver has inspired countless studies on mechanisms associated with the process [43]. It is beyond the scope of this review to summarize that enormous literature, although important aspects of liver regeneration are addressed in other chapters of this book (see

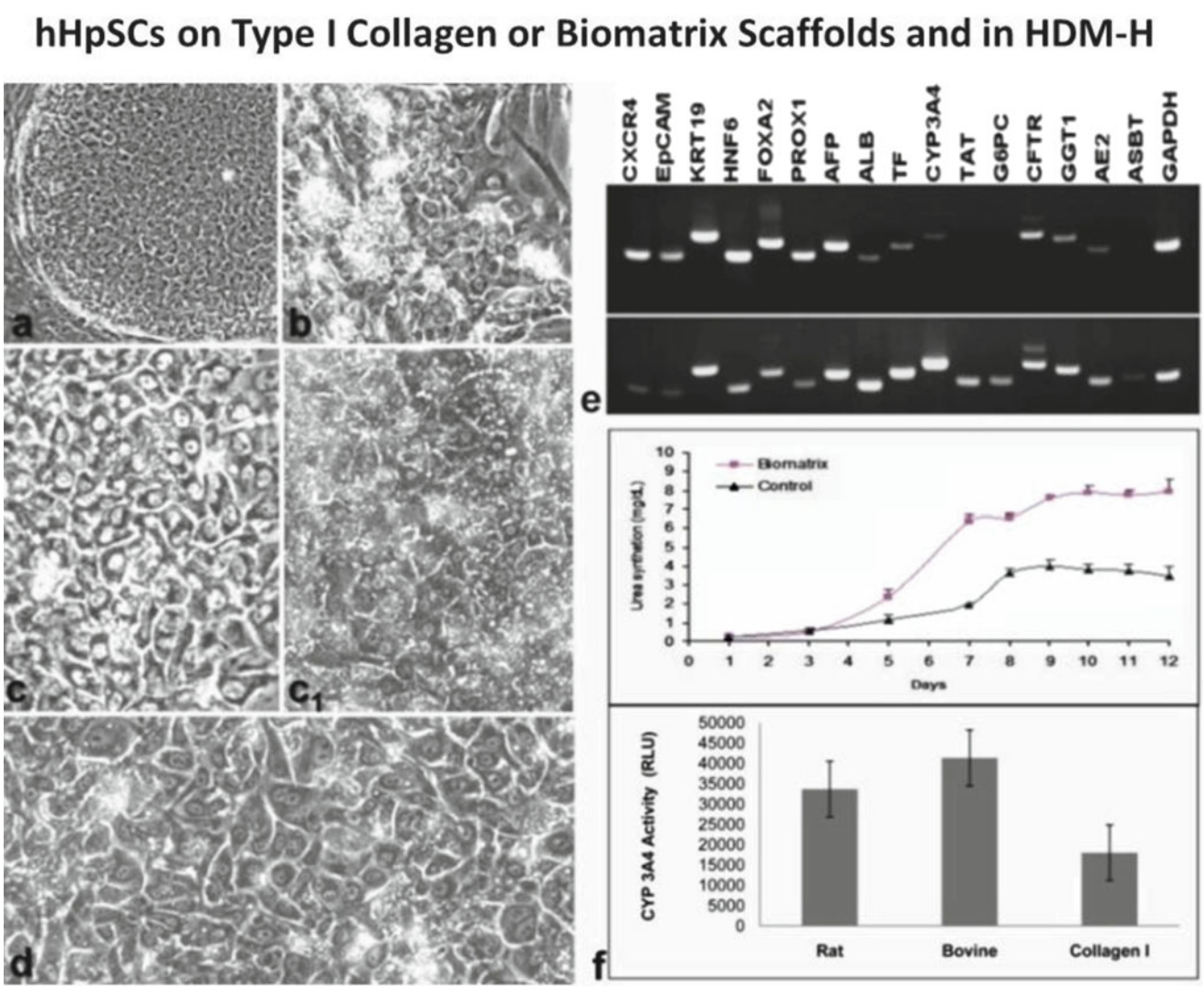

Fig. 22 Lineage restriction of hHpSCs to adult fates is made efficient by culturing the cells on liver biomatrix scaffolds and in HDM-H. (**a**) The hHpSCs on culture plastic and in Kubota's Medium. (**b**) The cells on type I collagen and in HDM-H after 10 days. The cells on liver biomatrix scaffolds and in HDM-H after 10 days (**c**, $\mathbf{c_1}$) and after 21 days (**d**). (**e**) RT-PCR assays of cells on plastic and in Kubota's Medium (*upper panel*) vs. in HDM-H and on liver biomatrix scaffolds (*lower panel*). (**f**) Functional assays (urea and cytochrome P450) of cells on type I collagen vs. liver biomatrix scaffolds. By the time of these assays, the cells on culture plastic have died. The figure is from Wang et al. (2011)

also Chaps. 22 and 34). Here we will note only the known responses of the stem cells and progenitors in two distinct forms of liver regeneration, namely that after partial hepatectomy and that after selective loss of cells in acinar zone 3 (the pericentral zone). (We assume that a parallel process occurs in pancreatic regeneration, though it has been studied in far less detail.)

A key to understanding the responses of the early lineage stage cells, including the stem cells, is recognition of *feedback loop signals*, factors produced by the most mature liver cells, those in zone 3 of the liver acinus, and secreted into the bile. The bile flows from pericentral zone to periportal zone and then into the biliary tree and finally into the gut (Fig. 24).

The signaling molecules include bile acids and salts that affect differentiation [83]; acetylcholinesterase [84], which is produced by mature hepatocytes and serves to inactivate acetylcholine produced by periportal cells [85, 86]; and heparins, which are produced by mature hepatocytes [87] (J. Esko, A. Cadwallader, and L. Reid, unpublished observations) and are relevant in control of stem cells and of tissue-specific gene expression [88, 89]. In addition, the flow of the bile mechanically affects primary cilia on periportal cells and thereby influences signal transduction processes mediated by these organelles [90–92]. In the presence of feedback signals, the stem cells remain in a quiescent state. Diminution or loss of these signals results in disinhibition of the stem/progenitor cell compartments. This leads to hyperplasia of

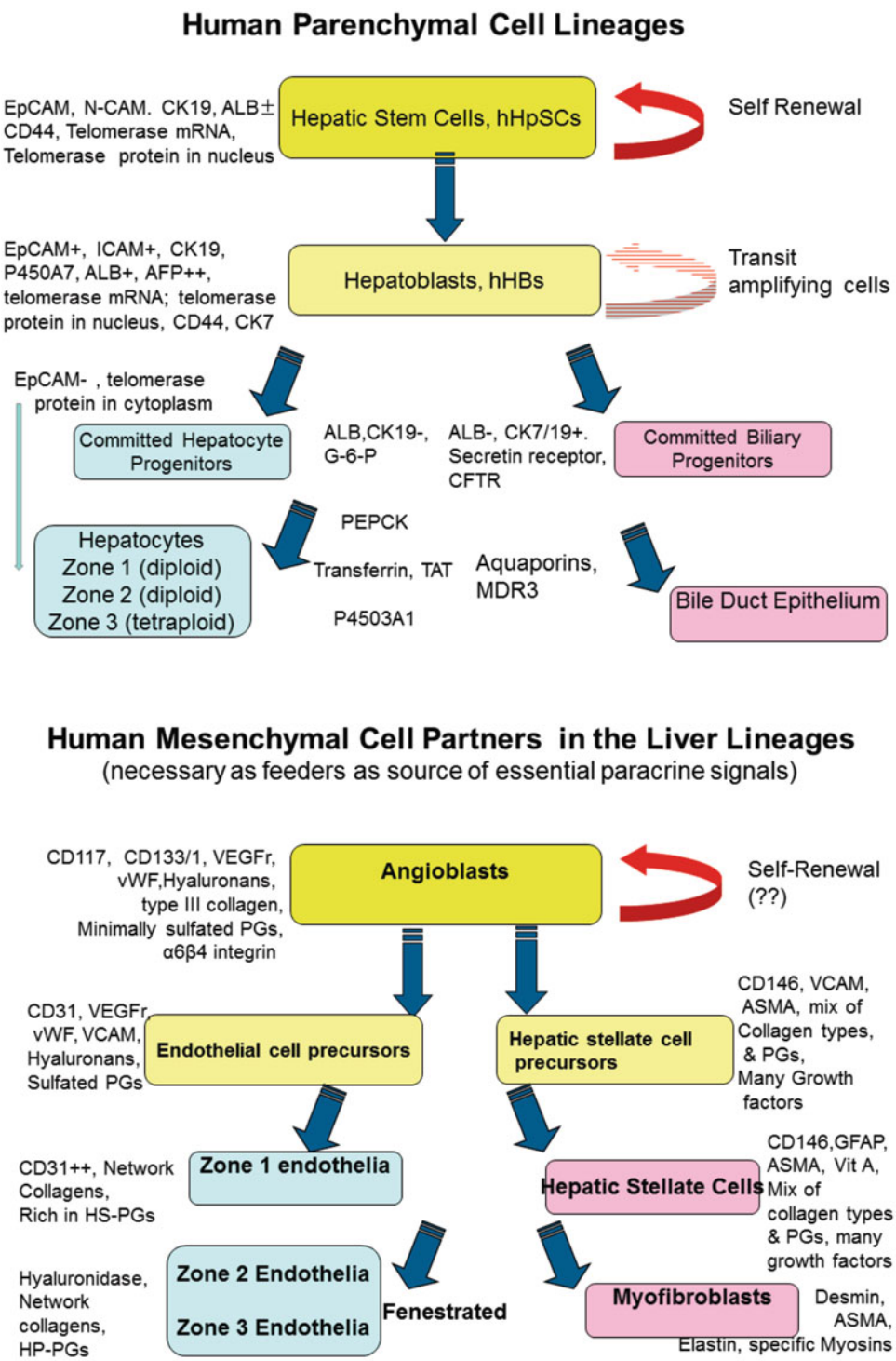

Fig. 23 Epithelial-mesenchymal cell partners in the maturational lineages of the liver. Modified from figure in the online supplement of Turner et al. (2011). *P6s* = proteoglycans; *ASMA* = - α-Smooth muscle actin; *VCAM* = Vascular cell adhesion molecule; *Vit. A* = Vitamin A; *G-6-P* = Glucose-6-phosphate; *GFAP* = Glial fibrillary protein; *CFTR* = Cystic fibrosis transmembrane conductance regulator

the stem cells and other early lineage stage cells. Factors that may release the stem cell compartment from the normal feedback signaling control loops include viruses, toxins, or radiation that selectively kill cells in zone 3, the pericentral zone of the acinus. The hyperplasia transitions into differentiation of the cells. The resulting fully mature cells produce bile, and the restoration or enhancement of feedback loop signals then inactivates the proliferative response.

The regeneration of the liver after partial hepatectomy is distinct from that described above and has been the subject

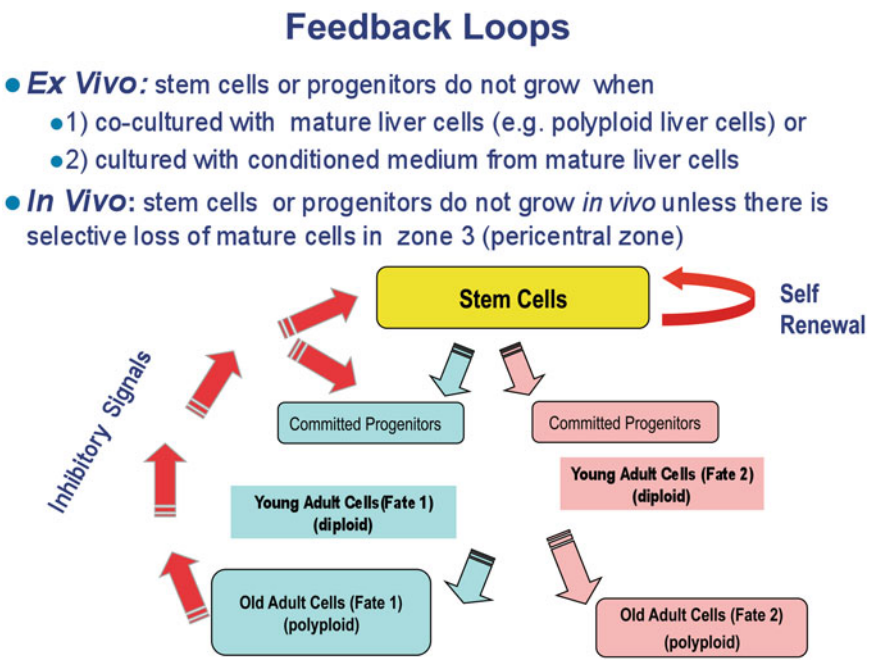

Fig. 24 Feedback loops of signals regulating the quiescent vs. proliferative status of the stem cells and progenitors. The signals *in vivo* are present in bile. In cultures, they are secreted into the medium and so are available in conditioned medium

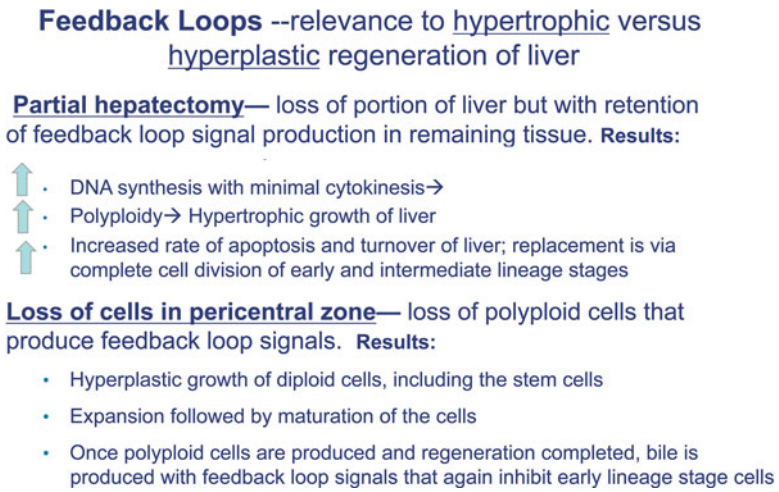

Fig. 25 Feedback loops of signals found in bile (*e.g.*, acetylcholinesterase, bile salts, heparins, and other factors) and the mechanical effects of bile flow influence whether the stem cells and other early lineage stage cells will divide or will assume a quiescent state. The feedback loop signaling processes are the primary mechanisms dictating liver size (i.e., whether the liver is the size for a mouse or human)

of many reviews [43, 93, 94]. The tissue remaining after surgical removal of a portion of the liver (*e.g.*, two-thirds of its mass) continues to have feedback loop signals, and the early lineage stage cells remain competent to respond to these signals. The depletion below threshold levels of various liver functions and secreted products triggers DNA synthesis as a wave across the liver plates [94]. However, the DNA synthesis in most of the cells of the liver (especially those in zones 2 and 3) is not accompanied by cytokinesis [95]. So these cells increase their level of ploidy and demonstrate hypertrophic growth [96]. The polyploidy triggers an increased rate of apoptosis resulting in turnover of the liver. With the loss of the apoptotic cells, there is a low level of proliferation of the stem cells and early lineage stage cells to replace those cells eliminated during apoptotic processes. In mammalian species examined, this turnover occurs in weeks (Fig. 25).

Clinical Programs in Hepatic Stem Cell Therapies

Clinical programs for hepatic stem cell therapies are in their very early stages. To our knowledge the only clinical trials of hepatic stem cell therapies that have been completed to date were carried out in Hyderabad, India, under the management of Dr. Chittoor Habibullah and associates in the Liver

Institute. These investigators found considerable value for hepatic stem cell therapy in treatment of patients with inborn errors of metabolism, cirrhosis, hepatitis B or C, and other liver disorders [73, 97, 98]. They used immunoselected EpCAM+ cells from fetal livers, comprising both hHpSCs and hHBs. Remarkably, immune suppression was not required, although donors and recipients were not matched for histocompatibility antigens. In a portion of the studies that have been published to date, 25 subjects with decompensated liver cirrhosis from various causes received cell infusions into the liver via the hepatic artery. At a 6-month follow-up, multiple diagnostic and biochemical parameters showed clear improvement, and there was a significant ($p < 0.01$) decrease in the mean Mayo End-stage Liver Disease (MELD) score, an accepted metric for clinical severity. The clinical trials, which have been conducted for more than 5 years, were completed in June 2012 and the findings provided the basis to apply for regulatory approval in India, which remains pending.

Future efforts to employ hHpSCs and/or hHBs clinically will be facilitated by large-scale manufacturing of the stem and progenitor cell populations. The sourcing of donor cells may be fetal tissues in those countries that permit their use, as demonstrated by the Habibullah group. However, postnatal tissues also can be used as a source and may have distinct advantages, both ethically and practically. Neonatal tissues, including the liver and biliary tree, adult livers not suitable for whole organ transplantation, and adult biliary tree may serve as the source of stem cells. The cells may be utilized as directly isolated or after expansion in culture (subject to additional levels of regulatory review). It is expected that the grafting strategies discussed above [65, 74], such as transplantation of cells using hyaluronans, possibly in combination with other extracellular matrix components, will greatly improve engraftment, minimize ectopic distribution of cells, and hasten the improvement of liver functions.

Even though immunological issues did not appear limiting in the highly encouraging first trials of fetal liver-derived hepatic stem and progenitor cell therapy by Khan and coworkers [73], it yet may be desirable to match, to the degree possible, the HLA (major histocompatibility) types of donor cells and recipients. Given sufficient expansion, it should be possible to bank large numbers of cells from a modest number of carefully selected donors and achieve a beneficial degree of HLA matching for the large majority of recipients [99] (Figs. 26 and 27).

Acknowledgments Findings from these studies have been included in patent applications belonging to Sapienza University (Rome, Italy) and/or to UNC (Chapel Hill, NC) and licensed to Vesta Therapeutics (Bethesda, MD). The authors do not have equity or a position in Vesta and are not paid consultants to the company. The authors declare no conflicts of interest. Almost all of the figures are reproduced from various publications with permission of the administrators of the journals in which they first appeared. The review was written primarily by Mark Furth and Lola Reid, with input and editing by all of the authors. All of the authors have contributed to the investigations and established the interpretations that are summarized in this review.

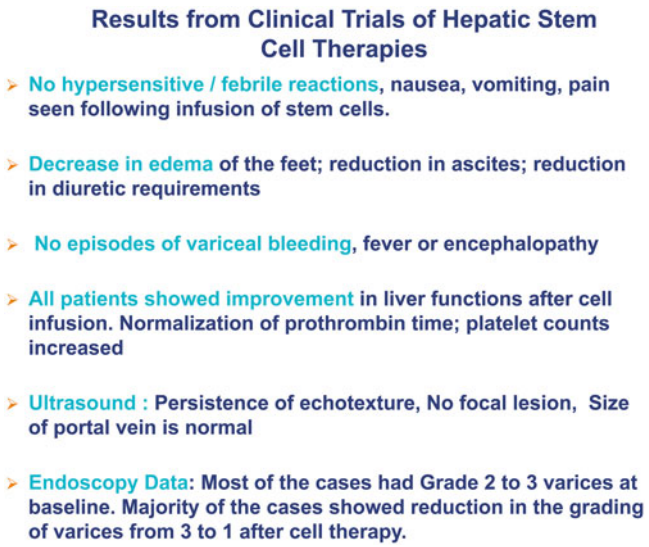

Fig. 26 Summary of the initial findings from clinical trials and that were published [73]. After this and other publications from the initial findings, the clinical trials continued. They ended in the summer, 2012. Filings have been made to regulatory agencies for approval of clinical products based on hepatic stem cell therapies. Therefore, further information on the findings from these trials will soon be available

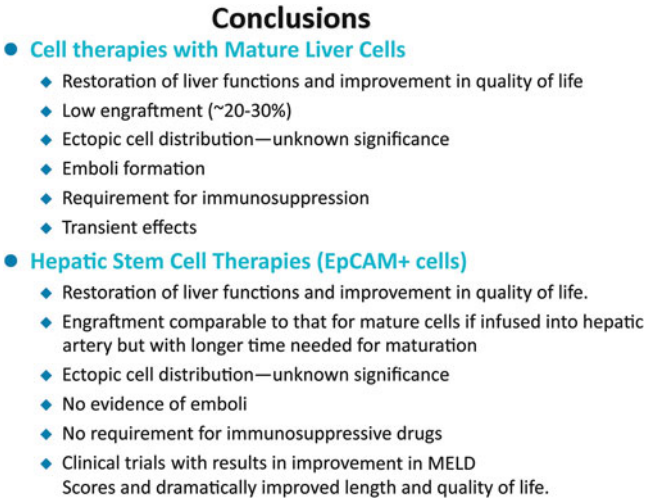

Fig. 27 The conclusions thus far on liver cell therapies with mature cells [45] vs. with hepatic stem cell therapies [73]

Authors' note: As this book goes to press, we acknowledge the publication on "liver buds" (Takebe et al. 2013). The investigators mixed 3 different stem cell populations in culture under appropriate conditions to form the liver buds.

Their findings demonstrate the importance of epithelial-mesenchymal interactions and the resulting paracrine signals in liver formation

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Liver Regeneration in Health and Disease

Malcolm R. Alison and Shahriar Islam

Introduction

Normally the liver has a low level of hepatocyte turnover, but in response to modest hepatocyte loss, a rapid regenerative response occurs from all cell types in the liver to restore organ homeostasis [1]. More severe liver injury, particularly chronic repetitive injury (e.g., chronic viral hepatitis), often associated with hepatocyte replicative senescence, activates facultative stem cells of biliary origin that give rise to cords (the "ductular reaction") of bipotential transit-amplifying cells (named oval cells [OCs] in rodents and hepatic progenitor cells [HPCs] in man) and that can differentiate into either hepatocytes or cholangiocytes. Moreover, the major primary tumors of the liver (hepatocellular carcinoma [HCC] and cholangiocarcinoma [CC]) invariably arise in a setting of chronic inflammation that is accompanied by both hepatocyte regeneration and ductular reactions, and while it seems that the founder cell of CCs is a proliferating cholangiocyte, the morphological heterogeneity often observed in HCCs suggests that these tumors can arise from bipotential HPCs as well as more mature hepatocytes. HCCs also appear to possess subpopulations of cancer stem cells (CSCs), responsible for continued tumor propagation and metastasis, and a number of phenotypic markers have been proposed for their identification.

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Liver Turnover and Regeneration

Kinetic Organization

The healthy liver in adults is essentially proliferatively quiescent with levels of proliferation suggesting a turnover time in excess of a year. Nevertheless there is still considerable debate as to how the liver is organized. Most studies concur that hepatic stem cells are located in the periportal region; for example, in the mouse, bromodeoxyuridine (BrdU) pulse-chase analysis following two rounds of acetaminophen intoxication has observed so-called label-retaining cells (LRCs), considered to be slowly dividing stem cells, as both interlobular cholangiocytes and peribiliary hepatocytes [2]. In man, rare putative stem cells expressing STAT3, Oct4, and Nanog are also periportally located [3], while Turner et al. [4] find $EpCAM^{+}NCAM^{+}$ hepatic stem cells in the periportally located canals of Hering; this latter group has also identified eight maturational lineage stages moving from the periportal (stem) region to the perivenous region.

Thus, is the liver organized like the intestine with a unidirectional flux of cells, cells being born in the portal area and migrating along a trajectory leading to the hepatic veins? This idea, the so-called "streaming liver" hypothesis, was first advocated by Zajicek et al. [5]. Examining the location of labeled hepatocytes in intact adult rat livers over time after a single injection of tritiated thymidine, it was suggested that hepatocytes moved at a speed of over 2 μm/day from the periportal region to the central vein. A recent murine study by Furuyama et al. [6, 7] appears to support the idea that hepatocytes migrate centrifugally from portal areas. They examined the expression of the embryonic transcription factor Sox9 (Sry [sex-determining region Y] box 9) in the liver. In human liver, immunohistochemistry identified interlobular bile duct cells as Sox9-expressing cells, and a similar pattern was seen in adult mice when a reporter gene, either enhanced GFP or LacZ, was knocked into the *Sox9* locus. Adopting tamoxifen-inducible genetic lineage tracing from the *Sox9* locus, detecting

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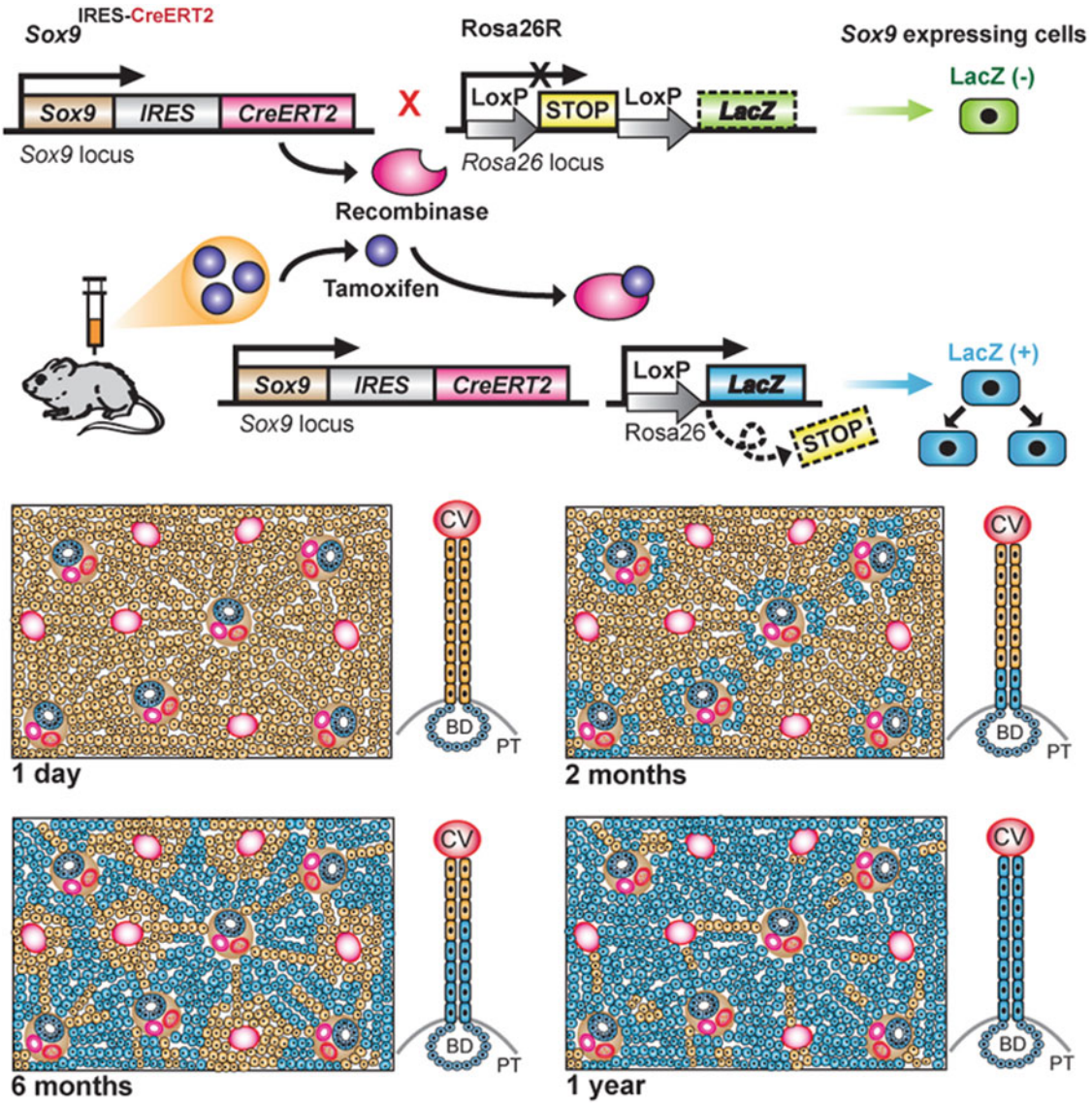

Fig. 1 *Top*, strategy of the genetic lineage-tracing study employed by Furuyama et al. [6] using tamoxifen-induced Cre-mediated cell tracking using *Sox9*$^{\text{IRES-CreERT2}}$, Rosa26R mice. *Bottom*, schematic illustrating the spread of X-gal staining after 8-week-old mice were injected with tamoxifen. After 1 day, only intrahepatic bile duct cells are labeled, but later X-gal-positive hepatocytes gradually spread from the portal tracts to the central veins, thus supporting the streaming liver hypothesis. See Alison and Lin [7] for further details. Reproduced with permission from *Hepatology*

Sox9-lineage cells by X-gal staining, it was found that X-gal positivity spreads out from the portal areas towards the hepatic veins until the majority of hepatocytes were labeled within 8–12 months (Fig. 1). Thus, cells "streamed," but more importantly hepatic replacement was from cytokeratin (CK) 7/Sox9-positive biliary cells, identifying cells within the biliary tree as drivers of not only hepatocyte replacement when regeneration from existing hepatocytes is compromised (see below) but also normal hepatocyte turnover. However, other studies of mice have failed to find evidence for the normal liver parenchyma being "fed" from the biliary system; Malato et al. [8] heritably labeled all mouse hepatocytes and singularly failed to find any evidence for an input of hepatocytes derived from biliary cells in normal liver homeostasis, thus refuting the Furuyama study.

Carpentier et al. [9] also employed lineage labeling in mice, this time from Sox9-expressing ductal plate cells in late embryonic development (E15.5), finding that these cells gave rise to interlobular bile ducts, canals of Hering, and periportal hepatocytes, but again finding that liver homeostasis did not require a continuous supply of cells from Sox9 progenitors. Iverson et al. [10] have sought to quantify the dynamics of mouse liver turnover by lineage labeling following activation of an albumin/Cre transgene, calculating that 0.076 % of hepatocytes had differentiated from albumin-naïve cells over a 4-day period. If these new cells have not

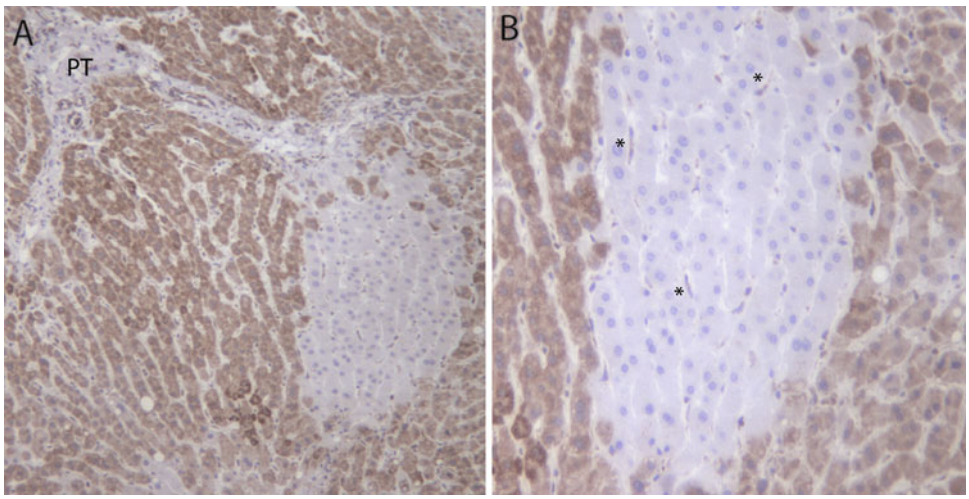

Fig. 2 (**a**) A single cytochrome-*c* oxidase-deficient patch, appearing to emanate from the portal tract (PT). (**b**) High-power magnification illustrates that within the patch there are cytochrome-*c* oxidase-positive sinusoid-lining cells (*asterisks*) indicative of different cells of origin from hepatocytes. See Fellous et al. [13] for further details. Reproduced with permission from *Hepatology*

arisen from biliary cells or periportal stem cells, are there any other cellular sources? Perhaps they could have arisen from small hepatocyte-like progenitors (SHPCs) first described by Gordon et al. [11]. These can be seen in rats that are pretreated with retrorsine, a pyrrolizidine alkaloid metabolized by the mature hepatocyte's cytochrome (CYP) P450 system to metabolites that form DNA adducts, which are then subjected to a regenerative stimulus such as a 2/3 partial hepatectomy (2/3 PH) or a necrogenic dose of CCl_4 [12]. The resistance of SHPCs to the antiproliferative effects of retrorsine is probably related to their undifferentiated state, lacking CYP enzymes.

In human liver Fellous et al. [13] have identified clonal populations of hepatocytes based upon finding large patches of cells deficient in the mitochondrial DNA (mtDNA)-encoded cytochrome-c oxidase (CCO) enzyme, all sharing an identical neutral mutation in the *CCO* gene indicating derivation from a single cell. Significantly these CCO-deficient patches were all connected to portal areas and had a portal vein to hepatic vein orientation (Fig. 2), suggestive of a "streaming" nature, but without information of whether derived from a periportal stem cell or an interlobular biliary cell. Notably in the rat, experimental observations have not been consistent with hepatocytes migrating along a portal vein–hepatic vein axis [14].

Liver Regeneration

The regenerative capacity of the liver is impressively demonstrated when two-thirds of the rat liver is surgically removed (2/3 PH), with the residual liver undergoing waves of hyperplasia and hypertrophy to restore preoperative liver mass within about 10 days [1]. After a 2/3 PH in healthy adult rats, all the normally proliferatively quiescent hepatocytes leave G_0 to semi-synchronously enter the cell cycle. DNA synthesis is first initiated in the periportal hepatocytes at about 15 h after PH with a peak in the hepatocyte DNA synthesis labeling index of ~40 % at 24 h [15]. Mid-zonal and centrilobular hepatocytes enter DNA synthesis at progressively later times, but the hyperplastic response in hepatocytes is essentially complete by 96 h, to be followed by a phase of hepatocyte hypertrophy. Elegant labeling studies have identified three groups of regenerative hepatocytes in mice, with all cells dividing at least once but with the periportal hepatocytes that divide first, dividing maybe three or more times after PH [16].

As might be expected, age has an adverse effect on the response; in old rats (>2 years old) a significant number of hepatocytes do not proliferate after PH, seemingly becoming reproductively senescent [17]. To maintain liver homeostasis, the non-parenchymal cells (cholangiocytes, endothelial cells) must also expand their numbers, and their cell cycle entry is delayed a few hours behind that of hepatocytes [1]. Partial hepatectomies of >70 % are not well tolerated due to lack of liver function, but in mice a 90 % PH can be tolerated by suppressing the MEK/ERK pathways, slowing down regeneration, and reducing liver dysfunction [18], a strategy that could be useful for improving survival in patients with small liver grafts from living donors that could be susceptible to small-for-size (SFS) syndrome.

Molecular Regulation of Liver Regeneration

Numerous cytokines, growth factors, and signaling pathways have been implicated in (1) the initiation (priming) of hepatocytes in order to be responsive to liver mitogens, (2) the proliferative response itself, and (3) the curtailment of the

response. The "priming phase" in the first few hours after PH, probably instrumental in the G_0 to G_1 transition, is associated with upregulation of many genes not expressed in normal liver and is essentially cytokine driven [19–21], with activation of transcription factors such as AP-1, NFκB, and STAT3 being particularly important. The ultimate cause of cytokine accumulation is unclear but enteric lipopolysaccharide may be the master regulator of the innate immune response, and liver injury can be associated with a defective intestinal barrier leading to exposure to lipopolysaccharides and complement fragments. Such exposure activates the NFκB pathway in Kupffer cells resulting in the production and secretion of IL-6 that activates the JAK/STAT pathway, leading to the initiation of DNA synthesis in hepatocytes. In mice, complement activation, in particular C3a and C5a, leads to the recruitment of natural killer T (NKT) cells and the production of IL-4 by these cells [22]. IL-4 maintains IgM levels and deposition in the liver, leading to increased C3a and C5a accumulation that in turn stimulates liver macrophages to produce IL-6. On the other hand, the relationship between IL-6 and hepatocyte proliferation after PH may not be so straightforward; Myd88-null mice (an adaptor protein for the Toll-like receptor family) actually show an accelerated start of proliferation after PH, possibly linked to the loss of the antiproliferative effects of IL-6 mediated by suppressor of cytokine signaling 3 (SOCS3) [23]. The cytokine interleukin-1 receptor antagonist (IL-1ra) is also important in the early phase of regeneration, reducing inflammatory stress and thus promoting proliferation [24].

The proliferative response itself appears to be driven by a number of growth factors/signaling pathways including IL-6, TNF-α, HGF, amphiregulin, SCF, IGF-1, T3, BMP-7, Wnt/β-catenin, Hh, and phosphoinositide 3-kinase (PI3K), although no one factor or pathway appears crucial to the process [19–21, 25]. Some of these signals are autocrine and others are paracrine; for example, in mice sinusoidal endothelial cells are involved in hepatocyte regeneration with VEGFR-dependent upregulation of the transcription factor *Id1* leading to the release of hepatotrophic factors such as Wnt2 and HGF [26]. Moreover, it seems that endothelial progenitor cells recruited from the bone marrow after PH provide the richest source of HGF [27]. Hepatic stellate cells also support liver regeneration and are activated by massive upregulation of delta-like 1 homology (Dlk1) that represses *Pparγ* in stellate cells [28]. Regenerative competence in mouse and man also appears to be maintained by activation of telomerase activity in regenerating hepatocytes [29]. MicroRNAs (miRs) are also involved in regeneration after PH; for example, in mice there is upregulation of miR-21 in the priming phase that targets a proliferation inhibitor facilitating cyclin D1 translation and downregulation of miR-378 that targets *odc1* mRNA, ornithine decarboxylase activity being essential for DNA synthesis [30, 31]. In rats after PH, there are also dramatic changes in miRs, with upregulation of 40 % of investigated miRs in the priming phase and downregulation of 70 % of miRs at 24 h after PH, presumably facilitating maximal proliferation [32]. Rat liver regeneration is also associated with upregulation of mRNAs that encode for transcription factors associated with pluripotency such as c-Myc, Nanog, Klf4, and Oct4 [33].

Equally important are the molecular mechanisms that curtail the regenerative response, ensuring the liver does not overcompensate for lost mass. TGF-β produced by stellate cells inhibits hepatocyte replication, and several mechanisms are involved in its production. In mice, serotonin acts on 5-HT_{2B} receptors in stellate cells, leading to phosphorylation of JunD via ERK, resulting in recruitment of JunD to AP-1 binding sites in the promoter region of the *tgf-β1* gene [34]. The multi-domain matrix glycoprotein thrombospondin-1 (Tsp-1) is also involved in TGF-β1 production in mice; Tsp-1 is expressed by endothelial cells in response to reactive oxygen species (ROS) shortly after PH and binds to latent TGF-β1 complexes, converting them to active TGF-β1 [35]. The IL-6 response is negatively regulated through transcriptional upregulation of SOCS3, but SOCS3 is not crucial for curtailing proliferation for although SOCS3 knockout mice have higher levels of hepatocyte proliferation after PH than wild-type mice and restore preoperative liver weight 2 days earlier, proliferation stops after 4 days, and liver weight does not go above normal [36]. The Hippo pathway seems particularly important for curtailing liver size; the kinases Mst1 and Mst2 (the mammalian orthologs of *Drosophila* Hippo) are responsible for phosphorylating the Yes-associated protein (Yap) at Ser127, the mammalian ortholog of *Drosophila* Yorkie which is a transcriptional activator of cell cycle proteins such as Ki-67 and c-Myc—phosphorylation blocks its ability to translocate to the nucleus [37]. Thus, overexpression of Yap in mice leads to massive liver weight increases (25 % of body weight vs. 5 % normally) [38], and likewise Mst1 and Mst2 double knockouts also have massive livers and eventually develop HCC [39, 40].

A Second Tier of Regeneration: Oval/HPCs

Massive acute liver injury, chronic liver injury, or large-scale hepatocyte senescence results in the activation of a reserve or potential stem cell compartment located within the intrahepatic biliary system [1]. Replicative senescence can occur in conditions such as chronic hepatitis [41] and fatty liver disease [42]. In man [43] and mice [44], the extent of the HPC response is proportional to the degree of parenchymal damage. HPCs are derived from interlobular biliary cells and/or the canal of Hering, and in human liver, the canal of Hering extends beyond the limiting plate, even perhaps throughout the proximate third of the lobule [45].

A number of animal models have been described to activate this progenitor response. In rats, a very effective model has been to pretreat the animals with 2-acetylaminofluorene (2-AAF) before performing a 2/3PH (the 2-AAF/PH protocol). 2-AAF is metabolized by the hepatocyte's cytochrome P450 system, producing metabolites that form DNA adducts, thus preventing hepatocytes from entering the cell cycle in response to PH. Under these constraints, oval cells/HPCs are activated since they lack the CYP enzymes necessary for 2-AAF metabolism [46]. In the mouse, dietary regimes are often employed including a choline-deficient, ethionine-supplemented diet (the CDE diet) that inflicts hepatocyte damage or the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) regime that damages cholangiocytes [47]. An oval cell response is also seen when hepatitis B surface antigen transgenic mice (a model of chronic liver injury) are treated with retrorsine, a pyrrolizidine alkaloid that blocks hepatocyte regeneration [48]. The potential of oval cells for liver repopulation in the mouse can be seen after genetic deletion of damaged DNA-binding protein 1 (DDB1), an E3 ubiquitin ligase, one of whose substrates is p21. This effectively abolishes hepatocyte turnover resulting in massive oval cell-driven regeneration [49]. The exact location of stem/progenitor cells within the biliary tree is unclear or indeed if all cells in small caliber biliary ducts and canals of Hering are capable of giving rise to oval cells, but in the mouse a small subset (3–4 %) of antigenically defined biliary cells that express Sox9 give rise to most oval cells in the DDC model [50].

A wide range of markers has been used to identify ovals cells/HPCs (Table 1, [51–67]). Many factors, often produced by cells of hepatic niche that intimately accompanies the reaction, can influence the oval cell/HPC response. Autocrine and paracrine Wnt signaling is clearly involved in the oval cell response in mice [68], rats [69], and humans [70]. In the rat 2-AAF/PH model, oval cells display nuclear β-catenin and Wnt1 is essential for differentiation of oval cells to hepatocytes; exposure to Wnt1 shRNA blocked this differentiation and oval cells generated an atypical ductular reaction—perhaps as the default position [71]. As oval cells/HPCs are bipotential, what regulates whether they become hepatocytes or cholangiocytes? Boulter and colleagues have described the mechanisms in mice governing these critical cell fate decisions [47]. After biliary cell damage with DDC, the intimate association of myofibroblasts with HPCs facilitated Notch signaling ensuring biliary differentiation in oval cells, in essence recapitulating ontogeny. On the other hand, after hepatocyte damage with the CDE diet, adjacent macrophages in response to engulfing hepatocyte debris were involved in Wnt signaling to HPCs that not only turned Notch signaling off but also specified hepatocytic differentiation in oval cells. On the other hand, in the rat 2-AAF/PH model, Notch1 may be important for hepatocytic differentiation since exposure to a γ-secretase inhibitor delayed the maturation process [72]. HGF signaling is also important for the oval cell response; genetic deletion of *c-met* from oval cells in the DDC model results in a diminished response with decreased hepatocytic differentiation [73]. Moreover, a failure to express SDF-1 leads to less recruitment of macrophages and associated MMP-9 secretion that is crucial for oval cell migration and liver remodeling (see below). Hedgehog (Hh) signaling is another important pathway, and ligands acting through the receptor Patched (Ptc) on murine oval cells and human HPCs are required for progenitor cell survival [57]. Perhaps most significantly, inflammatory cells produce a range of cytokines and chemokines that initiate the response [63, 64]; SDF-1 attracts $CXCR4^+$ T cells, and these cells express TWEAK (TNF-like weak inducer of apoptosis) that stimulates oval cell proliferation by engaging its receptor Fn14, a 14 kDa transmembrane receptor [61]. Tirnitz-Parker and colleagues employed the CDE diet and found that expression of Fn14 is markedly elevated [74]. Fn14 is not a receptor tyrosine kinase, but rather ligand occupancy activates NFκB signaling as shown by the presence of active (nuclear) NFκB in a progenitor cell line upon TWEAK stimulation. The early oval cell response to the CDE diet was delayed in Fn14 knockout mice, though interestingly there were comparable numbers of oval cells in wild-type and knockout mice after 3 weeks on the CDE diet. Significantly, recombinant human TWEAK (rhTWEAK) directly stimulated the in vitro proliferation of a progenitor cell line in a dose-dependent manner. This observation could have

Table 1 Some of the markers used in the identification of oval cells/HPCs in the damaged mammalian liver

A6 antigen (mouse marker)
ABCG2/BCRP1 (breast cancer resistance protein)
AFP (alpha fetoprotein)
Cadherin 22
CD24, CD133
Chromogranin A
CK7 and CK19
c-Kit (CD117)
Claudin7
Connexin 43
Dlk1 (delta-like protein 1)
DMBT1 (deleted in malignant brain tumor 1)
E-cadherin
EpCAM/TROP1 (epithelial cell adhesion molecule)
Flt-3 ligand/flt-3
Fn14 (fibroblast-inducible factor 14 kDa protein; TWEAK receptor)
GGT (gamma-glutamyltranspeptidase)
GST-P (placental form of glutathione-S-transferase)
M2-PK (muscle-type pyruvate kinase)
NCAM-1/CD56 (neural cell adhesion molecule-1)
PTHrP (parathyroid hormone related peptide)
TACSTD/TROP2 (tumor-associated calcium signal transducer)

Many of these markers are also expressed on normal biliary epithelial cells.

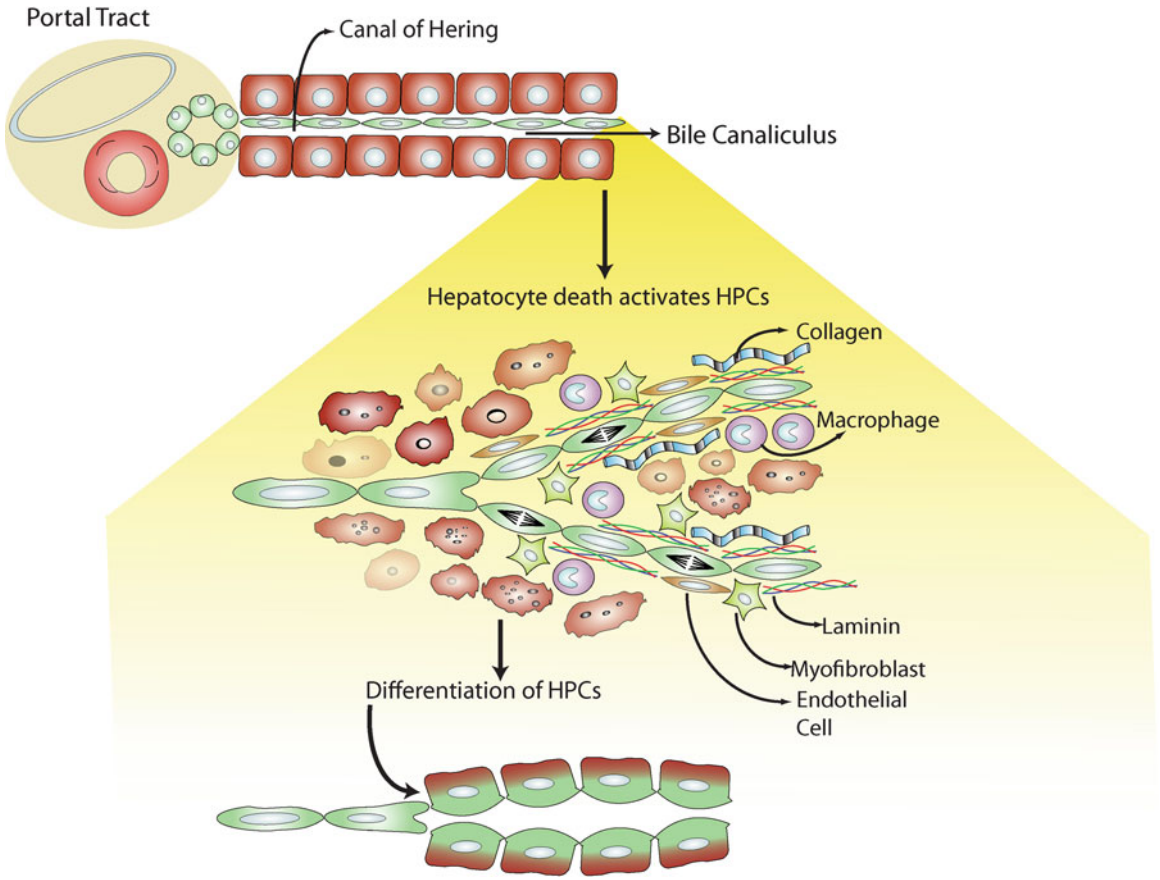

Fig. 3 Cartoon of the HPC response in man. HPCs are believed to have their origin in cells lining the canals of Hering. After hepatocyte damage, HPCs proliferate and are surrounded by an expanding niche composed of cells (mainly macrophages, myofibroblasts, and endothelial cells) and ECM components (principally laminin and collagens) that regulate HPC fate. Ultimately HPCs can undergo hepatocytic differentiation, losing biliary phenotypic markers such as CK7 and CK19

far-reaching clinical consequences, since the rapid activation of the HPC response after acute liver failure may represent an important lifeline. Other components of the inflammatory response that can stimulate oval cells include lymphotoxin-β, IFN-γ, TNF-α, and histamine [62]. Resistance to the growth inhibitory effects of TGF-β may allow oval cells to proliferate under conditions inhibitory to hepatocytes [75].

In terms of negative regulators of the oval cell response, the neurofibromatosis type 2 (*Nf2*) gene product Merlin appears critically important [76]. Genetic deletion of *Nf2* leads to massive oval cell expansion and the development of CC and HCC; Merlin appears to control the availability of EGFR and other growth factor receptors. Stem cells reside in a specialized supportive microenvironment known as a niche, and oval cells/HPCs not only have such a niche but this niche seems to migrate hand in hand with the expansion of oval cells (Fig. 3). For example, with the CDE diet, the activation of stellate cells (upregulation of αSMA expression) and deposition of collagen precedes the oval cell response suggesting the extension of the niche is a prerequisite for oval cell expansion [77]. In fact mouse and rat models of oval cell activation and HPC reactions in man bear a striking similarity, both in terms of the deposition of extracellular matrix (ECM) (particularly laminin) and cells (macrophages and αSMA^{+} myofibroblasts) that accompany progenitor reactions suggestive of a stereotypical niche [78]. Further support for the idea that the ECM adjacent to oval cell reactions is not merely a passive bystander comes from studies of the oval cell reaction in mice that produce mutated collagen I that is highly resistant to MMP degradation [79]; here a failure to remodel collagen stunts the reaction, seemingly through a failure to establish a laminin-rich progenitor niche. In the 2-AAF/PH model, blocking the activation of stellate cells with L-cysteine was a potent suppressor of the oval cell response, probably related to loss of cytokines such as TGF-β1 and the fibronectin matrix, that amongst other properties can concentrate cytokines such as CTGF for which oval cells have receptors [80].

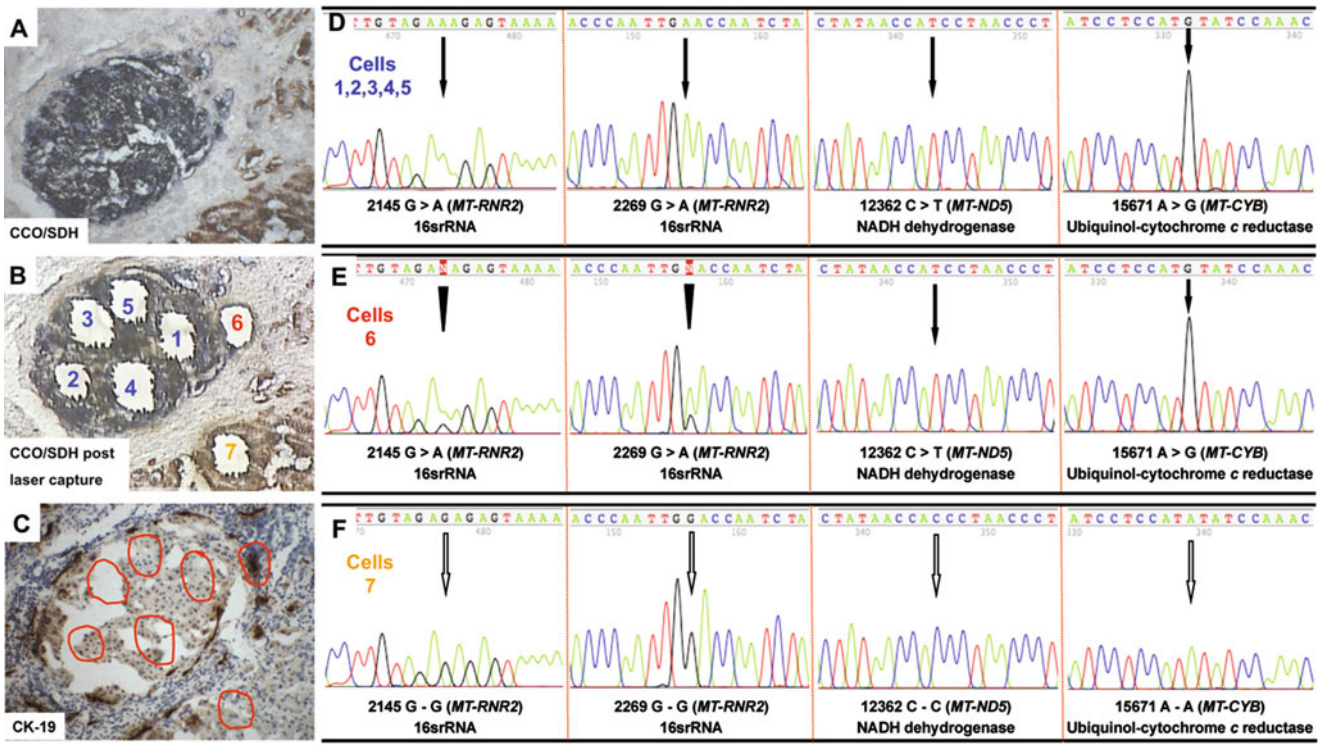

Fig. 4 Mitochondrial DNA genotyping indicates that regenerative nodules can be derived from CK19-positive HPCs. (**a**) An entirely CCO-deficient nodule (stained *blue* for succinate dehydrogenase activity). (**b**) Five groups of cells (*1–5*) from the same CCO-deficient nodule; cells (*6*) from the adjacent CCO-deficient ductular reaction, confirmed by CK19 IHC on the next serial section ((**c**) *brown* staining); and cells (*7*) from the CCO-positive nodule were laser capture microdissected and the entire mitochondrial genome was sequenced. (**d**) Cell areas *1–5* all contained four different transition mutations: 2145G>A, 2269G>A, 12362C>T, and 15671A>G (*black arrows*). (**e**) Cell area *6* from the abutting CCO-deficient ductular reaction had exactly the same mutations. Heteroplasmy was detected at location 2145 and 2269 (*arrowheads*), while the mutations at location 12362 and 15671 were homoplasmic (*black arrows*). (**f**) Cell area *7* from the CCO-positive nodule had no mutation (*white arrows*). See Lin et al. [84] for further details. Reproduced with permission from *Hepatology*

Chronic viral hepatitis is, of course, invariably associated with cirrhosis and hepatocyte senescence [41, 81, 82]; thus, activation of HPCs in this setting is common. In the fibrous septae that surround regenerative nodules (RNs), differentiation of CK19-positive HPCs to form buds of intraseptal hepatocytes (ISHs) is often observed [83]. In cirrhosis we observed that RNs are invariably clonally derived (Fig. 4) suggesting that they are not simply created by fibrotic dissection of the preexisting parenchyma; moreover, they are clonally related to the abutting HPCs and thus have been derived from them [84]. Thus, RNs may well represent the further expansion of buds of ISHs.

Stem Cells and Liver Cancer (Founders and Propagators)

Whereas CCs are believed to arise from either established biliary ducts or HPCs, the origin of HCCs is more problematic. Clearly hepatocytes are the cell of origin of many HCCs in experimental models where tumor yield is directly related to hepatocyte proliferation or where oncogenic transgenes are driven by the albumin promoter. On the other hand, HPC activation is commonly seen in models of hepatocarcinogenesis and invariably accompanies chronic liver damage in humans, thus making it quite likely that HPCs are the founder cells of many HCCs [85]. An origin of HCC from HPCs is often suggested because many HCCs contain an admixture of mature hepatocyte-like cells and cells resembling HPCs [1]. If tumors do arise from HPCs, then this indicates a block in HPC differentiation, a process that has been termed "stem cell maturation arrest" [86]. This hypothesis is supported by the fact that murine HCCs induced by a CDE diet have a mixture of neoplastic phenotypes recapitulating stages in normal development, suggesting intermediate states between bipotent oval cells and hepatocytes [87]. Likewise in humans, four prognostic subtypes of HCC have been identified equating to liver cell maturational steps [88]. The poorest prognostic groups had a significant proportion of either $EpCAM^{+}AFP^{+}$ cells (hepatoblast-like) or $EpCAM^{-}AFP^{+}$ cells (HPC-like), whereas

those with $EpCAM^{-}AFP^{-}$ cells (mature hepatocyte-like) or $EpCAM^{+}AFP^{-}$ cells (cholangiocyte-like) had a better prognosis. Gene expression profiling has identified a subset of HCCs with a profile consistent with an origin from HPCs, and these patients have a poor prognosis [89]; moreover, counting of CK19-positive cells in HCC can identify a poor prognosis group [90] that may be related to enhanced EMT [91].

A detailed discussion of CSCs in HCC is beyond the scope of this chapter, but a number of phenotypic markers have been proposed for their isolation including CD13, CD90, CD133, ALDH activity, and the side population [92]. As in other organs, HCC CSCs seem relatively resistant to therapy and strategies to either reduce ABC transporter function [93–95] or induce differentiation [96] have increased CSC sensitivity. For a detailed discussion, see [97].

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Human-Induced Pluripotent Stem Cells, Embryonic Stem Cells, and Their Cardiomyocyte Derivatives: An Overview

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Abbreviations

ACE	Angiotensin-converting enzyme
ALCAM	Activated leukocyte cell adhesion molecule
ANP	Atrial natriuretic peptide
ANT	Altered nuclear transfer
AP	Action potential
APD	Action potential duration
APMAAm	Aminopropylmethacrylamide
ATP	Adenosine-5′-triphosphate
BC	Beating cluster
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine (5-bromo-2′-deoxyuridine)
CDM	Chemically defined medium
Cdx2	Caudal type homeobox 2
CICR	Calcium-induced calcium release
CM	Cardiomyocytes
CNV	Copy number variations
CPC	Cardiac progenitor cells
CpG	Cytosine–phosphate–guanine
CTNT	Cardiac troponin T
CVD	Cardiovascular disease
Cx4	Connexin 4
DAD	Delayed after-depolarization
DCM	Dilated cardiomyopathy
DKK1	Dickkopf-related protein 1
DNA	Deoxyribonucleic acid
EAD	Early after-depolarization
EB	Embryoid body
ECG	Electrocardiography/electrocardiogram
ECM	Extracellular matrix
EGF	Epidermal growth factor
EM	Electron microscopy
END-2	Visceral-endoderm-like cells
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FCM	Fibroblast-conditioned medium
FGF	Fibroblast growth factor
Flk-1	Fetal liver kinase 1
GMP	Good manufacturing practice
GO	Gene ontology
HCM	Hypertrophic cardiomyopathy
hERG	Human ether-à-go-go-related gene
HES-2	Hairy and enhancer of split 2
hESC	Human embryonic stem cells
hiPSC-CM	Human-induced pluripotent cell-derived cardiomyocytes
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
ICM	Inner cell mass
IGF	Insulin-like growth factor
IKr	Rapid delayed rectifier potassium (K^+) current
IKs	Slow delayed rectifier potassium (K^+) current
IL	Interleukin
INa	Inward sodium (Na^+) current
iPSC	Induced pluripotent stem cells
IVF	In vitro fertilization
KDR	Kinase insert domain receptor
KLF4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
MAP(K)	Mitogen-activated protein (kinase)
MEA	Microelectrode assay
MEF	Mouse embryonic fibroblast
MEF2c	Myocyte enhancer factor 2C
mESC	Mouse embryonic stem cells
MHC	Myosin heavy chain
MI	Myocardial infarction

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MLC	Myosin light chain
MSC	Mesenchymal stem cells
NFAT (C4)	Nuclear factor of activated T-cells (cytoplasmic 4)
NKX2.5	NK2 transcription factor related 5
NOD	Non-obese diabetic
OCT-4	Octamer-binding transcription factor 4
O-S-K-M	OCT3/4 SOX2, KLF4, C-MYC
PAS	Peptide-acrylate surface
PDGF	Platelet-derived growth factor
phESC	Parthenogenic human embryonic stem cells
PMEDSAH	Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]
PSC	Pluripotent stem cells
PTPN11	Tyrosine-protein phosphatase non-receptor type 11
RA	Retinoic acid
RNA	Ribonucleic acid
ROCKi	Rho-associated kinase inhibitor
RPMI	Roswell Park Memorial Institute (medium)
RT-PCR	Reverse transcriptase polymerase chain reaction
RYR	Ryanodine receptor
SCID	Severe combined immunodeficiency
SCN5A	Sodium channel voltage-gated, type V, alpha subunit
SCNT	Somatic cell nuclear transfer
SERCA2a	Sarcoplasmic–endoplasmic reticulum calcium (Ca^{2+}) ATPase
SeV	Sendai virus
SFM	Serum-free medium
SIRPA	Signal-regulatory protein alpha
SOX-2	SRY (sex determining region Y)-box 2
SSEA	Stage-specific embryonic antigen
Tbx	T-box transcription factor
TdP	Torsades de pointes
TGF-β1	Transforming growth factor beta 1
TM	Transmembrane
TNFα	Tumor necrosis factor alpha
TRA	Tumor rejection antigen
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

Introduction

Cardiovascular diseases (CVDs) encompass different pathologies that impair the ability of heart to pump blood to maintain physiological functions and are the most frequent cause of death in adults and main noninfectious cause of death in children in the United States and Western Europe [1]. About 43 % of all CVD-related mortalities worldwide are due to the ischemic heart disease, which is characterized by irreversible loss of functional CMs. About one billion CMs are destroyed in a single event of myocardial infarction (MI) [2]. While the available treatments with pharmacological and surgical means are only of limited help, heart transplantation is currently the only effective therapy for heart failure providing 1- and 5-year graft survival in 85.5 % and 70.6 % of cases, respectively [3]. Due to organ shortage, chronic graft rejection, and toxicity of the immunosuppressive therapy, there is an urgent need for alternative treatments. One alternative approach for improving the contractile function of a failing heart is the replacement of damaged heart cells by transplantation of cells from various sources such as fetal heart cells, skeletal myoblasts, and multipotent adult stem cells as well as CMs derived from pluripotent stem cells (PSCs) [2].

The use of CMs isolated from aborted fetuses for human therapy is problematic because of ethical concerns, limited accessibility, and risk of immune rejection of heterologous cells. Bone marrow-derived stem cells and skeletal myoblasts have a great advantage of being available from autologous sources and have, therefore, already been tested for treatment of patients with MI [4]. Functional improvement of infarcted heart has been reported in animal studies and clinical trials with these types of cells [5–9]. However, in contrast to earlier belief [6, 10, 11] bone marrow cells cannot differentiate into CMs [12–14] and skeletal myoblasts are incapable of integrating electrically with the host myocardium [15–17]. In addition, their use may be accompanied with serious side effects such as ventricular tachycardia caused by transplanted myoblasts [17] or bone formation from intramyocardially implanted bone marrow-derived mesenchymal stem cells (MSCs) [18]. The modest, functional, benefits of myogenic and bone marrow cell transplantation are now believed to be mediated by factors acting in paracrine manner through anti-apoptotic, proangiogenic, or anti-inflammatory mechanisms [16, 19–22]. Since the engrafted cells should form functional syncytium with the host myocardium to contribute to systolic pump function, adult stem cells or skeletal myoblasts alone cannot be considered as an ideal and only source of cells for cardiac repair because they are incapable of replacing lost contractile cells. Pluripotent embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) represent an alternative source of clinically useful CMs because they are easily accessible and expandable in culture, have broad developmental potential, and have high capacity to reproducibly differentiate into spontaneously beating cardiac muscle cells in vitro.

Embryonic Stem Cells

The first pluripotent ESCs were derived from the inner cell mass (ICM) of mouse blastocysts in 1981 by Evans and

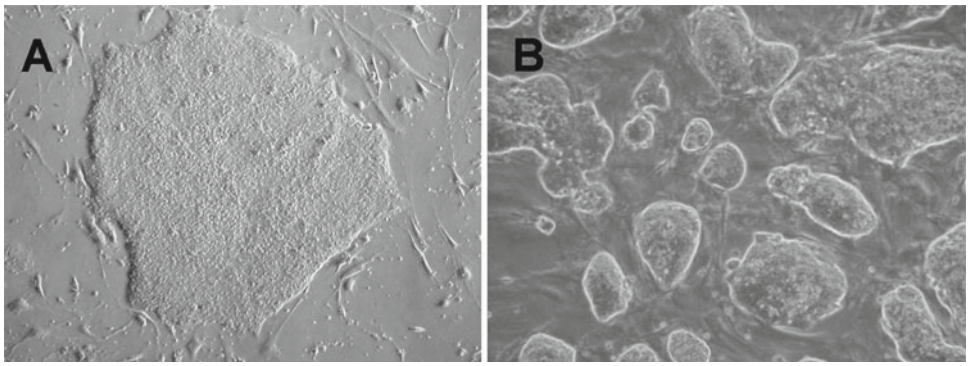

Fig. 1 Microscopic images of undifferentiated human (**a**) and murine (**b**) ESCs. Colony morphology of human and murine iPSCs resembles that of the respective ESCs (not shown)

Kaufman, and Martin and coworkers [23, 24]. Initially, murine ESCs were of interest almost exclusively to developmental and cell biologists and mostly served to elucidate the function of specific genes in vivo through generation of transgenic or knock-out animals. Establishment of in vitro pluripotent cell lines from human blastocysts had significantly lagged behind their murine counterparts due to their different cell culture requirements. However, the successful derivation of primate ESC lines [25, 26] and improvements in culturing human embryos obtained by in vitro fertilization (IVF) [27] led to subsequent isolation of human ESC lines from human preimplantation blastocysts in the late 1990s by Thomson and his group [28], Reubinoff's group [29], and Bongso and coworkers [30]. After this high-profile discovery, the interest in ESCs literally exploded and their basic properties and potential for cellular replacement therapy began to be extensively explored.

Human ESCs (hESCs) and murine ESCs (mESCs) differ in morphology (Fig. 1), cell culture requirements, cell surface marker expression, signaling pathways, and differentiation ability. However, they share the two fundamental properties of stem cells: self-renewal (ability to remain undifferentiated by symmetrically dividing into the same non-specialized cell types over long periods of time) and pluripotency (ability to differentiate into cells of different lineages). The pluripotency of mESCs is typically assessed, in the order of increasing stringency, by their ability to (a) differentiate in vitro into lineages of all three primary germ layers, (b) form teratomas after transfer to immunodeficient [29, 31] and immunocompetent syngenic animals [32], (c) contribute to chimera formation, (d) germ line transmission, and (e) allow tetraploid complementation [33]. A measure of pluripotency based on blastocyst manipulation, such as chimera formation and tetraploid complementation, cannot be applied for ethical reasons on human or primate ESCs. Consequently, developmental potential of these ESCs is typically demonstrated by in vitro differentiation and by the formation of all three germ layers in teratomas generated by transplantation of ESCs into immunodeficient animals [28, 34]. Routinely, the quality control of ESCs in culture is performed by more practical cytochemical, flow cytometric, or RT-PCR detection of various ESC-specific proteins and transcripts and in a more comprehensive manner by global transcriptional profiling. These later data can be applied to determine the degree of pluripotency of human cells by using an open-access bioinformatics assay called PluriTest [35].

The capability of ESCs to give rise to a wide variety of specialized somatic cell types render them an unlimited, scalable, and easily accessible source of various cell types for in vivo tissue replacement, cell-based high-throughput drug screening, and functional genomics applications, which aim to identify the pathways underlying lineage commitment. Culturing of undifferentiated ESCs on feeder layer of mouse embryonic fibroblasts (MEFs) [28, 29] or human feeder cell alternatives sustains their propagation in the undifferentiated state [36]. The mouse feeder cells can be replaced by leukemia inhibitory factor (LIF) [37], which helps to maintain the pluripotent state of mESCs while the hESC lines require feeder cells and the basic fibroblast growth factor (bFGF) to maintain pluripotency [28, 29]. The bFGF facilitates the clonal growth of hESCs on fibroblasts feeder layers [38, 39] as well as in the fibroblast-conditioned medium (FCM). At elevated concentrations, the bFGF permits the culture of human ESCs in the absence of MEFs or FCM. In unconditioned medium supplemented with 100-ng/mL bFGF, the hESC lines H1 and H9 were maintained for up to 164 population doublings without losing their pluripotency [39]. Addition of bone morphogenetic proteins (BMPs) to hESCs cultured in FCM containing bFGF promoted trophoblast differentiation. hESCs cultured in unconditioned media showed greater BMP signaling activity than those cultured in FCM, and this activity was inhibited by the addition of Noggin (a BMP antagonist), bFGF, or both. Noggin combined with high bFGF concentrations supports the long-term undifferentiated proliferation of hESCs in the absence of fibroblasts or FCM [40].

Maintenance culture of ESCs can be performed either on feeder cells or under feeder-free conditions. Feeder-free culture systems were developed to overcome the need for animal feeder cells and allow the hESC scaling up for maintenance and proliferation. Xu et al. successfully showed that feeder-free hESC cultures maintained the undifferentiated state for at least 130 population doublings over 6 months [41]. These investigators evaluated the capability of several matrices in FCM to sustain hESC growth in the absence of feeder cells. Cells grown on Matrigel, a mixture of extracellular matrix (ECM) proteins secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, showed that hESC possesses morphology similar to those obtained from feeder-fed cultures.

Protocols for expansion of ESCs that rely on the use of poorly defined substrates for cell attachment, such as Matrigel, carry the risk of irreproducibility, pathogen contamination, and immunogenicity. Therefore, several groups tested various recombinant ECM proteins [42, 43] and synthetic surfaces [44–48] for their ability to support long-term culture of hESCs. Laminin is the ECM protein expressed in very early stage embryos and component of all basal laminae in vertebrates. Human ESCs interact with matrix via laminin-specific receptors and α6β1 integrins. Laminin is the first ECM protein shown to support the undifferentiated growth of hESCs in FCM and maintenance of hESCs in feeder or feeder-free conditions did not alter the expression of the integrins α6 and β1 [41]. Feeder-free hESCs grown on laminin or Matrigel exhibited normal karyotype, stable proliferation rate, high telomerase activity, expressed OCT-4, hTERT, alkaline phosphatase, surface markers stage-specific embryonic antigen 4 (SSEA-4), and tumor rejection antigens TRA 1-60 and TRA 1-81, similar to hESCs cultured on MEFs [41]. Laminin isoform 511 was found to be secreted from the hESC-supportive feeder cells [49] and hESCs cultured on the recombinant form of human laminin-511 retained their pluripotency and normal karyotype for over 20 passages under this condition in chemically defined medium [43]. In a process of standardizing the generation of more defined hESC attachment substrates, several groups found that synthetic peptides derived from protein ligands of cell surface receptors supported hESC growth over prolonged passages under xeno-free conditions [45]. In addition to peptides, the synthetic polymer coating poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), peptide-acrylate surfaces (PAS), and hydrogel interface of aminopropylmethacrylamide (APMAAm) sustain long-term hESC growth in several different hESC culture media [46–48]. Establishing xeno-free culture conditions of PSCs on defined substrates will help in the derivation of clinical grade hESCs and in derivation of more reproducible and efficient cardiac differentiation protocols. Indeed, newly developed protocols for generation of CMs from human PSCs in high yields include preconditioning step that involves feeder-free cultivation of hESCs prior to the embryoid body (EB) formation step [50].

Alternative Sources of Human ESCs

To obviate the use of ESCs, adult stem cells have been proposed as an alternative source of cells for regenerative medicine. However, adult stem cells are difficult to culture for extended periods of time, and they seem to possess only limited ability to differentiate into a variety of tissue-specific cells. Therefore, derivation of many clinically useful cell types is still dependent on the availability of well-characterized human PSCs. Virtually all existing hESC lines have been derived from the ICM of the healthy blastocyst-stage embryos. Due to ethical objections to the use of hESCs, many investigators and legislative bodies examined the alternative ways for producing ethically, scientifically, and therapeutically acceptable PSCs [51]. Various approaches for generation of PSCs differ in their technical complexity as well as in the functionality of established cells, their safety, ethical acceptability, and clinical applicability. These approaches can be grouped into three categories depending on the source of cells used for derivation of PSCs, which will be shortly discussed in the following chapters.

The first alternative way for generation of PSCs includes derivation of PSCs from early embryos in a way that may be considered ethically acceptable. This concept includes derivation of ESCs from single blastomeres or from poor-quality IVF embryos that would otherwise be discarded (Fig. 2). For example, Zhang et al. generated hESC lines from IVF embryos that have stopped dividing or dead embryos and these cells exhibited properties of traditionally derived hESCs [52]. Verlisky et al. generated hESC lines from embryos with genetic disorders, which were discarded as medical waste, to establish in vitro models of human disorders such as muscular dystrophy, Huntington's disease, thalassemia, Fanconi's anemia, Marfan syndrome, adrenoleukodystrophy, and neurofibromatosis [53]. Chung and coworkers [54] were first to isolate mESC lines from a single cell removed from preimplantation embryo. Later, hESCs were derived from single blastomeres belonging to unused embryos from IVF for clinical purposes with full, informed consent [55].

Methods in the second category make use of adult germ cells (e.g., oocytes or spermatogonial stem cells) for obtaining PSCs. Revazova et al. [56] and Sung et al. [57] reported protocols to obtain parthenogenic hESC (phESC) lines. Parthenogenic embryos are created from an embryo without fertilizing the egg with a sperm by tricking the egg into believing it is fertilized, so that it will begin to divide and form a blastocyst. The phESC lines retained the genetic information of the egg donor and pluripotency. This alternate method does not require destruction of a fertilized embryo and offers to derive tissue-matched hESCs.

In the third category, adult somatic cells are the source of PSCs. Approaches in this category have the capability for derivation of autologous stem cells and include nuclear reprogramming by somatic cell nuclear transfer (SCNT),

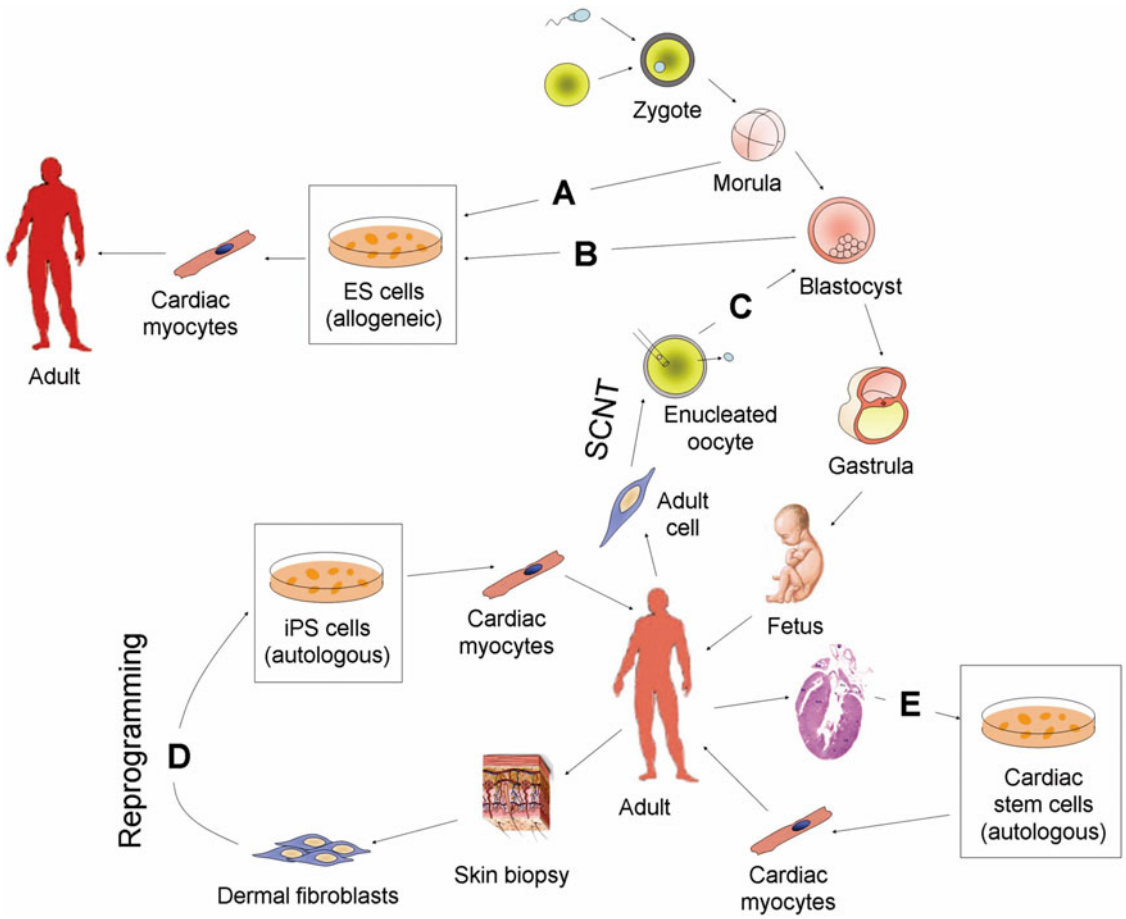

Fig. 2 Some promising sources of contractile cardiomyocytes for heart repair. Cardiomyocytes for heart regeneration can be derived from PSCs or endogenous cardiac stem cells. The PSCs can be generated from early embryos, fetal, newborn, or adult somatic cells. *A*. In a single-cell biopsy concept, a single totipotent blastomere is removed from an eight-cell embryo (morula) to derive a new ES cell line and the remaining seven-cell embryos are transferred into surrogate mothers, in which they develop into normal organism. *B*. The second approach is based on extracting pluripotent cells from the inner cell mass (ICM) of poor-quality embryos generated in the process of in vitro fertilization (IVF). These embryos are unsuitable for uterine transfer or cryopreservation and would therefore be discarded. *C*. Somatic cell nuclear transfer (SCNT) involves the transplantation of a somatic cell nucleus into an enucleated unfertilized oocyte. Pluripotent ES cell lines can be established from cloned blastocysts (therapeutic cloning, as shown in *B*) and used to generate cells for autologous cellular therapies. *D*. Complete reprogramming of somatic cells can be induced by ectopic expression of transcription factors required for pluripotency. This approach holds great promise for science and medicine because it circumvents the use of oocytes as well as embryos and may allow for patient-specific autologous therapies and establishment of human in vitro disease models. *E*. Cardiac repair can also be induced by activation of endogenous cardiac stem cells in situ or their isolation, in vitro expansion, and subsequent transplantation into injured heart of the donor patient

altered nuclear transfer (ANT), cell fusion, or ectopic expression of pluripotency factors. In SCNT (Fig. 2), the nucleus of a differentiated cell is transferred into an enucleated unfertilized oocyte with the purpose to establish SCNT-derived ESC lines. Such lines were derived so far from cloned murine [58] and recently, primate SCNT blastocysts [59]. These cells appear to be indistinguishable in their proliferative, developmental, and therapeutic potential from ESCs derived from fertilized embryos and are regarded as a potential source of patient-specific cells for custom-tailored tissue repair or gene therapy [60]. Although no human SCNT ESC lines have been created, yet, this technique is highly controversial and ethically disputable [61]. Therefore, this method seems impractical for derivation of PSCs for therapeutic purposes.

The ANT technology involves temporal inactivation of a gene required for normal development, such as Cdx2, before transfer of a somatic nucleus into an oocyte. This gene is essential for trophectoderm formation in mouse. Blastocysts with disabled Cdx2 lack trophectoderm and cannot implant, but can serve as a source of normal ESCs after removal of a transgene producing the Cdx2-interfering RNA [62]. Murine ANT-derived stem cells can self-renew in culture and are pluripotent as demonstrated by teratoma formation and tetraploid complementation. Although the concept of ANT was presented as ethically acceptable, the validity of the ANT for production of ideologically acceptable hESC lines has been strongly questioned [63]. Moreover, it is not clear if the Cdx2 would have the same function in human development as it

has in a mouse and testing this concept in the human system would not be possible for ethical reasons.

The method of cell fusion is based on polyethylene glycol-induced or spontaneous heterokaryon formation between adult somatic cells and undifferentiated ESCs. Fusions between murine or human ESCs and various types of adult cells such as lymphocytes [64, 65], neurosphere cells [66], fibroblasts [67, 68], or myeloid precursor cells [69] have been reported to promote the epigenetic reprogramming of the adult genome to a pluripotent phenotype. The resulting fusion hybrids were morphologically indistinguishable from normal ESCs, had the potential to differentiate into multiple lineages, contributed to all three primary germ layers of chimeric embryos, and exhibited transcriptional activity and epigenetic profile similar to that of ESCs. The method of cell fusion does not require the use of oocytes and preimplantation embryos, but its utility for the generation of cells for clinical use is limited by the tetraploid character of hybrid cells and the inability to separate the ESC genome from a somatic partner.

In 2006 and 2007, Yamanaka's [70–72], Thomson's [73], and Rudolf Jaenisch's groups [74, 75] demonstrated, in a significant breakthrough, that ectopic overexpression in somatic cells of a defined set of transcription factors crucial for maintaining pluripotency leads to their conversion into ESC-like cells, the so-called induced pluripotent stem cells (iPSCs) (Fig. 2). While technical limitations remain, this strategy is the promising path to generate ethically unobjectionable and autologous PSCs overcoming the limitations associated with human ESCs and providing the cells vital for drug development, human disease modeling, and regenerative medicine. The iPSC technology will be discussed in detail, in the following chapter.

Induced Pluripotent Stem Cells

Takahashi and Yamanaka [70] were first to show that murine embryonic and adult fibroblasts transduced with retroviral vectors to express transcription factors OCT3/4, SOX2, C-MYC, and KLF4 (Kruppel-like factor 4) can be reprogrammed into iPSCs. This combination of factors is usually abbreviated as O-S-K-M. Although slightly different combination of genes (OCT4, SOX2, NANOG, and LIN28) has also been shown by James Thomson's group to work for reprogramming human fibroblasts [73], almost all murine and human iPSC lines generated so far were produced with the original O-S-K-M combination in different perturbations. All stably reprogrammed iPSCs are highly similar to ESCs as demonstrated by highly comparable gene expression profiles, DNA methylation status, and chromatin configuration [71, 76]. Furthermore, when injected into immunodeficient mice, these cells form teratomas composed of various mature somatic tissues of all three germ layers. Moreover, murine iPSCs contribute to tissues in viable chimeras when injected into diploid blastocysts and are germ line competent. In a most stringent test for developmental potency, murine iPSCs injected into tetraploid blastocysts generated live embryos and adult animals composed entirely of injected iPSCs [77, 78]. Molecular similarity between iPSCs and ESCs is also evident at the proteomic level [79–84]. For example, Phanstiel et al. found that only about 300 proteins and phosphoproteins differed between human iPSCs and ESCs [83] and Munoz and coworkers showed that the similarity between human ESC and iPSC lines is about 97.8 %. Only a small group of 58 proteins belonging to metabolism, antigen processing, and cell adhesion were differentially expressed [82].

In 6 years since the establishment of iPSC technology, tremendous progress has been made toward generation of iPSCs that are more amenable for scientific and clinical use. The efficiency of reprogramming has been greatly increased by combining viral expression systems and small molecules (reviewed by Wang et al.) [85–92]; somatic cell types that are more susceptible to reprogramming have been identified [87, 88, 93] disease-specific iPSCs and various human in vitro disease models were established and characterized [93–96]; and newly designed compact vectors have significantly reduced the number of viral insertions in iPSCs [97]. Since first derivation of iPSCs from fibroblasts, many other cell types, such as mature B-lymphocytes, liver, stomach cells, primary hepatocytes, pancreatic β-cells, cord blood cells as well as mesenchymal and neural stem cells, have been successfully converted to iPSCs (reviewed in [98]). In addition, iPSC lines have also been obtained from other species, such as macaque monkey, rat, pig, dog, horse, sheep, cow, and even some endangered species, such as the silver-maned drill and the white rhinoceros [99–103]. However [104], the evidence for a full pluripotential nature of some of these iPSC lines was lacking, and in some cases, the continuous expression of the exogenous factors was required for maintenance of iPSCs in an undifferentiated state. These iPSC lines may serve as a useful complement to human iPSCs in preclinical evaluation of strategies for cell replacement therapies.

The use of retroviral vectors to introduce reprogramming factors into somatic cells raises safety concerns for iPSCs, because infection with retro- and lentiviruses results in their stable integration at multiple random sites in the genome, which may disrupt essential genes by insertional mutagenesis and lead to malignant tumor formation. In an attempt to generate safer iPSCs, different groups have produced iPSCs with only two reprogramming factors, but the efficiency was strongly reduced [73, 87, 88, 105–109]. Despite reduction in number of factors required for reprogramming and generation of iPS cells with only a single integration per cell of a polycistronic vector expressing O-S-K-M combination of

factors [97], clinical grade iPSCs must be generated without viruses and stable integration of foreign genes. Therefore, alternative approaches that permit transient factor delivery into somatic cells have been developed. Integration-free iPSCs have been generated by transient delivery of reprogramming factors using plasmid transfection [72, 110], the Cre/loxP system [111, 112], adenoviruses [113], transposon-based delivery systems [114, 115], episomal vectors [110, 116–118], minicircle vector [119], mRNA [120], or transfection of somatic cells with ESC-specific microRNAs [121]. Highly efficient and non-integrating RNA Sendai virus (SeV) vectors were also used to generate human iPSCs from fibroblasts [122] and blood cells [123]. Recombinant SeV vectors replicate only in the cytoplasm of infected cells and do not integrate into the host genome. To generate safer iPSCs, sustained cytoplasmic replication of viral vectors after the iPSCs have been established must be inhibited. To shut down viral replication, Ban et al. [124] used temperature-sensitive SeV vectors, which could be removed, at nonpermissive temperatures. Macarthur et al. [125] reported the generation of transgene-free iPSC line by using SeV vector in feeder- and xeno-free conditions with StemPro hESC serum-free medium (SFM). The technology based on direct delivery of recombinant reprogramming factors fused to protein transduction domains into somatic cells fully circumvents the use of genetic material in the process of iPSC production [126, 127]. However, the major limitations to this and most other virus-free methods are low efficiency, the need for repetitive transfection of cells during reprogramming and production of iPSCs with insufficient excision of integrated vectors. Therefore, more efficient and simple methods to generate human iPSCs with no noise of integration or remaining factors are needed. Nevertheless, these developments are crucial for the generation of therapeutically acceptable iPSC lines and will facilitate clinical translation of iPSC-based technologies.

Although iPSCs strongly resemble ESCs both at functional and molecular levels, comprehensive analyses of various undifferentiated ESC and iPSC lines revealed that iPSCs may not be perfectly identical to conventional ESCs at the molecular level [116, 128, 129]. These studies demonstrated that iPSCs can be distinguished from ESCs by unique gene and miRNA expression signatures as well as a CpG methylation pattern. Clinical utility of iPSCs may be also diminished by findings by Maysher et al. [130] and Laurent et al. [131] that specific genetic aberrations were associated with both hESCs and hiPSCs. ESCs showed gains while iPSCs are frequently associated with deletions. Laurent et al. also showed that the reprogramming process favored deletions that affect tumor-suppressor genes, while maintenance of the cell lines is associated with oncogene duplications [131]. In addition, Hussein et al. [132] showed that genetic reprogramming is associated with high mutation rates, which resulted in high copy number variations (CNV). CNVs were more frequently observed in early passage human iPSCs than intermediate passage human iPSCs, fibroblasts, or human ESCs. These CNVs are generated de novo and result in genetic mosaicism, in the early passage iPSC lines, but expansion of these iPSCs in culture selects against mutated cells to the advantage of more genetically intact iPSCs. However, long-term passaging per se could lead to genetic and epigenetic instabilities. Such genomic instability has already been reported in ESCs [133], which may become cancerogenic via accumulation of mutations and genomic rearrangements [134, 135]. Therefore, the safety aspects of iPSCs must be stringently evaluated before any therapeutic application of their differentiated derivatives.

Cardiac Differentiation of ESCs and iPSCs

Differentiation into Cardiomyocytes

When mouse or human ESCs and iPSCs are cultured in the absence of factors that normally maintain their pluripotency, such as mitotically inactivated MEFs, LIF, or bFGF, they spontaneously develop in vitro into spherical cell aggregates called embryoid bodies (EBs) [136]. These multicellular aggregates resemble early postimplantation embryos and contain a mixture of differentiating cells of endodermal, ectodermal, and mesodermal origin. In individual EBs, these cells are organized in clusters of specific cell types. Differentiation of PSCs into cardiomyocytes (CMs) in vitro is a spontaneous process and was first described by Doetschman and coworkers for murine [137] and by Reubinoff and coworkers for human ESCs [29]. Within the EBs, the CMs form between an outer epithelial layer with characteristics of visceral endoderm and a basal layer of mesenchymal cells. CMs emerge in murine EBs approximately 7–9 days and in human EBs 8–21 days (depending on differentiation protocol) after induction of differentiation and form clusters characterized by spontaneous rhythmic contractions. Depending on culture conditions, CMs can be maintained viable for months and can even survive few days of shipping at 4 °C over long distances [138], which may be of importance when organizing supply of these cells for clinical use.

CMs can be generated from PSCs using various approaches such as hanging drop [139, 140], suspension [141], monolayer [142], methylcellulose culture [143], and clusters [144, 145]. Gerecht-Nir and coworkers introduced, for the first time the formation of differentiating human EBs in rotating bioreactors with a threefold enhancement in the yield compared to Petri dish cultures [143]. Crucial limitation to currently existing differentiation protocols from stem cell-derived human CMs is the efficiency which is low, 1–3 % from mESCs, and <1 % from hESCs with the presence of other contaminating proliferating cells [146]. Therefore, identification of extrinsic and intrinsic

factors promoting cardiomyogenesis is critical for inducing efficient and reproducible cardiac differentiation of PSCs.

During differentiation of PSCs to CMs, a number of other tissue-specific cell types codifferentiate. Among those, the endoderm plays the most important role in inducing CM differentiation from PSCs. This has been particularly evident in cocultures of human ESCs with visceral-endoderm-like cells (END-2) from the mouse, which were first reported by Mummery and coworkers and resulted in induction of differentiation of hESCs into CMs [147]. Passier et al. described a protocol for differentiation of the HES-2 cell line (hESC) into CMs by coculturing with END-2 in serum-free media. The number of beating clusters increased by 24-fold compared to cultures containing 20 % serum. When ascorbic acid was added to serum-free cocultures, an additional increase in the number of beating areas was observed [148].

Andre Terzic's group elucidated the molecular basis of the inductive effect of the endoderm [149]. This group has demonstrated that tumor necrosis factor alpha (TNFα) promotes cardiac differentiation of transplanted undifferentiated ESCs in vivo as well as in EBs, in vitro. The procardiogenic action of this cytokine required an intact endoderm and was mediated by secreted endodermal growth factors including TGF-β1, BMP-2, BMP-4, activin A, VEGF-A, IL-6, FGF-2, FGF-4, IGF-1, IGF-2, and EGF. Addition of these recombinant factors to ESCs induced their differentiation to cells possessing an intermediate cardiac progenitor cell phenotype, which completed the cardiac differentiation program in the presence of these factors. Removal of these growth factors after 4 days of stimulation resulted in continued proliferation of cardiac progenitor cells without differentiation into CMs. This study suggests that directed and scalable production of CMs for heart repair under highly controlled culture conditions might be possible.

Cardiac differentiation of PSCs can be also achieved by addition of other growth factors, hormones, or small molecules thought to be involved in cardiogenesis [142, 150, 151]. These factors include the platelet-derived growth factor (PDGF) and sphingosine-1-phosphate as extrinsic cardiomyogenic factors [152]. Retinoic acid (RA) has been also reported to promote cardiac differentiation of mESCs [153]. In one study, RA-treated cultures contained approximately twice as many cardiac cells as compared to the non-treated flasks [154]. A small molecule inhibitor of p38 MAP kinase, SB203580, which is a potent promoter of hESC cardiogenesis induced more than 20 % of the differentiated cells to become CMs and almost doubled the yield of CMs when compared to controls [155]. Wu et al. identified a class of diaminopyrimidine compounds (cardiogenol A–D) using a cellular screening of a large combinatorial chemical library. This new class of compounds selectively and efficiently induced mESCs to differentiate into CMs [156, 157].

Signaling pathways such as Wnt/β-catenin and TGF-β family members regulate differentiation of CMs and cardiac progenitors [158, 159]. Yao et al. [160] reported a simple chemically defined medium (CDM) that supports efficient self-renewal of hESCs grown on a Matrigel over multiple passages. Treatment of hESCs with the combination of activin A (50 ng/mL) and BMP-4 (50 ng/mL) in the N2/B27-CDM (N2 and B27 are GIBCO supplemental media) for 3–4 days and continued culture for additional 8–10 days in the basal N2/B27-CDM induced marker expression associated with cardiac muscle lineage. hESCs were efficiently induced to form CMs using serum-free CDM and obtained more than 30 % CMs using activin/BMP protocol [150], which is a clinically scalable system for generation of human CMs. The CM yield can be further enriched to 80–90 % using Percoll® density-gradient centrifugation [150, 160, 161].

Burridge and coworkers developed a highly efficient protocol for differentiation of hESCs and hiPSCs to CMs by systematic analysis of >45 experimental variables that were reported to affect cardiac differentiation efficiency of PSCs [50]. The optimized differentiation method employed the EB formation by forced aggregation in V96 plates in a chemically defined RPMI medium, along with staged exposure to 5 % oxygen, and optimized concentrations of mesodermal morphogens BMP4 and FGF2, polyvinyl alcohol, serum, and insulin. Reportedly, with this system the intrinsic variability in cardiac differentiation capacity of different human PSCs was eliminated. If adapted to a large-scale production in controlled bioreactors, this system could allow a potentially unlimited production of functional CMs for different applications.

Protocols for a large-scale production of human CMs in bioreactors are mostly advanced in the sphere of biotechnological industry and are capable of delivering millions of highly purified CMs almost on a daily basis. Cellartis AB introduced hES-CMC™ 3D in a 3D tissue-like format [162, 163]. These are spontaneously beating hESC-CMs in a cluster format, ranging from 200 to 300 μm in diameter. These CMs exhibit specific markers and functional similarities to native human CMs. They are excellent in vitro tools for studies of human CM function and for cardiac safety pharmacology assays. Cellartis AB also introduced well-characterized hES-CMC™ 2D, fresh CMs, which are ready for in vitro use and can be easily dissociated for single-cell applications. These hESC-CMs are prepared as monolayers in a 96-well plate format and exhibit spontaneous beating and electrical coupling. Industrial production of hESC- and hiPSC-derived CM by several companies has overcome the setback of inefficient CMs differentiation protocols, which were in practice until recently. Different approaches for directed differentiation of PSCs to CMs are reviewed in Hattori et al. [164].

Besides the above-mentioned factors, microRNAs also play a crucial regulatory role in the process of in vitro and in vivo cardiogenesis. Babiarz et al. showed that mature human iPSC-CMs express CM-specific miRNAs miR-1, miR-133a/b, and miR-208a/b [165]. Ivey et al. showed that miR-1 promotes differentiation of mESCs and hESCs into the cardiac

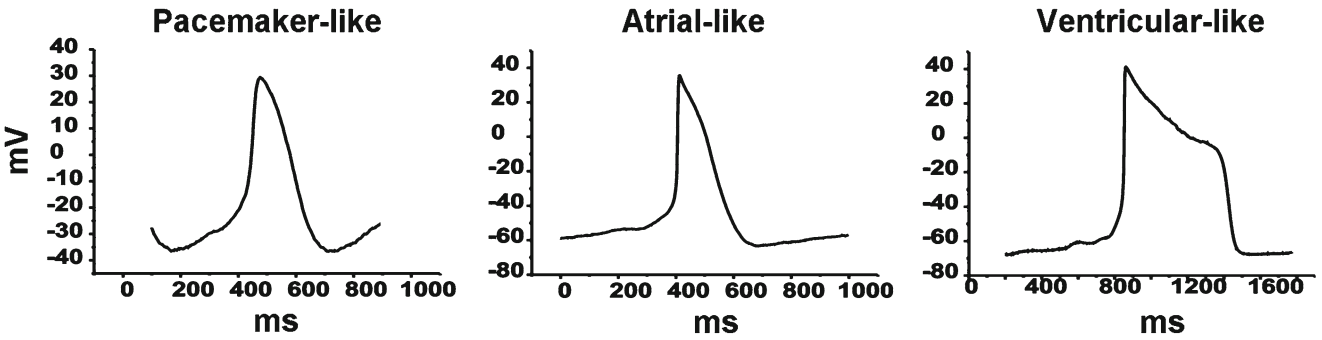

Fig. 3 The action potentials of three major cardiac cell types derived from human iPSCs. Action potentials in single cardiomyocytes differentiated from human iPSCs were analyzed by the whole-cell patch-clamp method. The classification of different cardiac cell types was based on the AP morphology and following AP parameters: V_{max} (~10 V/s for atrial- and ventricular-like cardiomyocytes, ~5 V/s for nodal cardiomyocytes) and APD90/APD50 ratio (~2 for atrial-like and <1.5 for ventricular-like cardiomyocytes)

lineage by enhancing expression of NKX2.5 and spontaneously contracting EB outgrowths [166]. Another differentially expressed microRNA miR-499 promoted ventricular specification of hESCs, while miR-1 facilitated electrophysiological maturation of hESC-derived ventricular CMs [167].

Murine and human ESC- and iPSC-derived CMs are capable of developing into the three major subtypes of cardiac myocytes, i.e., nodal/pacemaker-, atrial-, and ventricular-like cells [168–171] (Fig. 3). Derivation of protocols for directed differentiation of PSCs into a homogeneous population of specific subtype of CMs is of fundamental importance for their clinical use. Zhang and coworkers demonstrated that retinoid signaling determines whether hESC will differentiate toward atrial or ventricular CMs [172]. Human ESCs differentiated in the presence of Noggin and pan-retinoic acid receptor antagonist BMS-189453 yielded CMs of which 83 % had embryonic ventricular-like action potentials (APs). In contrast, in cultures containing Noggin and retinoic acid 94 % of CMs had atrial-like properties. The availability of homogeneous ventricular CM subpopulations will enable their use for myocardial repair without the risk for arrhythmias that may occur after ventricular transplantation of other types of CMs.

Differentiation into Cardiac Progenitor Cells

The only adult stem cells that have the capability to differentiate into beating CMs are the cardiac progenitor cells (CPCs) [173, 174] (Fig. 2). Several groups have also identified multipotent cardiovascular progenitor cells in mESC differentiation cultures [175–177]. The cardiovascular colony-forming cells were isolated from transgenic mouse embryos and differentiating mESC cultures using markers like NKX2.5 [178], lsl-1 [179], and VEGF receptor Flk-1 [175]. Like in mice, the three main lineages in the human heart are derived from a common progenitor cardiovascular colony-forming cell. Bearzi et al. recently identified C-KIT$^+$KDR$^-$ cells, in the adult human heart with cardiomyogenic potential [180, 181] and Yang et al. [142] reported a protocol for differentiation and isolation of similar early cardiac progenitors from human ESCs. Human ESC-derived EBs, when induced with activin A, BMP4, FGF2, VEGFA, and DKK1 in serum-free media, generated KDRlow/C-KIT(CD117)neg expressing progenitor population that displayed cardiac, endothelial, and vascular smooth muscle potential in vitro and, after transplantation, in vivo. The KDRlow/C-KITneg cells readily expressed cardiac troponin T (CTNT) and differentiated to generate populations consisting of >50 % contracting CMs in suspension or when plated in monolayer cultures. Most importantly, teratomas were not observed after transplantation of these cells. When qualitatively analyzed for field potential and whole-cell current, 80 % of the population resembled human atrial and ventricular myocytes in terms of voltage-gated and transient outward potassium currents. This population of cells was also coupled to each other [142]. When the KDRlow/C-KIT(CD117)neg cells were transplanted into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and analyzed for cardiac function after 2 weeks posttransplantation, the animals showed a 31 % higher ejection fraction than those injected with media alone. This improvement in cardiac function is consistent with previous studies [150, 182, 183] of rodent models of MI, where transplantation of hESC-derived CMs resulted in attenuation of post-MI scar thinning and left ventricular dysfunction.

Properties of ESC- and iPSC-Derived Cardiomyocytes

Cardiac Marker Expression Profile

The gene expression profiles of the hESC during cardiac differentiation [184, 185] and the differentiated hESC-CMs were studied by DNA microarray [144, 186, 187]. These studies revealed that the molecular signature of hESC-CMs

resembled the CMs from the human heart [188]. Human ESC-derived CM differentiation can be predicted by the transient expression of the early mesodermal marker Brachyury T [184] and cardiac regulatory markers such as Isl-1, Mesp 1, GATA-4, Nkx2.5, and T-box transcription factor 6 (Tbx6) can follow the cascade [142, 155].

RT-PCR and immunohistochemical studies demonstrated that both hESC-CMs and hiPSC-CMs express a number of early cardiac-specific transcription factors such as GATA4 and Nkx2.5, myocyte enhancer factor 2C (MEF2c), Tbx-5, and Tbx-20 [141, 189–193]. In addition, cardiac-specific sarcomeric proteins, which play a significant role in cardiogenesis in vertebrates, such as α-actinin, ANP, cardiac troponin I and cardiac troponin T, sarcomere myosin heavy chain (MHC), atrial- and ventricular-myosin light chains (MLC2v and MLC2a), desmin, and tropomyosin [141, 147, 168, 189, 191, 193] are expressed, and therefore used to confirm as well as characterize the cardiac phenotype of beating hPSC-CMs. Connexin 43 (Cx43), a gap junction protein, can also be used to determine the coupling between hESC-CMs [147].

Synnergren et al. revealed a comprehensive molecular signature of hESC-CM clusters using transcriptomic profiling by microarrays, gene ontology (GO) analysis, pathway analysis, and protein interaction network analysis [144]. This study improves understanding of biological processes, specific factors, and pathways that could induce and sustain CM differentiation from hESCs. 530 upregulated and 40 downregulated genes were identified in the hESC-CM clusters compared with undifferentiated hESCs [144]. Upregulated genes in hESC-CM clusters encompassed family of cardiac marker genes (MYH6, MYH7, TNNT2, and MYL7) as well as cardiac-related transcription factors (TBX5, MEF2C, GATA4, and ISL1), confirming the cardiac phenotype of the cell population and similarity to the human heart tissue. Out of fifteen genes enriched in the hESC-CMs and in fetal heart tissue, eight of these genes are also upregulated in hESC-CM clusters suggesting similarities between the CM cell populations obtained from hESCs independent of differentiation protocol and cell line used. Markers for undifferentiated hESCs (OCT4, NANOG, DNMT3B) were downregulated in the hESC-derived CM clusters. Genes upregulated in hESC-CM clusters were associated with GO terms muscle contraction, cell differentiation, development, focal adhesion, calcium signaling, and cardiogenesis. In another study, hESC-CMs exhibited similar transcriptomic profiles as fetal heart cells (20 weeks), thus making these cells a ready source for replacement therapies [186].

We have also extensively analyzed the transcriptional profiles of microdissected beating clusters (BCs) obtained from differentiating hESCs and hiPSCs [145]. Hierarchical clustering and principal component analysis of hiPS-BCs and hES-BCs showed that the transcriptional profiles are highly similar, and they differ in expression of only ~ 1.9 % of transcripts. Single isolated iPSC-CMs and ESC-CMs possessed similar sarcomeric organization, electrophysiological properties, and calcium handling. Gene ontology analysis of hiPSC-BCs revealed that among 204 genes that were upregulated when compared with hESC-BCs, the processes related to ECM, cell adhesion, and tissue development were overrepresented. Interestingly, undifferentiated iPSCs showed that 47 of 106 genes that were upregulated when compared with ESC cells remained enriched in iPSC-BCs vs. ESC-BCs. These data suggest that hiPS-BCs are transcriptionally highly similar, but may not be perfectly identical to hES-BCs since the former share some somatic cell signatures with undifferentiated iPSCs. The cellular composition of iPS-BCs and ES-BCs is different as they may retain some of genetic profile of somatic cells in differentiated iPS cell derivatives, or both.

Structural Properties

Structural properties of human PSC-derived CMs can be analyzed by immunofluorescence and electron microscopy (EM). In human iPSCs α-actinin and MLC2a labeling indicated clear striated pattern (Fig. 4) and MLC2a was localized to A-band of sarcomeres, similar to that seen in the hESC-CMs [194]. Immunolabeling for multiple myofilament proteins such as cTNT (myofilament-sarcomeric protein), MLC2v, α-actinin, αMHC, and tropomyosin showed comparable signals in both human ESC-CMs and iPSC-CMs [195–197]. These data indicate that both hiPSC-CMs and hESC-CMs have a well-organized sarcomeric structure.

Under the EM, the CMs showed a variable degree of differentiation. Large number of cells showed bundles of myofibrils. While most of the cells showed randomly distributed bundles, some cells clearly had organized, clear striated, and differentiated sarcomeres with Z lines, which is a characteristic feature of early stage CMs [141, 190]. Intercalated disks were also seen between adjacent cells. Cells contained several mitochondria in the proximity of sarcomeres and numerous polyribosomes. Immuno-EM showed troponin I immunoreactivity in the myofibrils. Thus, differentiated cells share characteristics similar to those of functional CMs regardless of the variation in the differentiation efficiency of human ESC and iPSC lines and the differentiation method [194]. Ki-67 (proliferating cell marker) immunostaining indicated that some of the differentiated CMs were still active in the cell cycle and immature [190]. Immature CMs could be beneficial for future cell therapy experiments as they might survive better, divide, and be influenced by the ectopic environment of the recipient heart [198]. In other studies, BrdU labeling also demonstrated that some of the hESC-CMs possess proliferative capacity [192, 199, 200].

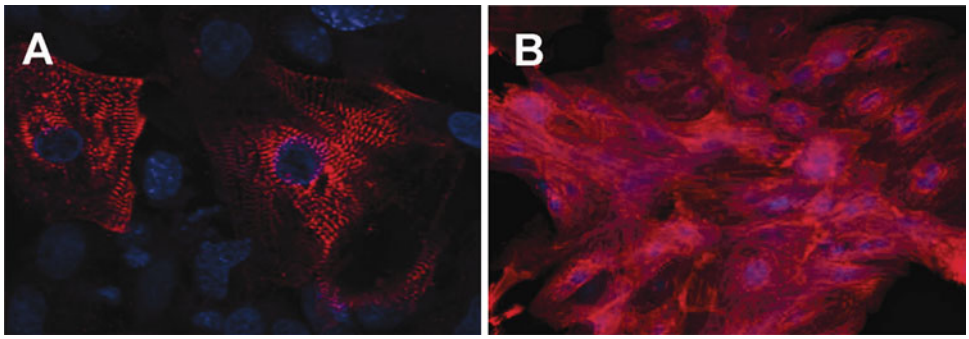

Fig. 4 Immunocytochemical analysis of cardiomyocytes derived from human (**a**) and murine (**b**) iPSCs. Cardiomyocytes were stained with alpha-actinin antibodies to show their typical sarcomeric cross striations.

Electrophysiological Properties

Ma et al. performed a detailed analysis of human iPSC-CMs by measuring current in seven ionic channels. These cells reproduced the electrophysiological properties, such as voltage dependence of channel gating underlying their APs and response to pharmacological compounds of bona fide human CMs. The seven ionic currents studied include sodium (I(Na)), L-type calcium (I(Ca)), hyperpolarization-activated pacemaker (I(f)), transient outward potassium (I(to)), inward rectifier potassium (I(K1)), and components of delayed rectifier potassium current (I(Kr) and I(Ks), respectively) [170]. Normal cardiac electrophysiological function of PSC-CMs is determined, but not their response to hormones and transmitters. Both hESCs and hiPSCs respond to adrenergic and cholinergic agents. β-adrenergic stimulation by isoproterenol leads to an increase in the spontaneous beating rate, a decrease in AP duration, and an increase in contractility in hiPSC-CMs [195–197]. These cells also show functional excitation–contraction coupling properties resembling those of adult CMs [202]. Based on multielectrode array (MEA) studies, hESC-CMs exhibit prominent Na^+ current at the 20–35 days post-plating [203, 204].

The cardiac AP of human PSC-derived CMs is considered as one of the powerful parameters for determining cardiac safety and efficacy of various drugs [205]. AP analysis of hESC-CMs generated from the hESC line H7 by sequential treatment with activin A and BMP-4 revealed that their electrophysiological phenotype was closer to the embryonic than adult myocytes [150]. The evidence for early developmental stage of PSC-derived CMs is provided by findings that transverse tubules are absent in mouse and human ESC-CMs [206]. Gap junctions contain, in addition to connexin 43, also the connexin 45, which is expressed in early stages of in vivo CMs. PSC-CMs express smooth muscle actin, which is normally found only in embryonic and fetal CMs, and dose–response curves in response to isoprenaline are similar to those of the fetal heart [207].

Inhibition of voltage-gated potassium channels of the hERG type, which mediate the IKr current by using the experimental class III antiarrhythmic drug E4031, induced early afterdepolarizations (EADs) in hESC-CMs [170, 205, 208, 209]. EADs could also be triggered in hESC-CMs by cisapride, a compound that was marketed as a gastrokinetic agent, but was withdrawn from the market because it also blocks hERG [170, 205, 208, 209]. Using voltage patch clamp, Jonsson and coworkers demonstrated that densities (pA/pF) of ion currents I(Kr), I(Ks), I(Na, peak), I(Na, late), and I(Ca, L) in hESC-CM were comparable to adult CM. However, I(f) density was larger and I(K1) not existent or very small in hESC-CM. Thus, hESC-CMs exhibit rather immature electrophysiological phenotype and lack of functional I(K1) channels and aberrant I(Na) channel activation may limit the potential of these cells to be used for testing proarrhythmic drugs other than hERG inhibitors [170, 205, 208, 209]. However, human PSC-derived CMs have been shown to respond to many experimental and therapeutic cardiac drugs in a highly predictable manner, suggesting that these cells may represent a suitable model for assessing the efficacy and toxicity of many existing and novel compounds [207].

The central event regulating cardiac muscle contraction is a rapid, transient elevation of cytosolic Ca^{2+} resulting from Ca^{2+}-induced Ca^{2+} release (CICR). In this process, depolarizing AP induces a small influx of Ca^{2+} through the plasmalemmal voltage-gated calcium channels (CaV1.2), triggering, in turn, much larger cytosolic release of Ca^{2+} from the sarcoplasmic reticulum (SR) into the cytosol through type 2 ryanodine receptors (RYR2). ESC and iPSC-derived CMs were reported to have functional SR as demonstrated by the existence of a functional CICR, spontaneous local Ca^{2+} events, and caffeine-releasable Ca^{2+} stores [210]. The load of RYR-mediated Ca^{2+} stores increases during in vitro maturation of ESC-CMs [211].

Pillekamp et al. introduced the first in vitro cardiac transplantation model where hESC-CMs (from H1 hESC line) were isolated and transplanted into ischemically damaged

ventricular slices of murine hearts [212]. This allowed the study of the functional integration and the isometric force development of mechanically loaded contraction of hESC-CMs using an isometric force transducer. In this model, hESC-derived cardiac clusters integrated in vitro into the matrix of damaged myocardial tissue with features that resembled irreversible ischemic injury in vivo. The hESC-CMs conferred force to the damaged myocardium and showed a positive length-dependent increase in tension. Force developed by cardiac clusters transplanted early after the onset of beating exhibited an immature phenotype. This model is well suited to assess the mechanical properties and functional integration of CMs into host that is suggested for cardiac replacement strategies.

In Vitro Disease Modeling

Tightly regulated temporal and spatial pathways representing electrical activation and subsequent mechanical contraction of CMs are essential for the functioning of a normal heart. Any disturbances may lead to serious life-threatening conditions or sudden death. Inherited cardiac disorders, such as the long QT syndrome, the Brugada syndrome, progressive cardiac conduction defect (Lenegre disease), catecholaminergic polymorphic ventricular tachycardia (CPVT), dilated cardiomyopathy (DCM), and others, are caused by mutations in genes coding for specific ion channels or structural proteins. Some of these diseases are known to have an extremely high degree of genetic heterogeneity. A single clinical phenotype may have different genetic background and a single gene may cause very different phenotypes acting through different pathways. Until recently, genetically engineered mice were the only experimental tools to investigate the molecular mechanisms responsible for human genetic diseases. Several murine models harboring human gene mutations leading to electrical and structural cardiac disorders have been developed, including channelopathies, familial conduction disorders, cardiomyopathies, and other inherited cardiac disorders [213]. However, the serious limitation of these animal models is that they cannot reliably reproduce the human pathology, especially when the genetic basis of the disease is extremely heterogeneous, the penetrance of the mutated gene is incomplete and the species-dependent differences in heart physiology and morphology exist. Another possibility to investigate human-inherited heart diseases is established by in vitro culture of human CMs obtained from patient-specific iPSCs. Such CMs may be also beneficial in drug discovery platforms to identify novel drugs and assess their toxicity. hiPSC models have been established for a number of inherited cardiac diseases encompassing cardiomyopathies and channelopathies, which will be discussed below [96, 176, 214–217].

Cardiomyopathies

In cardiomyopathies, there is insufficient or loss of cardiac muscle function that leads to heart failure. The two most common forms of inherited myopathies are hypertrophic (HCM) and dilated (DCM) cardiomyopathy. Various mutations in key sarcomere- or myofilament-related genes such as MYH7 coding for cardiac β-myosin heavy chain and MYBPC3 coding for myosin-binding protein C are associated with cardiac myopathy [218]. Lack of in vitro cardiac disease models had affected the understanding of mechanism behind the specific gene mutations and their resulting phenotypes.

Recently, patient-specific iPSC lines of LEOPARD syndrome (an acronym formed from its main features: lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth, and deafness) were established to serve as a model for investigating the pathophysiology of hypertrophic cardiomyopathy associated with it and identifying novel therapeutic measures [94, 219]. LEOPARD syndrome iPSC-derived CMs displayed large cell size, higher sarcomeric organization, and a preferential nuclear localization of the transcription factor NFATC4 (nuclear factor of activated T-cells, cytoplasmic 4) when compared to hESCs and wild-type hiPSC-CMs. The NFATC4 is known as an important regulator of cardiac hypertrophy. Active calcineurin dephosphorylates NFAT transcription factors and translocates them into nucleus. LEOPARD syndrome hiPSCs, the disease phenotype, resulted from a mutation in PTPN11 gene, which encodes for protein tyrosine phosphatase Shp2, and a defective Ras-MAPK pathway due to aberrant phosphorylation of several component proteins. Previously, Wu et al. established that Shp2 plays a role in modulation of intracellular signaling pathways that initiate differentiation in both hESCs and mESCs. A small molecular inhibitor of Shp2 or deletion of this gene resulted in impaired ESC differentiation [220].

Sun et al. have generated and characterized patient-specific iPSC for familial DCM [96]. Mutations in sarcomeric, cytoskeletal, mitochondrial, and nuclear membrane proteins along with R173W in sarcomeric cardiac troponin T gene (TNNT2) are the underlying mechanisms in DCM. Decreased Ca^{2+} sensitivity of myofilaments and Ca^{2+} ATPase activity (SERCA2a) decrease or impair force generation by CMs. Exogenous SERCA2 rescued the R173W mutation in DCM-iPSC-CMs. This study showed that abnormalities in Ca^{2+} sensitivity occur at an early stage of development in DCM-iPSC-CMs.

Channelopathies

Inherent, congenital, autosomal dominant cardiac channelopathies are associated with mutations in cardiac ion channels, which impair ion transport across the membrane ultimately resulting in changes in AP dynamics, arrhythmia, and heart failure [218]. They can also be acquired or induced by electrolytes, drugs, and genetic predisposition. Heritable cardiac channelopathies include the long QT syndrome (LQTS), short QT syndrome (SQTS), Brugada syndrome, CPVT, cardiac conduction disease, and sinus node dysfunction [221]. Mutations in the genes encoding potassium (KCNQ1, KCNH2), sodium (SCN5A), and calcium (CACNA1C) channels are the most common cause of the LQT syndrome. Ion channels are essentially transmembrane (TM) proteins, which form a pore in the membrane and control voltage gradients by selective and directional flow of ions. A cycle of depolarizing and repolarizing ion currents passing via these specialized ion channels produces AP in CMs [222]. Mutations in genes encoding any of these channel proteins will lead to the loss of function or loss of cell-to-cell electrical coupling through gap junctions and can evoke life-threatening cardiac arrhythmias, the most common cause of sudden cardiac death.

So far, patient-specific iPSC-CMs could be derived from patients with long QT syndrome [208, 215–217, 223–226], CPVT [227], LEOPARD syndrome [94], and Timothy syndrome [228]. LQTS is the most common electrophysiological disease with prevalence of 1 in every 7,000 people and has about 12 different subtypes [222]. Genetic and drug-induced LQTS are of foremost concern to patients and pharmaceutical companies because of a high risk of sudden cardiac death due to ventricular tachyarrhythmia. LQTS is associated with prolongation of the QT interval as seen on an ECG. Mouse models are not suitable since the resting heartbeat rate is 10 times greater than that of humans. Thus, human models must be used to scaling up high-throughput platforms for screening libraries of small molecules and drug sensitivity studies. The potential candidate drugs obtained are used in either ameliorating or reversing the disease phenotype to elucidate underlying molecular mechanisms in pathological diseases.

An autosomal dominant missense mutation (R190Q) in KCNQ1, a repolarizing potassium channel mediating the delayed rectifier I(Ks) current, resulted in type 1 LQTS (LQTS1). Patient-specific LQTS1-hiPSCs were generated from dermal fibroblasts from members of a family affected by LQTS1 and then induced to differentiate into LQTS1-hiPSC-CMs. The patient-derived cells recapitulated the electrophysiological features of the disorder by exhibiting characteristic APs, a significant reduction in I(Ks) current and altered channel properties [229].

Itzhaki et al. [215] generated disease-specific hiPSC line from a patient with type 2 LQTS (A614V missense mutation in the KCNH2 gene, codes for a potassium channel). Detailed analysis using patch-clamp technique and extracellular multielectrode recording revealed a prolonged AP because of reduced potassium current. These cells also exhibited EAD and triggered arrhythmias. This model evaluated the effects of existing and novel drugs that may either aggravate (e.g., potassium-channel blockers E-4031 and cisapride) or ameliorate the disease phenotype (e.g., calcium-channel blocker nifedipine, potassium ATP-channel opener pinacidil, and late sodium-channel blocker ranolazine) [215].

Yazawa et al. [228] generated and analyzed Timothy syndrome (TS) patient-specific iPSCs from dermal fibroblasts carrying a missense mutation in the L-type calcium channel Ca(V)1.2 leading to LQTS in these patients. Electrophysiological studies revealed that TS-iPSC-CMs possess irregular contractions, excess Ca^{2+} influx, prolonged APs, irregular electrical activity, and abnormal Ca^{2+} transients in ventricular-like cells. Roscovitine was identified as a new compound that increases the voltage-dependent inactivation of Ca(V)1.2 channel and rescues electrical and Ca^{2+} signaling properties of TS-iPSC-CMs.

Novel heterozygous autosomal dominant mutation p. F2483I in the cardiac ryanodine receptor type 2 gene (RYR2) leads to type 1 CPVT (CPVT1), which is a highly lethal form of inherited arrhythmias. It is characterized by stress-induced ventricular arrhythmia frequently followed by sudden cardiac death in young individuals. We have generated iPSCs from a patient with CPVT1 carrying the p.F2483I mutation in RYR2 [227]. Patch-clamp recordings of CPVT1-iPSC-CMs after catecholaminergic stimulation revealed arrhythmias and delayed after-depolarizations (DADs). hiPSCs are suitable in modeling RYR2-related cardiac disorders in vitro. Novak et al. [95] generated hiPSC-CMs from a type 2 CPVT (CPVT2) carrying a missense mutation p.D307H in the cardiac calsequestrin gene CASQ2 that causes an autosomal recessive form of the CPVT. CPVT2-hiPSCs were generated to investigate catecholamine-induced arrhythmias in the CASQ2-mutated cells. Isoproterenol caused DADs, oscillatory arrhythmic prepotentials, after-contractions, and diastolic $[Ca^{2+}]i$ rise in CPVT-hiPSC-CMs. Therefore, these patient-specific mutated iPSC-CMs are suitable for studying electrophysiological mechanisms underlying CPVT.

Davis et al. [230] demonstrated that both mESC- and hiPSC-CMs recapitulated the characteristics of genetic disorder affecting the Na^+ current INa with a combined gain and loss of function mutation in the Na^+ channel gene SCN5A by generating multiple iPSC lines. hiPSCs were generated from a patient with equivalent SCN5A (1795insD/+) mutation while mESCs were generated from mice carrying (1798insD/+) (Scn5a-het) mutation in Scn5a.

In a chemical screen, Peal et al. identified two compounds: flurandrenolide and the novel compound 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB). Both rescued type 2 LQTS in zebra fish model. Both of them shortened ventricular AP duration. A novel patient-derived LQTS-hiPSC model could analyze, establish, and confirm the toxicology profile of above-mentioned compounds [225].

Implications in Drug Discovery

Cardiovascular-targeting drugs and cardiotoxins have shown to have effects on the heart functions that involve changes in the contractility, cardiac rhythm, blood pressure, and ischemia. Such toxic effects have led these drugs to be withdrawn, requiring development of stringent rules on cardiotoxic testing. In vitro cardiotoxic testing applications using hESC-CMs are particularly advantageous and complementary to the existing repeat dose toxicity applications [231]. A balanced, concerted activity of several cardiac ion channels is vital in ventricular repolarization and any alterations may lead to ventricular arrhythmias [232]. Therefore, electrophysiological analysis of the proarrhythmic potential of a new drug is relevant and necessary in cardiotoxicity assays. Capacity for the in vitro development of calcium-handling mechanism, ion channels, and regulatory proteins, which are essential for a mature repolarization phenotype in CMs, is well established [231].

Cardiac toxicities such as ventricular arrhythmias that lead to life-threatening torsades de pointes (TdP) are a main concern during development of novel cardiac therapies [201, 232]. Early and accurate detection of such potential cardiac toxicities could save the pharmaceutical industry millions of dollars at an early stage. Cell-based screening with phenotypic endpoints has been successful [233, 234]. hESC-CMs represent a novel platform for electrophysiological drug screening and in vitro toxicity testing as the cells respond to a number of cardiac [208] and noncardiac drugs and the process is scalable using high-throughput technologies [232, 235]. Cardiotoxicity is analyzed in vitro using metabolic activity, membrane leakage, and energy content and intracellular calcium concentration. The evaluation of QT interval liability and the torsadogenic potential of novel chemical entities involve the analysis of ionic currents from engineered cell lines that express hERG, prolongation of the action potential from isolated, arterially perfused rabbit ventricular wedge preparation or canine Purkinje fibers, and rates of arrhythmia in animals [231]. Cardiotoxicity testing in vivo involves parameters such as blood pressure, heart rate, electrocardiogram (ECG), repolarization, conductance abnormalities, cardiac output, ventricular contractility, and vascular resistance.

hESCs and hiPSCs show unrestricted proliferation and, therefore, serve as an unlimited source for CMs. Stem cell-derived models will eventually reduce, replace, or refine current models of cardiotoxicity. For many toxicology applications, PSCs offer a homogeneously defined population. An in vitro toxicology platform for the hESC-CMs has been developed because of the lack of availability of human primary tissue material for cardiotoxic testing and their ability to overcome species variability [123].

Matsa et al. derived hiPSCs from skin fibroblasts of patients with a KCNH2 (IKr potassium ion channel) G1681A mutation, which were subsequently differentiated into functional CMs. Relative to controls, MEA and patch-clamp electrophysiology of LQTS2–hiPSC-CMs showed prolonged field/AP duration [208]. When LQT2–hiPSC-CMs were exposed to E4031 (an IKr blocker), arrhythmias developed and these presented as EADs in the APs. Isoprenaline treatment of LQT2–hiPSC-CMs also resulted in EADs and β-blockers propranolol and nadolol reversed this effect. Treatment of CMs with experimental potassium channel enhancers, nicorandil and PD118057, caused AP shortening and abolished EADs. Findings from this study demonstrate that patient LQT2–hiPSC-CMs respond appropriately to clinically relevant pharmacology and will be a valuable and novel human in vitro cardiac drug evaluation system.

Applications in Cardiac Repair

Müller-Ehmsen et al. [236] investigated the retention capacity of transplanted fetal CMs into the myocardium and found out that ~ 80 % of them disappeared between day 1 and 4 weeks after injection. Such loss or washout is probably caused by lack of adhesive ability of CMs toward ECM. This idea is supported by our findings that purified murine ESC-derived CMs were not capable of attaching quantitatively to any of 16 different two-dimensional biomaterials and did not morphologically or functionally integrate into collagen sponges, and dissociated cardiomyocytes did not reaggregate in hanging drops in the absence of collagen matrix or supportive MEFs or cardiac fibroblasts [237]. Laflamme et al. [150] used a proapoptotic cocktail to improve survival of transplanted cells and Kolossov et al. [238] have co-transplanted CMs with MEFs to improve survivability. Hattori et al. have reaggregated purified CMs into small balls of cells and these have resisted washing out and anoikis [239]. This strategy has improved the survivability and functionality of CMs by 90 % up to 8 weeks of posttransplantation into the myocardium of the immunodeficient mice. Paracrine factors are thought to be responsible for the improvement of function. Growth factors like FGF, EGF, PDGF-BB, and endothelin-1 stimulate the proliferation of connective tissue, smooth muscle cells, mesenchymal, glial and epithelial cells. These CM clusters exhibited growth and elongation after transplantation and reformed into intramyocardial sheet-like structures [164, 239].

One crucial clinical application of hESC-CMs is to repair heart damage in myocardial infarction by grafting (cell therapy). When uninjured hearts of immunocompromised nonprimates received transplants of hESC-CMs, the human myocardium exhibited grafting [138, 240, 241]. The ability of hESC-CMs to proliferate and form graft shows that if transplanted in subtherapeutic dose, they may be sufficient to reach a functionally meaningful cardiac implant over time. As reviewed in [20, 242, 243], transplantation of mESC-CMs improved contractile function of the infracted mouse heart. Laflamme et al. [150] have shown that, once grafted, partial remuscularization and attenuation of heart failure occur and it appears to normalize electrical conduction all through the infarct, manifested by reduced ventricular dilation, and improved global function (fractional shortening and ejection fraction). Most hESC-CMs grafted into infracted heart died, but the discovery of pro-survival cocktail helped the grafted CMs to survive [150]. Kehat et al. generated beating CM tissue from hESCs and showed that it integrates structurally, electrically, and mechanically with surrounding myocytes [244].

Nelson et al. showed that intramyocardial delivery of undifferentiated iPSCs into the infarcted hearts of mice following ligation of the left anterior descending artery yielded progeny that properly engrafted without disrupting cytoarchitecture [245]. In another proof-of-concept study, Mauritz et al. showed that injection of murine iPSC-derived Flk-1^{+} progenitor cells into the ischemic myocardium of mice improved cardiac function after MI and these Flk-1 positive cells differentiated into cardiovascular lineages (cardiac tissue) in vitro and in vivo [177]. Hence, iPSC-CMs are an autologous source as they can form a part of the myocardium and revascularize it.

Nearly three million people, now, harbor a man-made implanted pacemaker for cardiac rhythm disturbances worldwide and that number is predicted to increase in the future [246]. Liabilities like shorter battery life, discomfort, and lack of intrinsic responses to neural and hormonal regulations have led to recent interest in "biological pacemaker" via transplantation of CMs with pacemaking abilities [240]. Biological pacing concept is very close to being an alternative to the electronic pacemakers [247].

As a proof of principle, Kehat et al. [240], Xue et al. [248], and Potpova et al. [246] transplanted hESC-CMs, which survived, integrated, and functioned in vivo as they paced the ventricle in the swine model with complete atrioventricular block. iPSC-derived biological pacer can integrate with the host myocardial tissue and generate the electrical impulses. Ventricles of pigs with complete MI took implants of CMs derived from hESCs and the results [249] indicated that transplanted hESC-CMs successfully coupled with the host myocardium and were able to transiently pace the pig heart in an ectopic manner.

Challenges for Clinical Applications

Many challenges have slowed down the translation of stem cell models to human clinical trials.

Safety

Crucial safety concern using ESCs or iPSCs is their potential to form tumors upon transplantation. If the cells are injected directly into the organ, then undifferentiated hESCs or hiPSCs residing within differentiated cell population can produce teratomas at the host's transplanted site. Systemic administration of cells may result in development of disseminated teratomas in different organs [250]. This could be overcome by improving purification protocols that isolate homogenous populations of common progenitors.

Undifferentiated hESCs or hiPSCs should be destroyed, separated, or removed from their differentiated cell populations [250]. To ensure that no undifferentiated hESCs or hiPSCs are transplanted together with the hESC- or hiPSC-derived tissue to prevent teratoma formation, reliable methods are needed to eliminate contaminating undifferentiated hESCs or hiPSCs and to develop sensitive assays to detect such contamination in hESC- or hiPSC-differentiated tissues prior to transplantation [32, 250]. Encapsulation of hESCs and mESCs with membranes of 2.2 % barium alginate prevented the formation of teratomas up to 4 weeks and 3 months, respectively. The mESCs formed aggregates within the alginate capsules, but not the hESCs [251].

Transplantation therapy requires pure cells to eliminate the risk of teratoma formation. Phenotype-based purification approaches such as beating of CMs, reporter gene expression, and density-gradient centrifugation have enriched the purity by only five- to tenfold, but they are not scalable [2, 150]. In addition, selective pluripotent apoptotic agents like the ceramide analogues (sphingosine fatty acid family) are harmless potent selective apoptosis-inducing agents. Bieberich et al. [252] showed that a portion of proliferating Oct-4 positive ESCs in EB-derived cells could be eliminated by apoptosis-inducing ceramide or its analogues. The most widely used approach for isolation of pure CMs from ESCs includes genetic modification of ESCs to express a drug resistance and/or fluorescent marker protein under the control of a cardiac-specific promoter [187, 238, 253–257]. This strategy allows for isolation of CMs at purities around 99 % by either drug selection or fluorescence-activated cell sorting (FACS), and this method is highly scalable to bioreactors. However, the main drawback here is the need for insertion of an antibiotic selection cassette into the parental genome. Recently, protocols employing magnetic or fluorescent cell sorting based on expression of the cell surface

marker ALCAM on CMs [258] or their high mitochondrial activity [239] have also been described. The enrichment of CMs using FACS using mitochondria-specific florescent dyes resulted in >99 % purity in murine and human ESC differentiations. Although these strategies have the advantage of requiring no genetic modification of ESCs for enrichment of CMs, they suffer from limited specificity of ALCAM expression on CMs and FACS purification is not high-throughput and scalable enough. In addition, cells with less number of mitochondria such as immature hESC-CMs are difficult to be distinguished from other types of cells [259]. Dubois et al. identified the signal-regulatory protein alpha (SIRPA; CD172) as another cell surface marker, which enables isolation and purification of CMs from PSC cultures by FACS or MACS [259]. Alternatively, depletion of SIRPA-negative non-myocytes using their specific cell surface molecules using FACS can enrich SIRPA-positive cells. Uosaki et al. identified vascular cell adhesion molecule 1 (VCAM1) as a potent cell surface marker for robust, efficient, and scalable purification of CMs from hESCs/hiPSCs [260].

Yield

Yield of many desired cell types from undifferentiated PSCs is still very variable and inefficient, and factors affecting lineage specification are not sufficiently known. Considering that approximately 108–109 cells will be required for treatment of each patient [261], efficient protocols for reproducible differentiation of PSCs toward functionally intact CM at sufficient, clinically useful quantities are needed. Directed differentiation using BMP4 and activin A or BMP-2 and bFGF inhibitor can promote differentiation of hESCs into CMs. Upregulation of the Wnt3a signaling pathway resulted in expansion of the multipotent Isl11 progenitor cells. More robust cardiopoietic extrinsic factors and surface markers of specific cardiac progenitors are needed for purification, expansion, and differentiation.

Only one report on differentiation of human ESCs toward CM in large scale in fully controlled stirred suspension bioreactor has been reported to date [262]. This study demonstrated the importance of initial EB size and physicochemical parameters (oxygen tension) for successful CM differentiation. However, the cell yield was still low, and the differentiation was performed in serum-containing medium, which necessitates further adaptations of this protocol to GMP-compatible conditions. Steiner and coworkers showed that human ESC clusters propagated in suspension could be easily differentiated into neural spheres in suspension [263]. Small molecules will be crucial in developing defined media for large-scale production of CMs from human PSCs. A number of such molecules have been identified (e.g., cardiogenol, ascorbic acid, prostaglandin I2, p38 mitogen-activated protein kinase inhibitor SB203580, and cyclosporine A), but their broad applicability for clinically relevant cells in bioreactor systems has not yet been tested.

Current techniques for expansion of human PSCs in the undifferentiated state most commonly rely on adherent growth of PSC colonies on human feeders [264, 265], autologous feeders [266], and poorly defined and xenogeneic substrates (e.g., MEFs, Matrigel) [20, 267], and require mechanical dissociation for passaging, but can also be adapted to robotics [268]. These methods may expose human PSCs to xenogeneic factors and are labor intensive, inefficient, irreproducible, and thus not amenable to GMP-compatible industrial production of cells for preclinical and clinical studies. Culturing and expanding the human PSCs in suspension can circumvent these problems. These 3-dimensional culture systems included simple suspension [263, 269, 270] and microcarrier-based methods [271, 272] in combination with bioreactors [273]. These studies established the grounds for further development of culture technologies that are suitable for clinical application of human PSCs. The discovery of small molecules that increase the survival of human PSCs after dissociation into single cells, such as Rho-associated kinase inhibitor (ROCKi) Y-27632 and Thiazovivin, and the formulation of chemically defined culture media (e.g., mTeSR1 and E8 medium) [274] play a crucial role in establishment of these culture conditions.

Although these reports firmly established that human PSCs can be maintained as suspension aggregates in the undifferentiated state for a number of passages, the adaptation of these systems to fully controlled stirred suspension bioreactors has not yet been achieved. Furthermore, the maintenance of pluripotency over prolonged passages, cell viability, and yield are still not satisfactory. Whether current suspension cultures of PSCs promote any epigenetic or genetic changes that may affect the characteristics of PSCs and their therapeutic derivatives has also not been established. In addition, the variability in ability of different PSC lines to expand and maintain pluripotency in certain suspension cultures has been observed, emphasizing the need for assessment of the broad applicability of each reported method [273]. Therefore, conditions that allow mass expansion of PSC in suspension and their large-scale differentiation in controlled bioreactors require further development.

Immunological Rejection

Immunorejection of stem cell-derived cell populations in allogeneic settings is a major challenge. Immunosuppression by drugs becomes mandatory because any mismatch between the donor and the recipient cells would result in the rejection of graft. By establishing global repositories of large numbers of diverse human leukocyte antigen (HLA)-typed ESC lines for closest tissue match for a specific patient or by

reprogramming stem cells or derivatives to an embryonic state via patient-specific hiPSCs, one can overcome immunorejection. Since hESCs do not belong to the patient, immunosuppression is preferred. In case of patient-specific iPSC-CMs, immunosuppression may not be required [250, 275]. However, recent studies revealed that even iPSCs, and their derivatives, may be immunogenic and rejected from syngeneic hosts [276] presumably due to abnormal gene expression in some cells differentiated from iPSCs, which can induce T-cell-dependent immune response in syngeneic recipients. Therefore, the immunogenicity of autologous iPSCs should be evaluated before their clinic application.

Human iPSCs offer an ideal autologous source of cells for generation of desired therapeutic cell types for each individual patient. However, the feasibility of developing and applying autologous iPSCs in clinical practice will be greatly limited because of the complex, time-consuming, and costly nature of iPSC generation and the regulatory process required for approval of each individual cell line and derived products. Therefore, heterologous HLA-matched hiPSC lines may secure broad accessibility of iPSC derivatives to patients that would otherwise not be able to afford the individualized approach. In allogeneic transplantation, immunological rejection is due to mismatches in histocompatibility antigens, and the degree of immunological response from allogeneic transplants correlates with the extent of matching of HLA genes. Even a partial match in HLA types may result in beneficial HLA matching, thus reducing the dose requirement for immunosuppressive drugs. HLA genes are codominantly expressed, and the ones deemed the most important for matching are HLA-A, HLA-B, and HLA-DRB1. Derivation of iPSC lines from donors with homozygous HLA loci at the three main A, B, and DR sites, and their collection in the so-called HaploBank, thus represents a promising but yet to be validated strategy to make available clinically approved cellular products to the majority of a patient population on demand, and at affordable costs, while limiting the risk of immunological rejection.

Engraftment

Once sufficient amounts of transplantable cells can be produced at high purity, these cells must be reconstituted into injured or diseased tissues and organs with the purpose to engraft quantitatively, mechanically, and functionally for long periods. As mentioned above, the survival of murine and human ESC derivatives is very poor even if injected into healthy organ of a genetically identical recipient. In recent report, transplanted human ESC-derived CMs could be detected in only 18 % of infarcted rat hearts [150]. This observation illustrates the importance of developing strategies to minimize cell loss after transplantation. Engraftment of delivered cells could be increased by preconditioning the cells prior to transplantation to become more resistant to stress, by minimizing inflammation at the site of injection, and by co-injecting the cells with pro-survival factors or supportive noncardiac cell types to inhibit cell death and facilitate cell integration in the tissue [150]. In addition, it will be crucial to select the most appropriate method of cell delivery into diseased organ to avoid mechanical cell loss immediately after injection.

Excessive fibrogenesis by hESC-CMs is a major issue in engraftment [277–279]. The host can prevent integration of the graft by triggering electrophysiological uncoupling and increasing the risk of arrhythmias. A difference in electrophysiological properties and beating rate between grafted hESC-CMs and the myocardium of the host results in functional disintegration. Scar formation around the graft and graft healing could be promoted with the use of angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor blocker (angiotensin II promotes cardiac fibrosis) [277–279]. Expression of connexin 4 (Cx4) has been identified between the graft and the rat myocardium, suggesting the integration of remaining hESC-CMs that could survive into the rat heart [277–279].

Conclusions

Many studies [116, 144, 145, 163, 185] have revealed the overall similarity of gene expression and proteome profiles between undifferentiated ESCs and iPSCs and their derivatives such as CMs, while only a few studies have reported subtle differences in DNA methylation, RNA levels, and differentiation potential. Also, similarity of hESC- and hiPSC-CMs at the protein level remains to be explored in greater detail. Posttranscriptional and posttranslational modifications of proteins tend to control expression of cell phenotypes. This analysis needs to be extrapolated to studying differentiated CMs from both hESCs and iPSCs. Careful and systemic analyses of differences at RNA, miRNA, protein, phosphoprotein, and DNA methylation profiles will deepen the understanding of the molecular and functional properties of PSC-derived CMs and their suitability for experimental and therapeutic applications.

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Potential of Glial Cells

Magdalena Götz and Swetlana Sirko

Abbreviations

Aldh1L1	Aldehyde dehydrogenase 1 family, member L1
AP2γ	Activating enhancer binding protein 2 gamma
BBB	Blood–brain barrier
BBB	Blood–brain barrier
bHLH	Basic-helix–loop–helix
BLBP	Brain lipid binding protein
BMP	Bone morphogenic protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycan
DSD-1-PG	Dermatan sulfate-dependent proteoglycan1
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Emx2	Empty spiracles homeobox 2
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate aspartate transporter
GM	Grey matter
GS	Glutamine synthetase
Gsx1,2	Genomic screened homeobox 1, 2
Hes1,5	Hairy and enhancer of split 1, 5
HSPG	Heparan sulfate proteoglycan
LIF	Leukemia inhibitory factor
NPC	Neural progenitor cell
NSC	Neural stem cell
OB	Olfactory bulb
OPC	Oligodendrocyte progenitor cell
Olig1,2	Oligodendrocyte lineage transcription factor 1, 2
oSVZ	Outer subventricular zone
Pax6,7	Paired Box 6, 7
PDGFα	Platelet-derived growth factor alpha
PEDF	Pigment epithelium-derived growth factor
PLp	Proteolipid protein
RFP	Red fluorescent protein
RPTPβ	Receptor protein tyrosine phosphatase beta
SGZ	Subgranular zone
SHH	Sonic hedgehog
SVZ	Subventricular zone
Tbr2	T-box brain protein 2
TGFα	Transforming growth factor alpha
TNC	Tenascin-C
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
WM	White matter

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Introduction

Glial Cells of the Central Nervous System

Glial cells develop at the same time as neurons forming neural networks accumulate, and their number increases significantly during phylogeny [1–3]. In the early nineteenth century, Camillo Golgi identified glial cells as a distinct morphological cell type within brain tissue, and Rudolf Virchow was the first to describe glia as the connective tissue of the brain and called

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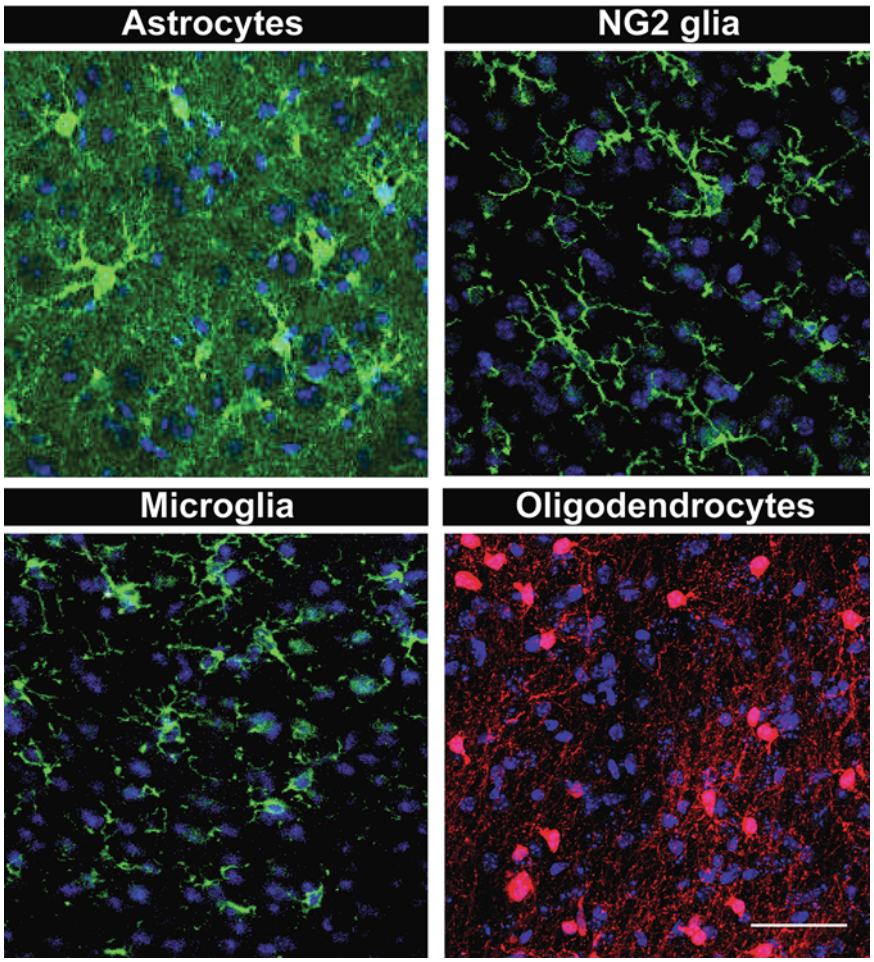

Fig. 1 Diversity of glial cell types in the adult mammalian brain. Glial cells of neuroectodermal origin, summarized as macroglial cells, were characterized as astrocytes (protoplasmic astrocytes are visualized in the cerebral cortex of the *Tg*Aldh1L1-GFP transgenic mice, expressing a green fluorescent protein (GFP) under the astrocyte-specific promoter of the gene encoding the aldehyde dehydrogenase 1 family, member L1 (Aldh1l1)), NG2 glia (immunostained for the carbohydrate-epitopes NG2 in the cerebral cortex), and oligodendrocytes (visualized in the cerebral cortex of the *Tg*Plp-DsRed transgenic mice, expressing a red fluorescent protein under the control of the proteolipid protein (Plp) promoter). Microglial cells are the major phagocytotic population in the adult brain and are derived from the mesoderm (immunostained in the cerebral cortex for the ionized calcium-binding adaptor molecule 1 (Iba1) that is specifically expressed in macrophages/microglia). All cell nuclei were counterstained with bisbenzimide and are shown in *blue*. Scale bar: 50 μm

them the "nervenkitt" or "neuroglia" [4]. Although the name survived, the original concept of cells filling the space between neurons was changed significantly with the identification of various glial subtypes (Fig. 1) and their functional specification and diversification. Microglia were identified as mesodermally derived cells of the macrophage lineage that enter the brain at early developing stages and remain there as resident microglia comprising between 2 and 5 % of all glial cells in various brain regions [5]. These tiny cells are the major phagocytic cells that provide the first line of defense for the CNS, acting as scavengers of cell debris, following either natural cell death or injury (for review, see [5, 6]).

In the years to come, several glial cell types of neuroectodermal origin, summarized as macroglial cells, were characterized as astrocytes, NG2 glia, oligodendrocytes, and ependymal cells with each of these cell types performing highly specific functions [7]. Astrocytes are among the most abundant glial cells in the adult mammalian CNS and possess many fine processes enwrapping synapses as well as contacting the basement membrane surrounding blood vessels and capillaries. Thereby, astrocytes form the link between synaptic transmission and blood flow, acting as connective units between capillaries and neurons. Astrocytes also control the level of ions in the extracellular space and have major roles in the function of the blood–brain barrier (BBB), which protects the CNS from unwanted substances in the general circulation [8]. In addition, astrocytes are an intricate part of synaptic transmission as part of the tripartite synapse consisting of the pre- and postsynaptic terminals and the astrocyte processes which remove and recycle neurotransmitters following release from nerve terminals and influence synaptic transmission in multiple ways (for review, see [9]).

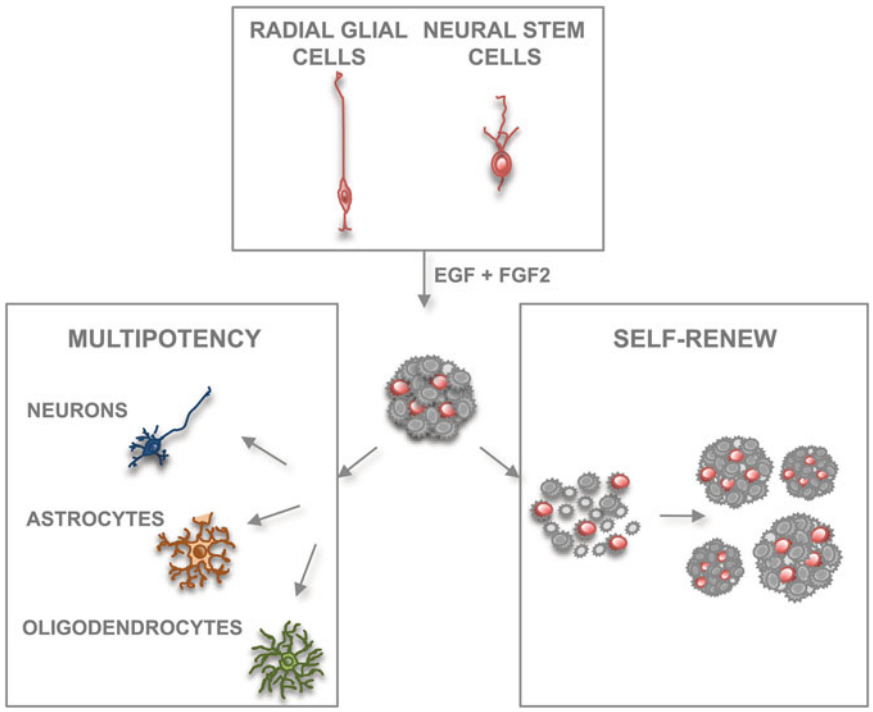

Fig. 2 The neurosphere assay—"the gold standard" to evaluate the cells with neural stem cell properties. Schematic outline of the neurosphere assay that examines two fundamental characteristics of neural stem cells: self-renewal (passage number) and multipotency (differentiation into multiple lineages). Single radial glial cells as well as the adult neural stem cells form free-floating clusters (small spheres, i.e., single cells had proliferated to generate a cell cluster) when removed from its normal environment in vivo and plated in a serum-free culture containing growth factors, such as the epidermal growth factor and the fibroblast growth factor 2 (EGF+FGF2, respectively). Some cells obtained from the dissociated primary neurospheres could be passaged for many times in a culture medium (self-renew), containing growth factors that promote proliferation. An individual neurosphere differentiates into neurons, astrocytes, and oligodendrocytes (multipotency), when exposed to differentiation conditions in a culture medium that promote cell differentiation

Oligodendrocytes, the other type of macroglial cells, form the myelin sheath, a highly specialized cellular structure consisting of tightly packed lipid bilayers thereby insulating axons and enhancing nerve fiber conduction velocity for rapid transmission of electrical signals in the brain as well as supporting axons by various intercellular signals (for review, see [2, 10]). Oligodendrocytes originate from NG2 glia, the progenitors of the oligodendroglial lineage that all express the transcription factors Olig1 and 2 and the proteoglycan NG2, and increase in number during vertebrate phylogeny [11]. This highly branched population of macroglial cells is distributed throughout the grey matter (GM) and white matter (WM) of the adult brain parenchyma, where they establish contacts to neurons, but also act as life-long oligodendrocyte progenitor cells (OPCs). Interestingly, they comprise the only progenitor population in the adult mammalian brain outside the neurogenic niches, and constantly generate new myelinating oligodendrocytes [12–14]. Finally, ependymal cells are the specialized glial cells lining the brain ventricle in vertebrates, possessing multiple motile cilia which may contribute to the local flow of the cerebrospinal fluid (CSF) and sometimes bearing long radial processes resembling radial glial cells from the developing brain [15].

Thus, all these glial cell types, of which further subtypes are constantly discovered, are highly specialized and perform distinct roles way beyond being a mere "glue" in the nervous system.

Glia as Neural Stem Cells: The Neurosphere Assay

In addition to the above described functions, glial cells also act as progenitors and even neural stem cells (NSCs) during development and in the neurogenic niches of the adult brain [16–19, 21–24]. For both, radial glia in the developing brain and adult NSCs, in vitro assays were first used to identify their surprising neurogenic lineage and even multipotency [18, 25]. Surprisingly, when two groups dissociated cells from the adult rodent forebrain and grew these cells in medium containing growth factors, such as the epidermal growth factor or the fibroblast growth factor 2, they observed the formation of small spheres, i.e., single cells had proliferated to generate a cell cluster (Fig. 2) [25, 26]. Most importantly, these cells exhibited the stem cell hallmarks of self-renewal and multipotency as they could be passaged many times and differentiated into neurons, astrocytes, and oligodendroglia when exposed to differentiation conditions [22, 25–27]. This was the discovery of cells with the potential to generate neurons even in the adult mammalian

brain. The neurosphere-forming assay has now become "the gold standard" to evaluate the potential of a cell to behave as a stem cell when removed from its normal environment as it assays for self-renewal (passage number) and multipotency (differentiation into multiple lineages) (for recent review and technical concerns, see [27]). Notably, various cell sources with this NSC potential have by now been identified in the adult brain parenchyma, including NG2 glia and astrocytes, as well as ependymal cells lining the ventricle [24, 28–31].

While this in vitro assay probes for potential, the source of these cells with NSC properties and their lineage in vivo became then the focus of attention. First, the hippocampus was identified as region of adult neurogenesis in the mammalian brain [32], then Doetsch and colleagues identified cells from the adult subependymal zone as source of neurosphere formation with astroglia/radial glia-like cells at the base of an adult neurogenic lineage in this region in rodents [16, 17, 32, 33]. Malatesta and colleagues soon after identified radial glial cells from the developing brain as a population comprising neuronal progenitors as well as NSCs generating neurons and glia [18], an observation confirmed immediately thereafter by live imaging in slice preparations in vitro [34, 35]. Finally, Seri and colleagues unraveled the radial glia nature of NSCs in the hippocampal dentate gyrus [36, 37]. Thus, while radial glial cells, a type of embryonic astroglia, are transient and largely disappear from the parenchyma of the mammalian brain at postnatal stages, a limited number of radial glial cells remain present in the adult neurogenic niches where they are the source of neurogenesis.

These exciting findings prompted great hopes for using NSCs from the adult brain for neural repair strategies (for review, see [24, 38]). However, most neurosphere-derived cells, or even neuroblasts isolated from the adult neurogenic niches convert to gliogenesis when transplanted outside the neurogenic niches [39–41]. Likewise, neuroblasts recruited from the sites of neurogenesis towards injury often succumb to cell death [38], highlighting the gliogenic environment throughout the adult brain parenchyma. Conversely, these data suggest that cells with a broader potential for neurogenesis might be hidden in the adult brain parenchyma, but fail to give rise to neurons due to their adverse environment. Indeed, such cells were discovered from various glial sources and will be subject of discussion below [24].

Astroglia as Stem Cells in the CNS of Vertebrates

Radial Glia as Stem and Progenitor Cells During CNS Development

The CNS forms from neuroepithelium lining the fluid-filled ventricle of the neural tube. This densely packed cell layer is initially composed of proliferative neuroepithelial cells, which maintain apico-basal polarity by contacting both the ventricular lumen and the pial surface with their radially extending cell processes. Neuroepithelial cells are coupled at the apical side by adherence junctions containing cadherins (first the epithelial, E-cadherin, later the neural N-cadherin) [42–46]. The decrease in the junctional proteins E-cadherin and occludin at the onset of neurogenesis (from embryonic day 8–9 in mouse embryos) is part of the developmental transition from self-renewing neuroepithelial to radial glial cells [44, 47, 48]. This transition constitutes a critical event in regulating the balance between brain growth and differentiation of the neural cell types. One of the key factors regulating this transition are the basic-helix–loop–helix (bHLH) transcription factors Hes1 and Hes5, both essential for the maintenance of radial glial cells, indicating significant differences between neuroepithelial and radial glial cells [49]. Notably, also at later developmental stages, Hes1-regulated activity of the Notch pathway plays a critical role in maintaining radial glial cells in an undifferentiated state in the developing mouse brain [50–52].

But why are radial glial cells referred to as glial cells? This is because they possess many hallmarks of astroglial cells, including gap junction-mediated intercellular communication, contact with blood vessels [53–57], and expression of Vimentin [58], astrocyte-specific glutamate transporter (GLAST), brain lipid-binding protein (BLBP) [18, 59–61], glutamine synthetase (GS) [62], tenascin-C (TNC) [63], and the receptor protein tyrosine phosphatase β long (RPTPβ long) [64]. They may also contain cytoplasmic glycogen granules [65] and glial fibrillary acidic protein (GFAP), an intermediate filament protein that is expressed in early radial glial cells of some species [66, 67]. During the later stages of rodent brain development (around E15 in mice or E17 in rat), most radial glial cells start to replace Vimentin expression with GFAP, consistent with radial glia having astrocyte-like properties [68, 69]. All these astroglia-specific molecular and ultrastructural criteria together serve to classify radial glial cells as the earliest type of astroglial cells, and the lack of these characteristics in neuroepithelial cells demonstrates the distinct nature of radial glia and neuroepithelial cells.

However, radial glial cells also still share some aspects with the former neuroepithelial cells [48]. For example, both cell populations share the expression of the intermediate filament protein Nestin and its posttranslationally modified isoform RC2 [61, 67, 70–73], as well as morphological features such as a radially oriented position between the ventricular zone (VZ) and the basement membrane at the pial surface [74–76]. To acknowledge both the radial morphology and glial nature of these cells, Pasco Rakic in the early 1970s coined the term "radial glia" of the previously termed "epithelial cells" or "spongioblasts" [77].

Both populations also are highly proliferative and exhibit interkinetic nuclear migration with the nucleus moving along the longitudinal cellular axis during the various phases of the

cell cycle. But, in contrast to neuroepithelial cells, the nuclear migration in radial glial cells no longer encompasses the entire length of the cytoplasm, and is restricted to the region lining the ventricle, the VZ [48, 78–80]. Interestingly, radial glial cells never withdraw their radial processes retaining a long radial process even throughout the cell cycle, which also enables them to guide simultaneously migrating neuroblasts [35, 81, 82].

Cell division of radial glial cells takes place at the apical surface, yet the mode of division and, hence, the nature of resulting progenitors depends on various signals, timing, and intrinsic fate determinants. Prospective isolation of radial glial cells by fluorescence-activated cell sorting (FACS) first demonstrated a notable heterogeneity among radial glial cells isolated from the developing mouse cerebral cortices at mid-neurogenesis [18]. While most radial glial cells generated only neurons, others generated only glia including further radial glia, and yet others generated both neurons and glial cells [18, 48, 83]. These differences in lineage and potential are defined by molecular determinants specifying the intrinsic responsiveness of dividing radial glial cells, the stage of the development and the local environment [83–90]. Indeed, the lineage of radial glial cells changes in the course of development towards more and more glial progeny, until neurogenesis is over at the time of birth in mice, a lineage that is accompanied by profound changes in the genome-wide transcriptome of these cells [83, 91]. Interestingly, radial glia biased to gliogenesis or neurogenesis can also be prospectively isolated by FACS at earlier stages, implying their coexistence at mid-neurogenesis [83, 92] and allowing to unravel candidate genes regulating this behavior by transcriptome analysis [83]. Moreover, distinct subsets of radial glial cells can be isolated in regard to generating neurons directly versus generating an intermediate set of progenitors, the Tbr2-positive basal progenitors [83]. Indeed, molecular regulators of these distinct lineages have emerged from this transcriptome analysis and proven to be highly functionally relevant, such as the transcription factor AP2γ [83, 92].

In addition, radial glial cells differ profoundly in different brain regions [91, 93]. For instance, radial glial cells in distinct CNS regions express different transcription factors involved in patterning of the CNS, such as Pax6 and Emx2 in the dorsal telencephalon which are both essential factors for specifying the features of the cerebral cortex [94], whereas they express Gsx1 and Gsx2 in the ventral telencephalon, key regulators of this region [93]. Interestingly, in this region radial glial cells also express retinoic acid binding proteins, while radial glial cells in the ventral telencephalon and ventral midbrain, in the floor plate region, express and secrete the key morphogen sonic hedgehog (SHH) [85, 93–97]. The regional differences between radial glial cells in the spinal cord and those in the telencephalon are given not only by differential expression of particular transcription factors (e.g., Pax7), but also in the fate of their progeny that are restricted to the glial lineage, while the neuroepithelial cells are the major neuronal progenitors in the developing spinal cord [60, 98–101]. Heterogeneity of radial glial cells further extends to heterogeneity in expression of RC2, GLAST, and BLBP, with, e.g., one-third expressing all these three proteins and a smaller subset expressing only some of them at mid-neurogenesis in the developing mouse telencephalon [61]. In addition, radial glial cells differ in regard to their mode of cell division (symmetric self-renewing or asymmetric) [35, 82, 102]. During neurogenesis, most radial glial cells undergo asymmetric divisions, generating one self-renewing radial glial cell and either a young neuron or an intermediate progenitor cell [34, 35, 48, 82, 102].

Interestingly, during corticogenesis of the more evolved cerebral cortices, such as ferrets or primates, a further type of radial glia-like cells, the outer or basal radial glia, resides at some distance from the ventricle in the outer subventricular zone (oSVZ) [103–106]. These cells have morphological similarity to radial glia cells and contact the pial surface by a basal process, but they lack apical processes and, hence, do not contact the ventricular surface. Contact to the basement membrane appears critical for proliferative capacity of these cells [104], which seem to contribute to neurogenesis [103], as well as to gliogenesis [105, 107]. Their amplification provides additional radial glial processes to guide migrating neurons, whose number is increased in brains undergoing gyrification [107]. Thus, understanding radial glial cell function and diversity is critical to understand brain ontogeny and evolution.

In most mammals, neurogenesis comes to an end in most brain regions at late embryonic or early postnatal stages. Radial glial cells disappear by various means at this stage. Either they are depleted by symmetric differentiative divisions generating two postmitotic neurons [82] or largely convert to gliogenesis, a change reflected by down-regulation of Nestin, but maintenance of GLAST and BLBP at the end of the neurogenic period [18]. While some radial glial cells differentiate at the ventricular site into ependymal cells, others delaminate from this surface as glial progenitors generating either oligodendrocyte progenitors or astrocyte progenitors, which then distribute throughout the brain parenchyma by migration and proliferation [11, 82, 108–117]. Modification of the ECM together with expression changes in intrinsic fate determinants, such as up-regulation of Olig2, and cell surface receptors, as well as in growth factor responsiveness have been proposed as multiple molecular events orchestrating the timing of cell fate and the later onset of gliogenesis [83, 118, 119]. Of particular relevance in regard to the transition of radial glial cells into astrocytes are the signaling pathways mediated by Notch-, ErbB2-, and FGF signaling, all of which have been implicated in the maintenance of radial glial cells, and are proposed to be particularly high in gliogenic radial glial subtype [87, 110, 112, 120, 121].

Thus, at the end of neurogenesis, the VZ vanishes while the subventricular zone (SVZ) enlarges. This region exists only in some forebrain areas during development, where it contains intermediate basal progenitors enlarging the number of neurons generated (for review, see [122]). At the end of neurogenesis, however, it becomes apparent throughout the CNS and comprises largely glial progenitors. The VZ conversely diminishes in size and becomes largely composed of multiciliated ependymal cells, generated from radial glial cells during development [123–127].

Interestingly, in the CNS of nonmammalian vertebrates, such as amphibians and fish, radial glia are maintained into adulthood and continue to line the ventricle [128–132]. This population of radial glial cells in zebrafish retains access to the ventricle their entire lifetimes and act as progenitors for adult neurogenesis also in amphibians, reptiles, and birds [129–134]. In contrast, in the mammalian CNS, the telencephalic radial glial cells are transient, and disappear or transform as described above with some also directly transforming into astrocytes [74, 125, 135–139]. The generation of all neuronal and major glial cells, i.e., astroglial, oligodendroglial and ependymal cells from radial glial cells underlines that radial glial cells may be considered less a specialized glial cell type and be considered more as precursors, or even stem cells as they are self-renewing and some are also multipotent. While only a small subset of them persists throughout the neonatal CNS, these then act as the source of the NSCs in the adult brain.

Adult Neural Stem Cells: A Unique Population of Astroglial Cells in the Neurogenic Niches of the Adult Mammalian Brain

While the adult NSCs resemble radial glia [31] and remain anchored at the ventricular surface of the lateral wall of the lateral ventricle, their progeny of fast proliferating transit-amplifying progenitors and neuroblasts form a densely populated SVZ [23, 31]. Also in the dentate gyrus, the other brain region where adult neurogenesis has been suggested for a long time in rodents [140] and more recently confirmed in many species [141–143], it is a radial glia-like or radial astrocyte-like cell that is at the source of adult neurogenesis [20, 36, 37]. In both regions, these cells have close contact with blood vessels via their endfeets [21, 124, 150–154] and have been identified as astroglia based on expression of GFAP, GLAST, calcium-binding protein S100β, glutamine synthetase, DSD-1-PG, TNC, and ultrastructural hallmarks (for review, see [16, 17, 20, 24, 33, 64, 144–149]). Moreover, the similarity between adult NSCs from the SVZ and glial cells has been confirmed by genome-wide expression analysis showing more similarities of NSCs with mature astrocytes and ependymal cells, rather than radial glia from the embryonic brain [23]. This is particularly surprising as adult NSCs share several aspects also with radial glial cells, such as expression of GLAST, Nestin, or Sox2 (for review, see [23, 83, 91]) and could be isolated by FACS using the same characteristics used for isolation of radial glial cells from the embryonic brain described above [83], namely the expression of GFAP and the membrane protein prominin1 at the apical membrane domain [23, 155]. The coexpreesion of GFAP and prominin1 is particularly important for separation of NSCs from other astrocytes present in the adult SVZ ("niche astrocytes") and the surrounding brain parenchyma.

Beyond these common anatomical and molecular features, NSCs are involved in homeostasis and gliotransmission, and display typical glial properties, such as a passive current profile, lack of action potentials, hyperpolarized resting potentials, gap junction coupling, expression of connexin 43 and hemichannels important for the propagation of Ca^{2+} waves in radial glia and astrocytes (for review, see [156–160]). The expression of the K^+ inward rectifying channels Kir2.1 and Kir4.1 in GFAP- and GLAST-positive NSCs are both important for the regulation of extracellular K^+-concentration. These hallmarks, together with the expression of transmitter receptors, such as $GABA_A$, but the lack of AMPA-type glutamate receptors led to the concept that GFAP/GLAST-positive cells in SVZ display a unique set of the cell biological and functional characteristics intermediate between astrocytes and radial glia [159, 161]. GFAP and GLAST co-expressing astroglial cells, partially embedded in the ependymal layer and possessing a radial glial-like morphology as well as a primary cilium projecting into the lumen of the lateral ventricle, are in fact the source of adult neurogenesis, and are able to self-renew in vivo and give rise to multipotent neurospheres in vitro [19–23]. In vivo, NSCs proliferate and give rise to neurons throughout life, whereby neurons born in the SVZ migrate a long distance through the rostral migratory stream to the olfactory bulb (OB), and enable the turnover of the olfactory neurons, while neurons born locally in the SGZ migrate a short distance to integrate into the dentate gyrus (for review, see [162, 163]).

In addition to their function as primary precursors of new neurons, astrocyte-like NSCs by themselves and together with supporting cells within the niche, also participate in the creation of the specialized microenvironment that stimulates neurogenesis. In this regard genome-wide expression analysis of genes specifically enriched in the SVZ NSCs revealed the dominant importance of genes involved in intercellular signaling, such as cilia-mediated signaling and Ca-signaling [23]. This is consistent with several signaling pathways influencing adult NSC proliferation which require cilia to mediate these signals, such as SHH, which also influences the progeny of NSCs such as transit-amplifying progenitors and neuroblasts and regulates patterning [164–168] or the platelet-derived growth factor alpha (PDGFα) [169, 170].

In addition, fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGFα), and pigment epithelium-derived growth factor (PEDF) all have been shown to regulate proliferation of SVZ NSCs [171–181]. Especially the role of EGF has led to the concept of activated and quiescent NSCs in the SVZ, suggesting that the activated NSCs express the EGF receptor and are responsive to EGF, while quiescent NSCs do not express it [182, 183]. In addition EGF mediates other functions such as proliferation and migration of neural progenitor cells (NPCs) in the SVZ [184]. The functional cell–cell interplay between transit amplifying NPCs and NSCs through a non-cell-autonomous mechanism involving EGFR-mediated regulation of Notch signaling has an essential role in maintaining the balance between these cell populations in the SVZ, which is critical to supply the brain with specific neural populations [52].

Bone morphogenic protein (BMP) signaling plays a key role for the progression of neurogenesis in the adult CNS. BMP signaling acts at endogenous levels as a proneurogenic stimulus within the SVZ [185] while, at the same time, it is important for NSC self-renewal in the SGZ [186]. The effects of BMP are age-dependent. In embryonic stem cells, BMPs act in combination with LIF to suppress differentiation and maintain self-renewal [187]. In addition to the signaling pathways described above, and in similarity to the nature of parenchymal astrocytes outside the neurogenic niches, NSCs (and other cells in the niche) contribute to the structure of the adult niche. They provide a source of ECM containing collagen IV, laminins, perlecan, and in some cases chondroitin sulfate and heparan sulfate proteoglycans (CSPGs and HSPGs, respectively). The latter two likely serve as important signals to the stem cells, including the presentation of FGF or EGF to receptors, and hence they promote either cell proliferation or cell fate determination, as has been shown for embryonic radial glial cells [118, 119, 188, 189].

Also soluble carbohydrate-binding protein lectins, such as Galectin 1 and 3, are involved in the molecular mechanisms that regulate behavior of the adult NSCs. Galectin 1 is expressed in a subset of slowly dividing adult NSCs in the SVZ and promotes the proliferation of these cells by the interaction with Integrin β1, thereby plays an important role in regulating the number of adult NPCs through mechanisms including cell adhesion [190–192]. Moreover, Galectin 3 is highly and specifically enriched in NSCs of the SVZ [23], suggesting that these two Galectins are a prominent hallmark of NSCs in this region. As such, NSCs provide a niche for their progeny, such as neuroblasts, which are affected in their migration in Galectin knock-out mice [193].

Since NSCs are also in contact with the cavity of the lateral ventricle via their apical primary cilia, a further potential source of regulatory signals in the SVZ is the CSF. Although the CSF has been shown to maintain survival, proliferation, and differentiation of neuroepithelial cells in vitro, the role of CSF in the regulation of adult NSC behavior in vivo is largely unknown [194, 195]. The flow of the CSF, generated by multiciliated ependymal cells in the adult CNS, is itself critical for the establishment of a molecular gradient, such as Slit, which is an important coordinator of directional migration of neuroblasts in the adult SVZ (for review, see [196, 197]).

Taken together, accumulating knowledge regarding the orchestration of astrocytic-like NSCs behavior already reflects the combinatorial nature of signaling in the regulation of their maintenance. Thus, cells classically considered to be of astroglial lineage, appear to serve as NSCs, and their function as primary precursors for new neurons is closely related to that of embryonic radial glia.

Reactive Astrocytes: A Novel Source of Cells with Stem Cell Potential within the Injured CNS

While the glial nature of NSCs in the developing and adult brain has been a challenge for the traditional view of glia as differentiated cell types, the recent discovery that apparently mature glial cells seemingly dedifferentiate into cells with stem cell hallmarks after brain injury is even more challenging the traditional view of glial cells. As the major cell type of macroglia, astrocytes occupy almost all regions of the CNS and are essential for the proper functioning of the CNS by maintaining the extracellular milieu in regard to ion homeostasis, clearance of extracellular glutamate, water transport, secretion of pro- or anti-inflammatory cytokines and chemokines, production of growth factors, glucose and other energy metabolites, as well as release and scavenging of free radicals [198–202]. Also in response to pathological changes astrocytes react with a wide spectrum of morphological and functional changes [148, 203]. This reaction of astroglia is, in general, referred to as reactive gliosis that is characterized by a phenotypic change in macroglial and microglia cells that occur in response to all forms of CNS injury or disease ranging from amyloid plaque deposition to inflammation or invasive damage. The basic process of reactive gliosis appears similar in vertebrates reminiscent of the wound healing response in other organs. In contrast to other organs, the access of hematogenous cells, immune cells, or other extrinsic cells as well as large immunoproteins is restricted by the BBB in the healthy brain or noninvasive injuries [204–207]. Therefore, the astrocytes have to take on some tasks performed otherwise by connective tissue cells and these features have been acquired during the evolution and during the maturation of the CNS (for review, see [206, 208]).

In the last 150 years much work has been done to elucidate the functions of reactive astrocytes, and both harmful and beneficial activities have been attributed to these cells. The initial anatomical description of reactive astrogliosis, called "fibrous gliosis," was performed by Rudolf Virchow in the nineteenth century, (according to Bignami and Dahl [209]), and it was Ramon y Cajal, who first proposed an abortive effect of "fibrous gliosis" on the growing axons. Following a series of studies by aid of the classical impregnation techniques, the major breakthrough came with the discovery of GFAP as "the marker" of astroglia. A general acceptance in the investigations of the glial reaction was gained with the immunohistochemical labeling for GFAP, revealing that "fibrous gliosis" was accompanied by the accumulation of this intermediate filament protein. This feature quickly became the standard marker for reactive astrocytes, providing a hallmark of the reactive gliosis and a highly sensitive, even though nonspecific indicator for brain injury [209].

It then became more and more clear that the glial reaction including the astrocyte reaction differs profoundly in different pathological conditions in regard to alterations in gene expression, morphological reorganization, and in some cases, proliferation [14, 148, 200, 210–215]. A key aspect of reactive astrogliosis, in regard to scar formation and sealing an invasive injury site, as well as in regard to a potential dedifferentiation, is their increase at the injury site, which has been suggested to be a result of proliferation and/or oriented migration towards the damaged tissue [14, 148, 211, 212, 216–218]. Given that astrocytes in uninjured adult CNS rarely divide outside the adult stem cell niches [14], the pathophysiological conditions seemingly trigger proliferation in previously quiescent or postmitotic astrocytes. Indeed, genetic fate mapping combined with viral vector injections demonstrated that the proliferating reactive astrocytes are not derived from the NSC niche of the SVZ or SGZ, but rather originate in the local grey matter of the cerebral cortex [24, 211]. Interestingly, the proliferative reaction of astrocytes occurs with a delay (5–7 days after the insult) compared to microglia starting to proliferate within hours after an insult or the NG2 glia that increase proliferative activity within 1–3 days [14, 211, 212, 219].

Notably, there is a profound difference between NG2 glia that constantly proliferate, albeit slowly, in the adult forebrain parenchyma, while astrocytes do not do so [12, 14]. Therefore reactive astrocytes enter the cell cycle de novo upon injury. In addition they reexpress proteins present in radial glia at earlier stages of development or in adult NSCs, but absent in mature astrocytes in the adult brain, such as Nestin, BLBP, Vimentin, DSD-1-proteoglycan, and TNC, as well as both lectins, Galectin 1 and 3 (Fig. 3) [14, 17, 33, 36, 37, 211, 212, 215, 218, 220–224]. This prompted the analysis of their stem cell potential by dissociating the tissue surrounding an invasive injury site and culturing the cells in neurosphere conditions as described above. Indeed, in this assay a limited fraction of reactive astrocytes shows long-term self-renewal and multipotency, suggesting that pathophysiological stimuli may trigger dedifferentiation of some mature astrocytes into NSCs [148, 211, 212, 218, 225–228, 235]. Interestingly, this potential to form self-renewing and multipotent neurospheres occurs in a time-dependent manner progression closely correlating to the proliferative reaction of astrocytes described above. For instance in response to acute injury (such as stab wound and focal laser lesion) or ischemia, the ability of reactive astrocytes to form neurospheres strongly increases during early post-injury stages (3–5 days after injury), but rapidly declines thereafter, such that neurospheres can be no longer observed as 14 days after injury [148, 211, 212, 228, 235]. Most importantly, genetic fate mapping using GLAST::CreER^{T2} mice revealed that neurosphere-forming cells originate from grey matter astrocytes both after stab wound injury and in amyloidosis conditions [211, 217, 235]. These cells exhibit stem cell hallmarks as they self-renew for many passages [211, 212] and approximately half of them are multipotent, since they are able to generate some neurons as well as astrocytes and oligodendrocytes in vitro [235].

While these data are very exciting in regard to a local origin of cells with NSC potential, it remains to be determined how this potential can be fully activated in vivo. While cultured reactive astrocytes derived from the injured nervous system fit the operational definition of NSCs—multipotency and the ability to self-renew—to which extent they are similar or differ from NSCs of the endogenous stem cell niches, the SVZ and SGZ, remains to be determined. Genome-wide expression analysis will certainly be a major step forward, and the recent analysis of adult NSCs [23], astrocytes from the normal brain parenchyma [229] and reactive astrocytes [215] paves the way towards a thorough understanding of the similarities and differences of these cells. Together with increasing knowledge on the molecular mechanisms mediating neurogenesis from adult NSCs and radial glia in the embryo [230–233], it has become more and more feasible to activate the neurogenic potential of reactive glia also in vivo [212, 217, 225, 234]. Thus, research on glia cells and NSCs is merging to unravel the key hallmarks of the wound reaction after injury and separate the beneficial from adverse function with one of the beneficial roles being a rejuvenated set of glia, which provide local sources for novel repair strategies.

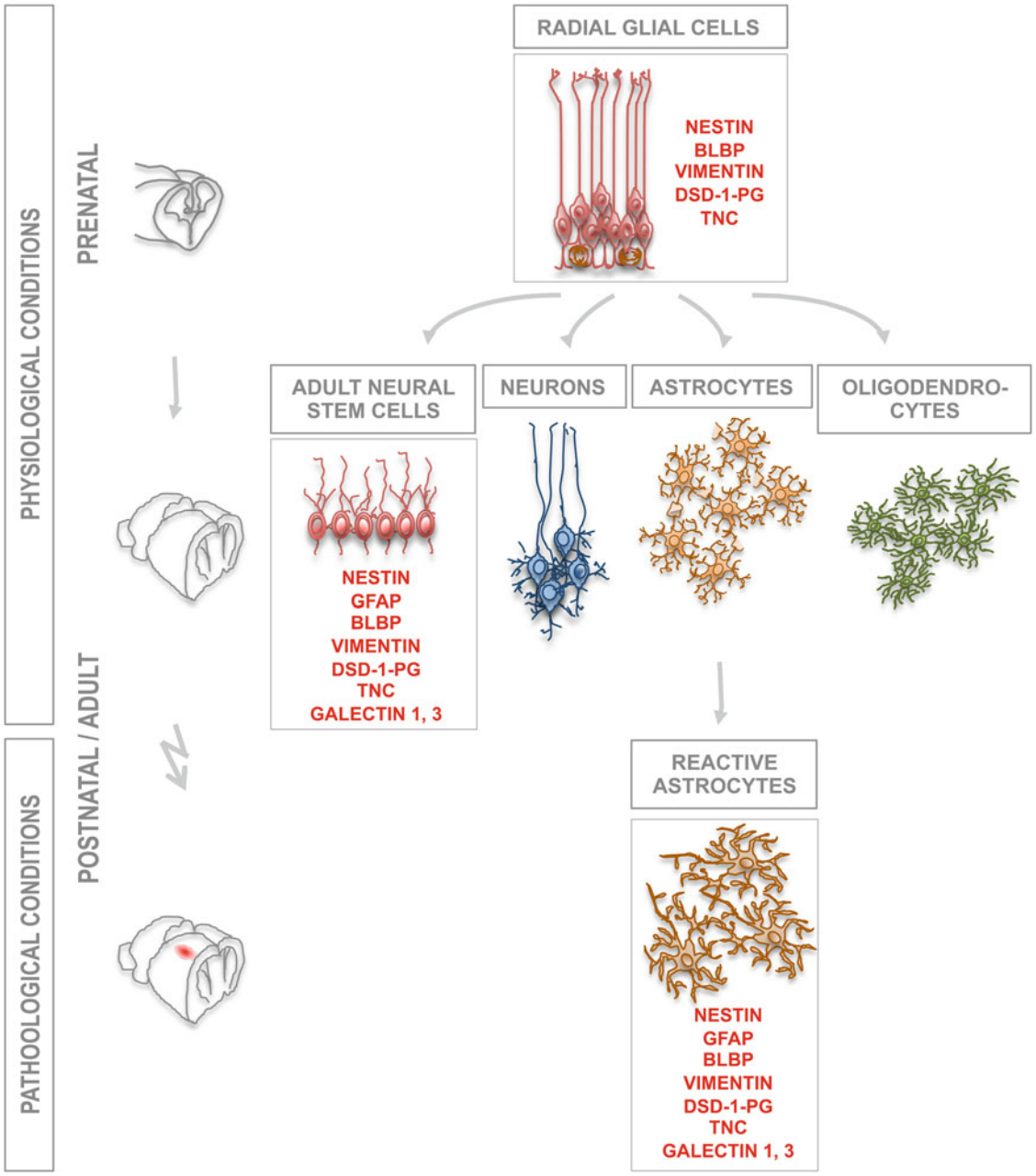

Fig. 3 Progression in development of the cell heterogeneity within neural tissue and the reaction of parenchymal astrocytes to brain injury. A schematic summary outlining the progression in development of the cell heterogeneity within neural tissue and highlights the reaction of parenchymal astrocytes to injury with focus on the molecular characteristics they share with radial glial cells and adult neural stem cells (NSCs), but absent in mature astrocytes in the adult healthy brain. During prenatal development, radial glial cells, which have cell bodies in the ventricular zone and radial fibers that reach the outer surface undergo cell division multiple times and gives rise to neurons, astrocytes, and oligodendrocytes. As development proceeds, some of radial glial cells persist into the adult brain and act as adult NSCs in the adult SVZ and hippocampal SGZ, where they proliferate to produce both neurons and glia. As consequence of brain injury, some mature parenchymal astrocytes become hypertrophic and upregulate the expression of intermediate filaments, including GFAP, nestin, vimentin as well as brain lipid-binding protein (BLBP), Galectin 1 and 3, proteoglycans, such as the DSD-1-proteoglycan and tenascin C (TNC). The pathophysiological conditions seemingly trigger changes in gene expression in mature parenchymal astrocytes indicative of dedifferentiation to a developmental, radial glia-like or adult NSCs state

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Stem Cells and Aging

Heidi Scrable and Susan Ashrafzadeh-Kian

Introduction

The Stem Cell Theory of Aging

With age, the ability to maintain organ homeostasis and cellular regenerative capacity diminishes. The stem cell theory of aging posits that this decline in homeostasis occurs when stem cells can no longer maintain their functionality, as defined by two characteristics, self-renewal and potency. Self renewal refers to the ability of a stem cell to divide asymmetrically such that one daughter cell is an exact copy that retains stemness, while the other daughter becomes a progenitor cell that gives rise to rapidly dividing precursors, which will eventually differentiate and perform specific functions [1]. Potency refers to the ability of a stem cell to give rise to a range of differentiated cell types. According to the stem cell theory of aging, the functional depletion of the adult stem cell pool by death, injury, senescence, cell cycle arrest, or differentiation results in an inability to regenerate old or injured tissues, leading to the organ dysfunction commonly observed in aged individuals.

The functionality of the adult stem cell pool depends upon conserved molecular pathways, which ensure the optimal balance between self-renewal, quiescence, and differentiation. In this chapter, we focus on molecular events that converge on a final common path through the tumor suppressor p53 and its downstream target, the cell cycle inhibitor p21Cip1/Waf1 (p21). Both intrinsic, nuclear defects such as unrepaired DNA damage or laminopathies that disturb the integrity of the nuclear membrane and chromatin, and extrinsic defects in the stem cell milieu that are transmitted to the stem cell by receptor-mediated signaling cascades, can activate p53 and turn on p21 transcription in stem cells. The increased load of p21 inhibits the regenerative potential of stem cells, limiting cell turnover, maintenance of tissue function, and, ultimately, life span.

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p21 and Stem Cells: The Goldilocks Effect

As the story goes, Goldilocks found the papa bear's porridge too hot and the mamma bear's porridge too cold, but the baby bear's porridge was just right. Eating the porridge that was just right sustained and rejuvenated her. Similarly, stem cells require just the right amount of p21 for long-term regenerative capacity, as illustrated in Fig. 1. In the absence of p21, stem cells fail to maintain quiescence, resulting in hyperproliferation and expansion of the progenitor cell population, followed by exhaustion of the stem cell pool. We discuss the essential role of p21 in maintaining stem cell quiescence in the next section. When stem cells accumulate excess p21, on the other hand, they can enter a state of prolonged or permanent quiescence, resulting in hypo-proliferation of stem and progenitor cells and exhaustion of differentiated progeny by attrition. We explore the consequences of having too much p21, a far more likely situation to arise during the normal course of aging, in a later section. Together, these examples illustrate how either too little or too much p21 can impair stem cell regenerative capacity and limit healthy life span.

Cell Cycle Arrest and the Maintenance of Stem Cell Quiescence by p21

Over the course of a lifetime, stem cell quiescence is critically important for the maintenance of tissue homeostasis and to prevent premature exhaustion of the stem cell pool under various conditions of stress [2]. p21, a member of the CIP/KIP

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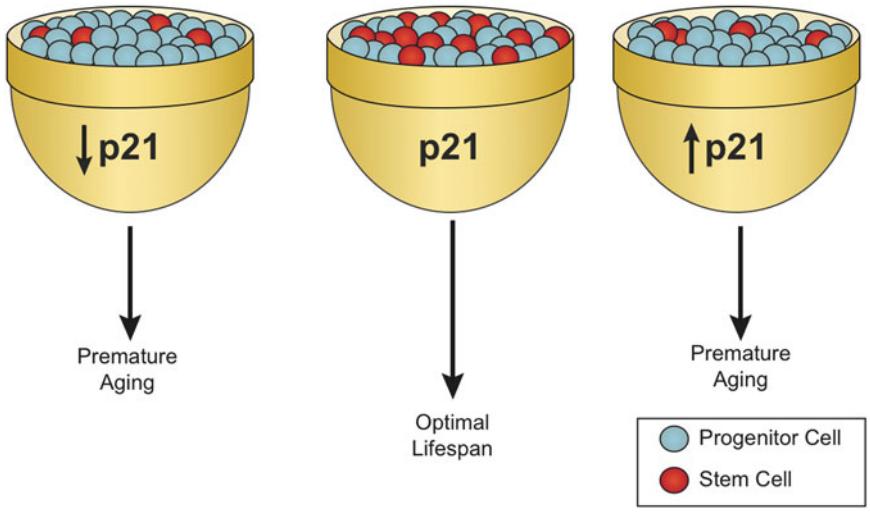

Fig. 1 p21 and the Goldilocks effect. Too much p21 leads to senescence and impaired proliferation, while too little p21 leads to the loss of quiescence and increased apoptosis. Both decrease stem cell regenerative capacity and compromise optimal life span

family of cyclin-dependent kinase inhibitors, appears to play a significant role in the protection of the stem cell pool by ensuring cell cycle arrest during quiescence. In small amounts, p21 acts as a positive cell cycle controller in that it is actually necessary for formation of the cyclinD/CDK4 complex [3, 4] and transit from G0 into G1. When expressed in amounts beyond what is needed for complex formation, however, it has a universally inhibitory role by blocking the activity of cyclin-dependent kinases (CDKs) necessary for cell cycle progression [5–8]. It does this in several ways (reviewed in [9]), as illustrated in Fig. 2. p21 prevents CDKs from working with their respective cyclin to phosphorylate the Rb protein, thus blocking the release of the E2F transcription factor and subsequent transcription of the E2F responsive genes needed for transit from G0 into G1 and from G1 into S phase [10] (Fig. 2a, b, and d). p21 can also function as a cofactor with other DNA binding proteins, such as transcription factors, to control the expression of genes essential for cell cycle progression. For example, p21 can interact with E2F directly and block transcriptional activation of the cyclin A promotor [11] (Fig. 2c). p21 can also bind the mismatch repair factor DNA polδ, inactivating PCNA-mediated DNA replication [12] (Fig. 2e). In addition, p21 indirectly affects transit from G2 into M by binding to and inhibiting CDK-activating kinase (CAK), which phosphorylates CDK1 on Thr161 and activates the CDK1-cyclin B complex (Fig. 2f). Inhibition of CAK is crucial for G2/M checkpoint activation [13].

Cell cycle control by p21 appears to play a particularly important role in stem cells. Studies on the hematopoietic stem cells (HSCs) and neural stem cells (NSCs) of p21-deficient mice provide convincing evidence of the protective role of p21 in the preservation of stem cell pools [14]. In the absence of p21, for example, the profile of hematopoietic cells in the adult mouse is maintained despite decreased cytokine-mediated proliferation of bone marrow progenitor cells [15–18], suggesting that p21 might play a dual role, simultaneously increasing progenitor cell proliferation while preventing stem cell proliferation. In a test of this hypothesis, Cheng et al. compared the time spent in G0 (quiescence) and G1 (cycling) phases of the cell cycle in stem cells from p21-deficient and wild-type mice. They found that p21-deficient HSCs spent less time in G0 and exhibited reduced repopulation capacity following 5-fluorouracil (5-FU) depletion of cycling bone marrow cells, as determined by cobblestone-forming assays (CAFC). Importantly, serial transplantation of p21-deficient bone marrow cells into lethally irradiated recipients resulted in greatly reduced survival compared to that conferred by normal bone marrow cells. Rather than remaining quiescent, p21-deficient stem cells prematurely differentiated, exhausting the pool of self-renewing stem cells and demonstrating the important role of p21 in maintaining HSC quiescence during times of stress.

Under steady state conditions, HSC quiescence appears to be maintained not by p21 but by growth factors, such as angiopoietin or thrombopoietin, which act through AKT or JAK/STAT pathways [19–21]. However, in NSCs, p21 appears to maintain quiescence under both steady state and stressed conditions. There are more NSCs in p21-deficient mice between postnatal days 60–240 than in their wild-type counterparts, and this is due to higher proliferation rates. At 16 months of age, however, NSC numbers drop in p21-deficient mice and display limited self-renewal in vitro, surviving only several passages before exhaustion [22]. This study highlights the contribution of p21 to the relative quiescence of adult NSCs, which might be more important than

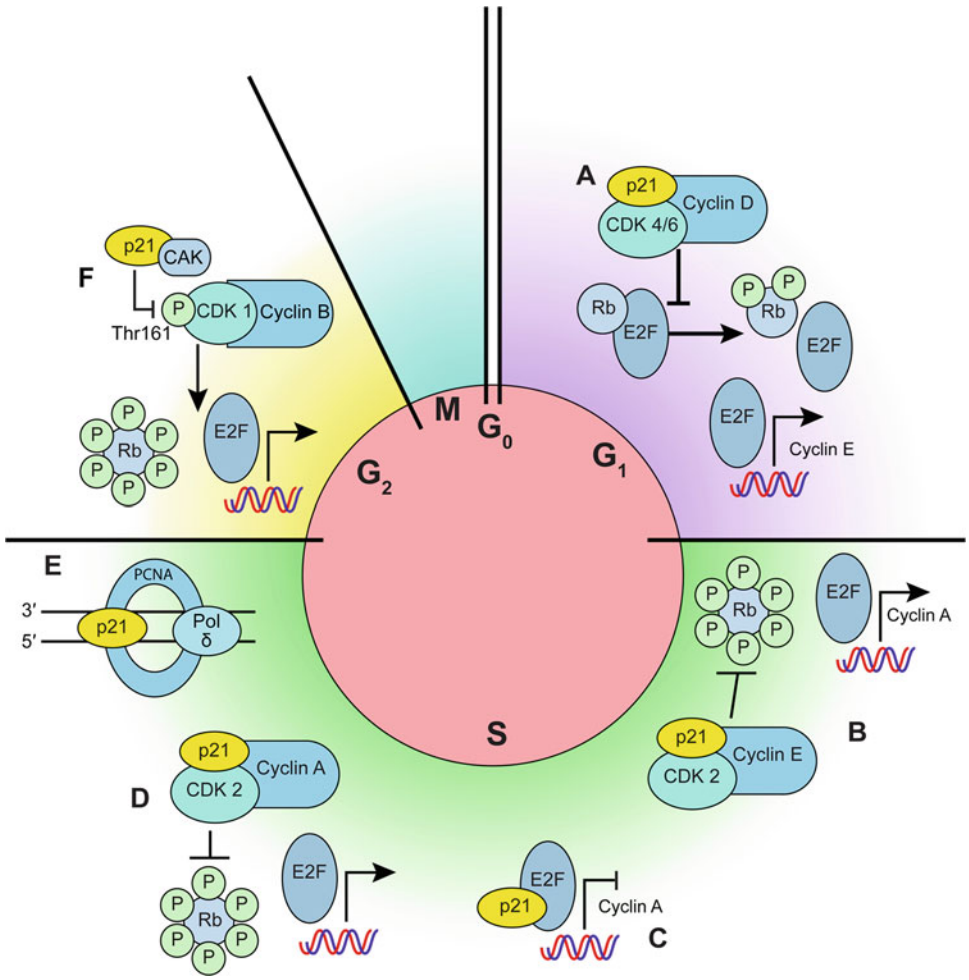

Fig. 2 p21 and the cell cycle. (**a**) p21 is required for complex formation of Cyclin D/CDK 4/6. This complex participates in the partial phosphorylation of Rb, reducing its binding affinity with E2F and allowing transcription of genes such as Cyclin E. p21 in amounts greater than the minimum for complex formation has an inhibitory effect on the process. (**b**) p21 inhibits the Cyclin E/CDK2 complex from completing the phosphorylation of Rb. This allows transcription of the Cyclin A gene and the G1/S transition. (**c**) p21 associates with E2F, directly inhibiting the transcription of Cyclin A. (**d**) p21 inhibits Cyclin A/CDK2 complex, preventing transcription of E2F responsive genes. (**e**) p21 inhibits PCNA, blocking polδ from replicating damaged DNA. (**f**) p21 inhibits phosphorylation and activation of CDK1 by CAK. As a result, transcription of E2F responsive genes is inhibited, blocking the G2/M transition

indefinite proliferation capacity for the life-long maintenance of NSC self-renewal.

By triggering cell cycle arrest and limiting transmission of damaged DNA, p21 can also protect against cell death [111], repressing caspases and other proteins needed for apoptosis [13] [23, 24]. In the case of cancer, this ability of p21 to repress apoptosis can maintain stem cells pools, with obvious deleterious consequences. In leukemogenesis, for example, p21 is actually critical for maintaining the leukemic stem cell pool [24]. A molecular model has been proposed in which the *CDKN1A* promoter is positively regulated by the tumor suppressors Miz and p53, which promote p21 expression and suppress apoptosis, and negatively regulated by the oncogene Myc (reviewed in [25]). When Myc is activated, it interacts with and suppresses Miz, blocking p53-mediated transactivation of *CDKN1A* and inducing apoptosis. Point mutations in Myc that render it unable to bind to Miz inhibit the induction of apoptosis in human fibroblasts [26]. When p21 was reexpressed in Myc-transformed cells, apoptosis was inhibited. When Zbtb4, a suppressor of Miz, was depleted in cell culture by siRNA, activation of p53 by vincristine promoted cell cycle arrest over apoptosis [27].

In summary, stem cells lacking p21 fail to maintain quiescence, resulting in expansion of the progenitor cell population and exhaustion of the stem cell pool. *Cdkn1a*-deficient mice exhibit deficits in HSC and NSC quiescence, leading ultimately to tissue deterioration and loss of function. Several tumor suppressors and oncogenes coordinately regulate the activity of the *CDKN1A* promoter, with potentially deleterious

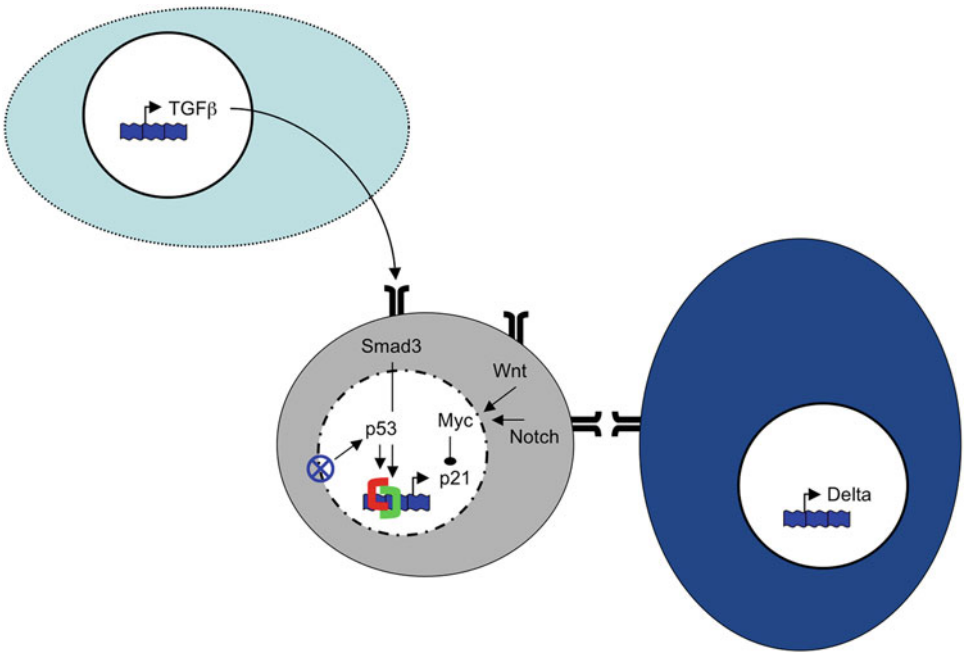

Fig. 3 Molecular mechanisms of p21 activation in aging stem cells. Cell intrinsic defects, such as the loss of nuclear architecture, which jeopardize the integrity of the genome or epigenome, activate p53, the principal means by which p21 is induced in stem cells. Surface receptors transduce extrinsic defects that affect the niche, such as a rise in systemic TGFb, to modulate critical intracellular pathways governing p21 expression. Both Wnt and Notch act in part through Myc, a transrepressor of the *CDKN1A* promoter. ⊗, nuclear (intrinsic) defects. T, receptor-mediated environmental defects ("niche")

effects on the ability of the organism to limit the regenerative capacity of tumor stem cells. In the next section, we explore the consequences on stem cell regenerative capacity of having too much p21 and how that can limit healthy life span.

Chronic Activation of p21 and Aging

Events that can result in excess p21 in stem cells fall into two broad classes, nuclear damage and damage to receptor-activated signaling pathways. With age, DNA mutations and epimutations can accumulate, the result of defective repair processes or a compromised nuclear membrane, for example, and cause chronic activation of the p53–p21 axis. Experimental hyperactivation of p53 in mice gives rise to a progeroid syndrome closely resembling normal aging at an accelerated rate. Pharmacologic inhibition of downstream effects of activated p53 returns p21 levels to normal and reverses senescence in fibroblasts derived from these mice. Mouse models of Hutchinson-Gilford progeria, a human progeroid syndrome, which are driven by defects in nuclear lamins, can be rescued by eliminating p53, which simultaneously normalizes p21 levels in fibroblasts and prevents senescence.

Age can also damage the microenvironment resulting in altered intracellular signaling pathways. For example, fluctuating levels of TGFb in the systemic circulation, coupled with defective Notch mobilization in the muscle stem cell, can upset the balance between activation and suppression of p21 and compromise muscle repair. The various effects of age that can chronically activate p21 in stem cells or their environment are represented in Fig. 3 and discussed in more detail in the following sections.

Nuclear Defects and the p53: p21 Pathway in Stem Cells

Nuclear damage, including single- and double-stranded breaks, telomere shortening, chromosome rearrangements, excessive mitogenic signals from oncogenes, and damage by reactive oxygen species [28–32], can trigger activation of the tumor suppressor p53 and induce p21 expression, with powerful consequences on cell proliferation [33]. The p53–p21 pathway is one of the two pathways that can induce cellular senescence (the other is p16–Rb), a cell culture phenomenon first characterized by Hayflick and colleagues, who demonstrated that normal cells had a finite capacity to proliferate in culture [34]. At the end of their proliferative life span, cells permanently halt cell division and become resistant to cell death (reviewed in [35]). Senescent cells can exhibit senescence-associated β-galactosidase activation [36] and

senescence-associated DNA damage foci (reviewed in [35, 37]) that can cause the loss of both potency and the ability to self-renew [38, 39]. We will discuss cellular senescence in the context of mouse models of progeroid syndromes that illustrate not only how induction of p21 by p53 can impair cellular regenerative capacity but also how constitutive activation of this pathway can limit organ homeostasis and life span.

Stabilization of p53 by D40p53 Causes Chronic Activation of p21

The first of these models was generated by introducing an ectopic, mutant allele of *p53* that codes for a protein missing the first 40 amino acids of full-length p53 into a background of wild-type p53 [40]. This protein, Δ40p53, is one of several naturally occurring isoforms of p53 normally produced by alternate promoter usage or alternative splicing [41]. Δ40p53 is unique in that its primary mode of production is by alternative translation initiation at a start site in exon 4 immediately downstream of an internal ribosome initiation site (IRES) [42]. Full-length p53 initiates at a start site in exon 2, resulting in the addition of 40 amino acids at the N-terminus of the protein, which make up the primary transactivation domain and the overlapping binding site for Mdm2. Other than this, p53 and Δ40p53 are identical, including in the tetramerization domain, where p53 monomers interact to generate the tetrameric form of p53 that binds DNA and functions as a transcription factor. The absence of the N-terminal domain and the loss of the Mdm2 binding site in Δ40p53 contribute to its longer half-life compared to p53 [43] and the increased stability of p53 in heterotetramers with Δ40p53 [44, 45].

p44Tg mice have two normal *p53* alleles that code for the full complement of p53 isoforms, as well as the ectopic allele on the transgene that codes for Δ40p53. The increased dosage of Δ40p53 in p44Tg mice leads to a progeroid syndrome characterized by a premature aging phenotype that can be observed as early as 4 months of age and results in an overall reduction in both mean and maximal life span by about 25% [40]. In addition, cells from p44Tg mice exhibit impaired proliferation, which results in fewer than the normal number of cells in adult organs as well as in embryos at all stages of development, and can be attributed at least in part to an increase in cellular senescence [40]. A model was proposed in which the extra dose of Δ40p53 in cells derived from p44Tg mice stabilized p53 and induced high levels of p21 [40], resulting in the state of permanent cell cycle arrest, resistance to apoptosis, and altered gene expression that defines cellular senescence [35].

This model was tested in NSCs and the effect of impaired proliferative capacity on their ability to contribute to the regenerative process of adult neurogenesis. Neurogenesis occurs throughout life in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus of the mammalian brain [46, 47]. In the SVZ, stem cells generate neural precursor cells, which then go on to migrate along the rostral migratory stream (RMS) until they reach the olfactory bulb (OB) where differentiation into granule cells and periglomerular interneurons occurs [47–49]. In the SGZ, stem cells generate intermediate precursors that eventually undergo differentiation into granule cells in the dentate gyrus (DG) [46, 50–52]. The continuous generation of neurons from these stem cells throughout life is crucial for maintaining odor discrimination and for learning and memory, functions subserved by the OB and DG, respectively [53–56].

Using BrdU incorporation into replicating DNA as a marker of NSC proliferation, p44Tg mice were found to exhibit significantly reduced proliferative capacity in both progenitor cell and stem cell populations with age [57]. At 2–4 months of age, there were no differences in the number of labeled cells in the SVZ, but between 9 and 12 months, there was a significant decrease in the p44Tg mice that did not occur in the NT mice until much later (30 months, Medrano and Scrable, unpublished data). In neurosphere (NS) culture, SVZ cells from p44Tg mice gave rise to fewer and smaller NS that could be serially passaged fewer times compared to cells from age-matched NT mice. NS from both prematurely old p44Tg mice [57] and normally aged NT mice (30 months, Medrano and Scrable, unpublished data) exhibited significantly higher levels of activated (Ser15 phosphorylated) p53 and p21 compared to controls. This can account for the 37 % increase in the length of the cell cycle that characterized p44Tg NSCs.

Consequences of impaired NSC proliferation were seen at all stages of neurogenesis, from reductions in the number of migrating neuroblasts in the RMS and new neurons in the OB to reduced density in the granule cell layer as dead or damaged cells failed to be replaced. The final consequence was a pronounced decrease in olfactory acuity in p44Tg mice relative to normal mice [57]. Thus, the loss of proliferative capacity of neurogenic cells in the SVZ can be linked directly to loss of function in the region of the brain they supply with new neurons, a clear validation of the stem cell theory of aging.

Defects in Nuclear Lamins Activate p53 and Chronically Induce p21

Hutchinson-Gilford progeria syndrome (HGPS) is the result of a mutation in the gene coding for lamin A (*LMNA* 1824C>T) in 90% of cases [58, 59]. The nuclear lamina is vital to maintaining the shape and size of the nucleus and is instrumental in regulating fundamental processes such as DNA replication, transcription, and repair [60]. These processes are compromised by progressive changes in nuclear lamina morphology that occur as a consequence of normal aging [43] and can explain the inevitable accumulation of cells with unrepaired DNA damage [61]. HGPS has been partially

recreated in mice by causing a deficiency in the gene coding for Zmpste24, a homolog of the zinc metallopeptidase STE24 found in yeast. STE24/Zmpste24 post-translationally cleaves prelamin A into mature lamin A, a necessary step in the generation of the nuclear lamina. Predictably, loss of Zmpste24 in mice led to profound abnormalities in the nuclear envelope, resulting in irregularly shaped nuclei with herniation-like blebs [62]. Similar defects have been shown to evoke a p53-mediated response, resulting in apoptosis or senescence [63]. And, in fact, *Zmpste24-deficient* mice exhibit increased numbers of senescent cells in tissues such as kidney and in cultured adult fibroblasts [64].

A second murine model of HGPS was generated by introducing a transgene expressing the most common *LMNA* mutation (1824C>T) under the control of a tet-inducible promoter [65]. Here, too, senescence was evident, this time in epidermal skin sections, consistent with depletion of adult epidermal stem cells and loss of regenerative capacity as early as 13 weeks after induction of mutant lamin A expression [66]. In addition to increased SA-b-galactosidase activity [36], isolated keratinocytes exhibited increased numbers of g−H2AX foci, evidence of increased unrepaired DNA double-stranded breaks [66].

In normal cells, recruitment of the DNA repair machinery to sites of double-stranded breaks is facilitated by acetylation of lysine 16 on histone H4 (H4K16), which converts the chromatin to a more relaxed state [67]. This chromatin modification is carried out by the histone acetyltransferase MOF in association with lamin A in the nuclear matrix [68]. Like keratinocytes from mice with inducible mutant lamin A expression, mouse embryonic fibroblasts (MEFs) from *Zmpste24*-deficient mice exhibit defective DNA repair. In the presence of excess prelamin A, there is reduced binding of MOF to the nuclear matrix and hypoacetylation of H4K16 [69].

The observation that cells from healthy elderly humans also exhibit nuclear abnormalities, prelamin A accumulation, and unrepaired DNA damage links progeroid laminopathies like HGPS and their mouse models to normal aging [70–72]. The same cryptic splice site that is used constitutively in Hutchinson-Gilford progeria to generate mutant lamin A (Δ50 lamin A or *progerin*)[58, 59] is used sporadically in old cells from normally aging humans [70]. Fibroblasts from individuals ranging in age from 81 to 96 years resembled cells from patients with HGPS, with increased numbers of g-H2AX foci at sites of unrepaired DNA damage, mislocalization of lamin A at the nuclear periphery, and nuclear abnormalities. Among the targets affected by abnormal lamin A processing in cells from normally aging elderly individuals, p21 was significantly increased. Inhibition of the cryptic splice site that gives rise to progerin using a morpholino oligonucleotide reversed these age-related defects, returned p21 levels to normal, and restored proliferative capacity [70].

Other Examples of Nuclear Changes That Increase p21 in Stem Cells

Aging affects processes in the chromatin such as DNA methylation and posttranslational modifications of histones, both of which are now thought to be reversible [73]. The epigenome is greatly affected by extrinsic factors, like diet, and intrinsic factors, such as double-stranded DNA breaks [74–76]. Increased DNA methylation, much of it at CpG islands in gene promoters, has been observed in intestinal, colon, and mesenchymal stem cells (MSCs) from old humans and mice [77–79]. Changes in histone methylation with age appear to be tied closely to the self-renewal and proliferation of stem cells because of their effects on the expression of cell cycle inhibitors, like p21. Polycomb group (PcG) and trithorax group (TrxG) complexes catalyze methylation of specific lysine residues on histones, resulting in repression or activation of gene expression, respectively [80, 81]. PcG and TrxG have been linked to organismal and stem cell aging [82, 83]. One such PcG that has been extensively studied is BMI1 can repress p21. Acute reduction of this protein by shRNA knockdown caused p21-mediated defects in adult mouse NSC self-renewal [82]. In human HSCs, loss of BMI1 affected the ability of HSCs to retain multi-potency by causing premature differentiation [84].

In summary, nuclear changes that occur with normal aging or with premature aging syndromes, such as HGPS, support the stem cell theory of aging. *Zmpste 24*-deficient mice exhibit osteoporosis, growth retardation, and premature death [62, 85], as well as reduced BrdU incorporation and cell proliferation and defects in cell cycle profiles [64], a phenotype very similar to that of p44Tg mice [40]. As with p44Tg mice, these defects are associated with activation of p53 and upregulation of p53 target genes, such as *Cdkn1a* [64]. p21 also appears to be a critically important target of aberrant lamin A splicing in normally aged cells, where it is associated with unrepaired DNA damage and reduced proliferation [70–72]. Loss of the chromatin modifier BMI1 in aging stem cells causes derepression of the *CDKN1A* gene promoter and increased expression of p21, resulting in defects in NSC and HSC self-renewal. Collectively, these examples highlight the central role of the cell cycle inhibitor p21 in mediating the effects of nuclear damage on the ability of cells, particularly stem cells, to maintain tissue homeostasis and healthy aging. That it is, in fact, an axis that acts through p53 to turn on p21 in affected cells is brought into even sharper focus by the finding that the phenotypes of both *Zmpste24*-deficient and p44Tg mice, including the increase in p21, are significantly rescued in the absence of *p53* [64].

Receptor-Mediated Transmission of Defects in the Microenvironment to Stem Cells

In addition to intrinsic changes to stem cells, such as breakdown of the nuclear membrane or impaired chromatin structure, age has important consequences on the availability of soluble ligands for several key signaling pathways, as illustrated in Fig. 3. We focus on one ligand, TGFb, which directly and indirectly controls p21 expression in stem cells, and two major pathways, Wnt and Notch, that modulate this activity of TGFb in stem cells. Notch blocks the binding of the TGFb effector Smad to the *CDKN1A* promoter, suppressing p21 expression. As Notch levels decrease with age, suppression is lost and p21 levels go up. On the other hand, TGFb counteracts the effects of an age-associated increase in Wnt levels by blocking transactivation of the *MYC* gene by the Wnt effector β-catenin. As systemic TGFb levels go up, increased suppression of Myc, which transrepresses the *CDKN1A* promoter, indirectly results in increased p21 expression. Thus, increased TGFb in the systemic "niche," combined with intrinsic defects in Wnt and Notch pathways in stem cells, results in impaired stem cell proliferation and regenerative capacity as the organism ages.

Decreased Notch Signaling Releases the Block on p21 Transcription Induced by TGFb

Notch is a transmembrane protein that is activated by contact of its extracellular domain with the extracellular domain of a transmembrane protein of the Delta family expressed on the surface of a second cell. Although the Delta–Notch pathway is known primarily for its role in specifying cell fate during development, it is also the critical mediator of muscle regeneration following injury [75, 86–91]. Notch is expressed on the surface of muscle stem cells (satellite cells), and Delta is expressed on both stem cells and myofibers [92], which make up the stem cell niche in adult muscle. With age, regenerative capacity is lost due to decreased Notch signaling in satellite cells, which in turn has been linked to reduced Delta expression in old muscle [86].

The interaction of a cell expressing Delta with a cell expressing Notch leads to cleavage of Notch into a transcriptionally active intracellular domain, which acts as a nuclear transcription factor for a number of genes, including *MYC* [93]. Myc acts as a pro-proliferative signal in part by suppressing expression of p21 [94]. Thus, in old satellite cells, one consequence of decreased Notch activation is reduced transrepression of the *CDKN1A* promoter by Myc, and elevated levels of p21. A second, and perhaps more significant consequence is on stem cell proliferation, which requires antagonism of TGFb-dependent upregulation of CDK inhibitors, including p21, by phosphorylated SMAD3. Young satellite cells display high levels of active Notch, which blocks binding of SMAD3 to the p21 promoter [95]. In old satellite cells with reduced Notch activity, this block is removed. Furthermore, p53 synergizes with SMAD3 to coordinately regulate the *CDKN1A* promoter, which requires p53 for full transcriptional activation [96]. The increase in p53 with age, as described in the previous section, along with systemic increases in TGFb, which occur in older mice and humans [97], would serve to increase promoter activation. In old stem cells, then, decreased Notch activity leads to increased p21 expression by both an indirect mechanism (reduced transrepression by Myc) and a direct mechanism (increased transactivation by SMAD3 and p53), the latter a direct consequence of age-dependent increases in the level of TGFb in the circulation.

In addition to these effects on p21 transcription, Notch and p53 also exert reciprocal effects on each other's signaling pathways (reviewed in [98]) that are not only sensitive to age but also can help to explain some of their age-associated defects. For example, one of the targets of Notch is *MDM2*, which binds to and ubiquitinates p53 [99], resulting in its proteasomal degradation. Decreased Notch signaling with age would result in less Mdm2-mediated p53 degradation and stabilization of the protein, which could help to explain the increases in p53 levels seen in aging cells. On the other hand, one of the targets of p53 is *PSEN1*, the gene encoding the catalytic component of g-secretase. g-secretase is the enzyme that cleaves Notch following its interaction with Delta, releasing the transcriptionally active Notch intracellular domain. p53 can suppress presenilin-1 expression by competing with Ets transcription factors for binding to the *PSEN1* promoter [100] or by binding to the *CDKN1A* promoter and inducing p21 expression [101–103]. Like p53, p21 is a negative regulator of presenilin-1 transcription [103, 104]. One result of age-associated increases in p53 and/or the p53–p21 axis would be decreased expression of presenilin-1, decreased catalytic activity of g-secretase, and decreased nuclear Notch activity. This in turn could account for the relative inactivity of Notch signaling seen in old satellite cells compared to young [86].

TGFb Can Induce p21 Transcription Even in the Presence of Increased Wnt Signaling

Wnt is a glycoprotein signaling molecule that binds to the Frizzled receptor and activates the transcription of cell cycle promoting genes, such as c-Myc, by β-catenin [105]. In an environment of increased Wnt, both intestinal crypt progenitor cells [106] and HSCs [107] are rapidly exhausted, presumably by an intrinsic mechanism of increased suppression of p21 by Myc. As stem cells require some p21 to maintain quiescence, as described in a previous section, too much Wnt, like too little p21, can cause premature reentry into the cell cycle and deplete the stem cell pool.

As the individual ages, however, systemic increases in TGFb exert powerful extrinsic effects on the ability of the

Wnt pathway to suppress p21 by Myc. Upon activation of the TGFb receptor, SMAD3 associates with the corepressor p107 in the cytoplasm, and the complex translocalizes to the nucleus, where it binds to and transrepresses the Myc promoter [108]. As Myc is a negative regulator of p21, repression of Myc by increases in systemic TGFb with age would cause p21 levels to go up. This can explain why MSCs exposed to serum from old rats exhibited both higher Wnt signaling and higher levels of p21 compared to cells exposed to young rat serum [109].

Although the upstream mediators of increased Wnt in the stem cell environment are not known, one possibility is suggested by a study of murine embryonic stem cells (ESCs), where genotoxic and non-genotoxic insults induced p53-dependent expression of five different Wnt ligands [110]. The age-associated increased in activated p53 seen in animal models of accelerated aging, such as that described for p44Tg and *Zmpste24*-deficient mice above, might be one factor contributing to an environment in which stem cells are exposed to increased levels of Wnt. The recent finding that, in an environment of increased Wnt, there is activation of the p53–p21 axis in MSCs [109] suggests another way a niche factor could induce senescence and compromise stem cell function, in this case by a mechanism that is still unknown.

Conclusions

A hallmark of aging is the loss of regenerative potential. The stem cell theory of aging posits that regenerative potential is maintained by stem cells, and that tissue homeostasis is compromised when stem cells fail. p21 is a universal cell cycle inhibitor that appears to be a principal regulator of the stem cell pool throughout adult life. Too little p21 and stem cell quiescence is lost resulting in premature exhaustion of the stem cell pool. Too much p21 and the self-renewal capacity of the stem cell is lost resulting in a state of permanent mitotic arrest that functionally depletes the stem cell pool. Optimal life span requires just the right amount (Fig. 1).

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Part IV

Stem Cell Transplantation

Translating Stem Cell Discoveries

Alan Trounson

Introduction

The linkage between discovery and translation is a tenuous one. Most academic researchers never venture into the space normally occupied by start-up and early stage companies that struggle to move new discoveries to the clinic. This is often referred to as "the valley of death" because of the scarcity of venture capital ("smart money"), longevity of the processes to marketable products, risk that the regulator will deny registration or impose costly holds and additional requirements for registration, and the general absence of accessible public funding. From the point of view of academic scientists, universities have been slow or neglectful of the recognition of activities necessary to translate basic discoveries. There may be no, or few, publications in high impact factor journals and translation doesn't fit well with the needs of postgraduate students. In fact, the absence of any recognition for product development by the universities has left the area without any inducement for academics to attempt translation. Medical centers recognize the need for translational research but are often underpowered for the needs for translation and tend to work more with autologous cell treatments (approved by institutional review boards/ethics committees) that often don't require the approval of the regulatory authorities.

In the face of discouragement, there are the compelling interests of the patients, who need new and effective therapies that are promised regularly in the media by scientists and university public relations announcements. The discoveries of embryonic stem cells (ESCs) [1, 2] heralded considerable optimism that effective cell therapies for a wide range of diseases and injuries would evolve. The difficulty of lifetime immune suppression for allogeneic transplants appeared to be solved by the discovery of human induced pluripotent stem (iPS) cells [3] that are genomically compatible with the donor patient. It is, however, a matter of debate in terms of the level of matching that will be required even with these iPS cell derivatives. HLA homozygocity may not be enough, as minor histocompatibility antigens; ex vivo culture of cells and gene mutations during ex vivo culture may generate neoantigen that is potentially immunogenic. Nevertheless induced pluripotent stem cell (iPSC)-derived tissues may require less immunosuppression or, at least, manageable immunosuppression.

The potential is clearly enormous but none of it can be captured without translation into acceptable products for patient therapeutics.

Discovery Drives Translational Opportunity

Basic research drives the opportunity to explore the translational merits of new discoveries. The discoveries of hematopoietic stem cell have led to effective therapies in blood diseases and recovery from chemotherapy and radiotherapy for patients with cancer. Likewise numerous clinical trials are underway with mesenchymal stem cells [4] and adipose-derived mesenchymal-like cells [5] that utilize their immunosuppressive and regenerative properties for a wide spectrum of disorders. Given the many important findings that are arising from basic research, it could be expected that many preclinical studies would be proposed to identify candidate cell products for evaluation in pharmacokinetic, safety, efficacy in animal models of human diseases, potency, stability, manufacturing suitability studies, and the many other parameters required by licensing authorities. Despite the accelerating number of publications in basic stem cell biology and the breadth of potential clinical applications, the spectrum of translational studies is rather limited. This may be partly due to the lack of adequate funding in this area.

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Translational medicine is a trendy area for development and is attracting public agency and disease foundation support but little private capital. The grants tend to be marginal for the expensive work needed. Organizations such as the California Institute for Regenerative Medicine (CIRM) (http://www.cirm.ca.gov/) have stepped into this space with a particular focus to enable discoveries in stem cell biology to progress to the clinic. CIRM has a portfolio of more than 60 projects in early translation (see Table 1). Grants range from US$3 to $25 million for 3–4 years, and some grants have been very significantly leveraged with additional collaborative funding from national and state agencies and foundations, creating a networked program of well managed translational projects. Other agencies have translational programs but are generally smaller and not usually focused entirely on stem cell medicine. It is critical that these projects are based on sound experimental data that have been published in major scientific journals and have independent validation from other laboratories. Milestones for progress are agreed to and projects may be canceled if teams are unable to achieve critical development targets.

The momentum of basic science is very impressive in stem cell research. New developments are occurring quickly and new opportunities for translation are evident in many situations. For example, the very recent demonstration of in vivo retroviral transcription factor-induced transdifferentiation of heart fibroblasts or stromal cells into functional cardiomyocytes [6, 7] and the lentiviral microRNA-mediated conversion of heart stromal cells to functional cardiomyocytes in vitro and in vivo [8] creates what appear to be, fascinating opportunities for translation where they may compete as a new clinical treatment for cardiovascular disease and heart repair of myocardial infarction (MI). It may be that the lentiviral approach could be more acceptable to regulatory bodies given the concerns about genome integrating retroviral constructs. This being so, the critical issue may be the relative efficiency of transduction of cardiac fibroblasts to functional cardiomyocytes and the approach needed to deliver the virus for maximum therapeutic effect and minimal side effects. If scar tissue in the heart can be converted to muscle and heart function regained there would be a strong interest in this type of translational research being funded for clinical trials. Many other examples may be found in the rich environment of basic stem cell research.

The major problem for blood stem cell research exists in the inability to reliably multiply the hematopoietic stem cell population. The production of large numbers of blood cells from more primitive pluripotential stem cells is the failure to discover how to mature blood cell progenitors into a marrow-engrafting lineage. In the meantime red blood cells and platelets can be produced in small numbers but production systems for clinically useful quantities are still awaited [9, 10].

In a number of tissue types, progress in translation has been slow and is very much dependent on generating sound basic research around the optimism of using cell-based therapies. In particular, research on the use of stem cell therapies for chronic kidney disease has been disappointing and most commentators believe that considerably more basic knowledge is required on endogenous cell repair and mechanisms of actions of circulating cells in these processes, as well as better differentiation protocols for pluripotent stem cells and their potential delivery into the kidney [11]. Similar issues are evident for repair and regeneration in the liver [12] and the lung [13].

Developments in tissue bioengineering using decellularized or polymer scaffolds have been evolving in clinical settings for tracheal replacement in patients with cancer [14] and will need to enter the translation pipeline to be registered for widespread use. There have also been very impressive developments in rodents for tissue engineering lungs [15] and human optic cups for replacement of stratified retinal epithelium [16]. It would be expected that these discoveries will also evolve into translational studies for clinical trials over the next few years, although some such as whole lung replacement have enormous challenges.

Academic and Industry Partnerships

Trounson et al. [17] proposed a model for academic and industry partners in early translation to accommodate the need to have sufficient research capacity to tackle issues raised in taking new stem cell therapeutics to the clinic. There is an additional need to have sufficient focus and experience to ensure the data for IND enabling submissions were rigorously obtained. Academics have skills and infrastructure for the former and companies have the experience in the latter. This has proven to be a very robust arrangement that avoids some of the difficulties of sparsely funded companies that need to raise funds at every milestone, and the wandering of academic interest that can prove difficult to focus on the regulator's demands in a timely and cost-effective fashion.

Public agencies find it difficult to fund industry for translation and early phase I and II clinical trials. Companies have their shareholders' interests at stake and are subject to board decisions that may not be in complete synchrony with the interests of public institutions. For example, the phase I clinical trial run by the Geron Inc. company for spinal cord repair, using ESC-derived oligodendrocyte progenitors, was terminated by a board decision to concentrate on cancer studies rather than stem cells. The spinal repair project had received support by CIRM. However, all the loan monies from CIRM were returned. There were no adverse events for the five treated patients and it is not known as yet if there will

Table 1 California Institute for Regenerative Medicine (CIRM) translational projects through August 2012

Disease	Award value ($M)	Project goal: Proof of concept[a]	Development candidate[b]	IND[c]	Phase I/II[d]
Blood diseases					
Fanconi anemia. Reprogram a patient's cells into induced pluripotent stem (iPS) cells and mature them into blood-forming stem cells. Then use a genetic tool to replace the defective hemoglobin gene with a normal gene	$6.6		→		
Sickle cell disease. Genetically engineer a patient's blood-forming stem cells to correct the gene that is defective in the disease. Then reinject those cells into the patient	$9.2			→	
Bone disorders					
Osteoporosis. Use a drug to make a patient's own mesenchymal stem cells better at repairing and replacing bone. The drug developed directs the stem cells to the bone surface and to form new bone	$20				→
Treat adult (mesenchymal) stem cells derived from bone marrow with parathyroid hormone for eventual transplantation at the site of injury	$1.9	→			
Spinal fusion. Starting with a patient's adult stem cells harvested from an area around blood vessels, treat them with a protein that encourage the stem cells to become bone, and seed them on a synthetic scaffold	$5.4		→		
Cartilage disorders					
Arthritis (*osteoarthritis*). Starting with embryonic stem cells or reprogrammed stem cells (iPS cells), mature them into progenitors of cartilage, and implant them into the defective joint	$3.1		→		
Determine which variant of the small molecule drug PRO1 is best able to get a patient's own adult (mesenchymal) stem cells to mature into cartilage	$6.8		→		
Take a patient's skin cells and convert them to an embryonic-like state. Mature those cells into cartilage precursors and use those to repair a person's damaged joint	$1.7	→			
Diabetes and complications					
Diabetes. Mature embryonic stem cells into cells that are the progenitors for the pancreas cells that produce insulin. Encapsulate them in a material that will protect them from immune rejection when transplanted into patients, where they mature into the pancreatic cells lost in the disease	$20			→	
Diabetic ulcers. Place adult (mesenchymal) stem cells on a synthetic scaffold where they can grow into a layer of skin for transplantation	$4.5		→		
Multiple diseases					
Multiple diseases. Alter a gene that induces a person's own stem cells to repair tissue so that it is active in stem cells in a sustained way over time	$5.8		→		
Eye disease					
Macular degeneration. Mature embryonic stem cells into a cell type in the eye that degrades in macular degeneration, then surgically implant those cells under the retina to replace the damaged cells	$15.9			→	
Mature reprogrammed stem cells from skin (iPS cells) into the cell type in the eye that degrades in macular degeneration	$5.9		→		

(continued)

Table 1 (continued)

Disease	Award value ($M)	Project goal: Proof of concept[a]	Development candidate[b]	IND[c]	Phase I/II[d]
Start with either reprogrammed adult cells (iPS cells) or stem cells from the eye, and mature those cells into the cell type in the eye that degrades in macular degeneration. Then engineer the cells so that they make a factor that turns down the overactive immune system that is thought to cause the disease	$5.5		→		
Retinitis pigmentosa. Create the specific cells that give rise to the retina starting with donor neural stem cells, with the eventual hope of replacing the damaged retina	$3.9		→		
Cornea damage. Develop a way to grow enough corneal stem cells, known as limbal stem cells, in the lab so there are enough to transplant as a possible therapy	$1.7	→			
HIV/AIDS					
Remove some of the patient's blood-forming stem cells and genetically modify them using a technology called siRNA to remove a protein from the cell surface that the HIV virus uses to enter the cell. Reinject those cells hoping the patient will develop mature blood cells resistant to HIV infection	$20			→	
Remove some of the patient's blood-forming stem cells and genetically modify them with a technology called zinc fingers to remove a protein from the cell surface that the HIV virus uses to enter the cell. Reinject those cells hoping the patient will develop mature blood cells resistant to HIV infection	$14.6			→	
Remove some of patients' blood-forming stem cells and genetically engineer them to carry multiple genes that help cells resist infection by HIV	$3.1		→		
Liver disease					
Liver failure. Mature embryonic stem cells into liver cells and transplant those into the diseased liver	$5.2		→		
Convert skin, blood, or fat cells into liver precursors and transplant those into people with liver failure	$1.5	→			
Metabolic disease. Take stem cells from the placenta that have liver function and use those cells to treat metabolic diseases of the liver in children	$1.8	→			
Skin disease					
Skin disease (*epidermolysis bullosa*). Reprogram skin cells from the patient into induced pluripotent stem cells, then genetically modify them to correct the genetic defect found in the disease. Mature the cells into sheets of skin that can be grafted onto the patient	$11.7			→	
Neurological disorders					
ALS (*Lou Gehrig's disease*). Genetically modify nerve stem cells so that they produce a protein that can protect them from the cause of ALS after transplant as well as protect any remaining undamaged cells	$17.8			→	
Mature human embryonic stem cells into the precursor of a cell that protects motor neurons and inject those into patients	$10.9		→		
Take skin cells from people with ALS and convert them to an embryonic-like state. Mature those cells into neurons and use them to screen for drugs that treat signs of ALS in the cells	$1.7	→			

(continued)

Table 1 (continued)

Disease	Award value ($M)	Project goal: Proof of concept[a]	Project goal: Development candidate[b]	Project goal: IND[c]	Project goal: Phase I/II[d]
Alzheimer's disease. Mature embryonic stem cells into three types of neural stem cells—either just the natural cells or cells modified to make them better able to mature into neurons or destroy proteins associated with Alzheimer's disease. Test those cells in animal models of the disease	$3.6		→		
Take skin cells from people with a hereditary form of Alzheimer's disease and convert them into an embryonic-like state. Mature those cells into neurons and use them to screen for drugs that treat signs of Alzheimer's in the cells	$1.9	→			
Mature human embryonic stem cells into neurons, and use those cells to find drugs that encourage the development of new neurons and protect the existing neurons	$1.7	→			
Spinal cord injury. Transplant nerve stem cells to treat spinal cord injuries in the neck, unlike prior studies that have worked on injuries in the back	$20			→	
Find the appropriate type of human neural stem cells to implant along with a scaffold at the site of injury in people with spinal cord injury	$4.7		→		
Mature human embryonic stem cells into a type of neuron that blocks the effects of other neurons. Transplant these into people with spinal cord injury to reduce pain and improve bladder function	$1.6	→			
Autism. Create reprogrammed stem cells (iPS) from people with autism, then mature those into neurons, and test drugs that alleviate symptoms	$1.5	→			
Canavan disease. Create reprogrammed stem cells (iPS cells), then mature them into intermediate neural stem cells, and genetically modify them to correct the inherited defect	$1.7	→			
Epilepsy. Mature embryonic stem cells into the very specific type of nerve cell found in the part of the brain thought to be malfunctioning in epilepsy	$1.7	→			
Huntington's disease. Use donor mesenchymal stem cells to deliver a growth factor to patients' damaged and endangered nerves. The growth factor is called BDNF	$18.9				→
Genetically modified bone marrow stem cell (mesenchymal) to turn off the mutated Huntington's gene in preparation for eventual injection into the brain	$2.8		→		
Maturing embryonic stem cells into neural stem cells with the goal of eventual transplantation into the brain	$3.8		→		
Parkinson's disease. Test the effectiveness of different types of stem cells including neuronal and cells derived from embryonic stem cells in an animal model of Parkinson's disease	$3.6		→		
Create reprogrammed stem cells (iPS) from people with Parkinson's disease and mature those cells into the neuronal type that degenerates in the disease. Then test drugs on those cells in the lab to find candidates that alleviate symptoms	$2.3	→			
Mature embryonic stem cells into the type of neuron that degenerates in Parkinson's disease and develop ways of creating enough of those cells to be therapeutically useful	$6.0		→		

(continued)

Table 1 (continued)

Disease	Award value ($M)	Project goal			
		Proof of concept[a]	Development candidate[b]	IND[c]	Phase I/II[d]
Spinal cord injury. Attempt to mature embryonic stem cells into intermediate nerve cells that can become either motor neurons or the neurons that control automatic activities like breathing	$1.6	→			
Spinal muscular atrophy. Create reprogrammed stem cells (iPS cells) and mature them into motor neurons. Then use a small molecule drug to coax the neurons into producing more of the protein that is deficient in the disease	$5.7		→		
Stroke. Mature embryonic stem cells into neural stem cells that would be transplanted at the site of the stroke alone or embedded in a biodegradable scaffold	$20			→	
Trauma. Mature embryonic stem cells into neural stem cells with the goal of transplanting the cells at the site of injury	$1.7	→			
Cancer					
Leukemia. Testing an antibody that blocks a protein on leukemia stem cells, dubbed the "don't eat me signal," that inhibits the immune cells that would normally destroy a cancer cell	$20			→	
Testing six existing drug candidates, three small molecule drugs, and three antibodies, that block the ability of leukemia stem cells to survive and replicate	$20			→	
Testing a small molecule drug that blocks a protein called BCL-6 that cancer stem cells need in order to survive	$3.6		→		
Testing a small molecule drug that blocks a protein called BCL-2 that cancer stem cells need in order to survive	$3.3		→		
Malignant melanoma. Use gene modification of a patient's own cells to make them better at seeking out and destroying cancer. They plan to give the patients modified blood-forming stem cells as well as modified mature T cells	$20				→
Brain tumors. Engineer donor neural stem cells so they carry a gene precursor of an anticancer drug. Those cells naturally migrate to the site of a tumor. Then inject a compound that converts the precursor drug to the active drug and kills the tumor	$18			→	
Develop donor adult stem cells (mesenchymal cells) engineered to carry a gene that kills tumors. The cells naturally migrate to the site of the tumor	$3.4		→		
Remove a patient's T cells and engineer them so that they home in on brain cancer stem cells. Reinject those cells into the patient hoping that the T cells will identify and destroy those cells	$5.2		→		
Solid tumor (*colon, ovarian*). Testing small molecules that attack two different targets on cancer stem cells	$20			→	
Muscle disease					
Muscular dystrophy. Reprogram skin cells from the patient into induced pluripotent stem (iPS) cells, then genetically modify them to correct the defective gene. Then mature corrected cells into skeletal muscle precursor cells that can be transplanted	$2.3	→			
Age-related muscular atrophy. Remove muscle stem cells from older people who are losing muscle strength. Multiply those cells in the lab, then reinject them into a patient's muscles	$1.8	→			

(continued)

Table 1 (continued)

Disease	Award value ($M)	Project goal: Proof of concept[a]	Development candidate[b]	IND[c]	Phase I/II[d]
Incontinence. Take skin cells from a person with incontinence and mature those into the smooth muscles that make up the bladder. Transplant those cells back into the person	$5.2		→		
Genetic disease					
Lysosomal storage disease. Transplant neural stem cells into the brains of children who have a genetic condition that damages the neurons of their brains	$5.5		→		
Vascular disease					
Limb ischemia. Genetically modify donor mesenchymal stem cells so that they secrete the growth factor called VEGF, which is known to stimulate blood vessel growth	$14.2				→
Heart disease					
Heart failure. Turn embryonic stem cells into what are called cardiomyocytes, the kind of cells that can become heart muscle for direct transplantation into patients	$20			→	
Harvest the patient's own heart stem cells, then grow them on the lab until there is sufficient quantity to inject back into the heart muscle	$5.6			→	
Mature human embryonic stem cells into heart muscle, and use those cells as a patch to repair damage after a heart attack	$4.8		→		
Develop a way of reprogramming heart cells directly into functional heart muscle as to repair damage after a heart attack	$6.3		→		
Isolate mesenchymal stem cells from bone marrow, and transplant those on a scaffold to repair damage after a heart attack	$4.9		→		
Mature human embryonic stem cells into heart muscle, and modify those cells so they won't be rejected by the immune system when transplanted into a damaged heart	$1.9	→			
Blood vessel growth. Develop a way of reprogramming cells of the body directly into cells that make up blood vessels	$2.3	→			
Danon disease. Take skin cells from a person with Danon disease and reprogram them into embryonic-like cells. Mature those into heart cells, and use those cells to screen for drugs that treat the disease	$1.7	→			
Immune disease					
SCID. Replace SCID patients' dysfunctional immune cells with healthy ones using a safer form of bone marrow transplant. Use an antibody to remove the bad immune cells instead of the dangerous chemotherapy and radiation used today	$20				→
SCID-A. Remove some of the patient's blood-forming stem cells and modify them to produce a protein that's missing in people with this disease. Reintroduce those cells so that the patient now has the missing protein	$3.9		→		
Multiple sclerosis. Mature human embryonic stem cells into neural progenitor cells and use these cells to treat people with MS	$4.8		→		
Develop a drug that promotes a patient's neural precursor cells to develop into the insulating sheath that is lost in people with MS	$4.3		→		

[a]Proof of concept in laboratory or animal model
[b]Development candidate chosen for clinical trial
[c]*IND* investigational new drug
[d]Phase I/II: clinical trials for safety (Ph I) and efficacy as a therapy (Ph II)

be any other investor willing to purchase the intellectual property and assets to continue these clinical studies. It is doubtful these events would have occurred in an academic–business partnership. Academics are wary of company-controlled projects because their own interests and that of the patients may be subjugated to financial priorities and changing industry priorities and focus, as occurred with Geron. However, it is possible that options for advancement of new discoveries become limited, particularly under economic downturns when venture capital evaporates. Presently stem cell company values often slide downwards even when early clinical success is reported because other forces may be operative in the marketplace, such as hedge fund share price interest. This makes it even more critical to ensure that publically funded partnerships enable both academics and companies to progress together to at least proof of concept (phase IIB) of the product in human subjects.

Knowing the Translation Pipeline and the Regulator

Understanding the translational pipeline is critical. The chance of succeeding with an Investigational New Drug (IND) registration depends on satisfying the agency that everything possible has been done to cover any potential risks. Arguably, the most important matter in translation is to communicate with the regulators. The sponsor or lead person/organization for the translation studies is responsible for the clinical investigations and needs to meet with the regulator for compliance, manufacturing challenges, the regulatory development requirements (critical in cell therapies), and the development plan proposed by the sponsor. Adequate information on why the candidate was selected, the data supporting safety in vitro and in a suitable animal model, bioactivity data, pharmacokinetic/pharmacodynamic data, cell distribution and survival, and cell potency are needed to enable the regulator to answer questions, provide feedback, and pose additional studies to ensure approval. Good planning is needed to be able to continue to derive data while awaiting the regulator's response. Holds that occur will often be for dose regimen and safety monitoring deficiencies, problems with the chemistry, manufacturing and controls (CMC) aspect of the IND (cell source, manufacturing and storage, and administration), and inadequate study design [18].

The regulators encourage communication, including pre-pre-IND meetings, pre-IND meetings, Workshops and Advisory Committee meetings, written submissions, and teleconferences [19, 20]. These opportunities enable sound planning and submissions that meet expectations. Since human cell populations are frequently heterogeneous, few industry standards exist and cell markers are still evolving. There are unique manufacturing requirements, cells may be genetically unstable and they may be delivered in variable and unique ways, hence it is critical to have an up-to-date read on the regulator's current position on these matters as it applies to the translational study under consideration. To avoid a clinical hold by the regulator, addressing major issues such as adequate safety assessments are done, quality of manufacturing meets expected standards, and monitoring potential safety and quality issues are met [18]. It is common that sponsors have major unrecognized issues that need to be addressed and guidance from the regulator can help focus the sponsor on areas that must be explored to reduce the regulator's concerns.

The wide range of cell types, diseases, and injuries that are being addressed by cell therapies makes it impossible to adequately address the specific issues that each and every study will face. The adequacy of animal models is often identified and it is critical that the field recognizes the limitations and appropriateness of the particular model proposed [21]. It is not always necessary to include a nonhuman primate model, but for example, care should be taken with monoclonal antibodies that may not show up as problems in rodents. The regulator may or may not be aware of the latest research findings and experts in particular fields can be helpful in guiding the sponsor's modeling and approach.

The added benefit that is apparent with combining biomaterials with cell therapy is attractive for the increased efficacy of stem cell therapeutic approaches, particularly for example, for the use of mesenchymal stem cells for bone repair [22, 23]. Despite the potential for scaffold and biomaterial approaches involving stem cells, for bone repair, the translational pathway is fragmentary and progress is limited [24]. It also needs to be appreciated that the use of biomaterials may lead to combination therapy, which will require both the cells and biomaterial to be subject to regulatory examination.

Cell Therapies in Translation

There are many areas moving into translation and early clinical trials. In several areas such as neurological and cardiac medicine, progress in translation and early clinical trials is informative of issues and challenges for cell therapies.

Neurological Disorders

The complexity of neurodegenerative diseases makes it extremely challenging to treat with single drug approaches. Damage and the ongoing pathogenesis of conditions that include stroke, spinal cord injury, and multiple sclerosis will require engaging multiple cell types and signaling pathways. The increasing incidence of Alzheimer's disease, the

recognition of the phenotypic penetrance of Huntington's disease with availability of genetic screening, and the lack of any treatment for motor neuron disorders demand that therapies be found. Approaches to multiple and simultaneous intervention to facilitate functional recovery make the translation challenges even more demanding. As explained by Miller and Bai [25] "cell therapies, because of their inherent complexity offer the opportunity to intervene at several points in the pathological process and thus may provide a more effective treatment strategy (than molecular approaches). Among the multiple cell types assessed as therapeutic treatment for neural insults, stem cells have emerged as possibly the most effective class. The particular characteristics of stem cells, namely their ability to self-renew and generate multiple cell types promoted their use as sources of cell replacement in the injured CNS." The progress in translation for some conditions that include Parkinson's disease (PD), Huntington's disease, and amyotrophic lateral sclerosis (ALS) is encouraging despite the acknowledged challenges [26, 27]. Despite the progress, the replication of benefit is not always shown when robust objective assessment is used, suggesting that the field needs to find agreement on the criteria for outcomes in translation, cell potency parameters, biomarkers for function, and the suitability of animal models. Otherwise success will be hampered. Aboody et al. [28] have recently provided a very comprehensive review of progress in neural stem cell translation. They identify areas such as remylination, promotion of host tissue regeneration, enzyme replacement therapy, and tumor-localized chemotherapy production as critical areas where translation is actively moving treatments forward towards clinical application.

Primary information on the causes of many of the major neurodegenerative disorders makes it difficult to design adequate translational protocols utilizing stem cell derivatives. In the case of ALS, the spreading of the disease along the anterior horn of the spinal cord involving both motor neurons and interneurons appears to mimic a prion-like pathological misfolding of proteins that is transmissible within and between healthy cells. The emerging scenario in ALS involves TDP-43/FUS RNA binding protein pathophysiology that triggers degeneration of motor neurons and also harbors a prion domain capable of pathological misfolding [29]. It seems unlikely that cell therapeutic replacement can address all the alterations in the various pathways affected in ALS. This will create further complications for designing translational strategies in such diseases.

Parkinson's disease (PD) has frequently been identified as a translational target for cell therapies because the transplantation of fetal ventral mesencephalic tissue into the striata of PD patients has provided proof of concept in the human that these cells can successfully return striatal dopaminergic function to these patients for many years [30–32]. There were, however, disconcerting side effects of dyskinesia due to the fetal tissue transplants in a subgroup of PD patients. The side effects may be due to graft-derived striatal serotonergic hyperinnervation that induces false dopamine release through an unfavorable serotonin/dopamine transporter ratio [33–35]. Fetal brain tissue contains both dopaminergic and serotonergic neuroblasts [36]. This has raised the possibility of using more purified dopaminergic neurons derived from directed differentiation of human embryonic stem cells (hESCs) [37], or possibly iPSCs. The opportunity is clear that provision of a better-characterized product may lead to an effective therapy that would reverse the pathological motor phenotype of PD. However, PD patients may also require additional grafts of serotonergic neurons to relieve non-motor symptoms by restoring serotonergic neurotransmission in specific cerebral targets [31, 32].

Neural stem cells have been isolated from fetal sources and expanded for use in a broad spectrum of clinical applications [38]. These neural stem cells have been demonstrated to be effective in clinical trials for allogeneic transplantation [39] and may also be very effective as vehicles for gene therapy including the delivery of neurotropic factor gene products for neuroprotective and pro-regenerative effects [40]. These approaches are currently in translation and clinical trials for correction of neural genetic diseases, macular degeneration, spinal cord repair, and stroke. The source of cells may vary but the translational processes are common. ESC derivatives are also in translation for neural regenerative applications for spinal injury, stroke, ALS, and macular degeneration [41, 42].

There remains debate about what are the best cells to use as the therapeutic candidate of choice. Some researchers believe that the best results are obtained with very specific and mature cell types rather than progenitor cells. In the case of spinal cord repair Noble et al. [43] have strong experimental evidence that transplanting specific astrocytes provides a far better outcome than for precursor cells. They also note that reproducibility between laboratories is a major challenge, suggesting the variability in models and lesions, delivery method, and experimental design makes it very difficult for the field to come to firm conclusions and move forward in a logical manner. There are certainly a wide variety of cell types and approaches presently under translational development and in clinical trials for many conditions such as stroke [44]. These include $CD34^{+}$cells from mobilized blood cells and umbilical cord blood, mononuclear cells from bone marrow, mesenchymal stem cells, neural stem cells, ESCs, and iPSCs. In the area of spinal cord repair, many different adult cell types have been explored including Schwann cells, olfactory ensheathing cells, neural stem cells, umbilical cord blood cells, mesenchymal stem cells, and ESC-derived oligodendrocyte progenitor cells [4, 39, 45].

There is as yet little agreement on the best cell type or approach and little evidence of significant patient benefit in early clinical trials to date.

Glaucoma is a progressive neurodegenerative optic neuropathy without reliable clinical effective therapy. The approaches under study in translation focus on neuroprotection and retinal ganglion replacement [46]. While there remain challenges for demonstration of an effective therapy for a range of eye diseases using a stem cell approach, there is considerable optimism that cell-based therapies will prevail because of the accessibility, range of surgical techniques available, and the impressive progress being achieved in translation [47]. The challenge will be to deliver this therapy to the widespread under-resourced communities in which glaucoma is a very serious problem.

Stem cells are popular therapeutic vehicles to attempt to destroy high-grade gliomas [48]. The therapeutic cell types under study include embryonic, neural, and mesenchymal stem cell derivatives delivering cytokines, enzyme/prodrug suicide combinations, viral particles, matrix metalloproteinase, antibodies, and chimeric antigen receptors [49]. Clinical trials utilizing neural stem cells that have strong homing properties to tumors are under way to deliver an enzyme/prodrug approach to destroy recurrent high-grade glioma resistant to conventional treatments [28].

Cardiac Repair

Considerable interest exists in the use of cell therapies for cardiovascular regeneration in ischemic heart disease and/or myocardial infarction (MI) for reducing mortality, improvement of quality of life, and reduction of the economic burden that exists for these patients. There are basically two different approaches being studied in translation at the present time; injectable cells and patch-based cell approaches [50]. There are merits to both approaches with considerable support for the cell-impregnated biomaterial scaffold approach using cardiac and induced pluripotential cell derivatives in translational research. However, success to date for cell therapies has been rather disappointing with inconsistent efficacy and modest benefits to patients, low conversion of donor cells to cardiomyocytes, and very limited engraftment [51]. The evolution of three-dimensional cultures and improving biomaterials are likely to have major impacts on the potential to effect repair of human myocardial infarction.

The necessity for myocardial remodeling and the associated processes of hypertrophy, proliferation, apoptosis, necrosis, and autophagy [52] have led to translational studies on the use of endogenous and exogenous cell-based therapies. The initial optimism that mobilizing bone marrow cells in patients using granulocyte colony-stimulating factor (G-CSF), could repair myocardium has failed to materialize into significant benefit for patients. Exogenous intracoronary infusion of bone marrow mononuclear cells, when compared to placebo controls, failed to improve global or regional ventricular function at 6 months in patients with myocardial infarction [53]. Considerable effort is being directed to the use of other cell types that include mesenchymal stem cells [4, 39, 54] with mixed results depending on the endpoints chosen. These translational studies underline the necessity for well-controlled trials and well-defined parameters for patient benefit. Interestingly c-kit$^+$ bone marrow cells but not mesenchymal stem cells augmented cardiomyocyte progenitor activity and cardiac performance in mice [55].

Studies on cardiac cells are evolving in translation with encouraging results. Bolli et al. [56] reported on a well-controlled study on autologous cardiac stem cells for reversal of heart failure months after it was diagnosed. Patients who had bypass surgery to try to improve cardiac function were assigned to treatment or control groups an average of 4 months after surgery. Treated patients had a biopsy to retrieve heart tissue and the cells were sorted to isolate those cells c-kit$^+$, a presumptive marker for cardiac stem cells. Those cells were expanded in vitro and reinfused into a cardiac blood vessel. Four months later the treated patients had significant improvement in heart function tests and the controls did not. In a subset of patients the benefit was even more pronounced 1 year later. Li et al. [57] compared expanded human cardiosphere-derived cells (CD105$^+$, partial c-kit$^+$, and CD90$^+$) to human bone marrow and adipose-derived mesenchymal stem cells, and bone marrow mononuclear cells for cardiac repair in mice. The cardiac cells had the highest myogenic and angiogenic potential in vitro, and after transplantation gave the best improvement in SCID mouse cardiac function after injection into infarcted hearts, highest cell engraftment, myogenic differentiation rates, and least abnormality of heart morphology (at 3 weeks post treatment). This strongly suggests that cardiac repair may be optimum with cardiac rather than mesenchymal cells or mononucleocytes.

Further data from the phase I clinical trial on autologous cardiosphere-derived cells by Makkar et al. [58] showed that intracoronary administration of expanded endomyocardial biopsy samples taken 4 weeks after myocardial infarction showed no acute complications and 28 % reductions in scar tissue as measured by MRI at 6 months and 42 % at 12 months. This despite no evidence of actual donor cell regeneration; indeed donor cells appear to be absent within 2 weeks of administration. It is proposed that the effects of cardiosphere-derived therapy are mediated via endogenous regenerative and reparative pathways, which is surprising that benefits up to 12 months were observed. As a consequence, the research group is now using allogeneic cardiac cells that are able to provide the same benefits without severe immune response. The transitory nature of the trans-

planted cardiac cells and relatively low dose apparently provide the same safety profile as autologous cardiac cells. The FDA has approved a phase I/II study to further evaluate this approach.

Regulatory bodies may have concerns about identification of stem cells following transplantation. Knowing the distribution and sites where stem cells accumulate can be very important. Are the cells transient, and removed by macrophages or other processes or do they take up residence in the target tissue or elsewhere, and multiply for regenerative impact? There are recent reviews that describe the tracking methods for stem cells transplanted for cardiac repair. These imaging modalities include MRI, PET, SPECT, and CT. The MRI agents include gadolinium chelates such as Cy3-labeled gadofluorine, super paramagnetic iron oxide nanoparticles (SPIOs) coated with an agent to enable cellular uptake in non-phagocytic cells, and ferumoxide-labeling [59]. The advantages and drawbacks of the various methodologies were described by Fu et al. [60]. Radionuclide imaging—PET and SPECT is the most sensitive and has the highest spatial resolution of imaging modalities for cell tracking, viability, and other cell functions. These include the uptake of fluorine ^{19}F [61].

Cell Therapies in Translation with CIRM

The CIRM has a large program of early translational studies that includes studies at the proof of concept stage that are seeking to demonstrate a viable candidate product for therapeutic use in human medicine, demonstration that a developmental candidate is effective in the appropriate animal or laboratory model of the human disease, and derivation of data necessary for submission of registration for a clinical trial (IND—Investigative New Drug) approval. The list as of August 2012 is shown in Table 1. The studies are listed under broad categories of tissue type and diseases and/or injuries. These involve all the translational techniques and modalities being explored in the field of stem cell science. These studies tend to be more complex than the majority of clinical trials presently registered with world regulatory agencies. This is due to greater emphasis being placed on new approaches by CIRM in order to enable more efficacious therapies to evolve with public–private financial support.

Studies are evolving rapidly, given CIRM has only been funding research for only 6 years [4, 39, 41, 42]. Studies that are presently evolving to clinical trials include allogeneic cardiac cells for heart disease, interruption of the CCR5 gene for prevention of HIV AIDS, ESC-derived insulin producing cells in a protective capsule for correction of Type I diabetes, ESC-derived retinal pigmented epithelium for correction of dry macular degeneration, gene correction using stem cell therapies—such as sickle cell disease, epidermolysis bullosa, and thalassemia [62], and ESC-derived neural stem cells for stroke. CIRM is also targeting the destruction of cancer stem cells [63]. This includes the destruction of glioma using neural stem cells transfected with an enzyme that will convert a cytotoxic prodrug into a highly toxic product at high levels in the localized area of the tumor(s). Other cancer stem cells in solid tumors and leukemias are being targeted with other cancer stem cell-specific drugs and monoclonal antibodies. This includes blockage of the antigen CD47—"don't eat me"—signal that inhibits macrophage phagocytosis of cancer cells.

Clinical Trials and the Need for Networked Alpha Clinics

There is an identified need for clinical trial sites to accommodate the rising list of translational studies progressing to early clinical trials as exemplified in Table 1. The foundation arguments an approach for developing a network of alpha stem cell clinics (proposed by Trounson et al.) [41, 42]. These should be sited in the tertiary medical centers and associated with universities to accommodate the need to provide for GMP cell treatment, manipulation, selection, and expansion technologies. These centers are the ones likely to provide the patient interface with discoveries evolving to clinical application in translation. The clinicians are likely to require modifications to fit clinical modalities and to address risk for patients not clearly apparent to translators of new therapeutics. The networking component enables rapid distribution of knowledge and success in meeting regulatory requirements and roadblocks that appear in new and highly variable approaches to cell-based therapeutics. These centers will need to develop a sustainable business plan and practice that should include both public and private financial support. The benefits for the insurance industry are obvious and the network may provide the infrastructure for the major biopharmaceutical industry to participate in a meaningful way in cell-based therapeutics. Ultimately, the prospect of a major new platform in medicine based on stem cell therapeutics is the success of correcting or curing serious disease and injury with strategies based firmly on sound scientific understanding of the mechanisms that cells provide in the regenerative process Many of the present clinical trials in progress lack any understanding of what role the administered cells play in these diseases or conditions and may become lost in translation [64].

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Mesenchymal Stromal/Stem Cell Transplantation: From Tissue Regeneration to Immune Modulation

Peiman Hematti

Introduction

The cells currently known as mesenchymal stromal/stem cells (MSCs), were first described by Friedenstein et al. as an adherent, fibroblast-like population of cells cultured from the bone marrow (BM) of rodents [1]. Through a series of elegant experiments, he showed that following implantation of these cells under the kidney capsule they generated rudimentary bone tissue that was capable of supporting hematopoiesis. Friedenstein's method was later used to derive similar cells from human BM [2], and then these cells were shown to support hematopoiesis in long-term culture assays [3]. Later Caplan proposed these cells to be stem cells for mesenchymal tissues and proposed "mesenchymal stem cells" could have therapeutic potential [4] in regeneration of many tissues. MSCs are calculated to comprise only a small population (<0.01 %) of adult BM cells [5], and one of the major challenges in this field is the lack of any reliable or widely accepted marker for direct isolation of them from BM aspirates. Thus, although markers such as Stro-1 [6], CD271 [7], and CD146 [8] has been proposed for direct isolation of these cells, there is still no consensus on which marker is the most representative of cells present in their in situ BM environment. The low number of MSCs in BM also means that for any in vivo use, either experimental or clinical, the cells have to be expanded ex vivo through extensive cellular proliferation. MSCs from BM are most commonly isolated by plating BM mononuclear cells in culture plates and passaging the adherent cells, which, after a few passages, leads to expansion of a homogenously fibroblast-like population of cells [9]. According to the widely accepted criteria by International Society for Cellular Therapy (ISCT), such cells can be labeled as MSCs if they (a) express a certain set of markers (i.e., CD105, CD73, CD90) and do not express hematopoietic markers (i.e., CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR) and (b) could differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [10]. Although the end point of this derivation methodology is a seemingly homogenous population of cells, they are functionally heterogeneous and comprised of different subpopulations with different differentiation capabilities at a clonal level [11]. Thus, the term "mesenchymal stem cell" could be only applied to a defined population of MSCs that fulfill the criteria for being true "stem cells," (i.e., to have the potential not only to self-renew themselves but also generate progenies that could differentiate into osteoblasts, chondroblasts, and adipocytes) [12]. Thus, in addition to the controversies surrounding the true in situ anatomical location and physiological role of these cells, there is also much controversy regarding their true stem cell properties [13]. This chapter discusses the use of ex vivo culture-expanded MSCs in different clinical settings, the rationale behind those approaches including their tissue regenerative and immunomodulatory properties, and unsolved issues and evolving concepts in this highly dynamic field.

Use of MSCs in Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell (HSC) transplantation was the first and is still the most common form of stem cell therapy. It is mainly indicated for the treatment of hematologic malignancies and nonmalignant conditions, such as immunodeficiency syndromes. For HSC transplantation, HSCs are harvested from BM, pharmacologically mobilized into blood followed by their collection, or more recently, HSCs from umbilical cord blood units are used to replace diseased HSCs of recipients (autologous or allogeneic). However, despite decades of clinical experience, HSC transplantation continues to be a high-risk procedure with a high rate of morbidity

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and mortality related to the toxic effects of pre-transplant preparative regimen, HSC graft failure and/or ejection, and immunologically mediated phenomenon of graft-versus-host disease (GVHD). MSCs are considered to be an essential constituent of the BM stromal microenvironment with an indispensable role in support of hematopoiesis [14]. Thus, not surprisingly, HSC transplant physicians were the first to use MSCs in a clinical setting. The Lazarus team was the first to conduct a phase I trial that showed the safety of intravenous infusion of ex vivo culture-expanded autologous human BM-derived MSCs [15]. These autologous MSCs generated from small-volume BM aspirates and expanded over several weeks were shown to be safe and not causing any adverse reactions. This, in addition to other pioneering studies by HSC transplant physicians, paved the way for the use of MSCs for treatment of a wide variety of other disorders in other disciplines of medicine.

A major goal of transplant physicians has always been to accelerate recovery of hematopoiesis after HSC transplantation. Due to the presumed role of MSCs in supporting hematopoiesis in the BM and evidence from animal studies in which co-transplantation of MSCs with HSCs improved the engraftment of the latter [16], one of the earliest indications for which MSCs were investigated was for their ability to promote hematopoietic engraftment. In a phase I–II clinical trial, ex vivo culture-expanded autologous MSCs were infused into breast cancer patients at the time of autologous HSC transplantation [17]. In another study culture-expanded allogeneic MSCs derived from BM of human leukocyte antigen (HLA)-identical sibling donors were infused to the respective allogeneic HSC transplant recipients [18]. Although these studies could not provide definitive conclusions regarding the engraftment-promoting effect of MSCs, they further reassured investigators that the use of culture-expanded autologous or allogeneic MSCs as a form of cellular therapy is feasible and safe. Since then autologous or allogeneic ex vivo culture-expanded BM-derived MSCs have been used in the context of HSC transplantation in a large number of patients with hematologic and non-hematologic malignancies for different purposes, such as reducing the risk of graft failure, preventing repeat rejections, or rescuing graft failure [19]. Most of these studies have provided encouraging results but have failed to prove their effectiveness in a clinically conclusive manner.

Aside from supporting HSCs, MSCs have also received much attention by HSC transplant physicians due to their effect on immune cells. MSCs have shown to modulate the immune responses in vitro and in vivo via their interactions with a plethora of immune cells. Such interactions lead to suppression of proliferation of activated T lymphocytes, an increase in the number of T regulatory lymphocytes, a decrease in activation and proliferation of B lymphocytes, suppression of cytotoxicity of natural killer cells, suppression of maturation of dendritic cells, modulation of neutrophil activities, and changes in the immunophenotype of macrophages [20–23]. One of the earliest of these effects to be discovered was the suppression of T cell proliferation and activation [23]. Moreover, this immunosuppressive effect of MSCs appears not to depend on the HLA compatibility status of the donor and recipient [24]. Since acute GVHD is a T cell-mediated process [25], years ago it was proposed that MSCs could be potentially used as a therapeutic modality, specifically for treatment-refractory GVHD [26]. Le Blanc et al. were the first to investigate the potential of MSC infusion for the treatment of refractory GVHD in a 9-year-old boy who had received a HLA-matched unrelated donor HSC transplant for leukemia [27]. Infusion of ex vivo expanded MSCs generated from the patient's mother, not the original donor, resulted in the resolution of GVHD symptoms. This seminal report was followed by larger studies from the same group in which MSCs were given to steroid-refractory GVHD from HLA-identical siblings, haploidentical family donors, and unrelated mismatched donors [28, 29]. These studies showed that infusions of MSCs to this group of very sick patients are safe, resulted in a significantly better survival rate compared to control patients, and the responses were independent of the source and HLA compatibility of MSCs. The latter point is very important, as the generation of patient-specific MSCs is very time consuming, costly, and in many instances impractical due to the urgent nature of the need for their use.

Since these original reports there has been a plethora of studies using BM-derived MSC in different doses and frequencies, made using different methodologies, and used in different age groups [19]. All these studies confirmed the original safety reports but with variably encouraging or successful results. The largest clinical trials performed with MSCs have been two Phase III double-blind, placebo-controlled, randomized trials evaluating (Prochymal) a proprietary formulation of MSCs derived from the marrow of a single third-party donor as a first-line treatment for acute GVHD or for the treatment of refractory acute GVHD. These double-blinded, placebo-controlled (in a 2:1 ratio) trials were designed to assess the safety and efficacy of Prochymal in multicenter international studies. To the surprise of most transplant physicians and investigators in the field, these studies could not show positive results in regard to their primary end points. Some potential confounding factors might have been the differences in treatment regimens administered to patients in conjunction with Prochymal in different centers. However, the use of the same Prochymal in pediatric patients with severe refractory acute GVHD resulted in more promising results [30]; indeed, this product indeed is now approved in Canada, but not yet in the United States, for

pediatric patients with GVHD as the first form of "off-the-shelf universal stem cell therapy product."

While MSCs have largely been used in the field of HSC transplantation for their ability to reconstitute the BM microenvironment and their immunomodulatory functions, another avenue for clinical use of MSCs is to utilize their tissue regenerative properties. Again in the field of hematology, these cells were used in patients who had a different spectrum of tissue and organ toxicities following allogeneic HSC transplantation, such as hemorrhagic cystitis, pneumomediastinum, and perforated colon and peritonitis with overall promising results [31]. Also, one of the earliest reported cases on use of culture-expanded, gene-marked MSCs was in conjunction with BM transplantation in six pediatric patients with osteogenesis imperfecta. This study, done based on the fact that MSCs are progenitors for osteoblasts, showed engraftment in five recipients and an acceleration of growth during the first 6 months post-infusion [32].

Use of MSCs in Non-hematopoietic Stem Cell Transplant Settings

Over the last decade, in addition to the field of HSC transplantation, MSCs have also generated a lot of excitement in other disciplines of medicine and surgery. Originally much of this enthusiasm was due to the assumption that these cells are capable of not only differentiating into mesenchymal tissues, such as bone and cartilage, but also transdifferentiating into many other types of cells, such as cardiomyocytes, hepatocytes, and pancreatic islets [33]. Although there were ample experimental and preclinical models that supported such assumptions, it was later realized that those early observations of such unorthodox plasticity were due to imperfect experimental tools, the use of animal models that were not representative of human biology, or other potential mechanisms such as cell fusion [34]. However, by then hundreds of patients had already been recruited into clinical trials based on those assumptions, many of them with preliminary promising results, albeit on a small scale and in nonrandomized formats. Nevertheless, by this time many new modes of action for these cells were discovered that initially were not appreciated. Indeed, the discovery of these new mechanisms of action was a major paradigm shift in the field and included tropism of MSCs for migration into sites of tissue damage or inflammation, their capability to support and stimulate proliferation and/or survival of resident tissue progenitor cells through secretion of a variety of cytokines and chemokines, and contribution to angiogenesis of tissues [35–38]. However, these newly discovered modes of action, in addition to their previously recognized immunomodulatory and anti-inflammatory properties, kept these cells at the forefront of use in many diverse groups of human pathologies. Indeed, there is a long list of phase I–II trials for a variety of non-hematological indications in which autologous, donor-directed, or third-party allogeneic MSCs had been used, including for treatment of patients with myocardial infarction, chronic obstructive pulmonary disease, amyotrophic lateral sclerosis, stroke, Crohn's disease, diabetes mellitus, systemic sclerosis, systemic lupus erythematosus, and refractory wounds among others [39–42].

In many of these disorders, allogeneic third-party MSCs were used without any immunosuppression, as MSCs are assumed to escape attack by cytotoxic T cells or NK cells and thus could be transplanted over major histocompatibility complex barriers in humans [43, 44]. This has been the rationale for the use of "off-the-shelf" ex vivo culture-expanded BM-derived MSCs from "third-party" donors, and the myriad of clinical experiences that have confirmed the impressive safety of this product has been reassuring. Also, use of "off-the-shelf," ready-to-use MSCs as a therapeutic entity, in contrast to production of patient-specific MSCs, is an important concept for large-scale production and commercialization of cellular therapeutics by biopharmaceutical entities [45].

MSCs Derived from Non-bone Marrow Origin

Although MSCs were originally isolated from BM, cells with similar phenotype, differentiation potential, and biological characteristics have now been derived from almost all adult tissues, including adipose tissue [46] and heart [47]; neonatal tissues such as placenta [48]; fetal tissues such as lung, liver, and blood [49]; and even embryonic stem cells [50]. Furthermore, it had been repeatedly shown that MSCs derived from non-BM tissues have immunomodulatory properties very similar to BM-derived MSCs [51–53]. Indeed, based on experimental data showing that AT-derived MSCs possess immunological characteristics similar to BM-derived MSCs, they have been used for treatment of GVHD after HSC transplantation [54]. However, adipose tissue MSCs are expected to find their highest clinical applications in the fields of plastic and reconstructive surgery [55]. Other types of MSCs that have reached the clinic include placenta-derived MSCs for the treatment of GVHD [56] and umbilical cord-derived MSCs for the treatment of severe and refractory systemic lupus erythematosus [57]. It should be no surprise to see other novel sources of MSCs to be tested for specific clinical settings based on their tissue of origin. For example, it could be argued that MSCs derived from pancreatic islets could be of more value for indications such as the protection of transplanted islets after cadaveric transplantation [58].

Unsolved Issues

In clinical medicine a confounding factor in interpreting results of clinical trials of pharmaceuticals is the heterogeneity of patient physiology, which affects the absorption, metabolism, and pharmacokinetics of the drug and the heterogeneity of the targeted diseases, which affects the potential responsiveness of the disease to the administered drug. However, in clinical trials of cellular therapeutics, particularly MSCs, another layer of complexity exists in the enormous heterogeneity in the final product (i.e., MSCs). MSCs are derived from tissues of autologous or allogeneic donors; therefore, from the very early stages of this process, there are numerous reasons for heterogeneity of the final product. The first issue is the appropriate donor source; uncertainties here include the fact that, theoretically and based on some evidence, the MSCs generated from tissues collected from the patients might not be the appropriate source, as these MSCs may be also afflicted by the patient's disease processes [59]. However, it can be also argued that autologous MSCs could avoid issues such as tissue incompatibility and rejection. However, even in the case of normal healthy allogeneic donors, we do not know if there are donor-specific characteristics that make certain allogeneic donors potentially more suitable for the production of MSCs, such as donors of younger age or special physical attributes. Additionally, in the case of allogeneic MSCs, we still do not know of the appropriate tissue sources of MSCs that could be most effective for specific indications. It is very well known that MSCs from different tissue sources, despite seemingly similar phenotypes, could have significant differences in their functional characteristics. Nevertheless, we do not know the impact of these variables between different preparations of MSCs on the intended clinical outcome.

One other major challenge in the field is the fact that currently there is no standard culture methodology for generating MSCs [60]. It is well known that culturing cells at different densities and using different types of growth media (containing fetal bovine serum, serum-free media, autologous serum, fresh frozen plasma, or human platelet lysates) could affect the phenotype and rate of growth of MSCs. Even the batch of fetal bovine serum used could have a major impact on the end product. All these subtle differences could result in clinically significant changes in the ultimate biological characteristics of the MSCs. Other factors that add to the complexity of interpreting results of clinical trials are the different dosages and frequencies of cells from different passages. MSCs have been used at different doses and frequencies, from different passages, either fresh or frozen, given at different stages of disease, and given alone or with different combinations of other medications and treatments. A major challenge in harmonizing the culture conditions is the fact that although these variations in the culture methodologies could affect the immunomodulatory and regenerative properties of MSCs, there is no clinically applicable potency assay that is widely accepted for MSCs. For example, the same MSCs that are used for treatment of GVHD could be also used for treatment of heart or lung diseases [42]. However, the latter disorders are not T cell-dependent disorders, and other potency assays or functional analyses of MSCs for those diseases are probably more appropriate.

Evolving Concepts

MSCs were originally promoted in the field of regenerative medicine for their potential to differentiate into many different types of cells and to replace lost or damaged cells based on a large body of literature in different animal models. However, these expectations were never realized in human studies. Indeed, despite seemingly encouraging positive results in numerous clinical trials, the durability of the infused MSCs is now a matter of great debate [61], and the new wave of studies report that the extent of MSC engraftment is usually very minimal and could not explain the observed clinical benefits [62]. Indeed, the lack of engraftability of BM MSCs has been well known to HSC transplant physicians for a long time, as most reports have shown that in BM transplant recipients, the MSCs remain of recipient origin and are not replaced by the donor MSCs carried in BM grafts [63, 64]. However, in these BM transplant scenarios, it could be argued that these cells were transplanted in low numbers and therefore were different from ex vivo culture-expanded MSCs. In any circumstance, there have been significant changes in our understanding of the potential mechanisms for MSC to exert their beneficial effects (Fig. 1). This new paradigm proposes that MSCs exert their beneficial effects through mechanisms such as paracrine effects prior to their demise. Thus, instead of "replacing" damaged cells, MSCs contribute to the "regeneration" of damaged cells and tissues indirectly, mainly via indirect paracrine effects. This low level of survival after administration of MSCs could also explain why repeated infusions of MSCs might be needed to achieve a clinical effect. This is in contrast to HSC transplantation, in which the administered HSCs usually engraft fully, and their beneficial effects depend on their persistent engraftment. It is ironic that one of the original attractive properties of MSCs was assumed to be their presumed lack of immunogenicity [43] and thus feasibility of transplanting across HLA barriers without the concern for rejection. However, it could be argued that lack of durability and persistence of MSCs, especially third-party MSCs, could be a desirable property of MSCs, as it could preclude any chance of tumorigenicity in the future.

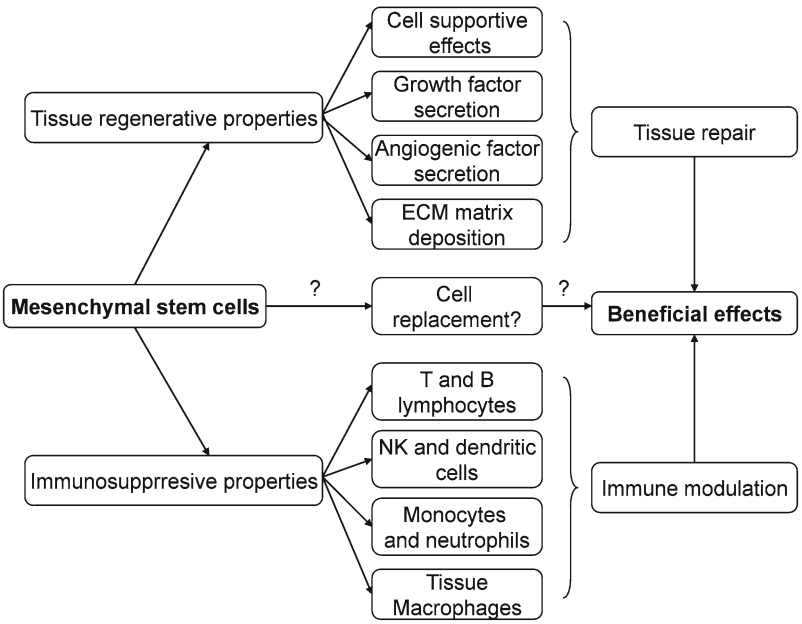

Fig. 1 Mechanisms of action and potential role of mesenchymal stem cells in tissue regeneration and immune modulation

A major question that has preoccupied the mind of clinicians and basic researchers alike is why these fibroblast-looking cells [65], derived mostly from BM, could have a therapeutic effect in such a wide range of conditions with seemingly unrelated pathophysiology, such as GVHD, myocardial infarction, heart failure, chronic obstructive pulmonary disease, Crohn's disease, diabetes, osteoarthritis, systemic lupus erythematosus, kidney transplantation, stroke, nonhealing wounds, systemic sclerosis, cirrhosis, and others. A common pathophysiological theme for all these disorders is the contribution of "inflammation." Now it is well known that a major way that MSCs exert their effects is through their interactions with macrophages, and this could provide a unifying rationale for the continued investigation of these cells in such a wide range of applications [66].

Conclusions

The original clinical trials pioneered by visionary HSC transplant physicians not only provided the initial safety data but also generated much excitement encouraging other disciplines of medicine to take advantage of the potential therapeutic effects of MSCs. A major factor in the expansion of MSC trials is the lack of any documented toxicity or long-term side effects and the unmet need for novel therapies for many degenerative diseases. Due to the inherent shortcomings of these small, nonrandomized clinical trials, a conclusive clinical benefit has been difficult to discern, and important questions remain to be addressed. However, although the final place of MSCs in regenerative biology is not clear, there is no other cell that has been applied to such a wide range of applications. There is a need for carefully designed clinical trials of sufficient size that examine the specific intended mechanism of action and the optimal source, dose, schedule, and route of administration. However, logistical considerations, including the cost for conducting such large clinical trials, are immense so a bedside-to-bench and back-to-bedside approach is also needed. Cell therapists are fascinated by MSCs as they are easy to produce according to good manufacturing guidelines, are not immunogenic but very safe to use, have multifaceted regenerative and immunomodulatory properties, and are therefore expected to remain very attractive to the field of regenerative medicine.

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Stem Cells and Tissue Engineering in Burns and Wounds

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Introduction

Cutaneous injuries as a result of abrasions, cuts, or burns trigger a dynamic and highly complex wound healing response. This process involves the coordinated action of an overlapping series of events by various resident cells and blood-derived cells, which include terminally differentiated cells and stem cells alike. Their roles in wound healing have been elucidated over the years and a more in depth understanding of this process has allowed for the development of new therapeutic strategies to enhance tissue repair. This chapter discusses the physiological wound healing response with a focus on the involvement of stem cells in natural wound repair events and highlights regenerative medicine techniques such as cell therapy and tissue engineering research aimed at harnessing the power of stem cells to contribute towards wound healing and tissue remodeling.

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Skin

The skin represents the largest tissue in the human body. In adults, skin has an average mean surface area of 1.6 m^2 and weighs approximately 4 kg. It accomplishes many vital functions such as barrier protection, immune function, thermoregulation, repair, and as a sensitive organ that plays an important neurosensitive and psycho-affective role [1]. Anatomically, skin is divided into two parts or layers: epidermis and dermis, which lie over the hypodermis or subcutaneous fat tissue (Fig. 1) [2]. The basement membrane or epidermal-dermal junction binds the epidermis and dermis [3]. Skin normally contains stem cells, which are responsible for its continuously renewing properties and which also act as a reservoir of cells to aid in tissue repair following injury [4]. It has been suggested that these multipotent stem cells not only produce skin, but also other cell types, such as nerve and bone cells [5]. The vast majority of resident skin stem cells are located in the hair follicle bulge [6].

Epidermis

The epidermis is the outer skin layer and has a thickness ranging between 0.4 and 1.5 mm, depending on the anatomical area [1, 7]. This ectoderm-derived layer constitutes an avascular poly-stratified, squamous, and keratinized epithelium. It contains four or five different layers or strata depending on the anatomical region (from the innermost to the outermost): stratum germinativum or basal layer, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum [1]. Keratinocytes, melanocytes, Langerhans cells, and Merkel cells are the predominant cells found in the epidermis.

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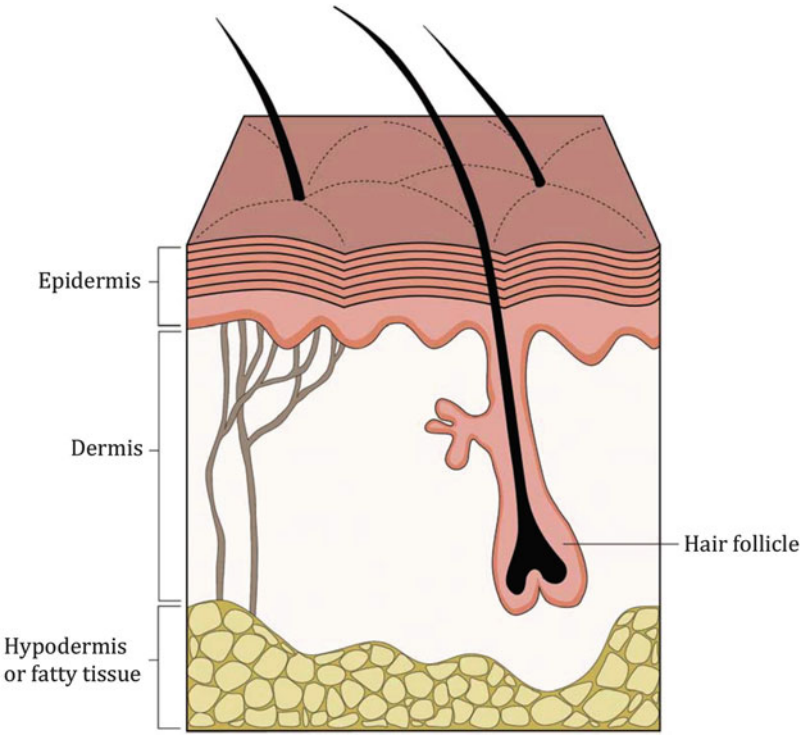

Fig. 1 The composition and various layers of normal skin. With permission from: Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells (iPSCs), by Trond Aasen and Juan Carlos Izpisúa Belmonte. Nature Publishing Group (2012)

Table 1 Primary differences between epidermal and dermal skin layers

Skin layers	Origin	Vascularity	Characteristics		Main cells
Epidermis	Ectoderm	Avascular	Keratinized stratified epithelium		Melanocytes
					Keratinocytes
Dermis	Mesoderm	Vascular	Contains extracellular matrix and skin appendages		Fibroblasts
					Adipocytes
			Superficial or papillary	Highly vascular	Macrophages
				Lax	
			Deep or reticular	Less vascular dense	

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Keratinocytes represent the most abundant epidermal cell type [7]. Melanocytes synthesize melanin, the pigment which is transferred to keratinocytes and is responsible for human skin color, and it is important to note that the color depends not only on the amount but also on the activation of melanocytes [8].

Dermis

In contrast to the epidermis, the dermis originates from the embryologic mesoderm and is a vascular skin layer (Table 1). The dermis is further subdivided into two parts: (a) the papillary or superficial dermis, which is loose and highly vascular, and (b) the reticular or deep dermis, which is denser and less vascular. This fibroelastic dermal tissue is composed of different fiber types (collagen, elastic, and reticular fibers), glycosaminoglycans, cells (fibroblasts, mastocytes, and macrophages), vessels, nerves, and adnexa (hair follicles, eccrine, and apocrine sweat glands, and sebaceous glands) [1, 7].

Hair Follicles

Hair follicles are ubiquitous, but not found in palms, soles, or mucosae. The hair follicle structure has an outer part or root sheath called the infundibulum, which goes from the epidermis to the point where the sebaceous duct empties the sebum, a middle zone called the isthmus or inner root sheath, and last but not least, an inferior part called the base or hair shaft [1, 4]. Hair originates from hair follicles and is composed of

hard keratin. The hair growth cycle is a 3-phase process: the anagen or active growth phase, which lasts 2–5 years; the catagen, involution or regression phase, which lasts 2–5 weeks, and the telogen or rest phase, which lasts 2–5 months. After this third phase, hair loss occurs and a new growth cycle begins [1]. The telogen-to-anagen transition is controlled jointly by bone morphogenetic protein (BMP), WNT, fibroblast growth factor (FGF), and transforming growth factor (TGF)-β signaling pathways. Loss of Fgf18 signaling accelerates anagen initiation, whereas loss of TGF-β2 signaling delays it [9].

Skin Stem Cells

Skin or cutaneous stem cells include epidermal stem cells (interfollicular and bulge stem cells), dermal stem cells, sebaceous stem cells, hair follicle stem cells, sweat gland stem cells, melanocyte stem cells, mesenchymal stem cells (MSCs), neural stem cells, and endothelial stem cells [10].

The more abundant skin stem cells are the epidermal hair bulge stem cells. Studies with murine bulge stem cells showed that they had the capacity to regenerate all epithelial cell types of the skin [11]. The hair follicle bulge is a well vascularized and innervated stem-cell niche located within the outer root sheath [4], at the deepest and most protected place within the contiguous epidermis [12]. It contains multipotent cutaneous and tissue-specific epithelial stem cells, such as melanocyte stem cells/precursors [13]. The hair follicle bulge cells are the slowest-cycling cells in the cutaneous epithelium; [14] they are normally quiescent and only proliferate at the onset of anagen, when a new follicle is generated [15]. Despite the fact they cycle slowly, their proliferation rate is very high, especially during fetal development and wound healing [16]. Only a small fraction of stem cells, the interfollicular stem cells, reside in the basal layer of the interfollicular epidermis. These stem cells maintain adult skin homeostasis and hair regeneration, but they also participate in the repair of the epidermis after trauma [4].

Wound Healing in Acute Wounds and Burns

Human adult cutaneous wound healing is a complex, multistep physiological process, which eventually serves to repair, but not regenerate, skin. The restoration of skin continuity after injury involves ectodermal and mesodermal repairing processes, including epithelial resurfacing or re-epithelialization, synthesis of connective tissue and biomechanics and wound contraction to reduce the tissue's gap [3].

Lesions involving epidermis and superficial dermis, such as donor sites or superficial partial-thickness burns, heal spontaneously by migration of epithelial cells from the wound edges, and from intact skin appendages. The scarring will be minimal, especially in areas where skin appendages are numerous. Deep partial-thickness or full-thickness burns may heal after 3 weeks or they may not heal at all, and they are associated with a high risk of infection and hypertrophic scarring. Therefore, these deep lesions usually require surgical intervention in order to aid in the natural wound healing process [3, 17].

Phases of Wound Healing

Wound healing has three phases: Inflammation, proliferation, and remodeling [3, 18] (Fig. 2). These steps include fibrin clot formation, cell migration, extracellular matrix deposition, dermal reconstitution, and re-epithelialization [19].

The inflammatory phase begins immediately after injury with vasoconstriction that acts to control bleeding and to achieve haemostasis. To accomplish this goal, vasoconstriction and platelet aggregation are followed by platelet plug formation and the activation of the blood coagulation cascade, resulting in fibrin deposition [3, 18]. After haemostasis is achieved, vasodilation occurs and vascular permeability increases, aiding the infiltration of inflammatory cells into the wound. This shift of vasoconstriction to vasodilation is due to the release of histamine, platelet-activating factor, bradykinin, nitric oxide, and prostaglandins [18].

Polymorphonuclear neutrophils are the first inflammatory cells to reach the site through chemo-attraction in response to platelet-released healing factors, such as PDGF and TGF-β [3, 18, 20]. These cells initiate wound enzymatic debridement, and their numbers peak at 24 h [3, 18]. Within 1–5 days after wounding, neutrophils are gradually superseded by macrophages, the main cell involved in this response, which peak in numbers by 48–72 h. Macrophages produce PDGF and TGF-β, thereby attracting fibroblasts and stimulating collagen production [18, 21]. Both fibroblasts and lymphocytes infiltrate the wound and peak at day 7 [22]. The arrival of fibroblasts to the wound occurs around day 3, and signals the beginning of the proliferative phase [18], although the inflammatory phase lasts longer in non-healing or problematic wounds such as deep partial-thickness burns or secondary intention wounds [3]. In the proliferative phase, collagen synthesis, angiogenesis, and epithelialization occur. Total collagen content increases for about 3 weeks, until the rate of collagen production and the rate of its degradation equalize, which determines the beginning of the remodeling phase. The remodeling phase lasts 6 months to 1 year. During this phase, fibroblasts and myofibroblasts cause wound contraction and vascularity decreases [18]. This last phase represents the maturation of the resulting scar, which is the result of normal wound healing or repair processes [23].

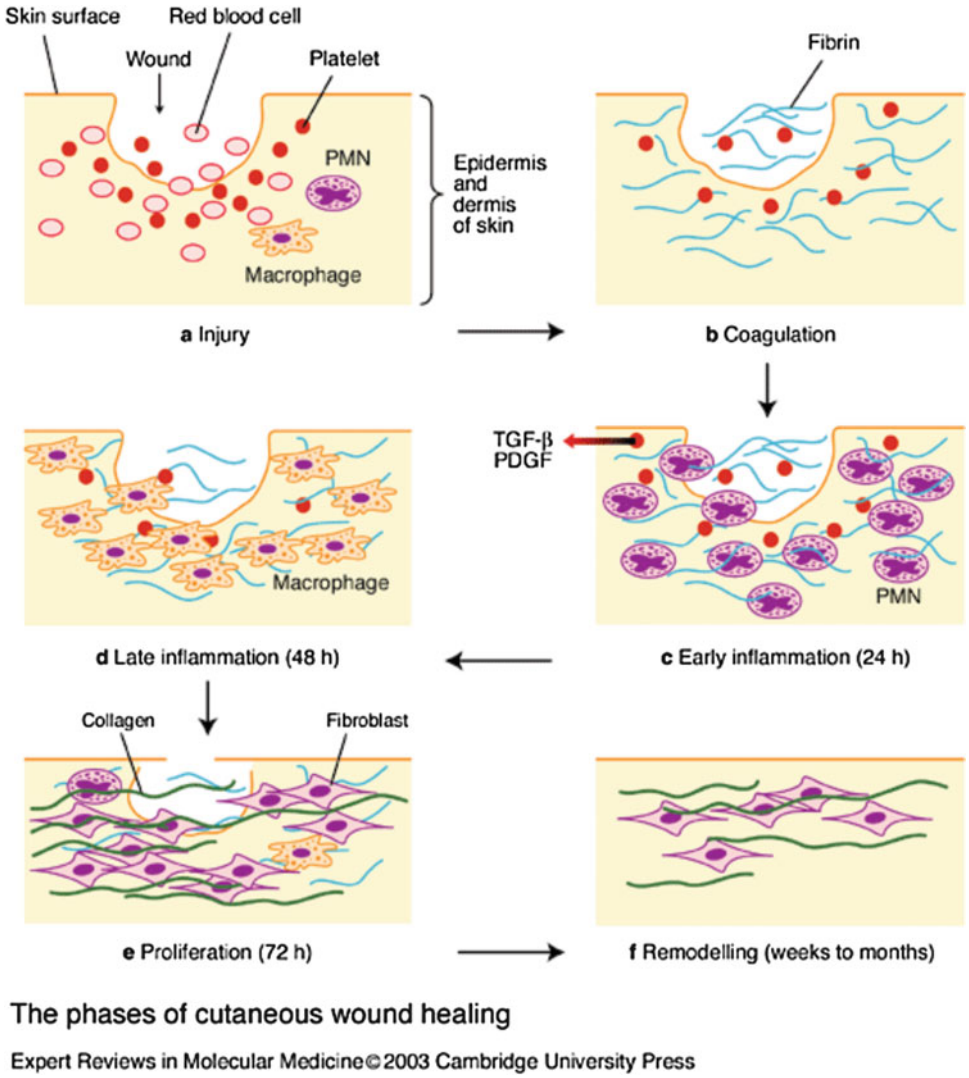

Fig. 2 Phases of wound healing. With permission from: Skin repair and scar formation: the central role of TGF-[beta], by Steven R. Beanes, Catherine Dang, Chia Soo, and Kang Ting Expert Reviews in Molecular Medicine, Volume 5, Issue 08 (21 March 2003), pp 1–22. Copyright © 2003 Cambridge University Press. Reprinted with permission

Stem Cells and Wound Healing

The skin is an organ with a highly regenerative capacity and thus, when healthy skin is injured or burned, it has the ability to repair itself through a cascade of highly coordinated events. Immediately after burn injury, resident endothelial progenitor cells and MSCs are also involved in the response and migrate from the bone marrow into the wound. Whereas the former cells primarily increase vascularization, the latter ones are reported to also differentiate into dermal fibroblasts and myofibroblasts contributing to re-epithelialization and dermis reconstitution [24]. In circumstances where the injury is severe and healing is delayed or inadequate, various therapeutic approaches have been utilized to enhance the inherent regenerative potential of the skin. One such approach to promote skin regeneration in chronic wounds or burns that has been championed by the scientific community consists of the delivery of exogenous stem cells. The homing capacity of exogenous bone-marrow MSCs to injured tissues such as skin, muscle, brain, lung, myocardium and bone, to name a few, suggests an important role for these cells in natural wound healing and supports the concept of using stem cells for therapeutic wound care [25–27].

Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the mammalian blastocyst that are able

to undergo unlimited self-renewal in vitro. The therapeutic potential of ES cells lies in their ability to undergo differentiation into endoderm, mesoderm, and ectoderm germ layers. Aberdam et al. have demonstrated the capacity of ES cells to differentiate into keratinocyte and fibroblast cell lineages and created a bioengineered skin composed of epidermal and dermal layers that holds potential for therapeutic applications in wound treatment [28, 29]. However, it is important to note that the use of embryonic stem cells has been met with ethical concerns pertaining to the harvesting of the cells from living embryos. Immune rejection and the potential for teratoma formation are other concerns raised with the use of ES cells for cell therapy applications. Thus, a subset of the scientific community has begun to redirect their focus away from embryonic stem cells and towards the use of adult stem cells.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) define a pluripotent stem cell population which has been reprogrammed in vitro from differentiated adult cells into an embryonic stem cell-like state by various factors. Takahashi et al. were the first to demonstrate the reprogramming of adult murine and human fibroblasts into pluripotent iPSCs [30, 31]. These iPSCs hold tremendous potential for cutaneous wound healing applications; skin fibroblasts could be isolated from the patient, induced to become pluripotent iPSCs, expanded in vitro, redirected to epidermal or dermal lineages and delivered to the wound. However, concerns have been raised regarding the use of potentially cancer-causing oncogenes and viral vectors for the cell reprogramming steps [32]. Moreover, although iPSCs are derived from a patient's own adult cells, Zhao et al. have raised concerns regarding their immunogenic properties by demonstrating that certain cells differentiated from iPSCs can induce T-cell dependant immune responses in syngeneic recipients [33]. Regardless of their flaws, iPSCs show great potential for regenerative medicine. The field of iPSC research is still very young and only through future studies will the true clinical impact of these pluripotent cells be determined.

Mesenchymal Stem Cells

MSCs describe a population of multipotent adult cells capable of differentiating into a variety of mesenchymal progeny. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed a standard by which to define MSCs with three important minimal criteria. First, the cells must be plastic-adherent under standard culture conditions. Second, they must express CD73, CD90, CD105 and lack the expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR surface markers. Third, the cells must have the capacity to differentiate into adipocyte, chondrocyte, and osteocyte lineages in vitro [34]. To date, MSCs have been isolated from a variety of sources including bone marrow, adipose, blood, amniotic, and umbilical cord tissues.

Bone marrow derived mesenchymal stem cells (BM-MSCs) have received the most attention as a therapeutic tool to enhance wound healing, based on their relative ease of isolation and their role in native tissue repair and remodeling. Mansilla et al. have reported a significant increase in the proportion of cells phenotypically identical to BM-MSCs circulating in the blood of burned patients as compared to non-burned patient controls. The authors also found that the percentage of cells circulating in the blood of burned patients which were phenotypically identical to MSCs correlated with the size and severity of the burn, suggesting that these multipotent cells play a role in homing to the wound and contributing to tissue repair [35]. However, BM-MSC isolation is still an invasive procedure and there is a significant decrease in available BM-MSCs with donor age [36].

As an alternative to BM-MSCs, phenotypically similar multipotent cells have also been isolated from amniotic and umbilical cord tissues [37–39]. Since these tissues are usually considered biological waste following parturition, they are considered a valuable untapped source of MSCs. Moreover, umbilical cord derived MSCs have been reported to have higher proliferation rates and a greater capacity for expansion in vitro than BM-MSCs, further supporting their potential for therapeutic use [40]. Adipose tissue represents another alternative tissue source for MSCs which is also considered biological waste in various cosmetic surgeries. Isolation of multipotent MSCs from these tissues is performed with relative ease and they have been shown to have high colony forming frequency as compared to the other tissue derived sources of MSCs mentioned above [36].

Regardless of their source, MSCs derived from adult tissues exhibit low immunogenicity; they exhibit mechanisms that enable them to escape the host response in transplant situations [41]. They are also not burdened by the ethical concerns associated with ES cells, and they are relatively easy to isolate and lack complex additional steps for clinical use as compared to iPSCs. These reasons explain the widespread use of MSCs derived from adult tissues, particularly BM-MSCs, in today's stem cell therapy applications.

Stem Cell Therapies for Wound Healing

In their seminal publication describing the role of transplanted BM-MSCs in homing to a site of muscle injury and differentiating to aid in its regeneration, Ferrari et al. demonstrated the use of a new tool for future therapeutic applications [25].

Table 2 Administration of exogenous mesenchymal stem cells for wound healing in animal models

Species/cell	Delivery method	Wound model	Results	References
Mouse bone marrow derived mesenchymal stem cells (BM-MSCs)	Intravenous injection into tail vein	A full-thickness 10 mm punch biopsy excisional wound model in mice	BM-MSCs contribute to wound healing through differentiation into keratinocytes, endothelial cells, and pericytes	Sasaki et al. (2009)
Mouse BM-MSCs	Intradermal local injection at four sites around the wound	A full-thickness 6 mm excisional skin wound model in normal and diabetic mice	Accelerated wound closure, enhanced angiogenesis and BM-MSC contribution to skin appendages	Wu et al. [43]
Mouse BM-MSCs	Topical delivery via fibrin spray	A full-thickness 1 × 1.5 cm elliptical excisional wound model in mice	Accelerated wound closure in diabetic mice	Falanga et al. [42]
Mouse BM-MSC conditioned media	Subcutaneous local injection and topical application of BM-MSCs conditioned media	A full-thickness 6 mm excisional skin wound model in mice	Accelerated wound closure and recruitment of endothelial cells into the wound	Chen et al. [54]
Mouse stromal progenitor cells	Local subcutaneous injection	An 8 mm diameter excision skin wound healing model in diabetic mice	Accelerated wound closure, enhanced granulation tissue production and neovascularization	Javazon et al. (2006)
Rat BM-MSCs	Intravenous injection	A 1 cm diameter excisional wound model in rats	BM-MSCs contribute to the regeneration of skin appendages	Li et al. [53]
Rat BM-MSCs	Intravenous injections in tail vein or local injections around wound	A full-thickness 5 cm-long incisional skin wound in normal and diabetic rats	Improved wound breaking strength, enhanced collagen production, and increased growth factor release	Kwon et al. [45]
Rat BM-MSCs	Local injection or systemic tail vein injection	A 5 cm-long incisional wound model in rats	Enhanced wound bursting strength of wounds	McFarlin et al. [44]
Rabbit BM-MSCs	Human amniotic membrane used as a cell carrier	A full-thickness 1.5 × 1.5 cm skin excisional wound model in rabbits	Accelerated wound closure and improved epidermal and skin appendage regeneration	Kim et al. [46]
Human BM-MSCs	Intradermal injection	A full-thickness 3-cm-long incisional skin wound model in rabbits	Enhanced wound tensile strength and inhibited scar formation	Stoff et al. [47]
Human BM-MSCs	Bilayer dermal substitute used as cell carrier	A full-thickness 1.5 × 1.5 cm skin excisional wound model in pigs	Accelerated wound closure and differentiated into epithelium at wound site	Nakagawa et al. (2004)
Human adipose derived MSCs	Topical application in a collagen gel solution	A full-thickness 7 mm diameter excisional skin wound in nude mice	Accelerated wound closure and re-epithelialization from the wound edge	Kim et al. (2007)
Human adipose derived MSCs	Human acellular dermal matrix used as a cell carrier	A 6 mm diameter excision wound model in athymic mice	Accelerated wound healing and contribution to neo-vascular network	Altman et al. [50]

As a means of treating osteogenesis imperfecta in children, Horwitz et al. demonstrated that allogeneic mesenchymal precursor cells could be delivered to the patient and these cells were able to home to the site of injury and increase total bone mineral content and promote de novo bone formation. In light of these results and the capacity of BM-MSCs to differentiate into various skin lineages, the administration of these multipotent cells for cutaneous wound healing therapeutic applications is self-evident. Moreover, in sharp contrast to the simple administration of growth factors or drugs, BM-MSCs can home to the wound and release important wound healing factors over a sustained period of time.

Recent studies have demonstrated pronounced defects on cutaneous wound healing associated with the exogenous administration of BM-MSCs in mouse [27, 42, 43], rat [44, 45], rabbit [46, 47], and pig [48] animal models (Table 2). An improvement in the wound tensile strength [44, 47], an increase in collagen production and cell recruitment to the wound [45], and an enhanced angiogenic response [43, 49, 50] are among some of the other beneficial wound healing-related responses reported with the application of MSCs.

These studies clearly show enhanced wound healing associated with the use of MSCs, however, the mechanism of action still remains to be determined. A debate as to whether MSCs exhibit their actions in skin regeneration primarily through differentiation into different cutaneous lineages or whether they play a more supporting role through cytokine release and paracrine signaling is still ongoing. Through the

use of green fluorescent protein-labeled MSCs or through MSC xenotransplantation techniques, various groups demonstrated the co-localization of MSCs with markers for epidermal [27, 48, 50], fibroblast [50], and endothelial cell [27] phenotypes, suggesting MSC differentiation into these skin cell types. Moreover, recent studies have reported on the contribution of exogenously administered MSCs to the regeneration of skin appendages, presumably through an environment-mediated differentiation process [43, 51–53]. The more indirect supportive role of MSCs in the wound healing process has also been reported in several studies. In vitro, Wu et al. demonstrated that BM-MSC conditioned media could promote an angiogenic response in human umbilical vein endothelial cell cultures through a paracrine effect [43]. Chen et al. expanded on this work and reported that BM-MSCs produce a greater amount of VEGF-α, IGF-1, EGF, keratinocyte growth factor, angiopoietin-1, stromal derived factor-1, macrophage inflammatory protein-1alpha and beta and erythropoietin than dermal fibroblasts. Moreover, using BM-MSC conditioned media as a therapeutic tool to study the effects of MSC paracrine signaling on wounds, the authors also reported accelerated wound healing through the recruitment of macrophages and endothelial cells [54]. Regardless of whether MSCs act primarily by direct differentiation into skin lineages or whether they act as support cells for the resident dermal and epidermal cells, their therapeutic value in wound healing applications has been clearly elucidated in animal models.

In human patients, the benefits of the administration of MSCs for wound healing applications is still not fully answered but several case reports and clinical trials have already demonstrated the safety and efficacy of the use of bone marrow aspirate and BM-MSCs (Table 3). Ichioka et al. reported granulation tissue formation within 2 weeks and subsequent complete wound closure with the application of autologous bone marrow aspirate in one patient suffering from a leg ulcer that had not healed despite 1 year of standard wound therapy [55]. Other case reports of one patient undergoing autologous BM-MSC therapies have reported similar wound healing properties in patients, with no adverse side effects observed [56, 57]. Other slightly larger clinical trials with patient enrolment sizes of three demonstrated that the administration of bone marrow aspirate or BM-MSCs directly to the wounds promoted granulation tissue formation, vascularity, and integrity of the dermis with complete wound closure within 4 months or less of the treatment (Badiavas et al. [51]; Rogers et al. [58]) [51, 52, 58]. In 2007, Falanga et al. [42] topically delivered autologous BM-MSCs to the wounds of 13 patients using a fibrin spray and a reduction in ulcer size, accelerated wound healing and even complete wound closure in some chronic wounds were reported. The authors also showed a strong correlation between the amount of MSCs delivered to the site and a reduction in ulcer size. In a case study of 20 patients with severe burns or intractable dermatopathies, Yoshikawa et al. [59] reported that the topical delivery of BM-MSCs to an injury site resulted in enhanced wound healing and the regeneration of subcutaneous tissue in 18 patients, with two patients dying of causes unrelated to transplantation.

In 2007, Badiavas et al. expanded on their previous studies and in a randomized control trial, they reported increased blood vessel growth in the patients' wounds, along with a reduction in wound size associated with the BM-MSC treatment [60]. In an important randomized control trial of 24 patients suffering from chronic ulcers of the lower limbs that did not respond to standard treatment, Dash et al. showed that BM-MSC therapy significantly improved patient pain-free walking distance and reduced ulcer size. The largest and most recent randomized control study of the use of MSCs for wound therapy was published in 2011 by Jain et al. [61]. In a study of 48 patients suffering from chronic lower limb wounds that had not responded to standard therapy for at least 3 months, the authors demonstrated that the administration of bone marrow aspirate enhanced the rate of wound healing for these chronic lower extremity wounds in the early weeks of treatment. Most case reports and clinical studies have reported beneficial effects of the use of MSC therapies for wound healing, however, a few studies have also shown some inconclusive results [62]. These differences may be a result of various factors associated with the procedures used in the studies such as cell isolation, the route of MSC delivery, the associated wound clinical care and the clinical endpoints chosen. For MSC therapies to become standard wound treatments, these factors need to be considered in more detail.

The delivery method of the stem or progenitor cells is an important point which deserves further consideration in the use of cell therapies for cutaneous wound healing. Systemic delivery of cells from the vascular system represents one of the simplest administration methods. This approach mimics the route of endogenous MSCs to the wounds, thereby taking advantage of the inherent homing capacity of BM-MSCs from the circulatory system to sites of injury. Intravenous tail vein injections of BM-MSCs in mouse and rat animal models have been widely employed and this technique has been shown to enhance wound healing [27, 45]. However, MSCs delivered via this systemic delivery approach risk being eliminated by the body's own clearance organs thereby reducing the total number of BM-MSCs reaching their destination at the wound site. Another widely used route of MSC administration is through local cell injections in or around the wound itself. This technique has been thoroughly used and shown to be just as effective towards promoting wound healing [43, 44, 47, 60]. However, it is important to note that this approach represents a very different scenario where cells are placed near the wound and migrate through tissues rather

Table 3 Administration of exogenous mesenchymal stem cells for wound healing in clinical trials

Cell type	Delivery method	Study design	Results	References
Autologous BM-MSCs	Intramuscular injections in the ipsalateral gastrocnemius muscle in the middle of the lower limb	Case report of a diabetic patient with ischemia induced chronic wounds on his foot	Improvement in perfusion and angiogenesis in the forefoot stump and complete wound healing 20 weeks after the treatment	Kirana et al. [56]
Autologous bone marrow aspirate	Topical delivery by using collagen scaffold as a cell carrier	Case report of one patient suffering from 85 to 65 mm^2 leg ulcer that did had responded to standard therapy for >1 year	Well vascularised healthy granulation tissue developed within 2 weeks and split-thickness skin was grafted onto it and wound completely closed. Free of complications for 1.5 years	Ichioka et al. [55]
Autologous BM-MSCs	Two local administration of BM-MSCs and covered with a collagen matrix	Case report of one patient suffering from a radiation included burn	Healing of the wound which persisted 1 year after therapy. No adverse side effects reported	Lataillade et al. [57]
Autologous bone marrow aspirate	Topical administration to the wound. In two patients, aspirate was also injected into the wound base and edges	Case series of three patients with chronic wounds of the lower limbs that had not responded to standard therapy for >1 month	All wounds demonstrated granulation tissue formation and complete closure within 60 days	Rogers et al. [58]
Autologous bone marrow aspirate and BM-MSCs	Topical delivery of aspirate to the wounds and injection into the wound edges, with three additional topical administrations of BM-MSCs to the wounds	Case series of three patients with chronic non-healing wounds that had not responded to standard therapy for >1 year	Improved thickness, vascularity, and integrity of the dermis and complete wound closure within 4 months or less of the treatment	Badiavas et al. [51]
Autologous cultured bone marrow cells	Injected into and applied directly over the wounds	Randomized control trial of four patients with chronic wounds that had not responded to standard therapy for >3 years	Increased blood vessel growth in wounds and a decrease in wound size was observed	Badiavas et al. [51]
Autologous bone marrow aspirate	3–5 cc of bone marrow aspirate was directly applied to wounds	Case series of eight patients with chronic non-healing lower extremity wounds that had not responded to standard therapy for >1 year	Results inconclusive with some patient wounds decreasing in size while others increased in size and then received alternative treatments	Mulder et al. [62]
Autologous BM-MSCs	Topical delivery via fibrin spray	Case study of 13 patients with acute wounds from removal of nonmelanoma cancers and chronic wounds that had not responded to standard therapy for >1 year	Accelerated wound closure, a reduction in ulcer size and complete wound closure in some chronic wounds and a strong correlation between amount of MSCs and reduction of ulcer size. No adverse events were reported	Falanga et al. [42]
Autologous BM-MSCs	Topical delivery by using collagen scaffold as a cell carrier	Case study of 20 patients with severe burns or intractable dermatopathies that had not responded to standard therapy for >3 months	Regeneration of subcutaneous tissue and wound healing observed in 18 of the 20 patients; the remaining two patients died of causes unrelated to transplantation	Yoshikawa et al. [59]
Autologous BM-MSCs	Intramuscular injections in the ischemic limb and wound edges, and topical administration to the wound	Randomized controlled study of 24 patients with chronic ulcers of the lower limb that had not responded to standard therapy for >1 month	Significant improvement in patient pain-free walking distance and a reduction in ulcer size	Dash et al. (2009)
Autologous BM-MSCs	Intramuscular injections in the ipsalateral gastrocnemius muscle in the middle of the lower limb	Randomized control trial of 30 diabetic patients with chronic foot ulcers (>6 weeks) that had not responded to standard therapy for >1 week	Improved vascular microcirculation and enhanced wound healing. No adverse events were reported	Kirana et al. (2012)
Autologous bone marrow aspirate	Injection into the wound edges and spraying onto the wound	Randomized control study of 48 patients with chronic lower limb wounds that had not responded to standard therapy for >3 months	Increased rate of wound healing for chronic lower extremity wounds in the early weeks of treatment	Jain et al. [61]

than through the circulatory system and thus circumvent extravasation through the endothelial cells of the vascular network. Falanga et al. successfully made use of fibrin sealant systems which incorporated MSCs as an alternative method for the topical delivery of cells to wounds [42]. This approach delivers MSCs onto the surface of wounds and entraps them in a fibrin mesh from which they can then migrate into the wound. An alternate method for the topical delivery of MSCs to wounds consists of incorporating cells into a carrier system such as a collagen scaffold that can be directly applied to the wound [55, 59]. Ideal methods for the engraftment of MSCs to the wound site still remain to be determined; however, the fibrin sealant and collagen scaffold approaches outlined above open a new door of opportunity into the use of both materials and cells for regenerative medicine applications.

Tissue Engineering

Tissue engineering is a field of medical research which lies at the intersection of biomaterials, cell therapy, and engineering for the ultimate goal of developing biological substitutes to restore, maintain or improve tissue or organ function. Skin tissue engineering approaches have the potential to provide off-the-shelf substitute skin for patients suffering from severe burns, cuts, and other cutaneous injuries.

Ideal biomaterials for skin tissue engineering should be biocompatible, simulate skin barrier function, and have controlled degradation rates which are matched to native skin regeneration. The materials should also not provoke a heightened inflammatory response nor cause any scarring. The biomaterial may also provide a support structure for the application of exogenous cells to the wounds or as a temporary template for the invasion of the patient's native cells to repopulate the wound site. To date, collagen-based materials have been widely used for the fabrication of commercially available skin substitutes such as Integra®, Transcyte®, Apligraf®, and Orcel®, to name a few [2]. Other materials such as hyaluronic acid, elastin, and nylon materials have also been widely employed in other commercially available skin substitutes; however, an exhaustive list of skin substitutes and their material composition is not the focus of this chapter. It is however important to note that several of these skin substitutes have incorporated dermal fibroblasts and epidermal keratinocytes to successfully repopulate the wound area. Unfortunately, poor integration into the host environment, scarring, and a lack of differentiated skin structures are still common problems with the application of today's commercially available skin substitutes.

The idea of incorporating dermal and epidermal cells into the skin substitutes has opened the door for more advanced therapies whereby stem cells can be incorporated into biomaterial scaffolds to promote skin regeneration. Nakagawa et al. reported enhanced cutaneous wound healing when MSCs were incorporated into a collagen-based bilayer dermal substitute (Pelnac®) and applied to rat wounds [48]. Using a collagen-glycosaminoglycan porous scaffold as a support structure for BM-MSCs, Liu et al. showed that when this skin substitute was applied to burn wounds it resulted in improved wound healing, epidermal formation, and vascularization with less wound contraction [63]. In yet another study, Wong et al. developed a pullulan-collagen hydrogel scaffold seeded with MSCs which was reported to promote cell survival and engraftment in wounds and ultimately enhance wound healing [64, 65]. The use of MSCs in skin substitutes is an area of study that is still in its infancy; however, these studies and others like them demonstrate that the marriage between biomaterial scaffolds and stem cell technologies may prove to be an essential step forward for the development of advanced wound healing therapies.

Conclusion

The skin is a complex stratified organ and upon injury, a highly integrated wound healing response is initiated to attempt to resolve the damage. Resident and bone marrow derived stem cells play an important role in enhancing this response and contribute towards proliferation and tissue remodeling. In the last decade, researchers have attempted to harness the regenerative capacity of native stem cells and novel exogenous MSC therapies for wound healing have come to fruition. Although great advancements in stem cell therapies have been achieved in recent years in the clinical setting, there are still no currently available therapies or skin substitutes that can lead to regenerated skin that completely mimic all the components of native healthy skin. Advancements at the interface of biomaterials, stem cell biology, and tissue engineering may lead us closer to reaching this goal.

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Derivation of Retinal Pigmented Epithelial Cells for the Treatment of Ocular Disease

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Age-related macular degeneration (AMD) affects an estimated 30–50 million individuals worldwide and is a leading cause of blindness in the elderly [1]. An estimated 7.2 million people in the USA suffer from AMD, which is responsible for as much as $250B in annual health care costs [2]. The disease primarily affects the macula, a small but crucial area in the central retina responsible for about 10 % of the central visual field, especially for high-acuity vision. As the disease progresses with age, patients experience blurred vision or loss of vision in the middle of their visual panorama. Early AMD can progress into two forms of mature disease. Atrophic, or "dry," AMD, which accounts for approximately 80–90 % of cases, results in areas of retinal "geographic atrophy" where photoreceptors and the retinal pigmented epithelial (RPE) cells are absent. Neovascular, or "wet," AMD is a more rapidly progressing, debilitating form characterized by choroidal angiogenesis. A majority of patients with wet AMD can be successfully treated with inhibitors of the angiogenic factor Veg-F [1], such as Lucentis or Avastin. Conversely, there are few treatment options for the dry form. Macular translocation and autologous transplants of peripheral RPE-choroid have lead to improved vision in some patients with dry AMD, but these methods are fraught with complications [3].

The appearance of abundant drusen, an extracellular deposit between Bruch's membrane (an extracellular matrix that separates the RPE from the choroid) and RPE, is an early indicator of AMD [4]. Subsequent loss or dysfunction of RPE is believed to lead to loss of photoreceptors and visual deficits [5, 6]. RPE cells are crucial support cells for the photoreceptors—they absorb stray light, transport nutrients from the blood supply in the choriocapillaris to the photoreceptors, and provide key barrier function as part of the blood/retina barrier. In addition RPE cells secrete a variety of important trophic factors, regenerate the required 11-cis retinal cofactor for rhodopsin, and phagocytose photoreceptor outer segments on a diurnal cycle [7]. Whether drusen deposits are a cause of AMD is not clear—they could be the result of defective degradative processes [8], but their presence is often a harbinger of subsequent progression of AMD. Accumulation of drusen and lipid deposits in Bruch's membrane may lead to the dysfunction and/or loss of RPE cells.

An important advance in our understanding of AMD pathology came from a flurry of papers that showed a variant of complement factor H, an inhibitor of the alternative complement pathway, predisposes individuals to AMD [9–11]. While the mechanism is still not understood, these studies suggest that a long-term inflammatory state and slowly progressing immune attack involving the alternate complement pathway may lead to loss of RPE cells, then photoreceptors.

In addition to AMD, certain forms of Retinitis Pigmentosa and Leber's Congenital Amaurosis are believed to be caused by defective RPE. Loss of the phagocytotic activity via mutations in the Mer Tyrosine Kinase (MERTK) gene or loss of the retinoid cycle enzyme RPE65 can result in subsequent loss of photoreceptors and blindness [12]. Exciting advances have been made for RPE65 defects using gene therapy [13], although these treatments are in early stages of clinical trial.

There is a clear, unmet medical need for new treatments for atrophic AMD that can replace the RPE via cell transplantation. Development of any cell therapy has the inherent challenges of isolating the cells of interest, ensuring cell survival and integration, preventing immune rejection, and precluding

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unwanted cell proliferation and tumor formation. However, the eye has distinct advantages over other tissues for establishing cell therapies. First, relatively low numbers of cells might be needed. It is estimated that about 120,000 RPE cells in a monolayer could cover the entire 6 mm diameter macular region. Second, refined surgical techniques are available for access to the back of the eye and the subretinal space where RPE cells might be delivered. Third, noninvasive methods for imaging the retina, such as Optical Coherence Tomography (OCT), Autofluorescence (AF), and novel methods using adaptive optics, may allow surgeons to follow transplanted cells and assess survival of photoreceptors. Finally, excellent endpoint parameters for measuring visual acuity exist, including old standards like the Amsler Grid and the Eye Chart as well as more sophisticated methods like microperimetry.

Proof of concept has been provided by previous studies in both animal models and human patients that have demonstrated efficacy. Several studies have been conducted using the Royal College of Surgeons (RCS) rat, which harbors a mutation in the merTK gene, leading to a defective phagocytosis by the RPE. These animals are born with vision, but lack of phagocytosis results in blindness due to photoreceptor degeneration over the first 3 months of life. While not an ideal model of AMD (rats actually lack a macula), the RCS rat has proved to be a useful model of RPE dystrophy. It has been shown that suspensions of ARPE19 cells (a spontaneously immortalized human RPE cell line), or genetically modified human RPE cells, transplanted into the subretinal space of the RCS rat can rescue visual function [14, 15]. A variety of cell types will rescue the vision in the RCS rat, including human cortical neural progenitor cells (hNPCs) [16]. In human patients, macular translocation of the retina and autologous transplantation of RPE-choroid have been used to replace macular RPE. While results are mixed and surgical complications can occur, some patients have received significant benefit [3].

A major limitation in these approaches is the difficulty of surgical technique and a lack of an abundant source of RPE. A number of investigations have used fetal tissues, which are limited in supply and can be of ethical concern, or immortalized cell lines, which may grow uncontrollably after transplant. Ideal would be a cell source that could be expanded indefinitely, with a final formulation that could be cryopreserved, shipped to the point of care, and easily administered. Human pluripotent stem cells offer an ideal source of RPE for use in cellular therapies, as they can be expanded in vitro and many have the potential to differentiate into these cell types.

In this chapter, we review methods for production of RPE derived from human pluripotent stem cells and discuss current transplantation strategies now rapidly progressing to clinical trials.

Strategies for Differentiation of Pluripotent Stem Cells to RPE

Human embryonic stem cells (hESCs) are obtained from 5-day-old blastocysts leftover from in vitro fertilization procedures by dissecting the pluripotent inner cell mass cells [17]. hESCs can be expanded indefinitely, and thus a single vial of early passage cells could potentially treat everyone in the world with AMD. Induced pluripotent stem cells (iPSCs) are somatic cells that have been reprogrammed to become ESC-like [18, 19]. iPSCs are likewise pluripotent and theoretically infinite in supply. Adding to their appeal for use in cellular therapies, iPSCs could be derived autologously and might avoid immune rejection. However, this patient-specific, personalized medical approach might be prohibitively expensive, especially if each cell population would be required to undergo lengthy safety studies for potential tumorigenicity in animal models. (An alternative approach involving the banking of HLA matched stem cell lines seems more feasible at this point.) Although there were initial concerns regarding the use of iPSCs in cellular therapies because of potential mutations caused by genomic insertion of reprogramming vectors, multiple non-integrative systems, including the use of small molecules, have been developed that make clinical translation more feasible [20–24]. However, iPS cells have "a dark side," harboring genetic and epigenetic abnormalities, including a higher number of mutations and copy number variations than ES cells, as well as abnormal DNA methylation patterns [25–29]. Despite these potential problems, iPSCs are likely to provide a potent source of RPE for future treatments.

hESCs were first shown to be capable of differentiation into RPE by Klimanskaya et al. [30] and this has now been replicated and refined by multiple groups [31–44].

The Continuous Adherent Culture Method

A variety of protocols have been developed, which fall into two categories (Fig. 1). The first method is to allow hESCs (cultured on feeders or on feeder-free extracellular matrix-derived material) to overgrow and then remove the basic fibroblast growth factor (bFGF) that retains hESC in the undifferentiated state. This causes hESCs to differentiate into a variety of cell types. Colonies differentiate, expand, and merge, sometimes becoming multilayered in this continuous adherent culture method [30, 31, 33–35, 41]. After 1–8 weeks without FGF, pigmented spots or rings made up of RPE can be discerned in the culture, which also contains many different unpigmented cell types [30, 31, 33–35, 41]. These pigmented cells replicate and expand to the point

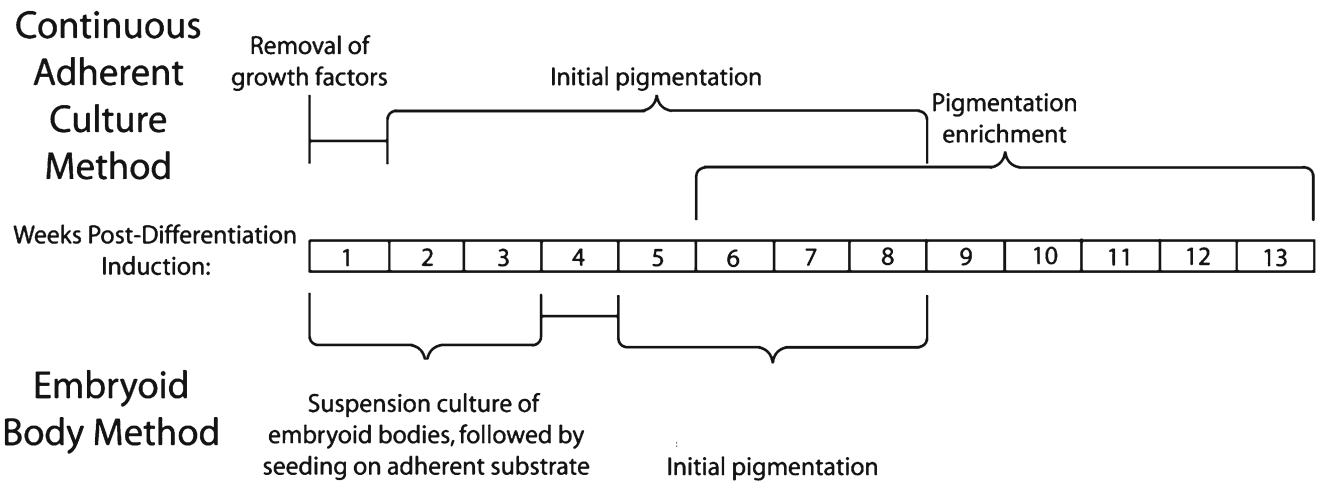

Fig. 1 Strategies for differentiation of pluripotent stem cells into RPE. In the continuous adherent culture method, cells are grown to confluence and bFGF is removed from the medium to induce differentiation. Colonies of pigmented RPE appear in a background of unpigmented cells, which can then be mechanically or enzymatically harvested. In the embryoid body method, embryoid bodies are created and cultured in suspension for 1–3 weeks, with media modified to induce differentiation, then seeded on an adherent substrate. Initial spots of pigmentation are observed between 4 and 8 weeks after the cells are placed in floating culture. Adapted from Rowland et al. [44]

where the pigmented colonies are large enough to dissect mechanically [30, 34].

One drawback to this approach is that mechanical dissection is difficult, but not impossible, to adapt to Good Manufacturing Practice (GMP) production. New methods are being developed that effect harvest and enrichment via judicious application of enzymes to selectively dislodge RPE. Such a protocol would be more amenable to GMP scale up and automation.

Once stem cell-derived RPE are isolated, it is important to demonstrate cell identity, purity, and functionality. Cells isolated using the continuous overgrowth method have been shown to be positive for RPE signature mRNAs and proteins, including cellular retinaldehyde-binding protein (CRALBP), PMEL17, and retinal pigment epithelium specific protein 65 kDa (RPE65), which are involved in the visual cycle; RPE transcription factors microphthalmia-associated transcription factor (MITF) and orthodenticle 2 isoform b homeobox protein (OTX2); tyrosinase, which functions in pigment synthesis; factors that are secreted by the RPE, such as pigment epithelium-derived factor (PEDF); membrane-associated proteins bestrophin, extracellular matrix metalloproteinase inducer (EMMPRIN), and zonula occludens 1 (ZO-1); and proteins involved in phagocytosis, including the integrin alpha V subunit and MERTK [30, 31, 33–35, 41]. The RPE65 gene is a particularly significant marker of a mature RPE phenotype, as robust expression is only detected in cultures that have formed a pigmented, cobblestone epithelial morphology. cDNA microarray analysis has revealed that hESC-RPE express a wide range of genes associated with cultured fetal human RPE, which is useful as a "gold standard" for bioinformatical comparison. Distinctions have been noted between stem cell-derived RPE and fetal RPE, and stem cell-derived RPE may represent a very early stage RPE population that matures with extended culture in vitro [41] (Hikita et al., unpublished). Care must be taken in these studies to control for differences in culture conditions, passage, and age of cultures.

hESC-RPE have also been shown to perform the functions of RPE cells, as determined by their ability to phagocytose rod outer segments or (less specific) latex beads [30, 34, 35, 41] and to rescue visual function in the RCS rat [31, 33, 36]. Some but not all iPS cell lines may differentiate into RPE using this method. Resultant iPSC-RPE expresses RPE marker proteins and mRNAs and carries out RPE functions such as phagocytosis and secretion of growth factors. Furthermore, they show visual rescue in the RCS model, with retention of the RPE phenotype and phagocytosis occurring after transplantation [34, 35, 41]. Most recently, iPSC-RPE has been shown to rescue vision in mice lacking RPE65 function, and, importantly, no tumor formation was detected over the entire lifespan of the animals [45]. iPS cell lines obtained via reprogramming with only a single factor (Oct4) in combination with small molecules have also been shown to differentiate to RPE [46].

Differentiation of RPE from hESCs was initially carried out on feeder layers of human or mouse fibroblasts, [30, 31, 33–35, 41] and it would be preferable to eliminate feeder layers. Several other substrate materials have been investigated. Gong et al. [32] used a feeder layer of mouse PA6

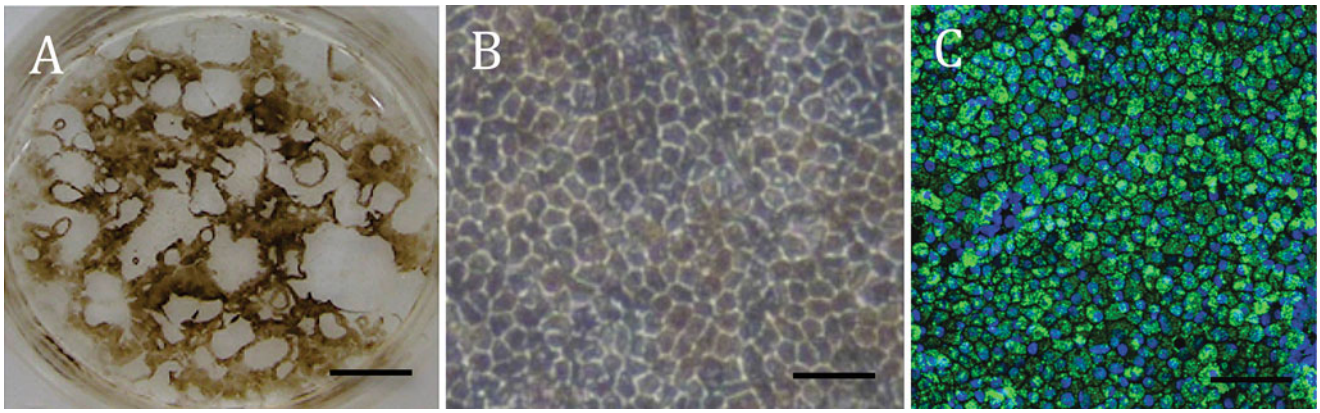

Fig. 2 Derivation of RPE from hESC. Using the continuous adherent culture method, abundant pigmented cells appear after ~100 days following removal of bFGF (**a**). These cells can then be mechanically or enzymatically harvested and expanded to generate homogeneous cultures of pigmented RPE with cobblestone morphology (**b**), which are positive for the RPE marker PMEL-17 (*green*). DAPI staining is shown in *blue* (**c**)

stromal cells to generate neural progenitor cells (positive for beta-tubulin III, paired box protein 6 (PAX6), neural filament, glial fibrillary acidic protein (GFAP), and vimentin). The neural progenitors were transferred to human Bruch's membrane explants or to Matrigel to generate pigmented cell clusters, but RPE65 and CRALBP were not uniformly detected. Other methods using Matrigel, laminin, and vitronectin have been more successful in inducing robust RPE phenotype [47]. In fact, depending on the hESC or iPSC cell line used, up to 50 % of the cells attain a pigmented, cobblestone phenotype (Fig. 2).

The Embryoid Body Approach

A second general strategy for differentiating pluripotent stem cells to RPE includes a step for embryoid body (EB), or neurosphere, formation [30, 36–40, 42]. This second approach is not as efficient as the spontaneous, continuous adherent differentiation protocol [33], but nevertheless it yields RPE. These protocols generate hESC EBs using standard methods employing low attachment plates in the absence of bFGF to induce differentiation. EBs are cultured in suspension and then aggregates are seeded onto matrix-coated plates [30, 36–40, 42]. This down-up-down strategy yields pigmented cells along with a mix of other cell types. hESC-RPE cells are generally dissected mechanically and analyzed. Studies show they express RPE markers including Mitf, ZO-1, RPE65, Bestrophin, PAX6, Otx2, and CRALBP, [36–40, 42] and manifest robust RPE phenotype as indicated from cDNA microarray analysis [36, 40, 47]. They carry out phagocytosis and rescue in the RCS rat and mouse models [30, 36, 39, 40]. IPSC also yield functional RPE using this approach [37, 39, 48, 49].

One question that arises is whether hESC or iPSC-RPE are superior for use in therapy. Feng et al. [49] reported that iPSC-RPE were significantly less proliferative than hESC-RPE. However, this may be cell line dependent as others have not reported such differences. Clearly there is great variability in the capacity for RPE differentiation among iPSC and hESC cell lines [37, 48]. For example, the H1 cell line does not pigment well using the continuous adherent culture method, yet the H9 and Shef-1 lines are champion RPE producers. Interestingly, Hu et al. [50] reported that some (but not all) iPSC derived from human fetal RPE showed a very high propensity to spontaneously redifferentiate into RPE, reflecting an epigenetic memory of their original state. Such an approach might be widely applicable as a strategy to generate large percentages of a desired cell type. Cell lines to be used for RPE manufacture must be carefully vetted to ensure reliable, reproducible differentiation.

Directed Differentiation to RPE

Efforts have been made to improve the efficiency of RPE differentiation by adding growth factors that play a role in vivo. Such approaches have been successful in a variety of cell types [51]. Starting with the EB protocol, several medium formulations have been reported to improve the frequency of RPE differentiation from hESC. Media have been supplemented with WNT antagonists (e.g., Dickkopf-1 [Dkk-1]) combined with NODAL antagonists (e.g., Lefty-A, also a TGF-beta ligand), which increases retinal progenitor cells that express the homeobox gene, Rx [38, 39, 42, 52]. Some studies have employed neural induction medium with N2 supplement and heparin while the embryoid bodies are in suspension [37, 38] or supplementation with B27 [36].

Alternatively, retinal differentiation medium formulations containing activin A or TGF-beta can be added after EBs are plated on an adhesive substrate [37, 40]. One factor that is especially effective is nicotinamide (vitamin B3), which is thought to increase production of TGF-beta superfamily factors in normal RPE development [40].

In general, the differentiation process of hESCs and iPSCs to RPE is not well defined and there is clearly room for improvement of protocols. More research will be required to understand the basic molecular and cell biology underlying RPE development. More robust protocols that achieve rapid conversion of high percentages of cells, using materials amenable to GMP production, are currently under development. It will also be important to establish assays to demonstrate RPE cell identity functionality and at the same time show that no contaminating cell types are present, especially undifferentiated stem cells.

Other Sources of RPE

An alternative strategy to using pluripotent stem cells would be to convert adult stem cells to RPE. However, to date, it is not clear that adult stem cells can generate bona fide RPE. Human adipose-derived stem cells (ADSCs) can be differentiated into RPE-like cells using protocols involving RPE conditioned media and vasoactive intestinal peptide (VIP) [53], which promotes RPE differentiation for some cell types [54]. The ADSCs express some RPE markers, including Bestrophin and RPE65, but the cells lacked the typical epithelial cobblestone morphology and low pigmentation was observed. Further analysis of these cells, with direct comparison to bona fide fetal RPE, is needed. Human retinal ciliary margin cells derived from early postnatal eyes can be induced to differentiate into RPE [55]. The cells were grown as neurospheres and then differentiated on laminin-coated substrates in media supplemented with FBS and different growth factors for 3 weeks. Approximately 1 % of cells were RPE65 positive, but further studies will be needed to characterize these cells. At present, pluripotent stem cells have a clear advantage over adult stem cells for the production of RPE.

Transplantation Strategies

A key question in the field is how best to deliver stem cell-derived RPE to allow cell survival and proper integration and function? When a suspension of RPE derived from hESCs [31, 33, 40] or iPSCs [56] is injected into the subretinal space of the RCS rat, photoreceptor death can be prevented and visual function rescued. Lu et al. [36] produced GMP- compliant suspensions of hESC-RPE and injected them subretinally into the RCS rat and a mouse model of Stargardt's disease. At least a few cells survived for over 220 days, maintaining some visual function during this time. Importantly, no teratoma formation or pathological reactions were observed over the lifespan of the animals [36].

One drawback of suspensions is that only a small percentage of the injected cells integrate into the endogenous RPE layer—most cells form aggregates in the subretinal space. These clusters are not positioned properly and only a small number can carry out phagocytosis [35, 56]. From studies of basic cell biology, it is well known that epithelial cells require an appropriate extracellular matrix substrate to survive and polarize, otherwise they undergo apoptosis in a phenomenon Ruoslahti and colleagues called anoikis (the Greek word for homeless) [57]. While difficult to quantify, it seems likely that the majority of cells die after bolus injection of suspensions [43].

To overcome these limitations, efforts have been made to develop scaffolds for transplantation to provide a substrate for RPE [58]. Such a strategy may be especially important in AMD patients, where Bruch's membrane may be compromised [59]. This would allow culture of mature, intact, polarized RPE sheets that could be implanted by surgeons. Natural biomaterials, such as collagen, fibrin, alginate, and hyaluronic acid, have the advantages of biocompatibility and biodegradability, and they may provide adhesive cues similar to those found in Bruch's membrane. RPE express abundant integrin ECM receptors and thus are capable of adhering to many substrate formulations. Human fetal and stem cell-derived RPE have been cultured on a variety of ECM extracts and proteins, such as Bruch's membrane explants [60, 61], corneal endothelial cell ECM [62, 63], RPE ECM [62], amniotic membranes [64–66], and Matrigel [67], which is derived from the mouse Engelbreth-Holm-Swarm tumor. Human RPE have also been cultured on substrates made of gelatin, collagens [60, 62, 67–70], vitronectin [60, 61], laminins [60, 61, 63], fibronectin [61, 63], or fibrinogen [71]. One concern about using natural materials is that many are of animal origin, which makes GMP compliance more difficult. Furthermore, sourcing and lot to lot variation presents an added challenge for manufacturing.

In contrast to naturally occurring materials, synthetic, biomimetic scaffolds can be modified and optimized for use with RPE. For example, adhesive peptides or growth factors might be embedded into biodegradable, biocompatible materials that could also sense and report cellular health. Synthetic materials would also have the advantage of scalable synthesis that would reduce lot to lot variability. Both biodegradable and nonerodable synthetics have been explored, including parylene, poly(lactic-co-glycolic acid) (PLGA), poly(L-lactic acid) (PLLA), polycaprolactone (PCL), poly(glycerol sebacate) (PGS), and polyhydroxyalkanoates

(PHAs) [58]. Human RPE has been successfully cultured on PLGA [72–76], PLLA [72, 76, 77], polyethylene glycol (PEG)-PLLA [74], polydimethylsiloxane (PDMS) [78], poly(hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) [79], polyether urethanes [80], methacrylate hydrogels, (meth)acrylamide hydrogels [81], and parylene [82]. Ideally xeno-free culture systems could be combined with xeno-free synthetic scaffolds for transplantation [48, 83].

Onward to Clinical Trials

Recently, Advanced Cell Technology received FDA approval for a Phase I/II clinical trial to treat patients with AMD by injecting a suspension of RPE derived from hESCs following the EB method described above, after showing its safety in preclinical models [36]. After 4 months, results from one Stargards patient and one atrophic AMD patient showed no sign of tumorigenesis or adverse effects [84]. Some modest improvement was reported, but this interpretation is controversial. Further, long-term follow-up studies are needed with proper control groups and larger numbers of patients. Other trials using a range of different approaches are also under way or will soon begin. Investigators engaged in the development of cellular therapies using stem cells face many difficult challenges. However, the eye may be a good place to start.

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Stem Cells and Diabetes

Der-I Kao and Shuibing Chen

Introduction

Diabetes is a polygenetic and chronic disease affecting approximately 346 million people worldwide. Uncontrolled diabetes results in hyperglycemia and over time leads to serious damage to many of the body's systems. In 2004, an estimated 3.4 million people died from the consequence of high blood glucose. The World Health Organization projects that diabetes deaths will double between 2005 and 2030 [1]. There are mainly two types of diabetes: type 1 and type 2. Pancreatic endocrine cells, particularly β cells, play a central role in the progression of both types of diabetes. It is believed that type 1 diabetes results from autoimmune destruction of β cells [2]. In the progression of type 2 diabetes, β cells undergo many complex changes and β cell mass declines gradually: β cells first compensate to hyperglycemia by secreting more insulin; once fail, β cells become dysfunction with the marked impairment of insulin secretion and phenotypic changes; finally, β cells become apoptotic [3]. Current data suggested that 72 % recipients of islets transplantation became insulin-independent [4]. However, the limited supply of immune-compatible cadaver islets/pancreas is one of the obstacles that must be overcome if islet transplantation is to benefit a larger number of diabetes patients. Establishing new and novel sources of islets is necessary for more general application of replacement therapy for diabetes.

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History of Islet and Pancreas Transplantation and Challenges

The first successful pancreatic transplant surgery in humans was achieved in 1966 for type 1 diabetes mellitus at University of Minnesota by Lillehei and colleagues [5]. Since then, numerous studies have been conducted to optimize the transplantation procedures and immunosuppressive protocols to improve engraftment and patient survival. The islets of Langerhans are the region of pancreas containing endocrine cells. Islet transplantation was developed later and the first clinical transplantation to treat diabetes patients was reported in 1977 [6]. In 2000, the development of Edmonton Protocol makes it possible to apply islet transplantation to broader applications [7]. However, the limited supply of immuno-compatible islets, which currently primarily depends on cadaveric organ donations, is the major obstacle that precludes its widespread application. This requires the development of efficient strategies to obtain functional pancreatic islet cells, particularly β cells.

Sources of Stem Cells to Derive Pancreatic β Cells

To overcome the shortage of available pancreas or islets for transplantation, several types of stem cells have been used to make pancreatic β cells, including human embryonic stem cells (hESCs)/induced pluripotent stem cells (iPSCs), pancreatic stem/progenitor cells, and non-pancreatic stem cells. There is also evidence of adult β cells regeneration through β cell replication and cellular reprogramming. In this chapter, we will discuss all of these approaches.

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Direct Differentiation of hESCs/iPSCs

Human pluripotent stem cells, including hESCs and iPSCs, have a virtually endless replicative capacity and have the potential to differentiate into most, if not all, cell types in human body [8]. In principle, they could provide an unlimited supply of starting material to derive functional pancreatic β cells, which could be used for both replacement therapy and disease modeling of diabetes. To realize this potential, it is essential to establish an efficient strategy to direct differentiation of hESCs/iPSCs into functional pancreatic β cells; this has not yet been achieved in vitro. Embryonic development provides important insights into key stages in hESC/iPSC differentiation [9, 10]. By mimicking signals used during embryonic pancreatic development, to the extent that they are known, a stepwise protocol is being explored to differentiate hESCs/iPSCs into functional pancreatic β cells. This involves directing ESCs first to form definitive endoderm, then pancreatic progenitors, followed by formation of endocrine progenitors, β cell precursors, and finally mature β cells (Fig. 1).

Two approaches have been used to establish the stepwise differentiation of hESCs/iPSCs into specific cell types. One is the candidate approach, recapitulating the key events of embryogenesis *in vivo* to guide hESC/iPSC differentiation into a specific lineage. The other one is the screening approach, which uses high throughput or high content screens to identify the small molecules and/or growth factors controlling stem cell differentiation.

The first stage of the stepwise protocol is the generation of the proper endoderm cell population that could be further specified into the pancreatic fate. D'Amour et al. showed that the treatment on hESCs with Wnt 3a and activin A results in ~70 % cells expressing SOX17, a marker of definitive endoderm [11]. In addition, chemical screening has been applied to search for small molecules to replace growth factor activin A. One high content chemical screen using mouse ESCs tested a library containing 20,000 compounds. This screen identified stauprimide, which substantially increased the number of endoderm cells in the presence of low level of activin A. The follow-up experiments suggested that stauprimide functions through sensitizing ESCs to a variety of differentiation signals [12]. Another chemical screen was performed in the absence of activin A to ensure that the identified small molecules induce endoderm differentiation without activin A. After screening 5,000 chemical compounds, two compounds, named IDE-1 and IDE-2, were identified. Both of them induced the differentiation of mouse and human ESCs toward definitive endoderm in the absence of Wnt 3a and activin A treatment. Alternations in cell fate produced by IDE-1 and IDE-2 compounds resulted from the activation of the TGFβ signaling pathway [13].

Following gastrulation, the pancreas derives from two patches of epithelium that bud dorsally and ventrally at approximately mouse embryonic day E9. Lineage tracing experiments [14] and gene targeting of *Pdx1* [15, 16] show that embryonic Pdx1$^+$ pancreatic progenitors are the common progenitors for the entire pancreas, including duct, exocrine, and endocrine tissues. Attempts to use FGF7 or FGF10, cyclopamine-KAAD (a hedgehog inhibitor), retinoic acid, dorsomorphin (a BMP inhibitor), SB431542 (a TGFβ inhibitor), and/or Noggin (a BMP inhibitor) for *in vitro* differentiation have produced a heterogeneous population from hESCs [17–20]. An unbiased chemical screen was performed using a starting population containing 70 % of endodermal cells. After screening a library containing 5,000 compounds, (−)-indolactam V was identified as a top candidate to increase both number and percentage of pancreatic progenitors. The mechanistic studies suggested that (−)-indolactam V functions through the activation of protein kinase C (PKC), although the most relevant PKC isoform has not been identified [21]. In addition, (−)-indolactam V has been used to promote the generation of pancreatic progenitors from iPSCs derived from type 1 diabetes patients [22] and healthy human fibroblasts [23]. The current differentiation protocols produce a heterogeneous population, containing 50–80 % PDX1$^+$ cells. This heterogeneous population is able to differentiate into glucose-responding cells and can protect mice against streptozotocin-induced hyperglycemia after transplantation into SCID-Beige mice [19]. More recently, the same group reported that the purified pancreatic progenitors show a similar differentiation potential as the heterogeneous population, which are able to become glucose-responding cells after being transplanted into immunodeficient mice and can protect mice from streptozotocin-induced glucose intolerance [24]. This result will allow us to study the components within the *in vivo* environment that support β cell maturation.

During pancreatic development, the pancreatic progenitors differentiate into endocrine, exocrine, and duct lineages [14]. A key regulator of endocrine development is the bHLH protein Neurogenin3 (Ngn3), which is expressed exclusively

Fig. 1 Stepwise differentiation from hESCs/iPSCs to pancreatic β cells

in endocrine precursors and subsequently down-regulated during differentiation [25]. Several signals are critical to endocrine development, including Delta-Notch and TGFβ [26–28]. Nostro et al. showed that inhibiting the BMP signaling pathway by using chemicals or proteins biased hESC-derived pancreatic progenitor population to become endocrine progenitors [29], which significantly increases the efficiency to make c-peptide$^+$ cells. C-peptide, the by-product of insulin biosynthesis, is commonly used as a measurement of insulin gene expression. In addition, Kunisada et al. suggested that the combinational treatment with forskolin (a protein kinase A activator to elevate cAMP levels), dexamethasone (a corticosteroid), Alk5 inhibitor II, and nicotinamide promotes differentiation to pancreatic endocrine cells [20].

The next stage is the specification of endocrine progenitors into insulin-expressing β cell precursors. β cells competence factors include the NK-homeodomain genes *Nkx2.2* and *Nkx6.1*. Glucagon-like peptide 1 (GLP-1) receptor signaling [30], insulin signaling [31], and PI3K/AKT signaling [32] are involved in the survival and proliferation of adult β cells. However, little is known about the extrinsic signal that directs the differentiation from endocrine progenitors to β cells during mouse embryogenesis. Although different growth factors or chemicals, including exendin 4 (a glucagon-like protein receptor agonist), DAPT (a g-secretase/Notch inhibitor), HGF, IGF-1, bFGF, or nicotinamide [17, 18], are expressed during the differentiation from PDX1$^+$ pancreatic progenitors to β cells, there is no strong evidence to suggest the effectiveness of these factors on hESC/iPSC differentiation.

The final stage is the maturation of β cells to acquire the activity of glucose-stimulated insulin secretion (GSIS). To accomplish GSIS, β cell precursors need to develop the machinery for glucose transport (such as GLUT2), glucose sensing (such as glucokinase), insulin processing, and exocytosis (such as PCSK1 and 2) [33, 34]. MafB and MafA may partially contribute to this activity during development or in response to glucose stimulation [35]. Recently, Blum et al. showed that functional β cell maturation is marked by an increase in the glucose threshold for insulin secretion and the expression of urocortin3, a marker specifically expressed in mature β cells [36]. Currently, GSIS cannot be achieved by any *in vitro* differentiation protocol to a level similar to adult islets, but only by *in vivo* implantation [19]. Therefore, the induction process of late stage β cell maturation is still unknown and needs to be studied further.

Pancreatic Lineage Differentiation of iPSCs

In 2006 and 2007, two laboratories using two different sets of transcriptional factors (*OCT4*, *SOX2*, *KLF4*, and *cMYC* from the Yamanaka group [37]; *OCT4*, *SOX2*, *NANOG*, and *LIN28* from the Thomason group) [38] independently showed that adult cells can be reprogrammed to a pluripotent stage. These cells, termed induced pluripotent stem cells (iPSCs), carry the genetic mutations of patients and, theoretically, have unlimited proliferation ability. In the last several years, iPSCs have attracted tremendous attention because of their potential translational applications in replacement therapy and disease modeling. To realize these applications, iPSCs need to be differentiated into functionally specific cell types. Insulin-expressing cells have been generated from iPSCs derived from mouse, human, and rhesus monkey, by applying the strategies combining growth factors and chemicals identified using both the candidate and screening approaches. The stepwise differentiation approach have been applied to human iPSCs (hiPSCs) derived from both type 1 diabetes patients and healthy controls to become insulin-secreting cells [22]. Using a similar stepwise protocol, Tateishi et al. showed that hiPSCs derived from healthy skin fibroblasts can be differentiated into c-peptide expressing cells under serum-free and feeder-free conditions [39]. Another group used slightly different protocols to make glucose-responsive cells from skin fibroblast-derived iPSCs [23]. More importantly, these c-peptide expressing cells are capable of secreting human c-peptide upon glucose stimulation although the secretion level is far lower than adult human β cells [39]. Santamaria et al. made c-peptide expressing cells from iPSCs derived from keratinocytes using an embryoid body-based protocol [40]. To evaluate the degree of maturation, it will be informative to compare glucose-stimulated insulin response in iPSCs-derived cells to human islets in the future. More recently, Alipio et al. differentiated iPSCs-derived insulin-secreting cells from mouse skin fibroblasts and successfully engrafted these insulin-secreting cells to restore hyperglycemia in types 1 and 2 diabetes mouse models [41]. In addition, an iPSC line created from rhesus monkey was shown to be able to differentiate into insulin-expressing cells through a stepwise process [42]. Although hESCs and iPSCs are derived using different approaches, no systemic comparison of pancreatic differentiation potentials between hESCs and iPSCs has been done yet. Based on the current data, the difference between hESCs and iPSCs is not more significant than the difference between hESC lines. The iPSCs carrying the genotype responsible for the human disease provide a disease model for further study of the initiation and progression of type 1 diabetes [43].

Challenges in hESC/iPSC Differentiation into Pancreatic β Cells

One future application of hESCs-/iPSCs-derived pancreatic β cells is to replace human islets in cell therapy for diabetes. The hESCs-/iPSCs-derived population will protect mice from glucose intolerance induced by streptozotocin [19] or rescue hyperglycemia in type 1 or type 2 diabetes models [41]. Despite the current successes of directing hESCs/iPSCs

into insulin-secreting cells in vitro, there are still challenges that need to be overcome to use hESC-derived cells for clinical therapy.

Firstly, the insulin-secreting cells derived using current published protocol often express multiple endocrine hormones, such as insulin and glucagon; therefore, these cells did not resemble mature pancreatic β cells. In addition, the amount of insulin produced in hESC/iPSC-derived insulin-secreting cells is much lower than adult β cells. These insulin-secreting cells do not respond to glucose stimulation in the same way as adult pancreatic β cells [24]. We need to further optimize the *in vitro* stepwise differentiation protocols. Our knowledge of pancreatic development after the organogenesis stage is limited. The application of current knowledge has not yet established an efficient protocol to derive mature functional β cells. Previous success in identifying the small molecules directing differentiation toward definitive endoderm and pancreatic progenitors is sufficiently encouraging to perform chemical screens at later stages during differentiation, including the generation of endocrine progenitors, β cell precursors, and mature β cells.

Secondly, the undifferentiated cells in the hESC/hiPSC-derived heterogeneous population might form teratomas after transplantation. Although identifying the novel signals required for increased differentiation efficiency can help to solve this issue, it is still challenging to establish a strategy to differentiate hESCs/iPSCs into functional pancreatic endocrine cells at 100 % efficiency. Identifying cell surface markers that are specific to adult human β cells for purification could be one approach to purifying a homogeneous pancreatic endocrine population. On the other hand, teratoma forming cells may be removed from heterogeneously differentiated cells by immunodepletion with antibody against SSEA-5 [44].

Thirdly, additional work is required to optimize the reprogramming process. Most of the iPSCs that are able to differentiate into insulin-expressing cells were derived using retroviral or lentiviral systems, which might introduce genetic changes resulting in deleterious consequences. Recently, several nonintegrating or excisable virus-free methods to deliver reprogramming factors, such as RNA delivery or protein transduction [45–49], have been created and need to be validated in cells of diabetes patients.

Lastly, the microenvironment needed to support and maintain grafted human or rodent islets has also been studied vigorously, and the results may be applied in future hESCs-derived pancreatic β cell transplantation. Vasculature niches provide an environment for insulin expression and β cell proliferation [50, 51]. Therefore, revascularization in implanted islets or hESCs-derived β cells is critical for not only nutrients and oxygen supply but also the intact functions of β cells. Revascularization in implanted islets may be achieved by enhanced expression of VEGF or co-transplant with mesenchymal stem cells (MSCs) [52–54]. In addition to vasculature, extracellular matrix components and 3-D scaffolds have been shown to facilitate the proliferation, survival, and insulin secretion of islets or purified β cells [55, 56].

Type 1 diabetes is an autoimmune disease. Although hESC/hiPSC-derived β cells will provide a novel resource to overcome the current shortage of human islets for transplantation, there are additional challenges needed to be resolved, particularly blocking the autoimmune response. As a promising strategy, encapsulation of grafted islets has been applied in human patients to minimize immune response [57, 58], especially to escape from autoimmune response in type 1 diabetes. In conclusion, the microenvironment of transplants needs to be carefully engineered to increase the success of hESC/iPSC cell therapy.

Other Stem Cell Sources to Derive Pancreatic β Cells

Adult Pancreatic Stem Cells

Adult pancreatic stem/progenitor cells with clonogenic potential and multipotency would be another useful resource to derive pancreatic β cells. The existence of pancreas adult stem/progenitor cells was mainly studied in β cells regeneration models, including injury, pregnancy, or insulin resistance. For example, the differentiation and proliferation of pancreatic duct cells have been suggested as the major source of β cell neogenesis using the pancreatic duct ligation model of injury [59]. The activation of $NGN3^+$ endocrine progenitors residing in the duct lining contributes to β cell mess after partial duct ligation in adult mice [60]. Lineage tracing experiment also showed that carbonic anhydrase II expressing duct cells could act as progenitors that give rise to both new islets and acinar cells after birth or after ductal ligation injury [61]. To make these adult pancreatic stem/progenitor cells a useful resource to derive functional β cells, efficient strategies need to be established to isolate and expand these adult pancreatic stem/progenitor cells and to differentiate them into β cells. Pancreatic duct epithelial cells were isolated and induced *in vitro* to become function islets, that responded to glucose challenge and reversed insulin-dependent diabetes [62]. Seaberg et al. reported the clonal identification of multiple precursor cells from adult mouse pancreas. Upon differentiation, individual clonal colonies produced distinct populations of endocrine, exocrine cells, as well as neurons and glia. The β-like cells showed glucose-dependent responsiveness and insulin release [63]. Suzuki et al. used perspective isolation and clonal analysis to identify pancreatic stem cells, which are able to differentiate into pancreatic endocrine and exocrine cells following transplantation [64].

Most of the current studies on adult pancreatic stem/progenitor cells are still at the proof of principle stage of manipulating endogenous adult pancreatic stem/progenitor cells into β cell lineage. More work needs to be done to solve the

challenges to purify, expand these populations, and induce β cell differentiation without genetic manipulation.

Adult Non-pancreatic Stem Cells

Adult stem cells, such as hematopoietic stem cells (HSCs) and MSCs, have the ability to proliferate and replenish damage tissues and dead cells. Adult stem cells also have the potential to be utilized in autologous cell replacement. HSCs are highly proliferative in adult bone marrow and there are well-established purification and transplantation protocols to treat multiple blood disorders. After autologous HSC transplantation in newly diagnosed type 1 diabetes mellitus patients, Couri et al. observed increased blood c-peptide levels and insulin independence [65]. In another study, Zhang et al. also suggested that acute HSC transplantation can improve the islet function in type 1 diabetes patients, but the mechanism may be through the elimination of the islet-specific autoreactive T cells but not transdifferentiation into β cells. Moreover, several other reports suggest that after transplantation, HSCs have a limited capacity to contribute to insulin-secreting cells, but may facilitate survival and stimulate proliferation of existing β cells [66–71]. Therefore, the ability of HSCs to directly differentiate into pancreatic β cells after transplantation is still controversial.

Bone marrow-derived MSCs are readily available and also possess the ability to differentiate to multiple lineages. Therefore, MSCs have been used to derive pancreatic β cells for patient-specific regenerative medicine. Ianus et al. showed that bone marrow-derived cells have the capacity to differentiate into pancreatic endocrine β cells with predicted cell markers and glucose-dependent insulin secretion activity [72]. Another group also showed that bone marrow-derived MSCs are capable of transdifferentiating into insulin-secreting cells under defined conditions *in vitro* and relieve hyperglycemia after transplantation [73]. However, other groups found that after autologous transplantation, MSCs induced sustained normoglycemia and recipient β cells increase by maintaining a microenvironment to support existing β cells survival and activity [74, 75]. Therefore, MSCs play supportive roles to restore hyperglycemia in diabetic animals and differentiation to pancreatic β cells remains to be documented.

Somatic Cells to Derive Pancreatic β Cells

Replication of β Cells

Although β cells have a very low turnover rate during adult life, adult β cells may be stimulated to proliferate to maintain β cell mess in response to physiological changes. Using the lineage tracing experiments, Dor et al. showed that preexisting β cells, rather than adult pancreatic stem cells, are the major source of new β cells during adult life and after pancreatectomy in mice [76]. In addition, Nir et al. used a diabetic mouse model and found enhanced proliferation of existing β cells played the major role in regeneration [77]. These studies suggested that terminally differentiated β cells retain a significant proliferative capacity. The β cell mass is dynamic and balanced by β cell formation (replication and neogenesis) and β cell death (senescence and apoptosis). Human pancreatic β cells have the ability to proliferate under specific physiological states, such as pregnancy [78], obesity, and in cases of insulin resistance [79]. Research done in a rodent islet model has shown that growth hormones, placental lactogen, prolactin, GLP-1, and glucose stimulate a small population of β cells to replicate [80]. Attempts at expanding human islet *ex vivo* are being done to obtain β cells for replacement therapy [81]. Human recombinant prolactin improves human β cell survival *in vitro* [82]. To identify small molecules that induce β cell proliferation, high throughput screening was carried out using immortalized mouse β cells. A number of structurally diverse molecules were identified that promote β-cell replication, including novel Wnt signaling agonists and L-type calcium channel (LTCC) agonists [83]. More recently, another group used small molecule screening and reported that a class of adenosine kinase inhibitors selectively enhances rodent and porcine islet β cells replication but not other cell types *in vitro and in vivo*. However, the β cell proliferation effect of adenosine kinase inhibitor was not tested in human islets [84]. In conclusion, more efforts need to be made to achieve β cells expansion *in vivo or in vitro* to treat diabetes mellitus.

Reprogramming of Pancreatic Lineage

Pancreatic exocrine cells, duct cells, and other endocrine cells have been investigated for their abilities to contribute to β cells because they share more development similarity with β cells than other somatic cells. The Melton lab introduced *Ngn3*, *Pdx1,* and *MafA*, which are essential for β cell function, to reprogram mouse exocrine cells into cells resembling β cells *in vivo*. The induced β cells can ameliorate hyperglycemia by remodeling local vasculature and secreting insulin [85]. In addition, rat exocrine cells are able to transdifferentiate into β cells in the presence of EGF and LIF *in vitro* at low efficiency. After transplantation, these exocrine-derived β cells restored normoglycemia [86]. Lineage tracing results suggested that mouse pancreatic acinar cells can transdifferentiate into insulin-secreting cells with secretory properties similar to those of native pancreatic β cells, and that activation of EGF signaling is required in such transdifferentiation [87].

Duct cells could be the source of adult pancreatic stem/progenitor cells, as mentioned earlier. Duct cells isolated from adult human pancreas can be reprogrammed to express islet β cell genes by adenoviral transduction of the developmental transcription factor *NGN3* [88].

Endocrine reprogramming of α cells to β cells can be induced by β cell loss and exogenous gene expression. Lineage tracing data showed a large fraction of β cells derived from glucagon producing α cells after β-cell ablation, revealing a novel level of pancreatic cell plasticity [89]. *Pdx1* could induce context-dependent α cells reprogramming to β cells [90].

Reprogramming from Other Adult Cells

Deriving β cells from other somatic cell types with proliferation activity also attracts attention and effort. The dogma in development biology is that once adult cells become terminally differentiated, they cannot turn to another cell fate. However, this has been challenged by several recent studies to show that adult cells can be reprogrammed again to other cell types, such as pancreatic β cells. Moreover, directly reprogrammed somatic cells have strong potentials in developing patient-specific regenerative medicine.

Adult hepatocytes, which share foregut endoderm origin with pancreas, have been successfully reprogrammed into β-like cells. In 2000, Ferber and Karasik et al. reported that by introducing the *Pdx1* gene, hepatocytes are able to express active insulin and to ameliorate hyperglycemia in diabetic mice treated with streptozotocin. The protein encoded by *Pdx1* gene plays a central role in regulating pancreas development and islet cell function [91]. In 2005, the same group engineered adult human liver cells to c-peptide-secreting cells in response to glucose concentration and rescue hyperglycemia in a rodent model, by introducing ectopic *PDX1* and supplementing EGF and nicotinamide [92]. The efficiency of reprogramming can be improved by the addition of exendin-4 and *NKX6.1* genes [93, 94]. Other groups used *NeuroD* and/or *Ngn3* together with the *Pdx1* gene to reprogram rodent livers *in vivo* to insulin producing cells, which corrected hyperglycemia in diabetic animals [95, 96]. In addition to hepatocytes, ectopic *Pdx1* expression reprogrammed keratinocytes to insulin-expressing cells [97].

Closing Remarks

Recent breakthroughs have exploited cell plasticity to generate pancreatic β cells from various cell sources. Currently, hESCs/iPSCs are the most promising stem cell source for deriving pancreatic β cells, but differentiation to mature β cells has not yet been achieved. Adult pancreatic stem cells need to be further exploited as a practical source in regenerative medicine. Adult non-pancreatic stem cells may support endogenous β cells survival and function through indirect effects rather than differentiation into β cells. Reprogramming of fully differentiated cells into β cells is a new field and still relies on viral delivery of transgenes, which raises another level of safety concern. Expansion of β cells could be a straightforward strategy but the efficiency will need to be improved for clinical application. In conclusion, the capability of differentiation, reprogramming, or replication of β cells will need to be improved to alleviate the shortage of pancreatic β cells in diabetes studies and therapy.

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Part V

Stem Cells and Cancer

Cancer Is a Problem of Developmental Biology: Maturation Arrest and the Hierarchical Model of the Origin of Cancer from Stem Cells

Stewart Sell and Pier Mario Biava

Introduction

In 1978, Barry Pierce et al. published a book entitled "Cancer is a problem of developmental biology" [1]. This implies that the key to understanding cancer lies in unraveling the process of how the undifferentiated cells of the embryo change into the differentiated cells of an adult (see Chaps. 1 and 3). Based on their work on teratocarcinoma, the principles cited in their book led to a hierarchical model (Fig. 1) for both normal tissue renewal and cancer [2]. In the hierarchical model, normal tissue renewal is accomplished by proliferation of transit amplifying cells which terminally differentiate into mature cells. The stem cells proliferate so that one daughter cell remains a stem cell and the other differentiates into a transit amplifying cell (asymmetric division, Fig. 2). In this way there is retention of the stem cell population as well as a continued renewal of transit amplifying cells during most of adult life. In response to increased demand for cells, such as the increased need for red blood cells when one moves from sea level to a high altitude, either stem cells will proliferate and produce more transit amplifying cells or the transit amplifying cells will produce more daughter cells before terminally differentiating, resulting in hyperplasia of the hematopoietic cell system. One theory of aging is that with time there is a gradual depletion of tissue stem cells leading to decreased production of new transit amplifying cells resulting in atrophy of aging tissues and eventually loss of tissue function.

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According to the hierarchical model, the main difference between normal tissue renewal and proliferation of cancer cells is that the transit amplifying cells of the cancer do not terminally differentiate as do normal transit amplifying cells, but continue to proliferate (symmetric division, Fig. 2). The difference between hyperplasia and cancer is that hyperplasia is self-limiting (for example, the increased production of blood cells seen at high altitudes will reverse when the individual returns to sea level), whereas cancer cells do not respond to physiologic control and continue to proliferate or do not die. However, Pierce and Wallace [3] found that even the proliferating stem cells of a squamous cell carcinoma could give rise to daughter cells that differentiate into mature keratinized cells. In normal tissue renewal there is equilibrium between the rate of proliferation and the rate of cell death so that the number of cells at any given time is relatively constant. In contrast, in cancer tissue the equilibrium is shifted in favor of proliferation over cell loss so that in cancers the number of cells continues to increase (Fig. 2). Because of this, the space occupied by the cancer tissue continues to enlarge, encroaching on and eventually forcing out normal tissue. In addition, the space occupied by the primary cancer tissue may be breached by the proliferating cancer cells, viable cancer cells released into the surrounding tissues and eventually the lymphatic or blood capillaries, resulting in growth of colonies of cancer cells at tissue sites distant from the primary cancer (metastases). The key concept is that cancers are maintained by cancer stem cells that give rise to daughter cells that do not mature at a normal rate and continue to increase in number. This is known as maturation arrest [4]. An even earlier model of cancer based on an origin from stem cells was the embryonal rest theory of cancer [5].

Embryonal Rest Theory of Cancer

The first theory of the origin of cancer was a "field theory." Field theories are based on the idea that the tissue surrounding the cells at risk (niche) provides a signal or environment

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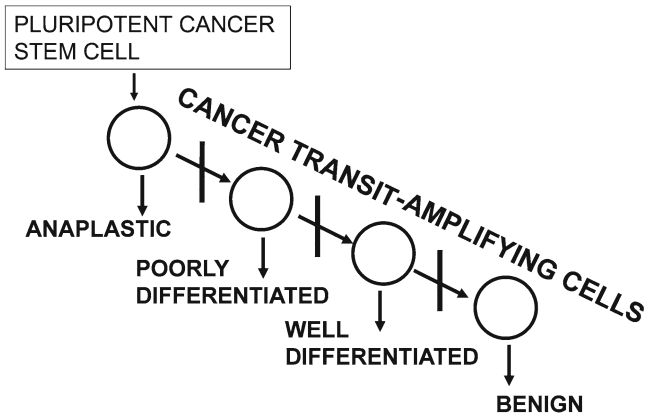

Fig. 1 Pierce's simplified hierarchal model of cancer based on concept of maturation arrest. In this model cancers arise because of a block in differentiation allowing immature cells at different stages of differentiation to continue to proliferate. If maturation arrest occurs at an early stage of differentiation the cancers will be poorly differentiated. If later, well differentiated, and even later benign tumors

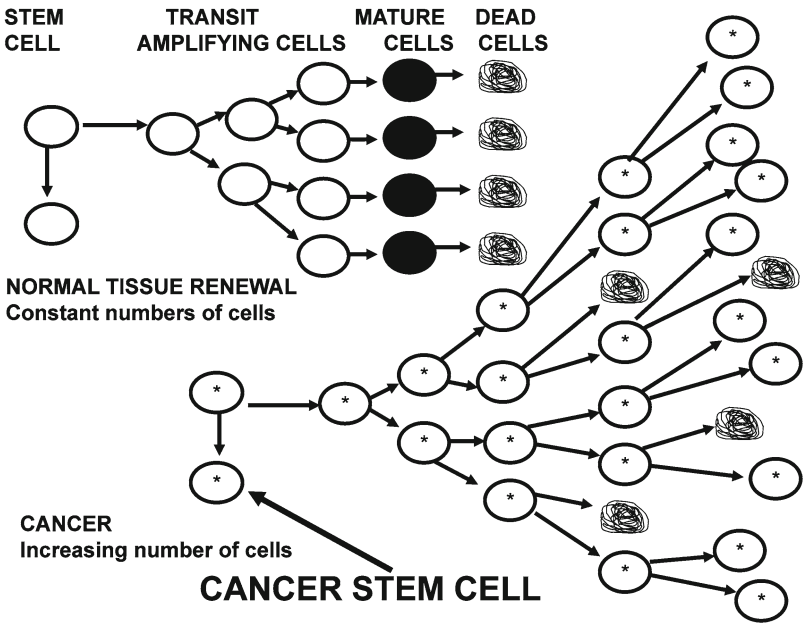

Fig. 2 Comparison of proliferation of cells during normal tissue renewal to that of cancers. During normal tissue renewal, tissue-determined stem cells divide by asymmetric division so that one daughter cell becomes a tissue amplifying cell and one remains a stem cell. The transit amplifying cells can divide by either symmetric or asymmetric division. Their proliferation contributes to normal tissue turnover when the progeny of the transit amplifying cells terminally differentiate. In cancers, some of the cancer transit amplifying cells continue to proliferate and so not terminally differentiate resulting in increasing size of the tumor

that acts to stimulate the cells to proliferate as cancer cells. In ancient Greece, at the time of Hippocrates, cancer was believed to be caused by an imbalance of humors, specifically, too much black bile. This concept held stage for over 2,000 years and variations of it remained in play well into the twentieth century [5, 6]. The first idea that cancers might arise from stem cells appeared in the early nineteenth century [7, 8]. Although the concept of stem cells was still far in the future, Durante [9] and Conheim [10] introduced the idea that cancers arise from embryonal tissue that survived in adult organs, i.e., embryonal rests. They proposed that disequilibrium between the embryonal cells in the "rest" and the surrounding tissue allowed these remnants of embryonic tissue to reassume proliferation and produce masses of cells that resembled fetal tissues. By that time the microscope was in use for examination of the cellular component of cancers and pathologists had noted that cancer tissue resembled fetal tissue. This mechanism of cancer development is consistent with a field theory, i.e., a change in the tissue stroma allows cancer to appear, but also identifies the cells of origin as stem

cells. However, by early 1900s the embryonic rest theory lost support [6] and in general interest in cancer research waned as the primary focus for research and clinical studies was infectious diseases [11]. It would take 50 more years before studies on teratocarcinoma would lead to a reassertion of the embryonic rest theory of cancer in the form of the stem cell theory of cancer.

Teratocarcinoma

Early pathologists noted that certain tumors contained mixtures of tissues that looked like malformed monsters, thus the term: Terato- (monster). Rudolph Virchow, the father of pathology, observed that teratocarcinomas were made up of an abnormal mixture of fetal and mature tissues, but he did not embrace the embryonal rest theory [12]. However, his student, Julius Conheim [10], used the resemblance of the tissue of teratocarcinomas to embryonic tissue to support the embryonal rest theory of cancer. Modern pathologists recognize that most teratocarcinomas consist of mature, differentiated tissues, as well as fetal components, such as yolk sac and placental elements [13, 14]. The production of alphafetoprotein (AFP) by the yolk sac component and of chorionic gonadotropin (CGH) by the placental elements suggests that the embryonal cells of a teratocarcinoma are totipotent, i.e., they can differentiate into both adult and embryonic cells [15]. The growth of these tumors may be followed by measuring the serum levels of AFP or CGH [16]. Most of the cells of a teratocarcinoma are mature and nonmalignant; the malignant cells are located in the embryoid body, a tissue structure that contains undifferentiated embryonal cells [17]. The cancer stem cell nature of these cells is documented by their ability to form cancers upon transplantation into histocompatible recipients.

Convincing documentation of origin of teratocarcinoma from normal tissue stem cells was obtained In the 1960s, when Leroy Stevens observed growth of malignant teratocarcinomas in recipient mice after transplanting normal germinal stem cells from the genital ridge of day 12 SIJ/129 male mice into the testicles of normal 129 adult male mice [18, 19]. In the testicular transplant niche the germinal stem cells grew abnormally and formed tumors, thus supporting both the stem cell origin of the cancer and the field theory of cancer. Then about a decade later it was demonstrated that teratocarcinoma stem cells did not grow into tumors when transplanted into a mouse blastocyst [20], but became incorporated into the developing embryos and developed into normal tissues [21–24]. The resulting adult mice had organs that were made up of a mixture of mature tissues from the normal blastocyst and from the cancer (Chimeras). The inner cell mass of the blastocyst is able to reprogram both mature tissue stem cells and cancer stem cells [25]. Thus, not only will normal tissue stem cells develop into cancer if these cells are placed in an environment which allows expression of the malignant phenotype but also established cancer stem cells may be converted to normal transit amplifying cells if placed into a reprogramming environment.

Barry Pierce and his coworkers extensively examined the cellular make up of teratocarcinomas [1, 25, 26]. From their studies, as stated above, Pierce hypothesized a hierarchical model of cancer, with cancer stem cells giving rise to cancer transit amplifying cells that would exhibit various stages of differentiation culminating in terminally differentiated cells (Fig. 1), and this hierarchical model was extended to provide a general thesis for the cells that make up any cancer [1]. They postulated that the differentiation state of a cancer depends upon the stage of maturation at which the majority of cells of the cancer become arrested. If maturation arrest occurs at an early stage the tumor will be poorly differentiated and most of the tumor transit amplifying cells will be able to divide; if at a later stage, the tumor will be well-differentiated and few of the tumor transit amplifying cells will divide. In either case, the tumor will be maintained by cancer stem cells which provide the self-renewing cells of the tumor [26]. A hierarchal model for germinal cell tumors is presented in Fig. 3. The property of cancer stem cells to resist therapy was demonstrated by the response of teratocarcinomas to differentiation therapy.

Many of the embryonic cancer stem cells of a teratocarcinoma can be induced to differentiate into mature nonmalignant cells after treatment with retinoic acids (RAs) [27, 28]. However, chemotherapy and surgery have proved to be more effective than differentiation therapy. Retinoic acids induce differentiation of the transit amplifying cells of the teratocarcinoma. However, following initial successful treatment, the tumor will regrow from the resistant teratocarcinoma stem cells. The putative teratocarcinoma stem cells are self-renewing and resistant to differentiation therapy. A general approach to more effective differentiation therapy is an approach that will activate the cancer stem cells to divide and differentiate so that their progeny will be sensitive to therapy. This may explain the effective treatment of leukemia by cycles of radiation or chemotherapy (see below).

The following principles of the stem cell theory of cancer were first demonstrated using teratocarcinoma (see review [29]):

1. Cancers arise from stem cells.
2. Location in an abnormal place (niche) allows cancer stem cells to express the malignant phenotype (field theory).
3. Cancers contain the same cell populations as normal tissues—stem cells, transit amplifying cells, and terminally differentiated cells (hierarchical model of cancer).
4. Cancers may be transplanted by cancer stem cells, but not by the transit amplifying cells of the cancer (tumor initiating cells).

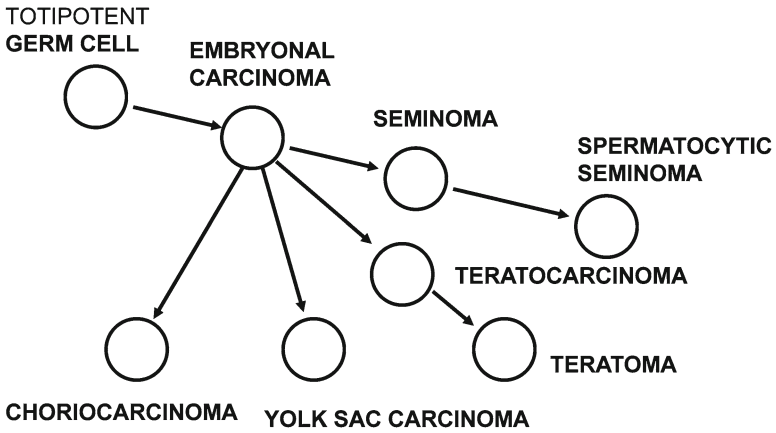

Fig. 3 Hierarchal model of male germinal cell cancers. The undifferentiated germinal stem cell gives rise to teratocarcinoma which may be composed of differentiated tissues derived from the totipotent embryonal cancer stem cells. Maturation arrest at later stages results in more differentiated cancers or even benign teratomas

5. Products of the cancer cells that reflect stages in fetal development may be used as markers for diagnosis, prognosis, and treatment (onco-developmental markers).
6. Malignant cells may become benign (differentiation therapy).
7. Differentiation therapy is directed to cancer transit amplifying cells; when treatment is discontinued, cancer regrows from resistant cancer stem cells. Resistance to therapy is a property of cancer stem cells.

Control of Differentiation by the Embryonic Microenvironment

As adumbrated above, the tissues of the developing embryo are able to induce differentiation of normal and cancer stem cells [30]. In addition, exposure of developing embryos to chemical carcinogens may lead to malformations, but not to cancer. Thus, embryonic cells that are mutagenized by chemicals that induce cancer in mature animals are prevented from expressing a malignant phenotype by the microenvironment of the developing embryo [31, 32]. As stated above, this blastocyst environment also controls growth of transplanted malignant cells, whereas malignant cells placed in other sites of the developing embryo, such as the perivitelline space, are not controlled [33–35]. In fact, the more differentiated tissues of the embryo may also regulate growth of cancer cells that are normally found in that tissue [36]. Diffusible factors produced by blastocyst cells may induce differentiation of cancer stem cells [35, 36]. The regulatory environment of the blastocyst is not limited to teratocarcinoma. Other cancers shown to be converted to normal developing tissue when placed in an appropriate embryonic microenvironment include leukemia, melanoma, hepatocellular cancer, and breast cancer [37–41]. Malignant melanoma cells placed into the extraembryonal membrane of the Zebrafish differentiated into normal neural crest-like cells [42]. Thus, different embryonal microenvironments may have different differentiation potentials related to normal cellular differentiation. For example, the placenta may also regulate transplanted leukemia cells [43] or other cancers [44, 45]. Embryo–mother cross talk may be very important in determining the arrest of tumor growth, because both maternal (decidua) and embryonic tissues contain substances with anticancer properties [46]. In addition, over 40 years ago the expression of the malignant phenotype by pluripotential nuclei from triploid frog cancer cells was reprogrammed when these nuclei are placed into an oocyte [47], and mouse embryos could be cloned from nuclei from brain tumors [48]. These results demonstrated that epigenetic regulation of tumor nuclei by nuclear transfer into an embryonic microenvironment is able to reverse the malignant phenotype: the transplanted tumor nuclei gave origin to postimplantation embryos undergoing tissue differentiation. These findings elaborate the concept that cancer is a disease of developmental biology. When we understand how the developing embryo is able to induce differentiation of cancer cells, we will have taken a major step in treating and preventing cancer.

In fact only few experiments have been made to investigate how the microenvironment of the embryo can determine the fate of cancer stem cells. These experiments, using factors taken at precise stages of cell differentiation from Zebrafish embryos, clarify which molecular events are involved in the mechanism of tumor growth inhibition. Treatment of cancer cells with proteins taken from Zebrafish embryos harvested during the stage at which totipotent stem cells are differentiating into pluripotent stem cells (50 % of cells during epiboly) induces activation of caspase 3, mainly by increasing the release of E2F-1, leading to c-Myc overexpression and the activation of a p73 apoptotic-dependent pathway. In vitro studies of different human tumor cell lines identify transcriptional or posttranslational modification of key-role cell cycle regulator molecules, such as p53 [49] and pRb [50]. Moreover, there is a concomitant increase in E-cadherin levels and normalization effect on the ratio of E-cadherin to β-catenin [51]. Based on the hypothesis that these embryo extracts induce differentiation of cancer cells,

the authors developed an agent-based model to optimize cancer treatment [52]. As already described, the concept of directing differentiation therapy to cancer stem cells or transit amplifying cells was established by principles established by studies of the teratocarcinoma model [4]. However, in the 1970s, teratocarcinoma was considered the exception to the rule. Investigators who extensively analyzed the cellular events during induction of cancer of the liver and skin were convinced that cancers arose from dedifferentiation of mature cells, but eventually more extensive study of these models revealed consistency that cancers of other tissues also arose from a block in maturation of cancer stem cells (the stem cell hierarchical model (see recent review [29])).

Chemical Carcinogenesis

Epidemiologic evidence provided the first indication that chemicals could cause cancer, and this was documented by extensive experimental work in animals. In 1761 John Hill noted the occurrence of cancer of the nasal cavity of snuff users [53]. Over 100 years later bladder and liver cancers were found to be increased in workers in the German dye industry [54]. The study of the induction of liver cancer in animals began in the 1930s and peaked in the middle of the twentieth century [55]. The finding of cellular changes in the liver during experimental hepatocarcinogenesis that preceded the development of liver cancer was interpreted as dedifferentiation of mature hepatocytes giving rise to liver cancer [56]. However, more recent observations are consistent with development of liver cancer from liver stem cells through maturation arrest.

Chemical hepatocarcinogenesis. Following exposure of rats to chemical hepatocarcinogens, foci of cells in the liver expressing fetal enzymes appear, followed by formation of nodules of large immature appearing hepatocytes that increasingly distort the liver and then cancer, sometimes appearing within the nodules [57, 58]. This sequence of changes was interpreted to indicate that liver cancer arises from dedifferentiation of mature hepatocytes through formation of foci and nodules that eventually progress to cancer. However, an alternative hypothesis is supported using a fetal protein, AFP [58], as a marker for cellular changes during chemical hepatocarcinogenesis [59, 60].

AFP is a serum protein that is barely detectable in normal blood but appears in elevated levels in the blood of newborn animals and animals with liver cancer [58]. It is also produced by the yolk sac during development and serves as a maker for teratocarcinomas that contain yolk sac elements (see above). If foci or nodules are the cellular precursors to cancer, then AFP should be found in foci and nodules. However, depending on the carcinogenic regimen used, AFP was not found in foci or nodules, but in small cells in the pericentral region of the liver (oval cells), in duct-like structures, or in adenomatous areas in the liver [58–60]. Based on the hierarchical model of Pierce, we concluded that, in adults, liver cancers could arise from stem cells (oval cells), transit amplifying cells (ducts), or mature hepatocytes depending on the stage of maturation arrest (Fig. 4).

The link between pluripotent stem cells and cancers arising from the liver lineage cells is a cancer known as hepatoblastoma [61, 62]. This cancer only appears in young children and not in adults. In fact treatment of rats at 3 weeks of age compared with 8 weeks of age results in a much higher rate of proliferation of small oval cells [63]. This result is consistent with a decrease in the number or potency of tissue stem cells with aging. In conclusion, in the liver, as in teratocarcinomas, cancers arise from arrest of cells in the hierarchy of normal maturation [1]. A similar hierarchical model may be used to classify human breast cancers (Fig. 5; [64]). The dynamics of development of squamous cell carcinomas of the skin after exposure to chemical carcinogenesis also convincingly identifies the epithelial stem cell of the skin as the cell of origin of squamous cell cancer.

Skin carcinogenesis. Cancer of the skin of the scrotum in chimney sweeps was reported in England in 1775 [65]. Chimney sweeps bathed infrequently and were constantly exposed to highly carcinogenic hydrocarbons present in soot. When bathing was recommended cancer in the chimney sweeps rapidly declined. In 1918, experimental squamous cell carcinomas were produced by painting the skin of rabbits with coal tar [66]. Chemical carcinogenesis in the rabbit skin was a two-step process: initiation and promotion. Peyton Rous painted coal tar on the skin of the ear of rabbits at a dose which did not cause carcinomas. After several weeks or more if he wounded the site where the coal tar was applied by scraping with a cork borer, skin cancers then developed [67]. The first step (initiation) is believed to cause mutations by binding of carcinogenic metabolites to the DNA of the skin cells (DNA adducts). The second step (promotion) induces proliferation as a repair response to wounding the skin. This second step activates proliferation of mutated cells that give rise to cancers. Without promotion the initiated cells remain dormant. It was later found that the time between initiation and promotion could be months or even years [68–70]. This is critical for identification of the cells involved in this process as the transit amplifying cells of the skin are replaced every 14 days [71]. Thus, if promotion is effective months or even years after initiation, the cancer must arise from the skin stem cells. It is only the self-renewing skin stem cells that are able to survive the long time between initiation and promotion. How the genetic changes in a leukemia cause accumulation of leukemic cells also proves the involvement of cancer stem cells with maturation arrest at various stages of differentiation depending on the stage of expression of the molecular lesions.

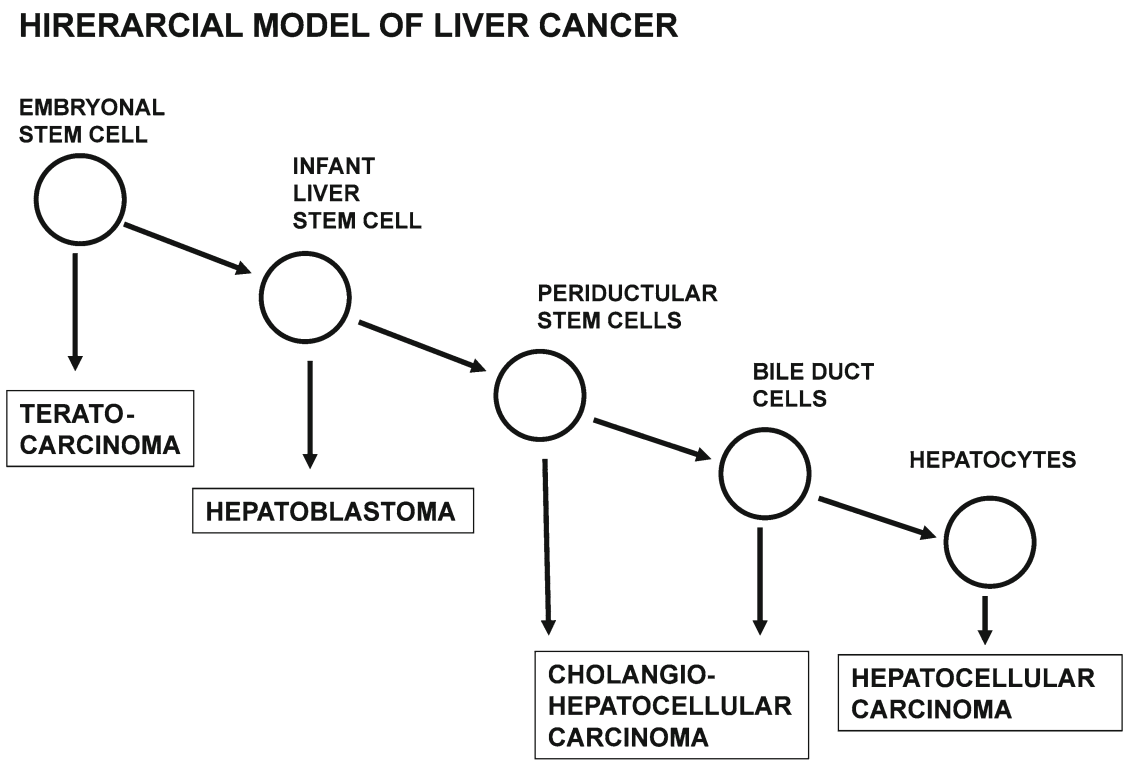

Fig. 4 Postulated hierarchal model of development of liver cancers. After exposure of rats to chemical hepatocarcinogens the tumors seen reflect the stages of maturation of liver cells

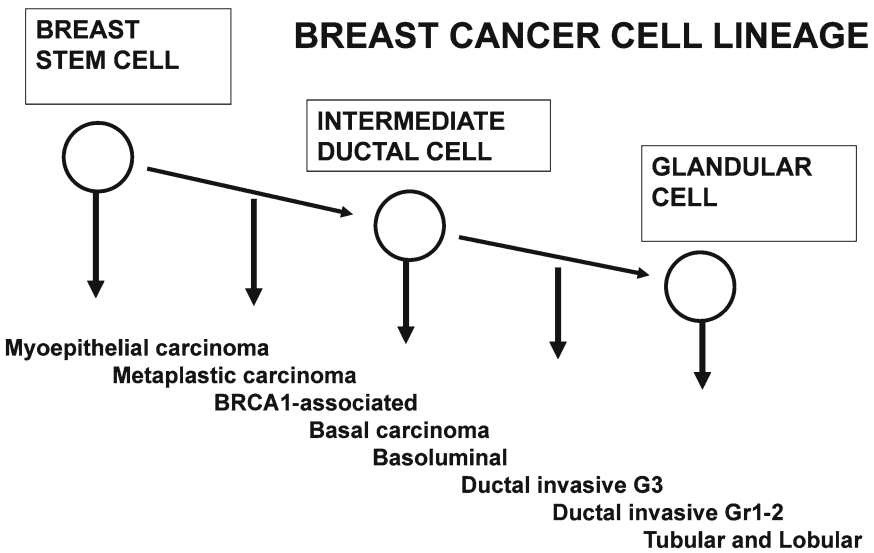

Fig. 5 Phenotypes of human breast cancers defined by cell surface markers and histology follow a hierarchal model of breast cell differentiation

Genetic Changes

There is compelling evidence that many cancers are caused by genetic lesions that produce the cancer phenotype. Examples of well characterized genetic lesions are gene translocations in leukemia and a series of genetic changes in colon carcinoma.

Leukemia. Peter Nowell and his coworkers in the 1970s [72] identified a specific gene translocation of the chromosomes of chronic myeloid leukemia (CML) cells, the Philadelphia

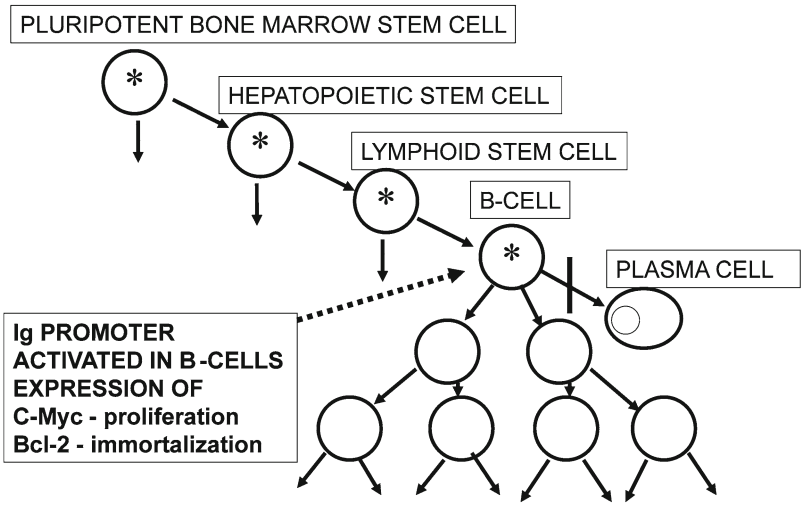

Fig. 6 Stage of maturation arrest of lymphoma in transgenic mice bearing Ig promoter linked to C-Myc and to Bcl-2 is determined by the stage of differentiation of the B-cell lineage where the Ig promoter is activated, B-cells. Although the transgene is present in all cells of the transgenic IgC-myc, Ig-Bcl-2 mouse the effects of the C-Myc and Bcl-2 are only manifested when the Ig promoter is activated

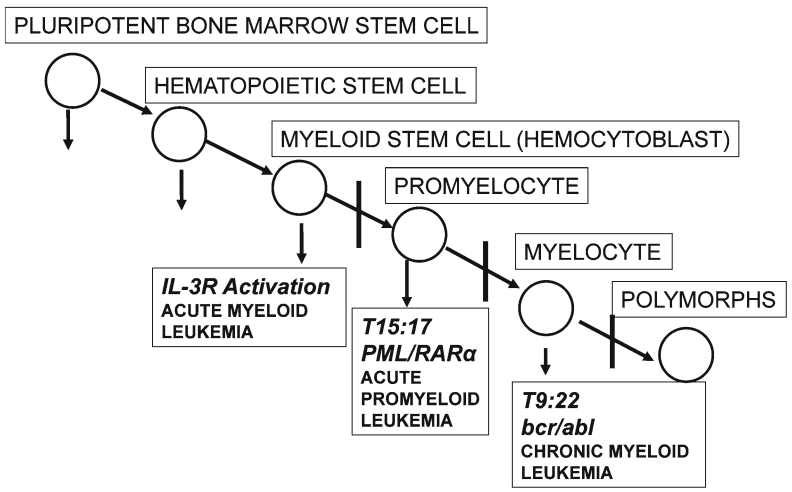

Fig. 7 Stage of maturation arrest of myelogenous leukemias is determined by activation of the genetic translocation responsible for the disease

Chromosome. For this translocation, the break point cluster region (bcr) on chromosome 22 is relocated next to a known oncogene (abl) on chromosome 9. This translocation results in production of a translocation gene product (bcr-abl) that increases proliferation, blocks apoptosis, and liberates the leukemia cells from their tissue niche. Since this discovery a large number of gene translocations have been identified in leukemia and lymphomas [73, 74]. Four will be used as examples in this section of this review: Ig-myc in Burkett's lymphoma (t8:14), bcr-abl in CML (t9:22), PML-RARα (t15:17) in acute promyeloid leukemia (APL), and the IL-3 receptor (t8:21) in acute myeloid leukemia (AML).

Burkett's lymphoma was discovered to be highly associated with infection by the Epstein-Barr virus in 1964 by Epstein et al. [75]. The continued proliferation of the cells in Burkett's lymphoma is due to translocation of the immunoglobulin promoter (Ig) next to the powerful oncogene, c-myc, with resultant proliferation of B-cells (Fig. 6). It is not clear how the virus infection results in this translocation, but an experimental model of this translocation illustrates how the function of activation of the expression of the transgene is related to the stage of maturation arrest of the lymphoma.

The experimental model is a transgenic mouse that has the Ig promoter linked to c-myc and bcl2 [76, 77]. When the immunoglobulin promoter is activated there is expression of one gene that increases proliferation (c-myc) and another that blocks apoptosis (bcl2) essentially delivering a double-whammy of cancer to increase the number of transfected cells. The key here is the linkage to the Ig promoter. The characteristic property of B-cells is production of immunoglobulin, i.e., activation of the Ig promoter. Although the transgene is present in all of the cells of the transgenic mouse it is only activated in B-cells. Thus, this translocation produces maturation arrest and proliferation at the B-cell level, a B-cell lymphoma (Fig. 6).

The translocations in myeloid leukemia also produce blocks in maturation arrest, proliferation, and survival of cells at various levels of maturation of cells in the myeloid hierarchy ([78], Fig. 7) and provide a model for application of differentiation therapy. For example, the t9:22 bcr-abl

translocation results in production of a fusion gene tyrosine kinase pathway that is constitutively activated at the myelocyte stage differentiation. This activation does not allow cells at the myelocyte level to differentiate further, so the cells continue to proliferate and accumulate (CML). This bcr-abl tyrosine kinase phosphorylates the tyrosine on a substrate in the next step of the signal transduction pathway. Brian Drucker has developed a small molecular inhibitor which blocks the adenosine triphosphate binding site of the tyrosine kinase so that it is unable to extract and transfer the phosphate. Treatment with this small molecule, Gleevec, allows the leukemic cells that reach the myelocytic stage to continue to differentiate and is an effective therapy for CML [79]. If therapy is discontinued the CML will regenerate from precursor leukemic cells in the myeloid series as the precursor cells are not affected by the therapy [80].

In acute promyelocytic leukemia (APL) cells the t15:17 translocation fusion protein (PML-RARα) is the promyelocytic leukemia protein linked to the retinoic acid (RA) receptor (RAR). PML is required for development of the cytoplasmic granules that begin to accumulate at the promyelocyte stage of myeloid differentiation. When this protein accumulates as the fusion product, it is unable to function, and the PML cells are unable to differentiate past the promyeloid stage [81]. RA (Vitamin A) binds with the RARα part of the fusion protein. This upregulates ubiquitination of the fusion protein, resulting in degradation of the fusion protein so that RA can now activate RA-induced transcription, formation of granules, and differentiation of the cells. Treatment with RA can produce complete remission in about 90 % of patients with APL [82]. Again, if therapy is discontinued, APL cells will regenerate from precursor cells containing the translocation.

In AML cells there is usually more than one translocation or mutation, one causing increased proliferation and one blocking apoptosis [83]. This results in rapid accumulation of cells at the myeloid stem cell level. Peter Nowell noted that development of one lesion leads to chronic proliferation of cells in the myeloid series setting the stage for a second mutation [83]. Thus, leukemias provide a "moving target" for finding directed therapies. One of the frequent mutations results in activation of the IL-3 receptor which is active at several stages of myeloid differentiation. Small molecular inhibitors are being developed for this lesion, as well as others, but positive clinical effects have proven elusive [84].

In any event, leukemic stem cells, like other cancer stem cells [84], are resistant to therapy and are responsible for treatment failures using radio-, chemo-, or differentiation therapy. For example, when a patient is first diagnosed with leukemia, we can estimate that there are approximately 10^{12} leukemic cells present. Assuming that treatment kills 99.9 % of the leukemic cells, after one cycle of treatment, 10^9 leukemic cells will be present. Treatment must be given in cycles as continuous treatment also kills normal blood and intestinal cells; so treatment is given in cycles to allow the normal cells to recover. Assuming there are 10^9 leukemia cells present when the next cycle is given (there may actually be more, but this is what is being assumed for the present example), treatment will result in reduction of the leukemia cell number to 10^6. So it can be seen that two additional treatments will be required to reduce the cell number to one. In about half the cases, four cycles may be curative, but in another half, the tumor will continue to regenerate from the tumor stem cells that resist therapy. In the case of cure, it is thought that destruction of the tumor transit amplifying cells by the therapy may activate division and differentiation of the tumor stem cells to tumor amplifying cells, which are affected by the therapy. For those that are not successfully treated, it may be possible to cure the leukemia by administering ablative therapy that will kill both normal and tumor stem cells and replace the function of the normal stem cells using a bone marrow transplant.

The lesions we have learned from myeloid leukemias are:

1. Molecular lesions determine the stage of maturation arrest of the leukemia.
2. Targeted differentiation can reverse the effects of the molecular lesion and allow terminal differentiation of the leukemic cells.
3. Leukemic stem cells contain the molecular lesion and restore the cancer transit amplifying cells when chemotherapy or differentiation therapy is discontinued. Note: there is a difference of opinion in regard to the nature of the therapy-resistant cancer cell. It may be a stem cell [85] or simply a tumor cell with proliferation capacity that is not in cycle at the time the therapy is administered [86]. For all intents and purposes, the resistance to therapy is essentially the same for either explanation.
4. Leukemic stem cells mutate to survive therapy and are thus a moving target for chemo- or differentiation therapy.

The key to development of cancer is a change that allows cells, that would normally mature and die, to remain in the tissues. This is illustrated by the Vogelstein model for colon cancer [87].

Colon cancer. The Vogelstein hypothesis [87] for the development of intestinal cancer states that there is a series of mutations and epigenetic changes responsible for a sequence of lesions culminating in expression of the malignant phenotype. The proffered sequence and the lesion produced are as follows: Absent in Polyposis Coli (APC)—hyperplastic polyp; hypomethylation—tubular adenoma; K-ras mutation—tubulovillous adenoma; Deleted in Colon Cancer (DCC)—villous adenoma; p53 mutation—adenocarcinoma (Fig. 8). The key item in this sequence is the effect of the first mutation, APC, in blocking the normal rapid turnover of the intestinal epithelial cells. Pulse DNA labeling studies reveal that there is a cohort of transit amplifying cells near the base of intestinal crypts that divide every day and migrate rapidly out of the crypt so that

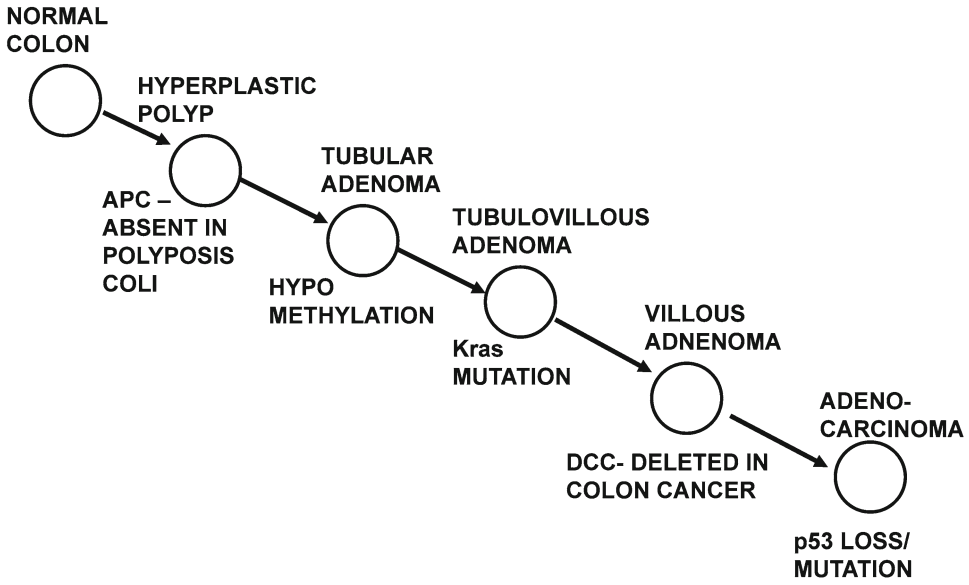

Fig. 8 Sequence of events proposed by the Vogelstein hypothesis for the development of colon cancer

the intestinal lining cells turn over in 4–5 days [71]. Because of this, if there was a mutation in one of these cells, unless there was some way to allow the cell to avoid this turnover, the mutated cell would be quickly lost. Thus, the first mutation (APC) must produce an effect than essentially immortalizes the immediate progeny of the stem cell.

The product of the APC gene targets β-catenin and survivin for destruction [88]. With a loss of the APC gene products β-catenin and survivin are not destroyed and accumulate. β-Catenin is a transcription activator for proliferation; survivin is an apoptosis inhibitor. Thus, loss of APC allows gastrointestinal progenitor cells to continue to proliferate without dying. This effect of loss of APC is demonstrated in APC knock out mice. The intestinal crypt cells of APC knock out mice do not migrate out of the crypt and continue to proliferate leading to accumulation of the intestinal cells and the formation of polyps [89]. The end result of the loss of the function of the APC gene is continued proliferation and survival of crypt epithelial cells resulting in a large number of cells available for additional mutations leading to malignancy. Thus, the first mutation in the Vogelstein sequence results in maturation arrest of progenitor cells in the intestinal crypt that do not die and are subject to further mutation.

Epigenetic Change

An epigenetic change is an inheritable alteration in the expression of a gene or genes that does not involve a change in the underlying DNA sequences. This is mediated by changes in methylamine of the DNA bases (usually Cog sites) or of the proteins of the histones associated with DNA. The state of methylation in turn controls the expression of genes critical for the normal homeostasis of tissue cells. The most frequent change associated with cancer is the loss of expression of the tumor suppressor gene p53.

Field cancerization of the skin. Sun exposure of the skin leads to development of damage to the skin expressed as multiple solar lentigos [90, 91]. These areas of the skin provide a setting for the development of skin cancer (field cancerization). The molecular lesion is believed to be an epigenetic change in the DNA leading to loss of expression of the ubiquitous tumor suppressor gene, p53 [92]. One of the recognized functions of p53 is to protect DNA against radiation damage by effecting removal of cells with damaged DNA. With the loss of p53, the cells with epigenetic change in areas of sun-damaged skin are not removed rapidly and are highly susceptible to second mutations, for example, by activation of c-myc. Thus, the lesion of field cancerization results in survival of cells that normally are rapidly turned over. The accumulation of these cells allows for additional mutations or epigenetic change leading to cancer.

Stomach cancer. H. pylori infection of the stomach is a major risk factor for development of gastric cancer. Areas of hypermethylation are seen in the gastric mucosa of patients with *H. pylori* infection and it is in these areas gastric cancers arise [93]. The pathologic sequence of events is the following: *H. pylori* infection leads to increased methyl transferase activity with resulting increased methylation of genes in gastric stem cells. This leads to repression of the p53 gene which is inherited in stem and transit amplifying cells with resultant increased proliferation and loss of apoptosis [94]. The identification

of both stem cells and transit amplifying cells as targets is based on the response to therapy. If the *H. pylori* infection is treated, some of the areas of hypermethylation disappear whereas others do not [93]. This finding is interpreted as follows: The areas of hypermethylation that disappear represent hypermethylation of transit amplifying cells. Since these cells turn over rapidly, the increased methyl transferase activity seen during the active infection is required to maintain hypermethylation. When the *H. pylori* infection is cured newly formed transit amplifying cells from non-methylated stem cells will not be hypermethylated. On the other hand, in areas where hypermethylation is not lost, this change must have occurred in the self-renewing stem cells that continue to give rise to hypermethylated transit amplifying cells [95].

Conclusions

In this review the contributions of tissue stem cells to the development of cancer is described for each of the major theories of the origin of cancer using specific cancer types as examples.

1. Field theory. Teratocarcinomas arise from normal germinal cells when placed in a tissue niche that does not enforce normal differentiation.
2. Chemical carcinogenesis. Chemicals that cause cancer of the liver appear to act at various stages of the differentiation of liver lineage cells. Exposure of the skin to chemical carcinogens causes mutations (adducts) in the long-term self-renewing basal stem cells.
3. Mutations: Translocations in myeloid leukemia produce fusion proteins that are activated at various stages of myeloid hematopoiesis leading to accumulation of cells at a specific stage of differentiation. The sequence of events in colon carcinogenesis begins with a mutation (APC) that results in a block in differentiation and continued proliferation of colonic stem cell progeny.
4. Epigenetic changes: Sun-damaged skin predisposes to development of cancer because of a loss of expression of p53. *H. pylori* infection of the stomach causes hypermethylation of the DNA of gastric mucosal stem and progenitor cells, loss of tumor suppressor gene function, and development of gastric cancer.

Each example follows a hierarchical model of tissue differentiation whereby the tissue stem cell contributes to cancer by expression of a phenotype in the progenitor cell progeny permissive for growth and inhibitory for differentiation.

In 1953 Harry Goldblatt and Gladys Cameron induced malignancy in normal cells from the myocardium of 5-day-old rats by culturing the cells under anaerobic conditions [96]. This approach was based on the hypothesis of Otto Warberg and his associates that cancer cells are able to grow under anaerobic conditions whereas normal cells are not [97]. Goldblatt hypothesized: "It seems possible that in all embryonic, and even adult, normal tissues there may be scattered cells, or groups of cells of potentially neoplastic sort, which naturally possess the ability to use the fermentative, glycolytic mechanism at least under anaerobic conditions, and that repeated, brief exposure of cultures of normal tissue contain such cells to an atmosphere deprived of oxygen, alternating with long periods when adequate oxygen is available, thus permitting recovery, might favor their multiplication and even interfere with the growth of the regional normal cells." Such cells might well be myocardial stem cells. Malignant transformation of tissue stem cells is a recognized problem for any cells derived for stem cell therapy protocols.

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Targeting the Stem Cell Plasticity of Tumor Cells

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Introduction

Myriad studies have demonstrated a high degree of plasticity associated with aggressive tumor cells. In fact, the multipotent phenotype expressed by these cancer cells is similar in many respects to embryonic stem cells and, therefore, contributes to the properties underlying cancer stem cells (CSCs). Molecular profiling of the plastic tumor cell phenotype has revealed highly upregulated genes associated with embryonic progenitors, extracellular matrix remodeling, hypoxia, and endothelial and lymphatic vascular formation; and downregulated genes generally associated with the respective, lineage-specific phenotype. For example, in the case of a poorly differentiated melanoma, several melanocyte-lineage genes are suppressed. Thus, the global perspective emerging of the plastic tumor cell phenotype is that these cancer cells are difficult to identify and challenging to target. Although these multipotent tumor cells implement similar stem cell-associated signaling pathways as employed by embryonic progenitors to sustain growth and plasticity, they lack major regulatory checkpoints resulting in the aberrant activation of embryonic pathways. Most noteworthy in this regard are the Nodal and Notch signaling pathways, which have been shown to underlie the stem cell-like phenotype, unregulated growth, and aggressive behavior of tumor cells [1].

Indeed, the evidence supporting the functional relevance of tumor cell plasticity consists of remarkable examples of tumor cell transdifferentiation and acquisition of survival properties providing a selective advantage to aggressive tumors. A growing body of literature indicates the diverse capabilities of tumor cells, ranging from the formation of vascular networks for perfusion, to reactivation of embryonic pathways, to the acquisition of drug resistance [2]. Therefore, therapeutic approaches targeting multipotent tumor cells should take into consideration the myriad phenotypes expressed and their biological interactions with the microenvironment relevant to tumor survival, including escape from immunosurveillance. Ultimately, the best anticancer strategy may well consist of a combinatorial approach—rendering tumor cell growth arrested, immunotargeted, and drug-sensitive. Thus, this chapter will review key studies constituting our knowledge base for tumor cell plasticity and the potential for effectively targeting this challenging phenotype (Fig. 1).

Biologic Significance of Tumor Cell Plasticity

Through the years of cancer research, two major developing theories on tumor initiation and progression have emerged, each with its own conceptual consequences on tumor cell plasticity. The clonal evolution theory postulates that tumors are initiated as a result of genetic insult to a single cell [3]. This clonal mutation may result in increased cell survival or proliferation, conferring growth advantages. Further carcinogenic changes in these cells, whether mediated through environmental or genetic factors, induce additional alterations which allow for more pronounced deviations from proper cellular regulation. This offers, in part, explanation for the well-documented heterogeneous nature of tumors, theorizing that multiple subpopulations of cancer cells exist within a tumor. Furthermore, the genetically unstable nature of tumors, due to alterations in cell cycle checkpoints, DNA repair mechanisms, and chromosome stability, adds to this, resulting in populations of cells with numerous abnormalities and high rates of malignancy [4]. The final step of the clonal evolution theory postulates that a cell has attained sufficient mutations which enable it to leave the primary tumor,

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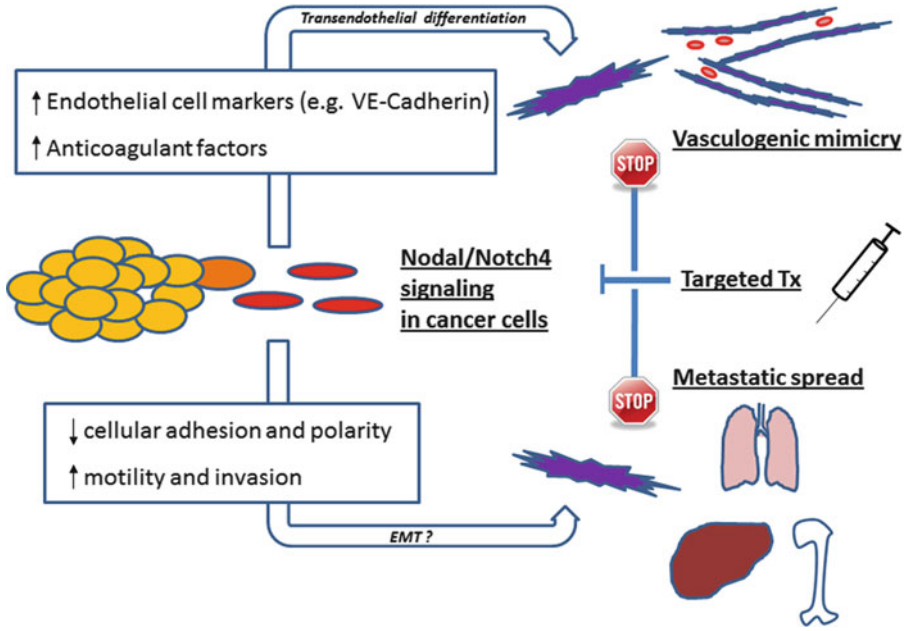

Fig. 1 Potential targets for tumor cell plasticity. A schematic diagram showing how Nodal/Notch4 signaling can play a role during cancer cell transendothelial differentiation with induction of endothelial-related markers characteristic of vasculogenic mimicry and, possibly, via regulation of epithelial-to-mesenchymal transition (EMT), whereby cancer cells lose polarity and adhesiveness to acquire a more motile and invasive phenotype. Inhibition of Nodal/Notch4 signaling has been shown to disrupt vasculogenic mimicry and reverse invasiveness in Nodal-expressing tumor cells

metastasize to distant sites, adapt to new microenvironments, and proliferate into a clinically relevant mass. This offers support for the statistical experimental results which demonstrate that only a small proportion of tumor cells are capable of completing the entire process of metastasis [5]. Thus, cell plasticity within a tumor increases over time and can be traced to a progressive, stepwise sequence of accumulated mutations.

The other major theory for the explanation of tumor development and progression, and cancer cell plasticity, suggests that tumors result from a specific underlying cell type, the cancer stem cell (CSC) [6]. These cells possess the ability to divide asymmetrically, allowing for self-renewal of the stem-like cell(s) as well as production of multiple lineages of differentiated cell types, together accounting for tumor heterogeneity. CSCs constitute only a subpopulation of the tumor, and as little as one CSC has been shown to be capable of recapitulating growth of an entire tumor. The CSC hypothesis was strengthened in the mid-1990s when subsets of tumor cells in leukemia patients were described to exhibit stem cell-like properties [7], and a hierarchy of lineage was traced to a single, tumor-initiating hematopoietic cell type [8]. These properties were also later noted in solid tumors from breast cancer patients where only a small portion of tumor cells possessed the ability to initiate tumors in mice [9]. Validation of each of these hypotheses has been debated, and it is likely, as has been suggested, that these two theories are not mutually exclusive [10]. However, the CSC theory has incredible implications for tumor cell plasticity and its biological significance, suggesting that the plastic nature of cancer cells is intrinsic to a subpopulation within a tumor.

Due to the importance of embryonic tissue generation and maintenance, stem cells are equipped with mechanisms that increase their survival capabilities by comparison to differentiated cells [11]. When these features are also present in CSCs or cells that possess stem-like qualities, differential responses in cancer treatment and relapse in patients have been observed, since, among other properties, these cells express multi-drug resistance pumps (ATP-cassette transporters) capable of removing cytotoxic compounds, including chemotherapeutic agents. Additionally, the ability of CSCs to enter a dormant state of quiescence reduces the effectiveness of therapies which target rapidly dividing cells. CSCs are also characterized by enhanced activity of DNA repair enzymes, modulation of apoptotic pathways, and increased capacity for long-term self-renewal. The ability of these cells to survive therapy and retain the capability to reproduce tumors from such small populations provides the basis for recurrence of cancer in patients in which the majority of the tumor burden has been eliminated.

The plastic phenotype of cancer cells often includes the ability to migrate and invade through local microenvironments and to travel to distant sites within the body. In tumors of epithelial origin, cancer cells may acquire this capability by undergoing a process of epithelial-mesenchymal transition (EMT). EMT is the result of a series of epigenetic alterations

which converts polarized, adherent epithelial cells to a more motile, invasive mesenchymal-like phenotype. Numerous signaling pathways regulate aspects of EMT in embryonic development and disease, including cancer [12, 13]. In cancer, cells undergoing EMT typically downregulate expression of intracellular adhesion components, such as E-cadherin, Occludin, and Claudins, and upregulate factors associated with a more motile, mesenchymal phenotype such as N-cadherin and Vimentin [13]. These motile and invasive phenotypes allow cells to intravasate into and extravasate out of blood and lymphatic vessels (often a means of transport throughout the host), as well as to invade through matrices encountered at both primary and secondary tumor locations. EMT may also give rise to cells with stem cell-like properties [14]. However, while the process of EMT and the functional abilities it confers have profound impacts on tumor cell plasticity, many of these characteristics may already be present in CSCs within a tumor.

In addition to mechanisms related to cell movement, it is well established that tumors require a blood supply to maintain growth [15]; however, proliferating tumors will quickly outgrow the local blood supply of the host. When oxygen is limited, cancer cells respond through signals mediated in part by hypoxia-inducible factors (HIFs) that result in the secretion of pro-angiogenic factors (e.g., VEGF, bFGF) that promote formation of new blood vessels [16]. Importantly, the highly plastic, cancer-initiating populations within tumors exhibit enhanced angiogenic potential over the bulk tumor and may be primarily responsible for endothelial cell recruitment [17]. In addition to the induction of vascular signals by CSCs, endothelial cells, in turn, secrete factors that support the survival and self-renewal capabilities of CSCs. Additionally, CSCs may employ other mechanisms to facilitate tumor perfusion, such as vasculogenic mimicry (VM) [18]. VM is defined as the de novo formation of perfusable, vascular-like networks by a subpopulation of aggressive tumor cells in in vitro 3D matrices, which resemble matrix-rich network structures in patients' tumors [19]. During VM, cancer cells acquire an endothelial-like phenotype that includes expressing vascular markers such as VE-cadherin [2]. VM can accompany vessel co-option of preexisting vessels, mosaic vessels, angiogenesis, vasculogenesis, and intussusceptive microvascular growth [20]. This ability of CSCs to adapt to and modulate the changing microenvironment in order to meet growth demands of a developing tumor exemplifies the dynamic plasticity of cancer cell subpopulations.

Another critical factor in cancer progression is the immune system, which has evolved not only as a protective barrier towards infectious disease but also as a hurdle for abnormal cell growth within the body. As tumor cells accumulate mutations, certain characteristics such as changes in antigen presentation are often recognized by the immune system as foreign, leading to cancer cell targeting and elimination. However, cancer cells may possess the capacity to inhibit immune responses or reduce detection. Evasion of the immune system can be accomplished through mechanisms that include downregulation of cancer cell antigen expression or dampening of innate and adaptive immune responses, e.g., by inhibitory cytokine release [21]. Recently, a report involving the study of T cell activation in melanoma determined that a subpopulation of tumor cells referred to as malignant melanoma initiating cells (MMIC) and marked by $ABCB5^{+}$ and stem cell-like qualities, were capable of decreasing T cell activation through IL-2. These cells further altered the immune system by increasing populations of immune suppressive regulatory T cells (Treg) and inducing secretion of the anti-inflammatory cytokine IL-10 from both Treg and peripheral blood monocytes [22]. These alterations led to the reduction of T cell-mediated killing of melanoma cells. Importantly, this effect was not induced by the $ABCB5^{-}$ population, demonstrating that the distinct MMIC tumor population alone was capable of mediating this effect. This study provides an example of intricate immune system modulation by tumor-initiating cells within a melanoma that is not shared by less plastic populations of tumor cells.

Since the resurgence of the CSC hypothesis, therapeutic and translational efforts have been made to target the stem cell-like subpopulations of tumors that exhibit the highest degree of cellular plasticity [23]. By targeting tumor-initiating cells, recurrence of cancer in patients may be reduced. In recent years, however, data have been accumulating that the hierarchal nature of stem cell lineage and division may not be as unidirectional as once believed. Initial reports demonstrated that differentiated cells could be reprogrammed to a stem cell-like state by specific transcription factors and embryonic cell culture conditions [24–26]. These pioneering studies are supported by subsequent reports of induced pluripotency in other cell types and initiated the field of induced pluripotent stem (iPS) cell biology. While these studies provided the molecular framework detailing how a differentiated cell can be induced to dedifferentiate into an embryonic-like phenotype, dramatically increasing its potential for plasticity, it remained unclear if this process occurred naturally. This idea was addressed when human mammary epithelial cells (HMECs) were shown to spontaneously convert to a $CD44^{hi}CD24^{low}ESA^{-}$ progenitor-like cell type with stem cell features [27]. Remarkably, these cells were capable of differentiation into multiple lineages of mammary cells and were able to reconstitute a mammary fat pad in NOD/SCID mice. Importantly, this study also demonstrated that oncogenic HMECs transformed with H-*ras* underwent spontaneous conversion to stem cell-like states. This latter observation provided evidence for spontaneous dedifferentiation to a CSC. This suggests that, rather than only a specific tumor subpopulation possessing the ability to undergo self-renewal, produce differentiated lineages, and

maintain plasticity, differentiated cells are also intrinsically capable of contributing to this population. Therefore, targeted CSC therapies could potentially be hindered when considering that CSC populations may spontaneously arise within a genetically diverse tumor.

Cell Signaling and Tumor Cell Plasticity

There is mounting evidence that signaling pathways critical for embryonic development and stem cell plasticity also play important roles in promoting cancer progression and tumor cell aggressive behavior. Studies focused on different tumor types have identified functions for multiple embryonic pathways, including TGFbeta, Notch, Wnt, and Hedgehog signaling [28, 29]. Two significant pathways that are both associated with the embryonic stem cell phenotype and cancer progression are Notch and Nodal signaling. Recent research suggests that these pathways are critical regulators of tumor cell plasticity and aggressive behavior, and, at least in melanoma, cross talk between Notch and Nodal signaling is an important driver of the plastic phenotype [1].

The Nodal pathway is a fundamental developmental signaling pathway involved in regulating pluripotency in embryonic stem cells, as well as in embryonic mesoderm formation and body plan establishment in amniotes [30]. Nodal, a secreted growth factor and member of the TGFbeta superfamily, is specifically expressed during embryogenesis, but is not typically detected in normal adult tissues. Importantly, Nodal expression is reactivated in aggressive cancers including melanoma, and breast, prostate, cervical, and pancreatic carcinomas [31]. Canonical Nodal signaling is propagated via Nodal ligand binding to heterodimeric complexes of activin-like kinase receptors: type I (ALK4/7) and type II (ActRIIB), either with or without the Nodal coreceptor, Cripto-1 [30]. Receptor activation leads to the phosphorylation of SMAD2 and SMAD3, which associate with SMAD4, and altogether translocate as a complex to the nucleus, where they regulate the transcription of target genes such as Lefty (a Nodal antagonist) and Nodal itself. During normal development, the feedback of signaling on both Nodal and Lefty maintains the pathway in a homeostatic balance [30]. In cancer cells, however, the Lefty promoter is heavily methylated, so Nodal but not Lefty is upregulated in response to Nodal signaling, such that the Nodal pathway is allowed to propagate without the normal regulatory controls [32].

Like the Nodal pathway, Notch signaling has important functions during embryogenesis and is involved in stem cell pluripotency and cell fate determination, as well as organ system development and maintenance [33]. Notch signaling is typically initiated by binding of a membrane-bound Notch ligand (DLL1, DLL2, DLL4, JAG1, or JAG2) to one of the four Notch receptors (Notch1–4) on an adjacent cell. Binding triggers a series of cleavage events that releases the intracellular domain (ICD) of the Notch protein into the cytoplasm. The Notch ICD, in complex with other proteins such as MAML and RBPJ, then acts as a transcription factor to regulate expression of target genes such as Hes and Hey [34]. During early vertebrate development, Notch signaling promotes a distinct subset of cells to express Nodal during left-right asymmetry determination, regulated via an RBPJ-dependent upstream enhancer element known as the Node-specific enhancer (or NDE) [35], thus adding Nodal to the list of Notch-regulated target genes. Work from our laboratory indicates that, at least in melanoma, Notch4 receptor expression correlates with Nodal expression in aggressive but not nonaggressive cell lines [1]. In addition, and similar to Nodal, Notch4 protein was more commonly detected in advanced-stage melanoma biopsies than in early-stage disease [1]. Importantly, Nodal expression in aggressive melanoma cells is dependent upon Notch4 signaling, since targeting Notch4 activity with neutralizing antibodies or Notch4 expression with siRNAs significantly reduced the expression of Nodal and the aggressive behavior of these cells. This regulation appears to be propagated via an RBPJ-dependent mechanism since a reporter of the NDE (the RBPJ-dependent, upstream Nodal enhancer element) was active in aggressive melanoma cells, but only when the RBPJ binding sites were intact. Cross talk between the Notch and Nodal pathways may be further regulated by the Nodal coreceptor, Cripto-1, since Cripto-1 can directly bind to the Notch receptors and can enhance the proteolytic maturation of Notch receptor ICD [36].

Work from our laboratory and others indicate a strong relationship between the expression and activity of Nodal and the plasticity of tumor cells. For example, Nodal is detected in advanced-stage breast cancer and melanoma more readily than in early-stage disease [1, 37, 38]. In cell lines, Nodal can regulate invasion into a defined extracellular matrix [37]. Furthermore, aggressive cancer cell lines cultured on 3D matrices can spontaneously form VM networks, cellular networks reminiscent of vascular channels that are perfusable with fluorescent dye [19]. Treatment with the ALK4/5/7 inhibitor SB431542 or with anti-Nodal antibodies diminished the ability of aggressive melanoma cell lines to form vascular-like networks on 3D matrices [37, 39]. Similarly, treatment with anti-Notch4 antibodies inhibited vascular-like network formation that could be partially rescued with inclusion of recombinant human Nodal, suggesting that this plasticity is dependent upon a Notch-Nodal signaling axis [1]. Importantly, Nodal expression appears to be specifically localized to the subpopulation of cells forming vascular-like networks in melanoma [40], and in aggressive melanoma and breast cancer cell lines, Nodal protein is detected only in a subpopulation of cells in culture [1, 41]. Of note, Notch4 protein is also limited to a subpopulation of

cultured melanoma cells and is enriched in VM networks [1, 42]. Coexpression of Notch4 and Nodal is restricted to approximately 10 % of cells in culture, suggesting the possibility that this may represent a subpopulation of cells with stem-like, plastic properties capable, for example, of transdifferentiation into vascular-like cells [1]. Certainly in pancreatic adenocarcinoma cells, Nodal expression and signaling are elevated in the $CD133^+$, anchorage-independent, sphere-forming stem cell subpopulation, and manipulation of Nodal signaling affected sphere formation, invasive capacity, and in vivo tumorigenicity [43]. Furthermore, in breast cancer cell lines, Notch4 activity is enriched in the breast CSC subpopulation (identified by the $ESA^+/CD44^+/CD24^{low}$ molecular signature), and inhibition of activity or expression reduced the tumor-initiating ability of cells in vitro and in vivo [44]. Whether Notch4 activity plays a role in the regulation of Nodal expression in pancreatic or breast CSCs has not been determined, but warrants further investigation.

Cripto-1, the coreceptor for Nodal, is a marker of embryonic stem cells and is associated with pluripotency [45]. Cripto-1 may also be involved in the regulation of tumor cell plasticity, since, in some cancers, it is detected in a subset of cells with stem-like properties [46–48]. For example, in the aggressive human melanoma cell line C8161, Cripto-1 cell surface expression is detected only on a subpopulation of cells in culture (<5 %) by fluorescence-activated cell sorting [46]. Interestingly, when these Cripto1-positive cells were isolated from the heterogeneous population and cultured separately, cells were smaller and slower growing than parental cells. Continuous culture in a human embryonic stem cell (hESC) medium resulted in melanosphere formation in Cripto-positive but not parental cells, concurrent with increased expression of pluripotency markers (e.g., Nanog and Oct4) and the drug resistance molecule, MDR1, suggesting that these cells are more stem cell-like. Importantly, Cripto-1 has also been described to regulate melanoma cell invasiveness, one indicator of tumor cell plasticity [49], as well as exhibiting the capacity to promote EMT in epithelial tumor cells [50]. Whether Nodal can also contribute to the regulation of EMT in cancer cells is not clear, but warrants further investigation, since Nodal can upregulate EMT-related transcription factors during embryonic development [51].

Typically, tumor aggressiveness and advanced-stage disease is associated with a less differentiated phenotype. Certainly in melanoma, more aggressive cells tend to lose expression of lineage-specific, melanocytic markers and exhibit a more primitive phenotype and an expression profile more typical of undifferentiated cells [2]. Interestingly, transplanting aggressive, GFP-labeled C8161 melanoma cells into the neural crest microenvironment of a chicken embryo resulted in the induction of a subset of these cells to behave as their ancestral neural crest neighbors and to migrate in typical neural crest patterns [52]. Importantly, a subset of these melanoma cells also appeared to be epigenetically reprogrammed, as they were able to reexpress the melanocyte marker, Melan-A/MART-1, or the neuronal marker, Tuj1. This phenomenon may be dependent upon Nodal signaling since C8161 cells in which Nodal expression was downregulated with morpholinos were unable to migrate away from the site of transplant, which was typically seen in control cell transplants where Nodal was still highly expressed [53]. Importantly, nonaggressive c81-61 cells which have little-to-no Nodal expression were not competent to migrate away from the transplant site, suggesting that this plastic behavior is specific to more aggressive, less differentiated melanoma cells that express the embryonic morphogen Nodal [52].

Tumor cells also exhibit the capacity to respond to other embryonic microenvironments, such as a hESC-conditioned microenvironment [54]. Exposure of aggressive melanoma cells to the extracellular matrix laid down by hESCs enabled a subpopulation of cells to form spheroids reminiscent of hESC colonies [55]. Importantly, this phenomenon was coincident with a decrease in invasive cellular behavior and an increase in expression of the melanocyte-specific lineage marker, Melan-A/MART-1. Further studies determined that this reprogramming capacity was restricted to hESC-conditioned matrix, and was behavior not recapitulated by other stem cell types, such as those derived from amniotic fluid and adult bone marrow [38]. Analyses of possible tumor-suppressive factors led to the identification of the Nodal antagonist, Lefty, as a factor secreted by hESCs into the 3D matrix. Most noteworthy, Lefty was capable of inhibiting the tumorigenic activity of Nodal-expressing, aggressive melanoma and breast cancer cell lines. Considering the anti-tumorigenic effects of inhibiting the Nodal pathway, exploiting targets such as Notch4, Nodal, or Lefty may prove useful strategies for therapeutic intervention, which is highlighted in the following section.

Potential for Targeting Tumor Cell Plasticity

It is becoming increasingly evident that cancer tissue is not comprised of cells with identical molecular and biologic properties, but rather consists of a heterogeneous population with distinct functional characteristics, which may enable cellular subpopulations to acquire survival advantages in specific environments. This paradigm has important implications for how we treat cancer.

Results of our studies and others have shown that embryonic pathways such as Nodal signaling are reactivated in various aggressive tumor types, and expression appears to be restricted to a subset of cells within a population that exhibit enhanced plasticity [1, 31, 41]. Since Nodal expression is not generally detected in normal tissues, it represents an exciting

potential anticancer target. Certainly, tumors in nude mice formed from orthotopically injected Nodal-positive aggressive human melanoma cell lines exhibited decreased tumor cell proliferation and increased apoptosis when mice were treated intratumorally with the hESC-derived Nodal antagonist, Lefty [56]. Similarly, aggressive melanoma cells injected retro-orbitally in nude mice and allowed to colonize to the lung formed fewer tumor cell colonies when treated with intraperitoneal injections of a function blocking anti-Nodal antibody compared with mice injected with isotype control immunoglobulin [39]. Furthermore, melanoma cells observed in the lungs of Nodal antibody-treated mice showed more frequent signs of cellular stress and apoptosis compared with melanoma cells in the lungs of control animals. Coupled with the in vitro observations that targeting Nodal or the upstream regulator Notch4 reduced cellular plasticity and VM formation in melanoma cells cultured on 3D matrix [1, 39], we suggest that targeting the Nodal pathway may offer a potentially attractive alternative treatment strategy for melanoma and other cancers.

EMT represents an example of tumor cell plasticity whereby alterations in the molecular and phenotypic profile of cancer cells can influence their biological behavior and affect therapeutic outcome. As such, EMT has been suggested as a key factor in determining the success rates of anticancer therapy. In certain cancers, specific inhibitors are more effective in epithelial-like tumor cells than in tumor cells that exhibit a more mesenchymal-like phenotype [57–59]. In one example, ErbB receptor inhibition in aggressive inflammatory breast cancer was associated with reversal from an aggressive mesenchymal phenotype to a less aggressive, more chemosensitive epithelial phenotype [60]. This and similar observations suggest that the reversal of EMT could lead to enhanced sensitivity of cancer cells to specific anticancer agents.

Considering the heterogeneity of tumor cells, one theory is that treatment of a particular tumor type with a certain anticancer drug may only affect the subset of malignant cells with sensitivity, while other cells within the cancer are unaffected either because they are resistant to the specific agent or do not express the drug target. This idea is exemplified in the treatment strategies for melanoma that target the mitogen-associated protein kinase signaling pathway associated with activating NRAS or BRAF mutations [61]. Although NRAS and BRAF inhibitors have shown promise in a subset of melanoma patients, a significant proportion of patients do not benefit from these novel targeted approaches, at least in part, because not all melanomas present these specific mutations or activated signaling pathways. Equally noteworthy, though, is the observation that most patients with an initial response to treatment, subsequently developed drug resistance [62], likely due to the evolution and adaptation of some cells. The treatment of breast cancer by direct targeting of the estrogen hormone receptor (ER) or the epidermal growth factor receptor, HER2, provides another example of the drawbacks of targeted therapies on heterogeneous cancer cells. Although these specific breast cancer therapies have significantly impacted patient survival, not all patients benefit from this approach since not all breast cancers express ER or HER2. Moreover, the expression of these receptors can vary dramatically over time. For instance, patients that were once HR positive may become HR negative and vice versa, thus significantly impacting treatment outcome [63].

In general, once cancer cells sensitive to a particular treatment have been eliminated, it is possible that a phenotypically distinct residual population of cancer cells with resistance to treatment will continue to proliferate unaffected. This is especially true given the support for the presence of cancer cells with stem cell-like characteristics (including chemoresistance and reduced proliferation rate) that can give rise to a plastic, tumor-initiating progeny with the potential to assume phenotypes distantly related to the original cancer tissue. This may explain why certain breakthroughs in anticancer therapy, although highly promising in initial clinical trials, have encountered difficulties and even failure when applied to a broader population of cancer patients. Our understanding of the molecular underpinnings of tumor cell plasticity responsible for the dynamic changes that regulate molecular and functional heterogeneity of cancer cells will help to provide the scientific rationale for adapting a more dynamic therapeutic approach capable of keeping in step with the ever-changing characteristics of the cancer being targeted.

Conclusion

The plasticity of aggressive tumor cells has presented a significant challenge in the detection and targeting of the metastatic, multipotent phenotype. Although the development of anticancer drugs has focused primarily on inducing tumor cell death and/or suppressing a tumor's blood supply by targeting endothelial cells, these strategies have been ineffective in eradicating the most deadly cancer cells. To address the reason(s) for this failure, we must first comprehend the biological evidence underlying the plasticity of tumor cells and its myriad implications—to drive the strategic development of new therapeutic approaches.

Tumors are composed of heterogeneous populations of cells with disparate cellular properties and unique targets. Based on this premise, recent studies are advocating an integrative approach to cancer therapy, including a combinatorial approach of targeting tumor cells and their microenvironment with immune-based therapies [64]. Other findings related to the pathways critically involved in tumor cell vasculogenic mimicry and the reactivation of embryonic

stem cell signaling pathways (such as Nodal and Notch4) constitute excellent, additional targets for suppressing tumor cell plasticity. Each of these pathways and properties contributing to tumor cell plasticity and escape from immune surveillance warrants serious scrutiny as potential new strategies to target the metastatic phenotype.

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Molecular Evolution of Leukemia Stem Cells

Leslie A. Crews and Catriona H.M. Jamieson

Abbreviations

5-LO	Arachidonate 5-lipoxygenase
AML	Acute myeloid leukemia
BAD	Bcl-2-related death promoter
BAK	Bcl-2 homologous antagonist killer
B-ALL	B cell acute lymphoblastic leukemia
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
BID	BCL2-like 11 (BIM) and BH3-interacting domain death agonist
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CSC	Cancer stem cells
CXCR4	C-X-C chemokine receptor type 4
EVI1	Ecotropic viral integration site 1
EZH2	Enhancer of zeste homolog 2
GMP	Granulocyte-macrophage progenitor
HDAC	Histone deacetylase
Hh	Hedgehog
HLF	Hepatic leukemia factor
HOX	Homeobox
HSC	Hematopoietic stem cell
ICN1	Intracellular NOTCH1
JAK2	Janus kinase-2
LSC	Leukemia stem cells
MPN	Myeloproliferative neoplasm
PcG	Polycomb group
PRC	Polycomb repressive complex
RUNX1	Runt-related transcription factor 1
Shh	Sonic Hedgehog
SIRT1	Sirtuin 1
STAT5	Signal transducer and activator of transcription-5
T-ALL	T cell acute lymphoblastic leukemia

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Introduction

Cumulative evidence suggests that many tumors are propagated by cells located in selective niches that have subverted stem cell properties, including self-renewal, survival, differentiation, and dormancy [1–9]. Although the majority of cancer treatments eliminate rapidly dividing cells, patients suffering from solid tumors and hematologic malignancies continue to relapse and develop resistance to standard therapies. A cancer stem cell (CSC) population has been described in a number of malignancies and may function as a reservoir of quiescent cells with tumor-regenerating capacity that ultimately reactivates, leading to therapeutic recalcitrance, relapse, and metastases—the leading causes of cancer-related mortality [10–12]. The concept that a subset of bulk cancer cells has the ability to propagate the malignancy is based on early studies in which a small number of self-renewing tumor cells were required to regenerate all aspects of the tumor [13, 14]. This CSC hypothesis posits that CSC harbor characteristic functional features of stem cells albeit in a deregulated manner and is not predicated on cells sharing the same cell surface characteristics as normal stem cells.

Cancer stem cells were first identified as leukemia stem cells (LSC) in human acute myeloid leukemia (AML) [15, 16], a disorder associated with accumulation of immature myeloid blasts in the marrow and decreased normal hematopoiesis. Initiating oncogenic events have been described at the level of the hematopoietic stem cell (HSC) in leukemias of both myeloid and lymphoid types. During leukemic progression, this abnormal progenitor cell compartment expands and evolves from a molecular standpoint, resulting in the activation of pro-survival and self-renewal signaling pathways and facilitating the acquisition of additional stem cell-like functional properties. In this chapter we will discuss

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LSC evolution at a functional, genetic, epigenetic, and transcriptomic level and the contribution of these alterations to the progression of myeloid and lymphoid leukemias.

Malignant Disorders of Abnormal HSC Development

Myeloid Leukemias

While AML LSC were originally identified as $CD34^+CD38^-CD90^-Lin^-$ immature cells [17], recent reports suggest that $CD34^+CD38^-$ or $CD34^+CD38^+$ fractions may contain leukemia initiating potential in myeloid malignancies depending on disease stage and the individual patient [18–20]. The phenotypic and molecular identity of LSC was initially established in robust xenotransplantation systems involving primary human AML samples (primagrafts) [21, 22]. These models recapitulate key aspects of the leukemic microenvironment and take into account some, but not all, niche-specific effects of gene expression. Despite groundbreaking advances in genetic and epigenetic classification of AML (Table 1) [23–28], the overall 5-year survival rate remains low at 25 % [29]. This is due in part to the fact that the current classification criteria do not define the self-renewal genes that fuel leukemic regeneration and relapse. Additionally, most effective therapeutic strategies, such as allogeneic hematopoietic cell transplantation, are limited to patients under 55. However, AML is a disease of aging, as 69 % of patients are diagnosed at age 55 or above [29]. Thus, better understanding of the molecular mechanisms of leukemic progression is needed in order to develop new therapies that can be used in patients who cannot be treated with current standards of care due to advanced age or therapeutic resistance.

The first tangible insights into the molecular pathogenesis of cancer stemmed from the discovery of the Philadelphia chromosome positive (Ph^+) and its constitutively active BCR-ABL protein tyrosine kinase product in the most highly studied myeloproliferative neoplasm (MPN)—chronic myeloid leukemia (CML). Studies of primary CML patient samples coupled with well-characterized xenograft mouse models of CML have furthered our understanding of the molecular evolution of LSC in myeloid malignancies [9]. CML represents an important paradigm for understanding the molecular evolution of cancer because it was the first cancer shown to be initiated at the HSC level by BCR-ABL; the first cancer found to undergo blastic transformation following malignant reprogramming of committed progenitors [9, 30]; and the first target of molecular therapy with the introduction of the BCR-ABL targeted tyrosine kinase inhibitor (TKI) imatinib.

It is estimated that there are approximately 4,800–5,200 new diagnoses of CML in the United States annually [31], and the estimated annual mortality, which before the advent of TKIs was approximately 10 % for the first 2 years and 20–25 % in subsequent years, has now been reduced to an estimated all-cause mortality of 2 % annually for the first 10 years of follow-up [31]. Moreover, as a result of these improved therapeutic outcomes, the prevalence of CML has started to grow annually reaching an estimated prevalence of CML that is approximately 70,000 patients in the USA in 2010. This is expected to increase to 112,000 in 2020, 144,000 in 2030, 167,000 in 2040, and 181,000 in 2050, when it will reach a plateau [31]. While current treatments are relatively effective at managing the disease in the chronic phase, TKI intolerance and noncompliance drive therapeutic resistance and disease progression in approximately one third of patients. Moreover, lifelong treatment with current standard of care TKI therapies comes at significant financial and physical costs. The estimated cost of imatinib today is approximately $54,000 annually, whereas the cost of second-generation TKIs is approximately $80,000 to $90,000 per

Table 1 WHO classification of AML subgroups with genetic and molecular characteristics and prognosis

AML subgroup	Genetic, molecular, and clinical characteristics	Prognosis
AML with recurrent genetic abnormalities	(a) Translocation of chromosomes 8 and 21 (*AML1/ETO*) (b) Inversion or translocations of chromosome 16 (*CBFβ/MYH11*) (c) Changes in chromosome 11 (*MLL*) (d) Acute promyelocytic leukemia with translocations involving chromosomes 15 and 17 (*PML/RARα*)	Good prognosis, responsive to therapy, high remission rates
AML with multilineage dysplasia	Preceding history of myeloproliferative neoplasm (MPN) or myelodysplastic syndrome (MDS) More than one abnormal myeloid cell type is involved	Poor prognosis, poor response to therapy
Therapy-related (or secondary) AML and MDS	History of radiation and/or chemotherapy (alkylating agents or topoisomerase-II inhibitors) Complex cytogenetic abnormalities	Poor prognosis, resistance to therapy
AML not otherwise specified (NOS)	Disease features are based primarily on the major cell lineage(s) involved and the degree of maturation (erythroid, monocytic, or basophilic cell types, among others)	Variable

patient per year [31]. Over 60 % of CML patients relapse within 12 months of TKI discontinuation supporting the existence of a quiescent LSC population [32]. Moreover, the acquisition of kinase domain mutations in the activating oncogenes may promote drug resistance [33]. Ultimately, a significant percentage of patients are expected to develop TKI resistance driven by quiescent LSC.

Additionally, due to the more effective treatment options currently available that delay the progression of the disease, the age at which patients progress to the accelerated phase or undergo blastic transformation is increasing. Unfortunately, this aging patient population is often unable to tolerate standard induction chemotherapy or myeloablative hematopoietic cell transplantation or even reduced intensity condition protocols. Notably, discontinuation of imatinib therapy produces a molecular relapse rate of approximately 60 %, and it is estimated that with current strategies, less than 5–10 % of patients may be able to discontinue imatinib therapy [31]. Thus, development of alternative treatment strategies specifically targeting the molecular drivers of the disease is of great clinical importance.

Lymphoid Leukemias

While in vivo xenograft studies of human myeloid leukemia demonstrate that LSC are capable of serially transplanting leukemia [9, 16, 18, 21], cellular subpopulations within precursor B cell acute lymphoblastic leukemia (B-ALL) samples demonstrate greater functional and genetic heterogeneity [34, 35]. Recently, DNA copy number alteration (CNA) profiling and in vivo analysis suggested that patients with BCR-ABL1 ALL have increased rates of early relapse [36]. In another leukemia subtype that often exhibits early relapse [37]—pediatric T cell acute lymphoblastic leukemia (T-ALL)—serially transplantable cells were found to be enriched in $CD34^+CD4^-$ and $CD34^+CD7^-$ fractions of newly diagnosed patient samples [38]. While T-ALL represents only 25 % of adult and 15 % of pediatric ALL cases, it provides another distinct example of a hematologic malignancy driven by specific molecular alterations. In approximately 60 % of T-ALL cases, activating mutations in NOTCH1 and FBXW7 result in constitutively active NOTCH1 signaling [39–41]. Somatic activating mutations in the NOTCH1 heterodimerization domain (HD) or PEST domain or alternatively loss-of-function mutations in FBXW7, a NOTCH1 E3 ubiquitin ligase, increase release or stability of cleaved intracellular NOTCH1 (ICN1). This, in turn, leads to transcriptional activation of genes that promote proliferation and survival such as MYC and HES1 [39, 42].

The LSC population has been reported to be an essential driver of therapeutic resistance and relapse in adult T-ALL [43]. In pediatric T-ALL the $CD34^+$ fraction of pediatric $NOTCH1^{Mutated}$ T-ALL samples possesses enhanced survival and self-renewal potential, characteristic of LSC, compared with their $CD34^+$ NOTCH1 wild-type ($NOTCH1^{WT}$) counterparts [44]. These $NOTCH1^{Mutated}$ LSC were susceptible to targeted inhibition with a therapeutic human NOTCH1 monoclonal antibody, while normal hematopoietic progenitors were spared [44]. These observations highlight the cell type and context-specific effects of NOTCH signaling [45–51] and the importance of oncogenic addiction to NOTCH1 signaling in T-ALL LSC maintenance [44].

Mutation and aberrant activation of members of several of these pathways including NOTCH1 are common among multiple forms of lymphoid and myeloid malignancies. A number of novel cancer genes (FBXW7, SF3B1) were recently identified in chronic lymphocytic leukemia (CLL), an indolent B-cell leukemia [52], suggesting that the molecular pathways driving the initiation and progression of disease may be quite similar across a variety of hematologic malignancies, while cell type and context-specific factors may dictate the clinical manifestation of each disorder. For example, CLL accessory cells, such as endothelial cells and activated T lymphocytes, have been shown to play a significant role in the maintenance of CLL tumor cells in vivo [53, 54]. Thus, intracellular alterations such as acquired mutations and epigenetic changes that ultimately result in deregulated signal transduction coordinate with extracellular, niche-derived cues to maintain malignant cell populations by influencing cell differentiation and enhancing cell survival, niche retention, and self-renewal. In this chapter we will discuss the functional characteristics of LSC and molecular mechanisms driving their evolution into self-renewing reservoirs of disease resurgence, along with alterations at genetic, epigenetic, and RNA levels that promote LSC generation and maintenance.

Functional Characteristics of LSC and Molecular Evolutionary Mechanisms

Populations of LSC were first functionally validated in AML, and later in CML and other hematologic malignancies. These functionally validated LSC populations possess multiple stem cell-like properties along with serial transplantation capacity in vivo—the gold standard for evaluating LSC self-renewal capacity. In CML, BCR-ABL translocation occurs generating the p210 form of this constitutively active kinase at the level of the HSC and initiates the chronic phase of the disease. However, BCR-ABL is necessary but not sufficient to enhance survival and self-renewal of human progenitor cells. Following initiating oncogenic events, such as BCR-ABL translocation in CML or chromosomal translo-

cations and inversions associated with the development of AML, genomic instability is increased. Subsequent acquisition of additional cellular abnormalities facilitates aberrant signal transduction through pathways that regulate cell differentiation, survival, self-renewal, and dormancy in protective niches.

Aberrant Differentiation

In myeloid malignancies, LSC progeny demonstrates skewed differentiation potential towards myeloid lineages, and in lymphoid malignancies, B-cell differentiation is favored. In advanced stages of CML, myeloid differentiation is favored at the expense of erythroid differentiation, and thus many patients suffer from anemia at varying levels of severity. The factors that skew cellular differentiation towards specific hematopoietic lineages in hematologic malignancies are the subject of intense investigation, and many important factors have been identified; however, the precise mechanisms and relative contribution of various molecular regulators remain unclear. Early seminal studies demonstrated that enforced expression of the oncogene and known reprogramming factor c-Myc can trigger the development of both myeloid [55] and lymphoid [56] neoplasias in mouse models. Interestingly, it appears that the expression level of MYC is a critical factor in determining the type of neoplasia that is established and the hematopoietic differentiation lineage that is favored [57]. c-Myc plays an important role in maintaining a healthy balance between HSC self-renewal and differentiation in normal hematopoiesis [58]; however, other factors may cooperate.

Another factor implicated in LSC differentiation is arachidonate 5-lipoxygenase (5-LO) gene (Alox5), which was identified as a critical regulator of LSC in BCR-ABL-induced CML [59]. In a mouse model, recipients of BCR-ABL transduced bone marrow cells from Alox5$^{-/-}$ donor mice failed to develop CML. Alox5 deficiency impaired the function of LSC but not normal HSC through effects on differentiation, cell division, and survival, resulting in a depletion of LSC, suggesting that 5-LO is an attractive target for pharmacological intervention [59]. In MPNs, activation of the Janus kinase 2 (JAK2) signaling pathway has been shown to play an important role in driving HSC towards erythroid differentiation [60], and signaling through signal transducer and activator of transcription-5 (STAT5) downstream of JAK2 has been more recently implicated in BCR-ABL-induced CML-like MPN and B-ALL phenotypes [61, 62]. In MLL-induced AML, a component of the polycomb repressive complex (PRC) 2 and a target of c-Myc transcriptional regulation—enhancer of zeste homolog 2 (EZH2)—inhibits differentiation programs in LSC likely through regulation of downstream genes relevant to the developmental and differentiation processes such as the transcription factor Egr1, thereby augmenting their leukemogenic activity [63]. Thus, transcriptional regulators and signaling molecules are important factors in driving skewed differentiation in leukemic disorders.

Enhanced Survival

During blast crisis transformation in CML, myeloid LSC arise from the expanded granulocyte-macrophage progenitor (GMP) population with amplified BCR-ABL1 expression [9]. These malignant GMP are reprogrammed via aberrant activation of survival and self-renewal pathways leading to therapeutic resistance [9, 15, 18, 64]. Previous studies have demonstrated that dormant BCR-ABL^{+}, CD34^{+} CML progenitors are resistant to apoptosis induced by imatinib [65] or dasatinib [66], suggesting that cellular pro-survival pathways may be aberrantly activated in CML LSC. Critical regulators of CML LSC include members of the intrinsic apoptosis pathway such as the B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins including Bcl-2, Bcl-XL, and Mcl-1, which promote cell survival and have been previously implicated in LSC maintenance [67, 68]. Additionally, factors that regulate Bcl-2 such as the stearoyl-CoA desaturase 1 (Scd1) gene, which was recently shown to have a tumor suppressive role in a CML mouse model [69], also influence LSC survival. Other related molecules with antagonizing, pro-apoptotic functions include proteins such as Bcl-2-associated X protein (BAX), Bcl-2-related death promoter (BAD), Bcl-2 homologous antagonist killer (BAK), BCL2-like 11 (BIM), and BH3-interacting domain death agonist (BID). Interestingly, upregulation of BIM is required for the induction of apoptosis in kinase-driven cancers, and a recent study identified a common intronic deletion polymorphism in the gene encoding BIM in CML patient samples [70].

Apoptosis-related genes have also proved to be useful prognostic indicators of AML outcome [71]. Specifically, high levels of Bad and Bax mRNA expression predicted adverse outcome in de novo and secondary AML [71]. In lymphoid leukemias such as CLL, tumor cells upregulate some anti-apoptotic proteins, such as pro-survival members of the Bcl-2 family (Bcl-2 and Mcl-1) and other programmed cell death factors [72]. Moreover, increased Mcl-1 expression has been associated with other poor prognostic markers [73]. Interestingly, in the past CLL was considered to be a disease of tumor cell accumulation due to impaired apoptosis rather than over-proliferation. However, more recent research clarifies that CLL is rather a dynamic disease with a higher leukemic cell turnover than previously detected, which is derived from proliferative pools in the bone marrow and lymph nodes [74, 75]. Thus, precise regulation of the balance between pro-survival and pro-apoptotic signals is a critical contributor to survival of LSC in both myeloid and lymphoid leukemic malignancies.

Increased Self-Renewal Potential

Self-renewal pathways that are commonly deregulated in LSC include canonical developmental pathways, such as the Wnt/β-catenin [9, 76, 77] and Hedgehog (Hh) signaling pathways [76, 78, 79], which are evolutionarily conserved and direct essential developmental processes. While these signaling molecules and developmental processes are tightly controlled and generally inactive in differentiated adult tissues, they play an important role in normal adult stem cells. During blastic transformation of CML, activation of Sonic hedgehog (Shh) [76, 79] and β-catenin [9, 77] and also inactivation of the negative regulator of β-catenin GSK3β through production of a misspliced kinase-deficient form [18] enhance the self-renewal capacity of leukemic GMP. Enforced BCR-ABL expression drives increased β-catenin levels, suggesting that the effects of β-catenin on CML LSC maintenance may be directly related to BCR-ABL kinase activity [80].

Deregulation of the β-catenin pathway appears to be a common event in leukemic transformation, as activation of β-catenin has been observed in AML [64]. Other signal transduction factors also contribute to LSC maintenance, such as constitutive activation of Flt3 and STAT5A, which has been shown to play a role in self-renewal and aberrant differentiation of AML LSC through a process that can involve downstream pathways involving STAT5, homeobox (HOX) genes, and hepatic leukemia factor (HLF) [81]. HOX genes are master transcription factors that are highly conserved and tightly regulated during embryonic patterning and development [82] and in tissue repair and regeneration. This family of genes also plays a role in hematopoietic development [83] and has recently been investigated for use in ex vivo reprogramming strategies targeting bone marrow-derived cell populations [84]. Other self-renewal factors involved in LSC maintenance include the polycomb group (PcG) protein Bmi1 along with cofactors Meis1 and Hoxa9 in MLL-induced AML [85, 86]. In T- and B-cell lymphoid malignancies, the NOTCH1 signaling pathway is an important driver of progenitor self-renewal [87], along with the activation of the LIM-only domain protein LMO2 and/or inactivation of the tumor suppressor Arf [88]. Thus, it has become increasingly clear that developmental stem cell pathways represent a common molecular thread linking a wide range of CSC-driven human malignancies. Aberrant activation of these pathways can facilitate the acquisition of stem cell-like properties, thus driving the evolution of LSC to favor more primitive phenotypes and functions (enhanced survival and self-renewal) throughout disease progression.

Niche Retention and Progenitor Cell Dormancy

Interactions between LSC and their specialized niches are vital for maintenance of the stem cell-like characteristics of LSC, and signals derived from the stromal microenvironment can alter the expression patterns of survival pathways implicated in LSC generation and maintenance. In leukemic disorders, LSC can become dormant in supportive microenvironments such as the bone marrow [89, 90] (Fig. 1). Bone marrow resident LSC harbor enhanced survival and

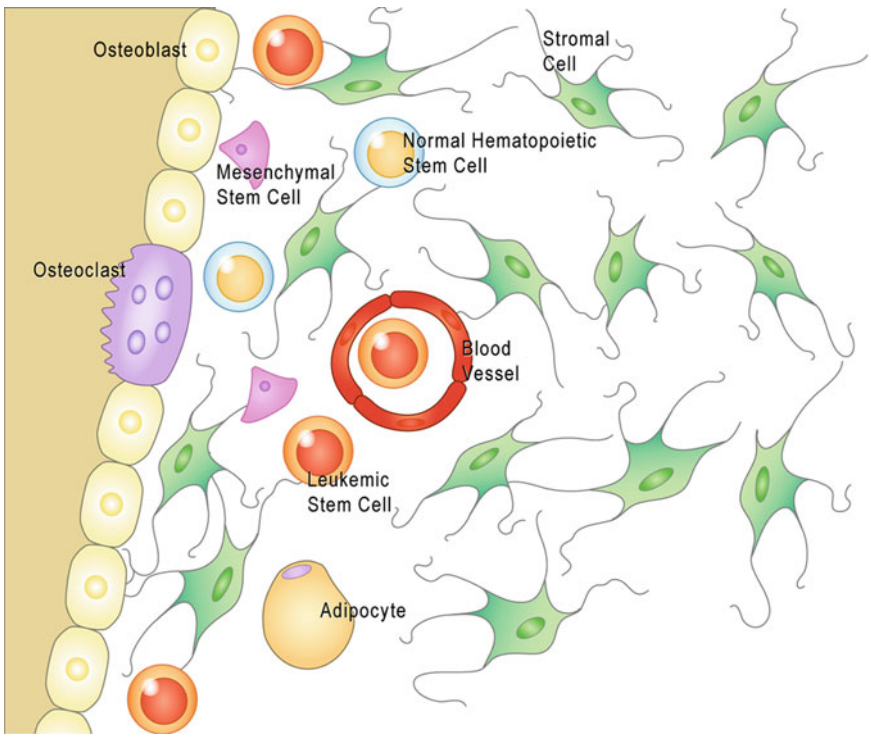

Fig. 1 Schematic diagram of components of the bone marrow microenvironment in leukemia. Leukemia stem cells migrate into the bone marrow where they can become dormant and reside preferentially in the endosteal niche. Aberrant intercellular signaling between LSC and other constituents of the bone marrow microenvironment (osteoblasts, osteoclasts, mesenchymal stem cells, adipocytes, endothelial cells, stromal cells, and normal hematopoietic stem cells) can disrupt microenvironmental homeostasis and promote LSC survival and self-renewal

self-renewal capacity and persist in a quiescent state [91], particularly in the endosteal niche [92] (Fig. 1). Notably, cellular quiescence induced by niche localization may also protect cells from radiation or chemotherapy [93], and poor disease outcomes can occur as a result of resurgence of leukemic cells derived from these resistant populations of LSC.

Numerous molecules can influence LSC interactions with the bone marrow microenvironment, including cell surface markers and signaling receptors. Preclinical studies identified the transmembrane glycoprotein CD44—a β-catenin target gene and putative CSC marker in a variety of cancers [94]—as an important mediator of the interaction between LSC and the bone marrow niche in both myeloid and lymphoid malignancies. Interestingly, antibody-mediated inhibition of CD44 resulted in a reduction of LSC burden in murine recipients of CD44-mutant BCR-ABL1-transduced progenitors [95] or human AML LSC burden in xenograft models [96]. Another cell surface receptor that is critical to HSC homing to the bone marrow and acquisition of quiescence is C-X-C chemokine receptor type 4 (CXCR4) and its ligand, CXCL12 (SDF-1) [97, 98]. CML cells express CXCR4 [99], and upregulation of CXCR4 by the TKI imatinib promotes migration of CML cells to the bone marrow, inducing cell cycle arrest and facilitating the survival of quiescent CML progenitor cells [100]. Together these results indicate that the bone marrow microenvironment provides a privileged sanctuary for LSC that protects them from TKI intervention and promotes the evolution of LSC towards therapeutically recalcitrant LSC.

Role of Additional Molecular Alterations in LSC-Driven Leukemic Transformation

Extensive research on molecular events fueling human leukemic progression has focused on acquired DNA mutations. Besides the known initiating oncogenic events that can occur at the HSC level in leukemia, such as BCR-ABL translocation in CML, NOTCH1 mutations in T-ALL, and in AML chromosomal abnormalities involving chromosomes 8 and 21 (translocations), 16 (inversions or translocations), 15 and 17 (translocations), or changes in chromosome 11 (Table 1), advances in DNA sequencing technologies in recent years have facilitated the identification of additional genetic abnormalities associated with leukemic transformation. Additional cytogenetic abnormalities, present in approximately 7 % of CML patients at diagnosis [101], may promote genetic instability and drive the development of additional DNA aberrations. Massive parallel sequencing studies of lymphoid leukemias such as CLL identified four mutated genes in coding regions (KRAS, SMARCA2, NFKBIE, and PRKD3) all in cases with progressive disease [102]. Acquired mutations or genetic polymorphisms in molecular regulators of HSC, such as the recently described BIM polymorphism that mediates TKI resistance [70], contribute to the molecular evolution of LSC by promoting cell survival, self-renewal, dormancy, and other functions.

Deregulation of epigenetic modifiers that control the acetylation and methylation status of DNA plays a pivotal role in leukemic transformation and the molecular evolution of LSC. Mutations in the DNA methyltransferase gene DNMT3a show age-related correlation with poor prognosis in AML [103], while activation of the lysine-specific demethylase KDM1a appears to sustain the MLL-AF9-induced oncogenic program in AML LSC [104]. Histone deacetylase (HDAC) enzymes, which regulate DNA expression by modifying histone proteins in chromatin, have been identified as therapeutic targets in several cancers [105, 106], and inhibitors of these enzymes have shown promise in eliminating LSC in CML alone and in combination with TKIs [107]. Similarly, a more selective inhibitor strategy targeting NAD-dependent histone deacetylase Sirtuin 1 (SIRT1) enhanced sensitivity of CML progenitors to imatinib-induced apoptosis but did not affect survival of normal progenitors [108].

Also in CML, translocations in ecotropic viral integration site 1 (EVI1), resulting in overexpression of this transcriptional regulator, appear to coordinate with BCR-ABL1 in the evolution of TKI-resistant blast crisis [109]. Notably, EVI1 is also deregulated in human AMLs and was recently identified as a target of transcriptional regulation by runt-related transcription factor 1 (RUNX1)—a frequent mutational target in myelodysplastic syndromes (MDS) and AML [110]. One of the mechanisms by which RUNX1 regulates the transcription of EVI1 is by acetylation of the histone H3 on its promoter region [110], and RUNX1 mutations in cytogenetically normal AML have been associated with poor prognosis and upregulation of lymphoid genes or lineage infidelity [111].

Although AML is quite heterogeneous in terms of its cytogenetic features in different forms of the disease, mutational analyses have begun to show promise for providing molecular diagnostic and prognostic markers. The 12 most frequently mutated genes in AML are FLT3, NPM1, CEBPA, KIT, N-RAS, MLL, WT1, IDH1/2, TET2, DNMT3A, and ASXL1 [112], and better understanding of the prognostic and functional significance of specific mutations may improve patient stratification and clinical outcome with more personalized treatment strategies based on molecular alterations. Base-pair resolution DNA methylation sequencing studies revealed profoundly divergent epigenetic landscapes in AML [113], and a number of the commonly mutated genes associated with leukemic transformation are factors involved in epigenetic modification of gene expression, particularly RNA processing, exon skipping, and splicing, and directly regulate stem cell function [114]. General changes in pre-mRNA splicing as a result of p210BCR/ABL kinase activity may contribute to CML pathogenesis [115]; however, the mechanisms promoting abnormal RNA processing in CML

remain to be elucidated. We have recently shown in whole transcriptome RNA sequencing studies that specific pro-survival splice isoforms of BCL2 family genes are enriched in LSC during CML progression [116]. Together with recent seminal studies identifying spliceosome mutations as harbingers of leukemic transformation and therapeutic resistance of leukemias, these findings have underscored the importance of elucidating the role of RNA alterations in cancer progression.

DNA sequencing and microarray gene expression results suggest that prognosis in human myeloid disorders is governed by specific mutations in splicing-related and RNA-processing genes that may generate splice isoform diversity more typical of primitive stem cells. Alterations in splicing factor genes [117] and epigenetic modifiers, such as *SF3B1* and *U2AF1*, have been increasingly found in hematologic malignancies [52] but with varying prognostic implications suggesting that effects of these mutations are cell type and context-specific. Recent genome sequencing research revealed a recurrent missense mutation at Ser34 of U2AF1, an essential component of the spliceosome that was associated with an increased risk of secondary AML transformation in patients with MDS [118]. Similarly, *SF3B1* mutations have been identified in approximately 10 % of CLL patients [119], and mutational status of this gene appears to correlate with disease progression and has prognostic value [120].

Essential differences between murine and human malignancies, particularly with regard to DNA repair and splicing, have been highlighted in recent years. Aberrant splicing could occur as a result of described leukemia-associated mutations in splicing factor genes but also as a downstream effect of deregulation of other RNA-processing mechanisms. An RNA-processing event that is relatively unexplored in the context of LSC maintenance and evolution is RNA editing, which occurs via enzymatic RNA editase activity targeting adenosines or cytidines in cellular mRNAs. RNA editing plays an important role in both embryonic hematopoietic cell fate determination and in maintenance of normal hematopoiesis [121, 122], and recent studies implicate increased expression of RNA-editing enzymes in the mutational evolution of cancer [123]. This family of enzymes is receiving growing attention in hematologic malignancies as overexpression of the RNA editase adenosine deaminase acting on RNA (ADAR)1 occurs in LSC during CML progression [124]. Furthermore, genetic knockdown of the ADAR1 gene in human or mouse cells prevents development of CML-like disease in in vivo models, highlighting the potential utility of novel therapeutic strategies targeting aberrant RNA processing activity in LSC [124, 125]. Furthermore, another poorly understood posttranscriptional modifying event of RNA—methylation of the N(6) position of adenosine (m(6)A)—is enriched in 3′UTRs and near stop codons, which may have additional implications for the epigenetic regulation of the mammalian transcriptome [126]. Thus, it is quite possible that aberrant posttranscriptional RNA alterations may contribute to remodeling of the cellular transcriptome and proteome of LSC that drive tumor progression and relapse; however, future studies will be necessary to elucidate the precise role of these events in leukemic progression and evolution of therapeutically recalcitrant LSC.

Conclusions

Recent advances in genomic technologies raise the possibility that the molecular pathways driving the initiation and progression of leukemia may be quite similar across a variety of myeloid and lymphoid malignancies, whereas cell type and context-specific factors may dictate the precise clinical presentation and outcome of each individual disease. Precise regulation of the balance between cell survival, differentiation, self-renewal, and dormancy is critical for the maintenance of LSC in both myeloid and lymphoid leukemic malignancies. Furthermore, the epigenetic and transcriptomic landscape of LSC represents a vital component of this process that is tightly integrated with genetic aberrations and molecular pathways that drive LSC generation and therapeutic resistance.

While current treatments are relatively effective at managing CML in early stages, TKI intolerance and noncompliance drive therapeutic resistance and disease progression in a significant fraction of patients. The development of effective therapies that eliminate LSC is predicated on a comprehensive understanding of a network of epigenetic and genetic mutations that alter differentiation, survival, dormancy, and self-renewal as well as the cellular context in which they arise. Future studies incorporating stem cell functional validation into cancer genomics, self-renewal, survival, and quiescence properties will be vital to facilitate evaluation of these essential CSC properties and will inform prognostication and therapeutic strategies that may avert relapse, particularly in patient populations that are poor candidates for current treatments.

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Rodent Models for Assessing the Role of Stem Cells in Liver Development, Regeneration, and Carcinogenesis

Jennifer A. Sanders and Douglas C. Hixson

Introduction

The concept of bipotent hepatic progenitor cells residing in the cholangioles, the smallest ducts that connect the bile canaliculi to the biliary tree, was postulated more than 50 years ago by Wilson and Leduc [1]. This concept has been supported by many subsequent studies demonstrating the activation and proliferation of small basophilic progenitor cells with ovoid nuclei after physical or chemical injury or treatment with hepatocarcinogens [2–7]. Because these so-called oval cells were only activated under extreme conditions, they were designated by Grisham et al. as "facultative stem cells," a designation suggested by their ability to undergo hepatocyte or ductal cell differentiation [8]. In the past the presence of a stem/progenitor cell population in the adult liver and their roles in injury, regeneration, and carcinogenesis had been a source of debate. This controversy is due to the lack of cell turnover in the normal liver and the demonstration that hepatocytes and bile ductal epithelial (BDE) cells have an immense capacity for self-renewal that is more than sufficient to regenerate and maintain the size and functional capacity of the liver [9, 10]. Now there is evidence from a number of recent studies that has led to the general acceptance of the existence of cell population(s) with stem-like properties in the adult liver [11, 12]. The term "oval cell" is commonly used to describe a heterogeneous population of liver cells that likely includes multiple cell types in addition to cells with stem-like properties. Controversy still remains over the origin, phenotypic identification, molecular traits, and role of oval cells in normal regeneration and carcinogenesis. It is also unclear whether oval cells from different species or that arise from different insults are comparable [13]. In the future it seems likely that the descriptions of various stem/progenitor populations in the liver will include cell-surface marker designations.

Under severe or chronic liver injury where the proliferative capacity of hepatocytes and ductal cells has been compromised, hepatic stem/progenitor cells play an essential role as a fail-safe system that can repair the liver [8]. Indeed, studies suggest that there are three and possibly four bi- or multipotent cell populations coexisting in the normal adult liver. Primitive periductal progenitors, designated as type 0 by Sell [3] and as "blast" or "basal" cells by Novikoff and Yam [14], are the first cells that undergo DNA synthesis and proliferation following treatment with liver carcinogens [14–16]. These cells lack lineage markers and reside either in the portal mesenchyme in close proximity to bile ductules or sequestered inside a basal compartment formed by bile duct epithelial cells (BDEC). Other bipotent liver progenitors such as those designated type I, type II (classic oval cells), or type III (transitional hepatocytes with ductal and hepatocyte features) are viewed by some as "transit" cells that rapidly proliferate and eventually differentiate into hepatocytes or ducts [3, 4, 6, 17]. The Sox9-positive cells isolated by Dorrell et al. using cell-surface antibodies seem to closely resemble those progenitors designated as type II [11]. In relating these adult stem cell types to fetal progenitors, the rat type 0 progenitors, the OC.10 positive, bipotent, fetal ductal cells isolated by Simper-Ronan et al. [18] from embryonic day 16 fetal livers, and the type II progenitors in adult liver would seem to most closely resemble the 12–14-day progenitors described by several groups [19–21]. In addition, Gordon et al. [22], Tateno et al. [23, 24], and others have also described what have been called "small hepatocyte progenitor" (SHP) cells. SHPs are present in normal liver in low numbers but expand rapidly in retrorsine-treated rats. SHPs would seem to fit the characteristics ascribed by Sell [3] for type III progenitors since they initially express both hepatocyte and bile ductal markers and seemed to be restricted to differentiation along a hepatocyte lineage.

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Because hepatocytes and BDE cells have an immense capacity for proliferation and "self renewal" [9, 25], it has been suggested that they are in reality "unipotent" stem cells [26], a concept that seems at odds with the basic properties ascribed to true stem cells. For example, proliferation of hepatocytes and BDE appears to involve symmetric cell division. In essence the two daughter cells are identical to their parent. True stem cells, in contrast, not only undergo symmetric cell divisions but also divide asymmetrically. This results not only in self-renewal but also in the production of daughter cells that can undergo further differentiation [4]. Recent investigations in *Drosophila* and *C. elegans* demonstrate that this process is mediated by factors that direct the orientation of the mitotic spindle and asymmetric localization of adaptor proteins and cell fate determinants into one daughter cell. After mitosis the proteins involved in cell fate determination act together to prevent self-renewal and proliferation and promote differentiation [27]. Although most of the key players involved in asymmetric cell division in these model organisms are conserved in vertebrates, their function still remains unclear. Herein lies a second distinction between "stem cells" and hepatocytes—the absence of differentiation and lineage progression, a hallmark of stem cells that is unnecessary for hepatocytes or mature BDEC. Under conditions where the replicative capacity of hepatocytes has not been compromised, such as restitutive proliferation following partial hepatectomy, regeneration appears to be a purely replicative process mediated by mature hepatocytes or ductal cells [25, 28]. In addition, true stem cells have a self-renewal capacity that renders them immortal relative to the normal life span of the animal. Hepatocytes, in contrast, have a finite longevity and, over the course of a year, are completely replaced by "new" hepatocytes through the normal process(es) of renewal [29]. In this respect, hepatocytes are more akin to progenitor cells, a bi- or multipotent cell population distinguished from stem cells by their more limited capacity for self-renewal [28].

When all of the current information on hepatic progenitor cells is viewed as a whole, the picture that emerges is a multitiered system of renewal designed to provide alternative pathways to liver regeneration that assure the retention or the reestablishment of liver functionality even when mature hepatocytes and ductal cells have been severely compromised. Various studies have documented the ability of hematopoietic stem cells (HSC) to differentiate into hepatocytes [30, 31]. Transplantation experiments using genetic markers demonstrate that the majority of HSC-derived hepatocytes contain genetic information from both the donor and the host indicating that bone marrow-derived hepatocytes are a result of cell fusion [32, 33] or of the uptake of hepatocyte microvesicles with the ability to induce the expression of characteristic hepatocyte genes [34]. However, the involvement of HSCs in the regenerative response remains controversial as studies have demonstrated that HSCs contribute less than 1 % to liver repopulation [35, 36].

Since hepatic stem/progenitor cells are few in number and thus not usually apparent in the normal adult liver, development of methods to induce their activation, expansion, and differentiation in a reproducible fashion is essential for understanding the molecular events that determine their ultimate fate, particularly during the course of liver carcinogenesis and chronic disease. In the remainder of this review, we discuss a number of the most commonly used animal models for studying hepatic progenitor cell populations and strategies used for lineage analysis.

Selective Activation of Rodent Liver Progenitor Cells

In most animal models, activation of progenitor cells requires treatment with agents that severely compromise the proliferative capacity of hepatocytes but have little or no effect on progenitor cells, presumably because progenitors lack the cytochrome P450s needed to generate mitoinhibitory metabolites. 2-acetylaminofluorene (2-AAF), a liver carcinogen that is effective in blocking the regenerative capacity of hepatocytes at noncarcinogenic doses, has been used for this purpose in a number of oval cell induction protocols [37–39]. When coupled with partial hepatectomy or treatment with CCl_4, 2-AAF induces a moderate oval cell expansion (Fig. 1) that peaks between 7 and 9 days after PH or CCl_4 [38, 40]. The reproducibility and short induction period have led to wide use of this protocol. A considerably larger oval cell

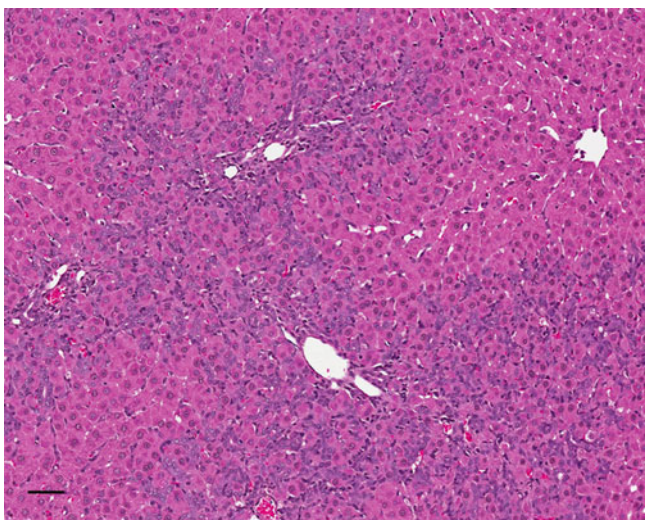

Fig. 1 Oval cells in the liver of Fischer rat 7 d after treatment with 2-AAF and partial hepatectomy. Hematoxylin and eosin staining of formalin-fixed paraffin-embedded liver. Oval cells are the basophilic cells emanating from the portal tracts and diffusing out into the liver parenchyma. Bar = 50 mm

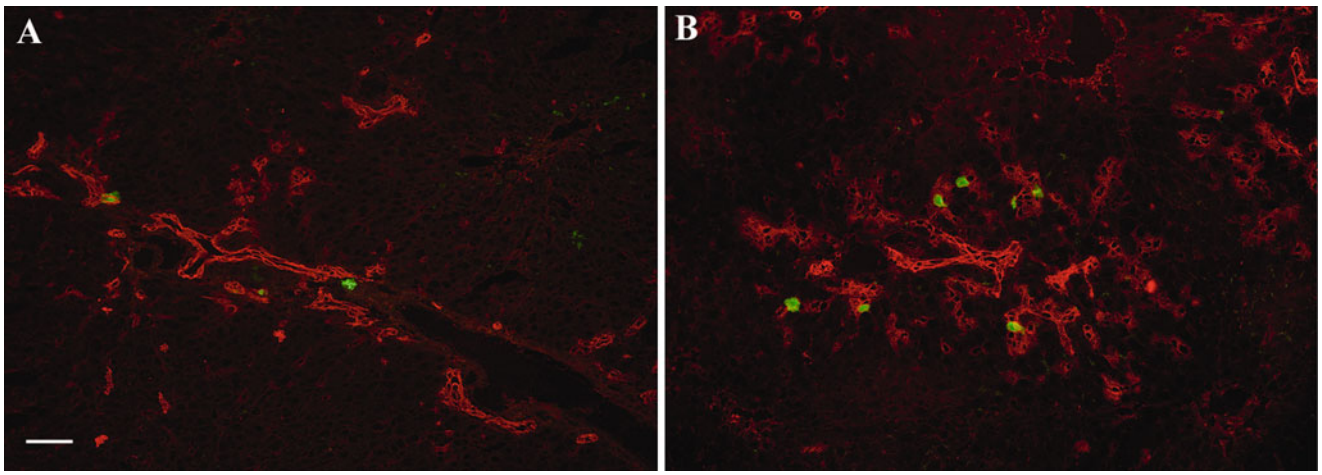

Fig. 2 Oval cells induced in rats fed a choline-deficient diet in combination with daily ethionine injection. Oval cells are brightly stained by indirect immunofluorescence with a monoclonal antibody OC.10 (*red*) that recognizes oval cells and bile duct epithelial cells and the proliferation marker phospho-histone H3 (*green*). Rats maintained on the protocol for (**a**) 11 or (**b**) 15 days. Bar = 50 mm

expansion can be obtained by combining 2-AAF with a choline-deficient (CD) diet [37]. With this protocol, as much as 80 % of the liver is occupied by oval cells and atypical oval cell ducts within 2–4 weeks. A major drawback to this system is the high mortality in treated animals (100 % by 5 weeks), a figure that does not improve significantly when animals are placed on a choline-sufficient diet [37]. It could be that in the AAF/CD-treated liver, an unfavorable microenvironment shortens the life span or hinders the differentiation of oval cells, the end result being inadequate liver function due to a failure to regenerate functional hepatocytes. Whatever the mechanism, the lethality appears to require the combined effects of 2-AAF and CD diet. This conclusion is based on our own studies showing that oval cells recovered from AAF/CD treated rats form GGT+ colonies of hepatocytes when transplanted into rats maintained on CD diet [41].

Oval cells can also be induced by placing animals on a CD diet containing 0.05–0.1 % ethionine (CDE), a low toxicity protocol with very low mortality [42, 43]. Alternatively, rats maintained on a CD diet can be administered ethionine via daily injection (Fig. 2). On this protocol, small periportal cells positive for phospho-histone H3 first appear on day 7. By day 11 oval cells are present along the portal tracts and by day 15 are distributed throughout the liver and account for 55 % of the total liver area [44]. Interestingly, CD diet with or without ethionine destroys the acinar pancreas. This causes the activation of ductal progenitor cells that for unclear reasons differentiate into hepatocytes instead of acinar cells. Ethionine in a CD diet appears to act indirectly to cause DNA damage by elevating levels of reactive lipid peroxidation products, free radicals, and reactive oxygen species [45]. Ethionine is also known to cause changes in gene expression by altering DNA methylation patterns which in turn alter differentiation and/or growth regulatory pathways [46]. In addition, there is evidence that CDE diet may decrease growth inhibitors and increase growth stimulators, a shift that could facilitate oval cell expansion [47]. As one might expect, combining ethionine with a CD diet greatly enhances formation of HCC and cholangiocarcinomas [48, 49], relative to CD diet [50–52] which by itself is also carcinogenic in Fischer rats [53, 54]. Studies from our and other laboratories have shown that many CDE-induced HCC express both hepatic and oval cell/ductal antigens, a phenotype consistent with a derivation from oval cells arrested at an intermediate stage in hepatocytic differentiation [42, 55].

From a practical standpoint, protocols that avoid the use of liver carcinogens would be highly desirable and several of these have been devised. Intraperitoneal injection of galactosamine, for example, induces extensive liver damage that results in marked oval cell proliferation beginning within 24 h postinjection, peaking at 5 days [56, 57] and diminishing thereafter as oval cells undergo hepatocytic differentiation or apoptosis. In Long-Evans Cinnamon (LEC) rats, an inbred strain that carries a defect in the Wilson disease gene, oval cell expansion occurs spontaneously in response to the acute hepatitis that occurs at 20–23 weeks of age [58, 59]. Survivors of the acute hepatitis (the end result of copper accumulation caused by the genetic defect) develop chronic hepatitis that ultimately leads to the generation of cholangiocarcinomas and hepatocellular carcinomas at a high incidence [60]. Yasui et al. have demonstrated that LEC oval cells differentiate into hepatocytes when transplanted into a normal rat liver, suggesting that at least a portion of HCC in this model system derive from oval cell progenitors [60]. Ethanol has also been reported to activate oval cells, but the time course of activation differs significantly from CDE or other rapid induction protocols. After 4 weeks of treatment with ethanol, oval cells are barely detectable but increase

steadily with time thereafter, multiplying by more than 15-fold over the next 23 months [61]. In comparison, animals maintained on a CDE diet attain oval cell densities at 4 weeks that are fourfold higher than those induced by ethanol after 24 months.

Although the rat has been the preferred animal model for studying oval cell activation, an increasing number of studies now take advantage of genetically manipulated mice to interrogate stem/progenitor cell activation and expansion. Overexpression of "TNF-like weak inducer of apoptosis" (TWEAK) stimulates oval cell proliferation [62] as does deletion of "damaged DNA binding protein 1" (DDB1) or deletion of upstream members of the Hippo-Salvador pathway [63–66]. Deletion of DDB1 abolishes hepatocyte proliferation leading to compensatory activation of oval cells, while inactivation of WW45, an upstream member of the Hippo pathway, leads to oval cell activation despite the retention by adult hepatocytes of a normal regenerative capacity [64]. The deletion of WW45 results in the development of hepatomas with an intermediate phenotype suggesting a progenitor cell origin [64], while deletion of the upstream kinases Mst1 and Mst2 leads to the development of hepatocellular carcinoma and cholangiocarcinoma again suggesting that the tumors may have arisen from progenitor cells [65]. SV40 transgenic mice offer a model system not only for studying the role of oval cells in hepatocarcinogenesis but also for examining the factors that lead to their spontaneous activation around the tenth week after birth [67]. Oval cell proliferation also occurs in mouse models of chronic hepatitis induced by infection with cytomegalovirus [68]. Many of these genetic models offer the advantage of specifically targeting hepatocytes in a background of very mild or no liver damage.

As in the rat, a number of mouse models take advantage of the oval cell expansion that occurs following chemical injury, pertinent examples being ethionine in a choline-deficient diet [69], *N*-acetyl-para-aminophenol (APAP) [70], 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) [71], diethylnitrosamine (DENA) [72], allyl alcohol [73], or *N*-nitrosodimethylamine in combination with Helicobacter hepaticus [74]. Interestingly, the repair process in the mouse following exposure to allyl alcohol differs significantly from the rat. For one thing, necrotic areas in the mouse are not restricted to the periportal zone. In addition, restoration of necrotic areas is primarily mediated by hepatocytes instead of oval cells, the major agents of repair in the rat. Mice and rats also differ in their response to 2-AAF/PH, a commonly used method for inducing oval cells in the rat that is ineffective in mice [13]. There is also evidence that there are distinct differences in the phenotypes of rat and mouse oval cells induced by CDE diet, a dietary regimen that induces oval cells in both species. Oval cells induced in the rat expressed cytokeratin, AFP, and Dlk/Pref-1 while only cytokeratin was expressed by oval cells in mice. A rare subpopulation of oval cells in the mice with a robust response transiently expressed Dlk/Pref-1[13]. These differences in phenotype urge caution when comparing progenitor-mediated regeneration between individual species and protocols.

Treatment with dipin in combination with partial hepatectomy has proven to be a useful model system for studying mouse oval cell activation, expansion, and differentiation. Of interest is the close resemblance between the dipin/PH protocol in mice and the retrorsine/PH regimen used in rats [75, 76]. Both dipin and retrorsine are potent alkylating agents capable of causing irreversible damage to liver cell DNA that severely inhibits the replicative capacity of differentiated hepatocytes [77]. This creates a dire situation that leads to the activation of proliferation-competent, bipotent progenitors capable of hepatocytic differentiation and restoration of the liver mass. Herein lies a major difference between the two model systems, the nature and origin of these bipotent progenitors. Results from both autoradiographic and morphological analyses indicate that regeneration in dipin-/PH-treated mice is mediated by oval cells in the canals of Hering that express A6 and A7, two antigens common to oval and biliary epithelial cells and transitional hepatocytes. In contrast, oval cells appear to play a relatively minor role in the retrorsine/PH model where reconstitution seems to be mediated almost entirely by a population of small hepatocyte-like progenitors (SHP) [76].

The origin of SHPs in retrorsine-/PH-treated rats is a much debated subject. Best and Coleman have shown that treatment with 2-acetylaminofluorene blocks the SHP response in retrorsine-exposed rats providing new evidence that these cells are not derived from oval cells [78]. A study by Avril et al. using a retroviral-based model to label mature hepatocytes with B-galactosidase showed that a significant number of SHP clusters expressed B-galactosidase in retrorsine-/PH-treated rats, leading these authors to conclude that mature hepatocytes give rise to SHPs [79]. However whether other cell types were labeled with the B-galactosidase gene was never specified, leaving open the question as to whether SHPs are the progeny of mature hepatocytes or represent a novel stem-like progenitor cell.

Significantly, SHPs are also present in healthy rat livers, suggesting they may play a role in normal repair and renewal processes [24]. SHPs are distinguished in several respects from oval cells, BDEC, and other liver progenitors (Fig. 3). At the time of emergence, SHPs already express the hepatocyte-specific proteins albumin and transferrin [22]. In addition, early SHPs transiently express the ductal markers OC.2 and OC.5 [22, 55] and display high levels of the transferrin receptor which is not detectable by IIF on oval cells or any other liver epithelial cell types [80]. SHPs are also distinguished from oval cells and oval cell progenitors by their expression of H.4, a hepatocyte-specific epitope that is never found on oval cells or fetal/adult BDEC [22].

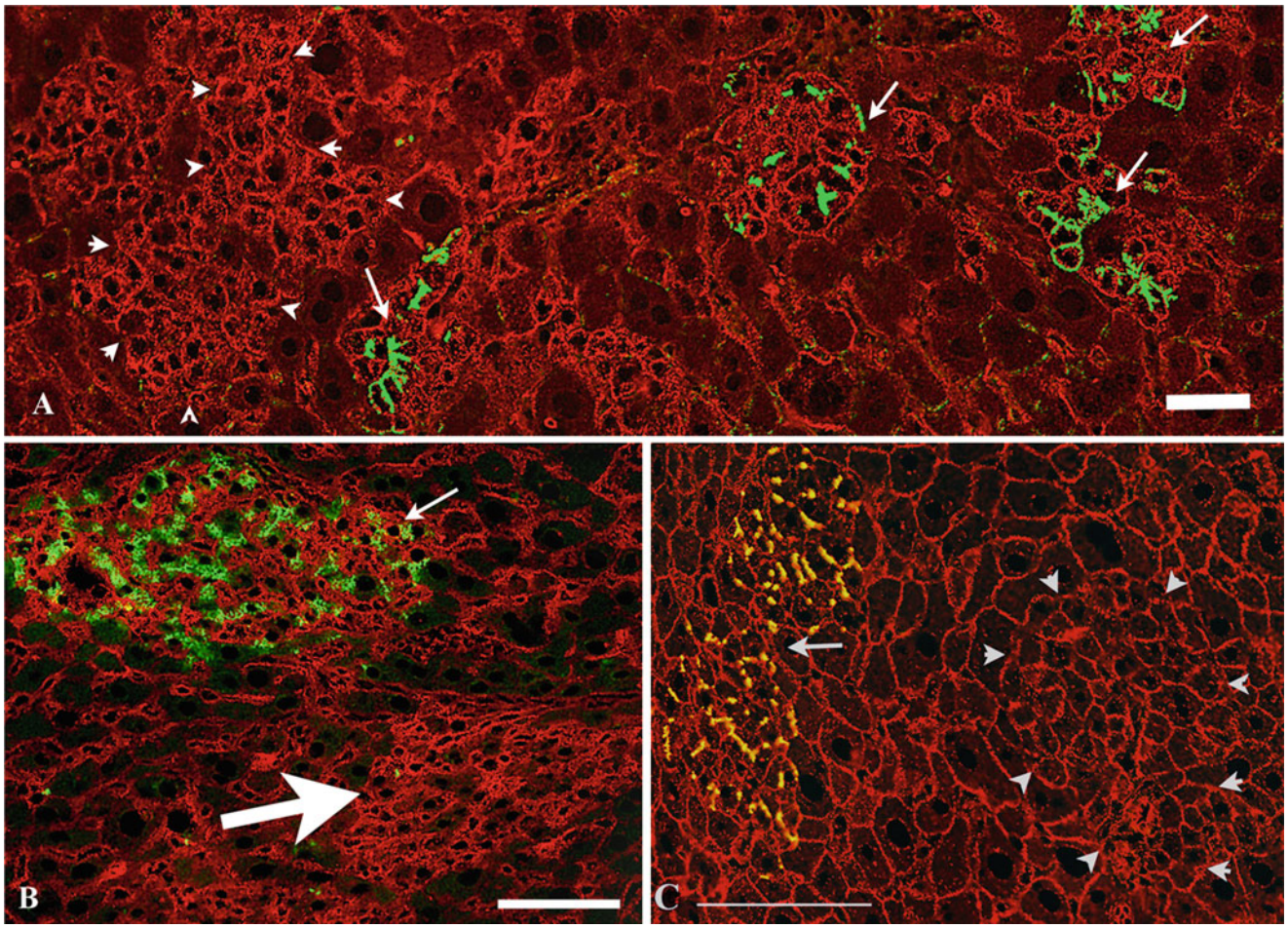

Fig. 3 Characterization of endogenous and transplanted small hepatic progenitor cells in the retrorsine partial hepatectomy model. DPPIV-negative hosts were subjected to the retrorsine/PH protocol and transplanted with freshly isolated small hepatocytes (SHPs) from normal DPPIV-positive rat liver. (**a**) An area containing colonies composed of exogenous dipeptidyl peptidase IV-positive (*green*) small hepatocytes (*arrows*) and endogenous DPPIV$^-$ SHPs (*arrowheads*) 7 days after PH. Both exogenous and endogenous SHPs show high levels of transferrin receptor staining (*red*). (**b**) Exogenous DPPIV-positive (*green*) small hepatocyte colony (*small upper arrow*) and an endogenous SHP colony (*large lower arrow*) with positive sinusoidal reactivity for transferrin receptor. (**c**) Expression of hepatic marker H.1 (*red*) in exogenous DPPIV-positive (*yellow*) small hepatocytes (*small arrow*) and endogenous SHPs (outlined with *arrowheads*) 21 days after transplantation. Bar = 100 mm. Photomicrographs originally published in Tateno et al. [80]

Human Model Systems for Oval Cell Activation

It is impossible, for obvious reasons, to treat human subjects with agents that induce oval cell proliferation. Consequently, studies of human liver have to rely on biopsy specimens, excess tissue from tumor resections, and tissue from liver transplants or donated livers that for logistical or immunological reasons went unused. When taken together, results from a number of studies suggest that progenitor cells arise in the human liver under conditions similar to those that activate their rodent counterparts. Oval cell expansion is a consistent feature of liver tissue taken from patients with alcoholic liver disease, genetic hemochromatosis, and NASH and from patients infected with HCV or HBV [81–84].

In human liver disease there is an association between severity and an increase in the number of progenitor cells [83]. Hepatic progenitor cells positive for c-kit and OV6 have been found in liver specimens from pediatric patients diagnosed with biliary atresia, alpha 1 anti-trypsin deficiency, or fulminant hepatic failure [85, 86]. Type II oval cells and type III intermediate hepatocyte-like cells have also been found in specimens from children with chronic HBV infection [87]. As in rodents, oval cell activation also occurs in humans exposed to aflatoxin, a potent human carcinogen that in mice works synergistically with HBV to produce a more rapid and extensive oval cell proliferation and a shortened time of progression to HCC [88]. The availability of human liver tissues containing proliferating hepatic progenitors raises the possibility of using strategies developed in rodent model systems for isolating these cells and

characterizing their growth and differentiation under both in vivo and in vitro conditions.

Targeted Liver Injury in the Rat

Hepatotoxins can be valuable tools to amplify rare pathways of differentiation. Their major drawback is a lack of specificity that results in varying degrees of collateral damage which can often confound the interpretation of results. This is exemplified by 4,4′-methylenedianiline (DAPM), a polycyclic aromatic amine that produces a time- and dose-dependent ductal necrosis [89]. In rodents, DAPM treatment has been shown to inhibit carcinogenesis initiated in the liver by ethyl-hydroxy-ethyl-nitrosamine, 2-AAF, and methyl-dimethyl-amino-azobenzene [90], suggesting that DAPM was destroying initiated ductal progenitors. Support for this conclusion was provided by Peterson et al. [38] who reported that treatment with DAPM prior to PH greatly attenuated oval cell expansion in response to AAF/PH. A recent study by Limaye et al. used DAPM in conjunction with bile duct ligation in chimeric rats that only express dipeptidyl-dipeptidase IV in hepatocytes [91]. This protocol resulted in the transdifferentiation of hepatocytes into BDEC. Taken together, these studies suggest that there are unrecognized variables that influence the effects of DAPM, raising uncertainties about the usefulness of this biliary toxin for assessing the nature and origin of biliary progenitors.

Allyl alcohol, another hepatotoxin that induces progenitor cells, is distinguished by its ability to target hepatocytes located in the periportal region [92]. Intraperitoneal injection of allyl alcohol initiates the proliferation of intraportal progenitors lacking both ductal cell and hepatocyte lineage markers. Following expansion into the region of periportal necrosis, these hepatic "stem-cells" repair the damage by undergoing hepatocytic differentiation [92]. The restriction of stem cell-mediated regeneration to the periportal region is the key advantage offered by this system. Unfortunately, the marked lobular and intralobular variability in the extent of damage and stem cell activation is a major limitation that hampers analysis and complicates the interpretation of results.

In a series of reports, Sirica and coworkers have shown that under extreme conditions of liver injury or toxicity where there is extensive damage to existing hepatocytes, progenitors other than oval cells can be activated. CCl_4 treatment of bile duct-ligated rats was reported by these investigators to produce duct-like structures composed of ductal cells and hepatocyte-like cells in various stages of differentiation, suggesting that under these severe conditions, interlobular BDEC had undergone hepatocytic differentiation [6, 93]. Severely hepatotoxic doses of furan also produced hepatocyte-containing ducts resembling the mixed ducts induced by BDL/CCl_4 treatment [10]. Unfortunately, the high mortality associated with this protocol severely limits its application for lineage analysis at the molecular level. However, at lower nonlethal doses of furan, a very different outcome was observed. Under these conditions, ductal cells in the right and caudate lobes entered into an intestinal lineage leading to the appearance of well-developed intestinal glands [94]. Prolonged treatment with furan led to the development of primary hepatic adenocarcinoma with small intestine cell differentiation in the right and caudate lobes only [95]. The intestinal metaplasia observed in this model is associated with the expression of the intestine-specific transcription factor CDX1 and overexpression of the growth factor receptors c-neu and c-met [96, 97]. Although the mechanism for this dramatic change in lineage commitment remains unclear, it seems likely that furan treatment resulted in the activation of multipotent periductular stem cells. Under the influence of a microenvironment that was apparently unique to the right and caudate lobes, these multipotent progenitors entered an intestinal instead of a hepatocytic pathway of differentiation. A similar change in commitment is also observed with pancreatic ductal cells following depletion/repletion of copper, in this case a shift from a pancreatic to a hepatocytic lineage.

Carcinogenesis Protocols Producing Progenitor Cell-Derived HCC

Numerous studies with human samples and animal models indicate that progenitor cell activation is a consistent feature of viral hepatocarcinogenesis. Patients with chronic HCV and HBV infections often exhibit extensive oval cell proliferation, usually in close proximity to regenerating nodules and HCC [82, 83]. Furthermore, approximately 50 % of the HCC found in HBV-positive individuals express oval cell and hepatocyte markers, a percentage similar to CDE diet-induced carcinomas in the rat. These observations suggest that a portion of HCC with a viral etiology arise from oval cell progenitors. In the Woodchuck experimental model system for viral hepatocarcinogenesis [42, 82], oval cells are nonpermissive for virus infection, suggesting that similar to other induction protocols, activation of progenitors in the Woodchuck model results at least in part from the ability of the virus to directly or indirectly compromise the viability/replicative capacity of hepatocytes. Indeed, based on their studies of the pathogenesis of hepatitis B virus in human liver and the livers of mice transgenic for the HBV surface antigen, Dunsford et al. [98] suggested that chronic liver injury and inflammation and the resulting compensatory mitogenesis play a central role in the carcinogenic process by causing mutations in proliferating hepatocytes or in chronically activated progenitors. A recent report by Lowes et al. indicates that similar mechanisms are also operative

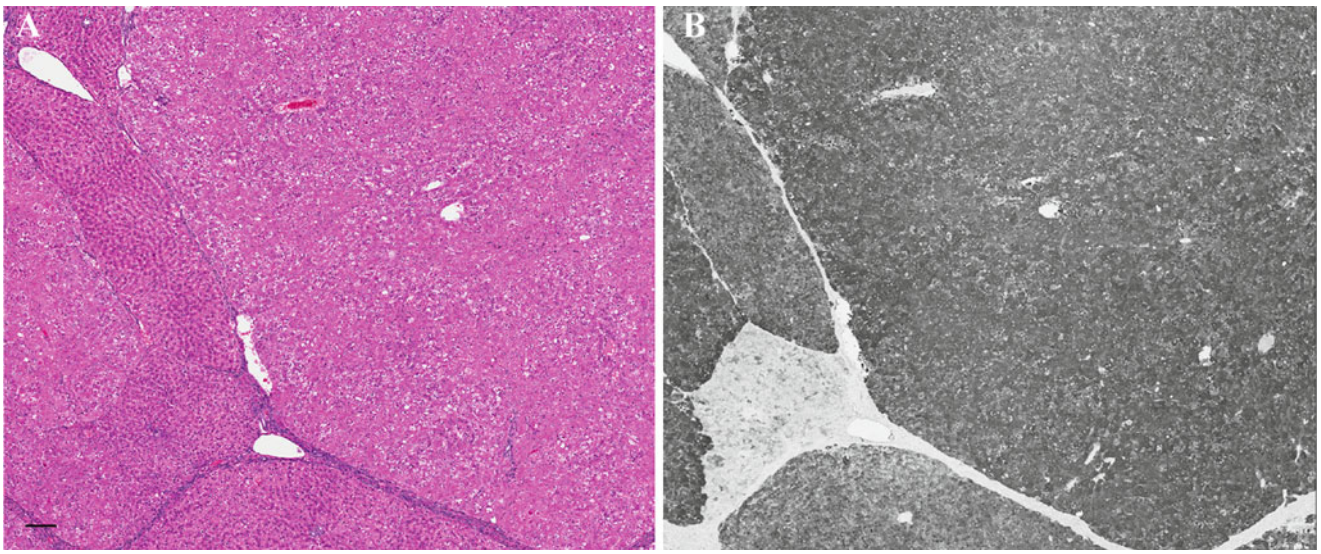

Fig. 4 Preneoplastic foci in liver of Fischer rat 70 d after starting the Solt-Farber protocol. (**a**) Hematoxylin and eosin staining of formalin-fixed paraffin-embedded liver showing dysplastic foci surrounded by oval cells. (**b**) Immunohistochemical staining for the preneoplastic marker GST-P showing homogeneously stained persistent foci and light heterogeneously stained remodeling foci. Bar = 50 mm

during hepatocarcinogenesis mediated by HCV [83]. At odds with these findings are results from a study by Tabor et al. demonstrating that HBV surface antigen was detected in oval cells and transitional cells, but not in bile ducts or ductule cells in nontumorous tissue adjacent to HCC in human liver [99]. More recently oval cell lines have been tested for their permissiveness to HCV infection. HepaRG human liver progenitor cells were susceptible to HCV infections only in an immature undifferentiated state but required differentiation along a hepatocyte lineage to support long-term production of infectious HCV particles [100]. These results suggest that oval cells in vivo may be permissive to HCV infection, and this could directly lead to hepatocarcinogenesis arising from oval cell progenitors that sustain viral damage sufficient to initiate neoplastic progression but insufficient to cause apoptosis.

In the rat, CDE diet (described above) and the Solt-Farber protocol have been the most widely used treatments for studying the progenitor cell origin of HCC. In the Solt-Farber protocol, initiation is achieved by a single intraperitoneal injection of DENA. The key element in this regimen is the delivery of a noncarcinogenic dose of 2-AAF to block hepatocyte proliferation following PH at 2 weeks after DENA (Fig. 4). This selectively promotes the growth of initiated cells that have become "resistant" to the mitoinhibitory activity of 2-AAF [101, 102]. Since oval cell proliferation occurs to a similar degree and with a similar time course as was described for oval cell induction with 2-AAF followed by PH, the initiation with DENA appears to have little or no effect on the proliferative response of progenitors. An analysis of the progression to HCC in this model demonstrated that 50 % of persistent nodules and all HCCs express cytokeratin 19 (Ck19) suggesting that this is a model of progenitor-derived HCC [103]. Moreover, comparative functional genomic analysis using the Ck19-positive signature accurately predicted HCC patient outcomes. Dumble et al. [104] have recently described a novel liver carcinogenesis model utilizing p53 knockout mice. These investigators showed that oval cells from p53 knockout mice maintained on CDE diet progressed to hepatocellular carcinoma following transplantation into nude mice, a finding that provided further support for oval cells as progenitors of HCC.

There are many additional hepatocarcinogens that induce oval cell expansion and their discussion is beyond the scope of this review. However, it is interesting to note that much of what is known about the process of liver carcinogenesis, e.g., initiation, promotion, and progression, has been acquired using carcinogenesis protocols that induce minimal activation of progenitors cells and produce almost exclusively HCC derived from initiated hepatocytes [105–108]. It is not surprising, therefore, that for many years, attention centered on hepatocytes, a bias that diminished the role of hepatic progenitor cells. Recent studies that classify patients with HCC according to progenitor cell markers have found that tumors positive for these markers represent a novel subclass of HCC with a unique genetic signature [109, 110]. These tumors are also associated with a more aggressive phenotype and a poor prognosis compared to progenitor marker-negative tumors [109, 111, 112]. However, both the progenitor cell and hepatocyte models of liver carcinogenesis may be closely linked if one considers the possibility that initiated oval cell progenitors undergo differentiation into hepatocytes prior to progression to HCC. This idea is suggested by recent studies demonstrating the ability of transplanted or endogenous oval cells to

differentiate into hepatocytes [113, 114]. It also explains the presence of HCC displaying hepatocyte and oval cell markers, a phenotype consistent with the blocked ontogeny model proposed by Sell and Pierce [115]. This is not contradictory to the classification of tumors as undergoing differentiation prior to HCC development and would likely result in tumors with a different genetic signature and phenotype.

If HCC produced by treatment with hepatocarcinogens arise from progenitor cells, it follows necessarily that progenitor cells may become initiated at some point during exposure to the carcinogenic agent. Initiation requires DNA synthesis/cell proliferation and appropriate enzymes for activating the carcinogen. Farber et al. have argued that oval cells cannot be progenitors of HCC in the Solt-Farber protocol, because they do not fulfill either of these requirements [107, 116] at the time of DENA injection. However, at least two studies, one in rats and one in mice, have observed a transient proliferation of oval cells following treatment with DENA [72, 117]. Rapid activation of progenitors also occurs following treatment with other liver carcinogens. Significantly, Bisgaard et al. and others have shown that oval cells undergo DNA synthesis within 24 h of a noncarcinogenic dose of 2-AAF [16]. There are also a number of reports indicating that progenitor cells express cytochrome P450 enzymes (CYPs) needed for activating carcinogens [118, 119]. Small hepatocytes, transitional cells described by Golding et al. [114], and BDEC all express CYPs capable of metabolizing 2-AAF and DENA, two of the most frequently used liver carcinogens [22, 120–122]. In addition, Yang et al. [123] and Ring et al. [124] have detected expression of CYPs by human embryonic liver cells at very early stages of gestation, raising the possibility that immature stem-like cells such as oval cells may also have significant levels of CYPs. It is well known that oval cells are a heterogeneous cell population composed of undifferentiated periductal/periportal progenitors and bipotent oval cells in different stages of ductal differentiation [125]. The most primitive oval cells, designated by Sell as type 0 and type 1 progenitors, would seem to be the least likely to undergo initiation because they are sequestered in a protected niche free of contacts with surrounding cells and most likely lack the CYPs needed for carcinogen activation [3, 14, 15]. In contrast, their hepatocyte or oval cell progeny, the type II and III progenitors [3], is more likely to express the appropriate CYPs. They will also upregulate CYP expression as they differentiate and integrate into hepatic cords, a location that will expose them to the promoting agent and hasten their progression to HCC.

Consideration must also be given to the possibility that cells lacking essential CYPs may become initiated by indirect mechanisms. Previous studies by Novikoff et al. have shown that oval cells can acquire a dual polarity by forming two apical domains, one with hepatocytes through a shared bile canaliculus and a second as part of the lumen of an oval cell duct [14]. Since the canalicular domain is delineated by specialized junctions shared with hepatocytes, it is conceivable that oval cells lacking appropriate CYPs could be initiated by metabolites produced by their hepatocyte partner. A similar situation may exist between oval cells and BDEC at the interface between oval cells and intralobular ducts. It should also be considered that oval cells are capable of expressing either connexin 43 or connexin 32, the gap junction proteins found on BDEC and hepatocytes [126, 127]. This raises the possibility that oval cells may be exposed to carcinogenic metabolites transferred through gap junctions. In addition, progenitor cells arise and proliferate in a microenvironment of chronic inflammation, reactive oxygen species, and growth factors [128], a microenvironment associated with the acquisition of mutations and promotion of carcinogenesis. If progenitor cells are the cell of origin for a subtype of HCC, it would be expected that the earliest lesions would contain progenitor cells, which is the case for 55 % of the earliest defined premalignant lesions that lead to HCC in humans [128].

Animal Models for Lineage Analysis

In the past, the fate of progenitor cells has been determined by inference using classical histochemical or immunological methods to identify oval cells and transitional hepatocyte/ductal cells with both oval cell and hepatocyte characteristics [129]. One of the most widely used markers for lineage analysis has been AFP, a fetal protein expressed at high levels by oval cells and many HCC. Since AFP is a secreted protein, serum levels can be used as a minimally invasive means to measure appearance and expansion of AFP+ oval cells and the growth of AFP+ HCC. More recently, epithelial cell adhesion molecule (EpCAM) which is expressed in small bile ducts, early fetal liver, and oval cells, but not adult hepatocytes, has been used to trace the fate of progenitor cells. These studies have shown that EpCAM is a marker of progenitor cells and that these cells transition into EpCAM+ intermediate cells with features of both oval cells and hepatocytes and finally to EpCAM+ hepatocytes [130, 131]. Labeling with tritiated thymidine or BrdU has also been used to provide evidence for hepatocytic differentiation of oval cells [37, 40, 132, 133]. In general, the labeled nucleotide is injected at time points during oval cell induction where there is minimal hepatocyte proliferation, thereby restricting labeling to oval cells. This timing is critical since the appearance of hepatocytes labeled with tritium or BrdU is the primary evidence in these studies for the differentiation of oval cells into hepatocytes. Significantly, transference of label from hepatocytes into oval cells or ductal cells has never been convincingly demonstrated, suggesting that the formation of Ck19 ducts by hepatocytes in primary culture may be a rare event that occurs

with a relatively high frequency in vitro because of the inability to accurately reproduce conditions in vivo.

A number of new strategies for following cell fates are based on the creation of subpopulations carrying endogenous or exogenous markers. Transduction in vivo with adenoviral or retroviral vectors has been used successfully by several investigators to transfer marker genes such as β-gal into hepatocytes, BDEC, and other nonparenchymal cell types. Yu et al. [134] used an adenovirus encoding β-gal to transduce normal and injured rat livers and found that nonparenchymal cells were transduced more efficiently than hepatocytes over a range of adenoviral titers. A major advantage of adenovirus is the high efficiency of transduction which can approach 100 % in vivo and in vitro. However, this high infectivity is counterbalanced by the transient nature of expression (7–14 days) and the possibility of an immune response against viral structural proteins. The latter problem can be overcome with a baculoviral vector carrying a reporter gene driven by a mammalian promoter and viral genes controlled by insect promoters that are inactive in mammalian cells [135, 136]. Although somewhat longer than adenoviral vectors, expression conferred by baculovirus is still transient in nature.

Retroviral vectors, on the other hand, produce long-term, stable expression following integration into the host genome. While the integration provides for stable expression, the integration site of the vector is often unknown, and off-target effects must be taken into consideration when interpreting data. In addition there is often extinction or attenuation of the promoter driving transgene expression in vivo [137], a problem that can be overcome to a great extent by using tissue-specific promoters such as the one for transthyretin which Ponder et al. [138, 139] showed was still driving the expression of activated *ras* in HCC 6 months after transduction [140]. To determine whether a retroviral vector could transduce the biliary tree, Cabrera et al. [141] introduced a gibbon ape leukemia virus (GALV) carrying the β-gal gene into the biliary tree of bile duct-ligated rats 24 h after ligation. Interestingly, their results showed that at 12 h after infection, most of the β-gal activity was located in Ck19-negative peribiliary cells. Although the basis for this selectivity remains to be determined, it could be that Pit 1, a sodium-dependent phosphate transporter that acts as the GALV viral receptor [142], is differentially expressed on hepatic progenitors and hyperplastic BDEC. Lentiviral vectors have been shown to transduce both proliferating and growth arrested cells at a very high efficiency and are an attractive system for transducing liver cells. Hepatocytes and hepatoblasts transduced with a lentiviral vector expressing GFP was used to assess the repopulating ability of these cells. Long-term GFP expression up to 4 months was attained when driven by the albumin promoter, while GFP expression was lost within 2 weeks when driven by the CMV promoter [143].

More recently, studies have identified putative liver progenitor cells in mice using genetic fate mapping. For these experiments mice are bred to contain a cell type-specific Cre and a conditional reporter allele (e.g., LacZ, GFP, or YFP) that is preceded by a *loxP*-flanked stop cassette which renders it inactive until acted upon by Cre recombinase [144]. This technique was used to identify Foxl1 as a marker of progenitor cells in DDC-treated mice that give rise to both hepatocytes and cholangiocytes [145]. In vivo lineage tracing using *Sox9creER*^{T2} and *Rosa26YFP* revealed that the cells that proliferate in response to a DDC diet in mice are progeny of Sox9-expressing precursors [11]. Although not conclusively proven, the Foxl1 cells isolated by Sackett et al. express Sox9 suggesting that these cells may represent a resident liver progenitor cell [12, 145].

Lineage Analysis in Chimeric Livers

As discussed above, transduction with various vectors can be used to generate what Cardiff has called "transgenic organs," a term originally invented to describe mammary glands formed in situ by mammary epithelial cells expressing a transgene of interest [146]. In the case of the liver, most strategies produce a mosaic of transgene positive and negative cells and thus fall short of a completely transgenic organ [138]. Another approach to producing organ chimeras that has yielded valuable information regarding organogenesis and cell lineage relationships has been chimeric mice generated by implanting a fusion of two 8-stage embryos into the uterus of a pseudopregnant female [147]. The resulting offspring have organs composed of cells from each of the two parental strains, thereby providing a means to identify morphological features that are clonally derived. Chimeras between mouse strains with H-2k and H-2b haplotypes, for example, were used to show that individual crypts in the large and small intestine were composed entirely of cells of a single strain, suggesting crypts were derived by clonal expansion of a single stem cell [147]. Villi, in contrast, often contained cells of both haplotypes, indicating that they were formed from progeny produced by two or more crypts. Khokha et al. [148] used a similar approach to analyze liver organogenesis in chimeras generated from congenic rat strains differing only in their major histocompatibility complex (MHC). Their results suggested that the liver was fashioned with patches of cells from each strain that were fractal in nature. For the liver, this meant that the parenchyma was generated by repetitive application of a simple cell division program which required no bias in the spatial arrangement of daughter cells. The end result was a patchwork of cells from the two mouse strains arranged in patterns that bore no relationship to either the lobular or acinar architecture of the liver. This model of parenchymal organization is thus at odds

with both streaming liver and stem cell-fed maturational models where hepatocytes are thought to continually stream from the portal areas to the hepatic veins, a movement that would require biased sitting of newly formed hepatocytes [149–151]. An even simpler mosaic liver model that avoids the complexities involved in the formation and implantation of fused embryos was recently reported by Shiojiri et al. [152]. In this model, mosaic livers form spontaneously as a result of random X chromosome inactivation in female mice heterozygous for a wild-type and an inactive OTC gene carrying the spf^{ash} mutation. Under specific immunofluorescence conditions, cells with wild-type OTC are intensely fluorescent while cells with mutant OTC remain dark. Using this system, Shiojiri et al. [152] confirmed earlier work by Khokha et al. [148] and showed that patches of negative or positive cells were connected with each other, forming cell aggregates with no definite orientations with regard to portal areas or central veins. However, data from Fellous et al. support the streaming model of liver development in humans. Clonal patches of hepatocytes identified by mutations in the mitochondrial genome were found to originate in the periportal region and spread to the hepatic vein [153].

Transplantation Analysis with Donor Cells Carrying Endogenous or Exogenous Reporter Genes

Transplantation of donor cells that can be distinguished from host liver cells by endogenous markers or exogenous reporter genes is another widely used method for generating chimeric livers suitable for lineage analysis and cell fate determinations. However, interpretation of events in chimeric livers generated by transplantation can be difficult since there are a number of confounding variables that can greatly influence the results. One issue is the stability of marker gene expression. If the marker is an endogenous gene unique to the donor cells, expression will be regulated in a normal manner. This is usually advantageous but can become a problem if expression of the marker gene is developmentally regulated, a characteristic that may preclude the analysis of early developmental stages. This is exemplified by DPPIV, a marker gene that appears late in fetal development [154] and is usually not expressed by progenitor cells in vitro. In vivo, expression levels can also vary significantly depending on the origin of the promoter driving the marker gene, with tissue-specific promoters providing more stable expression than those of viral origin (CMV, retrovirus LTR).

Assessment of differentiation potential by transplantation also requires the development of protocols for isolating subpopulations of putative progenitors. Purity thus becomes an important consideration since the presence of contaminating cells in significant concentrations complicates interpretation by raising questions about the origin of the engrafted donor cells. In a number of studies, this issue has been resolved by showing that at low doses, the number of contaminating cells cannot account for all of the donor-derived colonies. Purified donor cells must also be able to stably integrate into hepatic cords or ducts, an ability that seems to be inherent in most liver cell types, both normal and malignant. The efficiency of engraftment, however, can vary significantly depending on the origin of the donor cells and the status of the host liver. A key feature in the majority of available models is that a strong selective pressure exists in the host to promote donor cell engraftment and expansion. This selective pressure is attained by treating the host with an agent that impairs endogenous hepatocyte proliferation in combination with a growth stimulus or using genetic models that result in extensive and chronic liver injury. Inclusion of a proliferative stimulus, such as 2/3 partial hepatectomy, or repeated infusions of cells lead to an increase in liver repopulation [155]. The mode of transplantation also affects efficiency. For example, transplantation of donor cells into the liver via the spleen increases overall viability relative to portal vein infusion but delivers only 50 % of the injected cells to the liver [156]. Analysis in the liver can be further complicated by lobular differences in the distribution of donor cells and variation in density related to the distance from the liver hilum.

In spite of these caveats, transplantation continues to be the method of choice for analyzing the differentiation capacity of hepatic progenitor cells. Details of a number of current transplantation models are reviewed in Chap. 31. All of these models, by necessity, share common features. In most models, the donor cells express a gene product that distinguishes them from host cells. Transplantation models with wild-type donors and mutant hosts that lack or express an inactive form of a normal liver protein have been used extensively for lineage analysis. Transplantation of wild-type hepatocytes or progenitors capable of hepatocyte differentiation has been shown to restore serum albumin levels in Nagase analbuminemic rats (NAR) [157]. Engrafted donor cells were readily detected in tissue sections by in situ hybridization with albumin cDNA probes or immunohistochemically with anti-rat albumin antibodies. One of the advantages of this model system is the ability to quantitate engraftment efficiency, the extent and rate of expansion of donor cells, and the duration of engraftment by measuring temporal changes in serum albumin levels. The DPPIV transplantation model developed by Thompson et al. [158] offers similar advantages. In this case, the DPPIV-negative German Fischer 344 rats used as hosts produce an enzymatically inactive form of DPPIV. This allows for the localization of transplanted donor cells from wild-type American Fischer rats by a simple histochemical procedure for active DPPIV or by immunocytochemical staining methods with monoclonal antibodies that recognize only the active form of DPPIV (Fig. 5a). Although

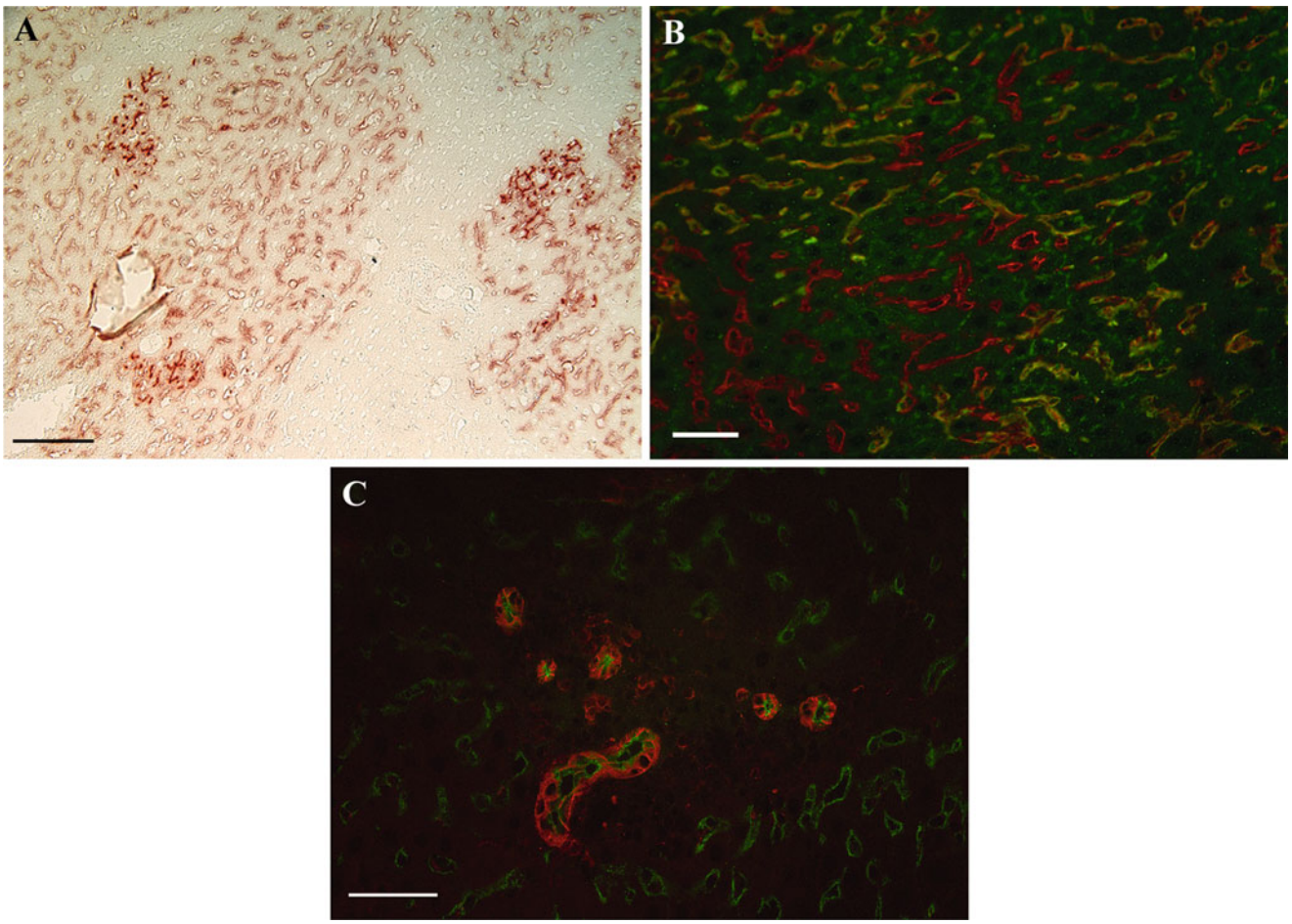

Fig. 5 Transplantation of DPPIV-positive newborn ACI liver isolates into DPPIV-negative Fischer rats treated with mitomycin C/PH and anti-CD3 antibody. Frozen liver sections prepared 1 mo after transplant (**a**). Mixed colony of donor endothelial cells and hepatocytes that were stained histochemically for DPPIV. (**b**) Dual immunofluorescence for active DPPIV (*green*) and the endothelial marker RECA (*red*) show donor cells completely and partially lining sinusoids in host liver. (**c**) Dual immunofluorescence for active DPPIV (*green*) and the bile duct epithelial cell marker OC.10 (*red*) showing engraftment of donor-derived cholangiocytes. Bar = 50 mm

DPPIV is a type II transmembrane protein, it is susceptible to cleavage by extracellular proteases that release a soluble 200 kDa, enzymatically active fragment into the plasma [159]. Plasma levels of DPPIV can thus be used to measure the same parameters noted above for albumin [160]. One limitation of the DPPIV model system is the late expression of the enzyme during fetal development [154] and its absence on most hepatic progenitors, a temporal pattern that limits its usefulness for analyzing early time points in liver development or progenitor cell differentiation. This is not an issue for albumin since it is one of the earliest development markers detected in fetal liver.

Another model system that is currently enjoying wide usage is the retrorsine model of Laconi et al. [75, 161]. In this system DPPIV-negative host rats are pretreated with retrorsine, a DNA alkylating agent that promotes the selective expansion of donor liver cells by impairing the ability of host hepatocytes to proliferate following PH. After several weeks, donor cells repopulate as much as 80 % of the liver. Although this model has proven valuable, the high cost, limited availability of retrorsine, and potent hepatotoxicity and carcinogenicity of retrorsine are major drawbacks that prompted Brilliant et al. to search for alternative models [162]. They described a rapid transplant model where DPPIV-negative host rats are administered a single dose of mitomycin C 1 week prior to PH and transplantation of donor cells. This model allows for the engraftment and rapid expansion of donor hepatocytes, endothelial cells, and cholangiocytes (Fig. 5) and can be extended to allogeneic transplantation by administering a short course of anti-CD3 antibodies to the hosts. Similar effects can also be obtained by pretreatment with galactosamine [163] or radiation, but the degree of repopulation by donor cells is considerably lower.

Other donor cell markers that have been used successfully include MHC/alloantigens [30, 31, 164] and alpha 1 antitrypsin [158, 165]. Models relying on alloantigens, an

approach pioneered by Hunt et al. [164], utilize F1 progeny as hosts for donor cells from either of two inbred strains differing in their MHC haplotypes (ACI and Long-Evans or Wistar Furth and Fischer F344) [41, 164]. Donor cells are subsequently identified using alloantisera produced by immunizing ACI rats or F344 rats with Long-Evans or Wistar Furth spleen cells, respectively. In the past, we have used this model system to demonstrate that oval cells induced by 2-AAF and CD diet formed GGT+ colonies of hepatocytes following transplantation into partially hepatectomized F1 hosts [41]. One drawback to tracing donor cell fates using alloantisera is that it is the host cells and not the donor cells that show positive staining with the alloantisera. Consequently, unstained donor cells have to be discerned against a positive background of host hepatocytes, making it difficult to detect small single donor cells or even small donor cell clusters.

In a number of studies, the fate of donor cells from male rats or mice following transplantation into female hosts has been determined by using fluorescent in situ hybridization (FISH) to detect cells bearing a Y chromosome [30]. In studies by Theise et al. [31], this approach was used to demonstrate that circulating stem cells, most likely of bone marrow origin, can integrate into the liver and differentiate into hepatocytes and cholangiocytes, an observation that challenges current concepts of differentiation and commitment. However, subsequent reports demonstrated that bone marrow cells can fuse spontaneously with other cell types and assume their identity [33, 166, 167], suggesting that the presence of the Y chromosome in hepatocytes of female hosts could represent a fusion event rather than differentiation of bone marrow stem cells into hepatocytes.

There are increasing numbers of excellent transplantation models that make use of transgenic mice carrying marker genes as a source of donor cells. These genetically marked cells are transplanted into hosts treated with chemicals that inhibit the proliferation of hepatocytes. Dorrell et al. transplanted donor *Sox9creER^{T2}-R26RYFP* progenitor cells into adult mice that were subsequently treated with tamoxifen to induce marking of the Sox9-expressing donor cells and their progeny with YFP [11]. Small numbers of YFP+ periportal hepatocytes and ductal cells were detected in the adult mouse liver even in the absence of a growth stimulus. A second option is the transplantation of marked donor cells into transgenic or mutant strains carrying genetic defects that severely compromise hepatocyte-/ductal cell-mediated regeneration and repair. Mignon et al. transplanted donor cells from transgenic mice expressing human Bcl-2 into immunosuppressed host mice treated with a nonlethal dose of anti-fas/CD95 antibodies to induce apoptosis [168]. Since the donor cells were protected from apoptosis by Bcl-2, they selectively expanded and gradually replaced as much as 16 % of the host liver. Wilson et al. developed a xenotransplant model using a similar approach. In this case, an anti-mouse agonistic fas monoclonal antibody (Jo2 mAb) was used by to promote engraftment of rat hepatocytes into immunodeficient beige mice [169].

Liver cells from β-gal mice have been used by Rhim et al. [170, 171] to demonstrate the expansion of hepatocytes transplanted into the livers of transgenic mice expressing the urokinase-type plasminogen activator (uPA) under control of the albumin promoter. Overexpression of uPA is cytotoxic to hepatocytes and greatly compromises their regenerative capacity, giving a large growth advantage to donor β-gal+ hepatocytes or host hepatocytes that lose the uPA transgene. The end result is complete clonal repopulation of the host liver by donor hepatocytes. Overturf et al. [9, 172] reported similar findings following transplantation of β-gal-positive mouse liver cells into host mice lacking the enzyme fumarylacetoacetate hydrolase (FAH). The lack of this gene produces symptoms similar to those observed for hereditary tyrosinemia type 1, a condition that produces extensive liver damage. This damage accelerates and promotes repopulation of the liver by wild-type b-gal+ and FAH+ donor cells.

An unanswered question in all of these animal models is whether the introduction of exogenous genes into donor cells or the abnormal host environment needed to promote donor cell expansion has any effect on the differentiation capabilities of donor cells. This may be particularly important for putative progenitor cells which are likely to be more sensitive to changes in microenvironment than mature hepatocytes or ductal cells. Introduction of Bcl2, for example, may enhance the ability of transplanted cells to survive genetic changes that would normally induce apoptosis, thereby creating a population of damaged cells at higher risk for neoplasia. Wesley et al. [173] have reported that DPPIV suppresses the malignant behavior of melanoma cells by reversing a block in differentiation and by restoring growth factor-dependent cell survival, activities mediated by serine proteases. Although previous investigations by Coburn et al. [174] led to the conclusion that DPPIV proteolytic activity was not necessary for immune competence, more recent studies suggest that DPPIV enzymatic activity is essential for certain T-cell activation pathways and is involved in the inactivation of chemokines [175].

Future Directions

There is a consensus among researchers that the ultimate goal for stem cell research should be to determine ways to induce regeneration by stem cells in situ rather than by transplantation. The ability to promote regeneration by endogenous progenitors eliminates many of the potential problems with transplantation therapy, such as rejection of the transplanted cells by the recipient, incomplete differentiation, limited availability of hepatic progenitors, poor engraftment, and limited expansion without prior treatment. Transplantation

therapy, on the other hand, offers the possibility of ex vivo gene therapy, the success of which will depend on the engraftment of transgene-bearing donor cells into a normal liver in sufficient numbers to produce a beneficial effect. Clearly, development of effective stem cell treatments will require an accurate inventory of genes and the signaling pathways that promote or inhibit stem cell expansion and/or commitment regardless of whether the therapeutic effects are mediated by endogenous or exogenous stem cells. The ability to commit HSCs or iPS cells to a hepatocyte lineage prior to transplantation, for example, could enhance liver colonization sufficiently to eliminate the need for bone marrow ablation and donor cell engraftment. Similarly, insight into the mechanisms controlling proliferation during development could lead to new methods for inducing expansion following integration into hepatic cords or ducts. It is important, however, not to lose sight of the fact that an understanding of critical molecular events cannot be achieved without first gaining insight into the temporal changes that occur in the developmental potential of progenitor cells as they progress along a hepatocyte or ductal lineage. Identifying periods marked by shifts in developmental or proliferative potential will define time points and cell populations that should be targeted for detailed molecular analysis by proteomic or genomic methodologies. The realization of endogenous stem cell therapy will also require a better understanding of the activators of hepatic progenitors and the expansion and differentiation of their offspring.

Another key issue in the future will be the development of better methods for assessing the extent of differentiation of hepatic progenitors under in vitro or in vivo conditions. Do hepatocytes derived from progenitor cells express the full complement of hepatocyte genes or are they only partially differentiated, a status that could not be discerned by examining a small handful of hepatocyte or ductal markers which may or may not be appropriately expressed. Functional differentiation is another important consideration. Even if donor cells express a large number of hepatocyte-specific genes, they may fail to perform critical functions if the proteins needed for important biosynthetic pathways are not expressed or are expressed inappropriately. It is also essential that donor cells respond to the microenvironmental cues that regulate cell growth and death, thereby, creating a steady state that maintains the size and lobular structure of the liver. In this regard, there is evidence that hepatocyte colonies derived from fetal donor cells do not require a selective growth advantage such as that conferred by retrorsine/PH but continue to increase in size even when transplanted into a normal liver [176]. This continued expansion is not observed with mature hepatocytes, suggesting a defect in the microenvironment or in the programming that prevents fetal progenitors from maintaining a G_0 status. Work by Curran et al. and Boylan et al. [177, 178] suggests a possible mechanism for this defect. These investigators have found that around gestation day 21, fetal hepatoblasts stop proliferating as they transition from a growth factor-independent to a growth factor-dependent status. Thus, donor fetal liver cells harvested at gestation day 14 have never undergone this fetal/neonatal transition which may involve the acquisition of a key step in growth regulation.

A final issue that needs further study is the ability to distinguish between phenotypic modulation and true differentiation. As we and others have shown, true differentiation involves the loss or gain of tissue-associated genes in a well-ordered and invariant temporal process [42, 179]. This process has been demonstrated for T cells, B cells, keratinocytes, and mammary epithelial cells and most recently BDEC. Phenotypic modulation, on the other hand, does not appear to follow well-defined patterns. Wu et al. [180] found that when a continuous line of mouse hepatocytes was maintained in serum containing medium, hepatocyte-specific gene expression was rapidly extinguished. After only 24 h in serum-free medium, however, a well-differentiated hepatocyte phenotype was restored. These findings suggest that one way to distinguish between phenotypic modulation and true differentiation is to determine if the appearance of tissue-specific markers occurs in a fashion that recapitulates fetal liver ontogeny. The epigenetic profile of transplanted cells may also be an indicator of the attainment of a fully differentiated state. The epigenome has been shown to be of vast importance to the regulation of gene expression and the response to environmental stimuli, thus reprogramming of the epigenome of transplanted cells may be important for the acquisition and long-term maintenance of the differentiated state. Differentiation in experimental systems must also be defined in the context of the microenvironment to which the cells of interest are exposed. This is exemplified by pancreatic ductal cells which can switch to a hepatocyte lineage when the acinar pancreas has been destroyed by copper depletion, thereby exposing ductal cells to a vastly different microenvironment [181].

All of these concepts are testable using currently available technologies and the animal models described in this review. Completeness of differentiation in vitro and in vivo could be tested using the DPPIV transplantation model. DPPIV-specific antibodies could be used to isolate DPPIV-positive hepatocytes and compare them by proteomic, genomic, and epigenomic analysis to DPPIV-host hepatocytes isolated from the same liver [18]. This would be particularly interesting for donor hepatocytes derived from fetal progenitors. The issue of phenotypic modulation versus a temporal process of differentiation could be analyzed using similar techniques.

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Breast Cancer Stem Cells: From Theory to Therapy

Sarah J. Conley and Max S. Wicha

Introduction

Most tumors display a striking degree of intratumor cellular heterogeneity. Recent studies in a broad range of malignancies have demonstrated a cellular hierarchy within cancers contributes to this heterogeneity. This evidence has become the basis of a relatively new biological model. The cancer stem cell (CSC) hypothesis holds that malignancies are generated from cells that either maintain or acquire the stem cell property of self-renewal. It also posits that the malignancies produced from CSCs are organized in a hierarchical fashion where CSCs drive the malignant process and generate the more differentiated cells that make up the bulk of tumor. The CSC theory has been garnering acceptance for almost 2 decades. The first evidence for a population of cancer cells displaying stem cell characteristics was reported in 1994 in acute myeloid leukemia (AML) by Laipot et al. [1]. This was followed almost 10 years later with the discovery that breast cancer [2], as well as many other solid tumors, contains cells with similar stem cell-like phenotypes. These studies demonstrated that rare populations of cells, identified by specific cell surface molecules, possessed the ability to form human tumors upon serial passage when implanted in immunocompromised mice, and these new tumors recapitulated the hierarchy of the original tumor. Further experiments demonstrated that these tumorigenic cells also had the capacity of self-renewal, an activity measurable by the ability to form several generations of tumorspheres in vitro. The CSC hypothesis has important implications for the clinical treatment of cancer. There is striking evidence that CSCs in most malignancies, including breast, are relatively resistant to conventional cancer therapies including radio- and chemotherapy. If the CSC model holds true, this indicates that the use of CSC-targeting agents will be required in order to produce a lasting response in patients. Indeed, many novel CSC-targeted agents are being developed and tested currently. Here, we will discuss the properties of breast CSCs as well as potential means by which to clinically target these cells.

Defining a Breast Cancer Stem Cell

There is much confusion in the scientific arena over the term "cancer stem cell." Much of this misunderstanding stems from misuse of terminology. The term CSC is often mistakenly used interchangeably with tumor-initiating cell (TIC). A TIC refers to the stem or progenitor cell type within the adult tissue from which the cancer originates. In contrast, CSCs mediate tumor progression and metastasis. These cells are also defined by their contribution to tumor heterogeneity by generating differentiated cells that constitute the tumor bulk. Finally, CSCs possess the capacity for self-renewal as demonstrated by their capacity to initiate tumors upon serial passage in immunocompromised mice. The CSC model was initially proposed several decades ago in response to two critical observations seen repeatedly in cancer research. The first was based on the evidence that most cancers contain a complex heterogeneous mix of tumor cells and secondly that only a fraction of cells from these malignancies are tumorigenic [3–5]. CSCs were first identified in hematological malignancies during the early 1990s. These initial studies demonstrated that rare populations of cells with surface markers similar to those expressed in normal hematopoietic stem cells could be isolated from human chronic myeloid leukemias and AMLs [1, 6]. Fluorescence-activated cell sorting (FACS) was used to isolate a specific population from patients defined by expression of the cell surface antigen CD34 that simultaneously lacked expression of the differentiation antigen CD38. Similar to normal stem cells, these cells displayed the ability to self-renew and differentiate, as

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well as possessed the capacity to initiate leukemias following implantation into non-obese diabetic mice with severe combined immunodeficiency (NOD/SCID). The first evidence for the existence of CSCs in solid human tumors came from studies in breast cancer [2]. CSCs have subsequently been identified in a wide variety of human cancers including brain, colon, pancreatic, head and neck, and others [7–10] based on cell surface markers as well as functional characteristics.

Using primary human breast cancers, Al-Hajj et al. identified a population of cells by FACS using antibodies to ESA$^+$/CD44$^+$/CD24$^{-/low}$/lineage$^-$ [2]. These cells were highly tumorigenic when injected into the mammary fat pads of NOD/SCID mice. When cells with this phenotype were isolated, as few as 200 cells could efficiently form tumors. In contrast, 20,000 CD44$^-$/CD24$^+$ cells failed to initiate tumors when implanted, and there was only ~25 % take rate when 10,000 unsorted cells were injected. Moreover, the CD44$^+$/CD24$^{-/low}$/lin$^-$ cells were able to be serially passaged, reinitiating tumors in subsequent mice. In addition to being able to form tumors with a low number of cells, when tumors were initiated from cells within the CD44$^+$CD24$^-$ fraction, the resulting tumors recapitulated the heterogeneity of the original tumors. Because these cells had the capacity for self-renewal, differentiation, and tumorigenicity, they were classified as breast CSCs.

Identifying and Measuring Breast CSCs and Characterizing Their Activity

The current "gold standard" for evaluating a breast CSC population is to determine the ability of these cells to initiate tumors following injection into immunocompromised mice [11]. An enrichment of CSCs can be determined when a population of cells isolated by one or more selective means demonstrates greater tumor-initiating capacity compared to unsorted cells from the original tumor. Several methods are currently used for isolating breast cancer cells with stem cell activity. The most widely utilized method for isolating CSCs from a variety of malignancies, including breast cancer, is with antibodies to specific cell surface markers using FACS. Additionally, assays that characterize intracellular enzyme activities such as aldehyde dehydrogenase (ALDH) are also often used. Less utilized are tests that evaluate the presence of transmembrane transporters or proteasome activities. Finally, the ability of cells to form spherical colonies from single cells under nonadherent conditions and be serially passaged is a measure of self-renewal capacity of CSCs in vitro.

Expression of cell surface markers is the most often utilized characteristic to isolate CSCs. These markers can demonstrate significant variation among different tissue types as well as between species. Although several different markers have been identified, there is currently no universal cell surface marker, or combination of antigens, for the isolation of breast CSCs by antibody techniques. Explanations for this may be that several different populations of breast CSCs may exist within tumors or that CSCs exist within different states characterized by expression of different markers. The existence of multiple stem cell populations with varying self-renewal capacity has been demonstrated in the hematopoietic system [12, 13]. Varying CSC characteristics might be dependent upon which signaling pathways are deregulated. Alternatively, the variation could be due to different cells of origin for these CSC populations.

The first combination of cell surface markers used to purify breast CSCs was ESA$^+$/CD44$^+$/CD24$^{-/low}$/lineage$^-$. This combination of antigens, originally established by Al-Hajj et al. [2], has since been frequently utilized to isolate CSCs from primary breast tumors as well as breast cancer cell lines [14–17]. Currently, the most commonly used combination of antigens for isolating breast CSCs is ESA$^+$/CD44$^+$/CD24$^{-/low}$ [18]. The CD44^{high}/CD24^{low} phenotype has also been used to enrich for stem cells from normal human mammary glands, demonstrating that this phenotype may be conserved in the carcinogenesis process. The percentage of cells isolated from cell lines with the CD44$^+$/CD24$^-$ phenotype can range from less than 1 to 85 % [16]. The disparity seen in the percentages found among cell lines is likely a reflection of the breast cancer subtypes these various cell lines represent. The CD44$^+$/CD24$^-$ phenotype appears to be a better marker of CSCs in luminal type cancer cell lines, where other markers may be better suited for isolating CSCs in breast cancer cell lines characterized as mesenchymal.

CD133 (prominin) has been a useful marker for isolating CSCs from brain and colon tumors [7, 19, 20]. Relative to these other tumor types, CD133 has been less utilized as a means to enrich for CSCs in breast cancers. One study demonstrated that distinct populations of CD133$^+$ cells with CSC characteristics exist within *Brca1* breast tumors [21]. As few as 50–100 CD133$^+$ cells isolated from these tumors initiated tumor formation in NOD/SCID mice. In contrast, 50- to 100-fold greater unsorted cells or cells depleted for this phenotype were required to generate tumors in mice. The expression of stem cell-associated genes, including Oct4, Notch1, Aldh1, Fgfr1, and Sox1, was increased in CD133$^+$ cells. However, when the CD133$^+$ cells were cultured in monolayers, they progressively lost their stem cell phenotypes. Since CD133$^+$ cells are located within the luminal compartment in the normal human mammary gland, CSCs exhibiting this marker may differ from the CD44$^+$/CD24$^-$ population that is more prominent in mesenchymal tumors. In any case, CD133 as a CSC marker in luminal breast cancer remains an interesting area for future studies.

In addition to cell surface markers, CSCs can also be enriched from both normal mammary gland and breast cancers by selecting for their increased ALDH activity [22].

The levels of this intracellular enzyme can be determined by FACS utilizing the ALDEFLUOR assay. ALDH is a detoxifying enzyme that catalyzes the oxidation of retinol to retinoic acid. In addition to being a marker of stem cells, it may also be involved in the early stages of stem cell differentiation [23, 24]. Many other types of stem cells have been enriched by virtue of increased ALDH activity, including hematopoietic and neural stem cells [25–28]. ALDH activity can also enrich for CSCs in ovarian cancers, further enriching CD133 as a CSC marker [29]. Similarly, further enrichment of CSC populations in breast cancer can be achieved through the use of multiple markers. ALDH-expressing breast cancer cells can be enriched for CSC activity by the use of $CD44^+$, $CD24^-$, and CD133. The CSC populations demonstrating the highest tumorigenic and metastatic potential are characterized as $ALDH^+/CD44^+/CD24^-$ or $ALDH^+/CD44^+/CD133^+$ [30]. Moreover, these studies demonstrated that none of the $CD44^+/CD24^-/lin^-$ cells that are ALDEFLUOR negative are capable of tumor initiation [22], indicating that the $CD44^+/CD24^-/lin^-$ population does not contain all of the CSCs in breast tumors. This suggests that the population of cells identified using the ALDEFLUOR assay is heterogeneous.

Antigens used for FACS identification of CSCs can also be used for immunohistochemistry (IHC) to identify these cells in situ [17]. However, this approach is somewhat deficient, as it cannot establish the functional stem cell activity of cells expressing the CSC markers. The advantage of FACS over IHC is that sorted cells remain viable and can be used in subsequent functional assays to verify the presence of CSCs and validate the efficacy of targeted drugs. Functional CSC assays also have limitations, as large samples of fresh cells are required, while IHC requires only a small fixed sample and can readily be carried out in the clinical setting. These factors must be considered when attempting to translate CSC assays into the clinic.

Both normal stem cells and CSCs have been demonstrated to possess drug efflux potential [6–10]. Investigators have employed this characteristic for many years to isolate stem cells from tissue types using the side population (SP) technique [31–34]. This method depends on the ability of stem cells to exclude vital dyes through increased expression of membrane transporters. Like their normal stem cell counterparts, CSCs express high levels of transporters, such as the ATP-binding cassette protein, ABC transporter ABCG2/BCRP1 (breast cancer resistance protein 1). These proteins efficiently exclude dyes such as Hoechst 33342 from the cells which can then be assayed for by FACS to separate the CSCs from the differentiated cells that retain the dye. The SP technique has been used to enrich for stem cells from both normal mammary tissue and tumors. A higher level of BCRP1 expression was demonstrated in cells isolated by the SP method from healthy tissue derived from reduction mammoplasties. These cells appeared to be undifferentiated since they expressed neither luminal nor myoepithelial markers [35]. The SP technique also enriches for tumorigenicity in breast cancer cell lines. Only 2 % of MCF-7 cells are within the SP population, and these cells are highly tumorigenic in immunocompromised mice, forming tumors that recapitulate the heterogeneity of the parental line [36]. Despite its qualities, the SP method is not the preferred approach for isolating CSCs. This is due to limitations in biological studies due to the cytotoxicity of the Hoechst dye, complicating the interpretation of functional assays.

The quiescent nature of breast CSCs can be utilized as another means for identification. A recently reported technique for detecting CSCs in culture uses PKH26 dye which binds irreversibly to cell membranes becoming progressively diluted during cell division. Thus, very little dye is lost in the stem cells that do not divide often. This assay provides an indirect measure of dormancy, and cells with high dye content identify the quiescent CSC population [37].

The ability of cells to form nonadherent colonies (spheres) under serum-free conditions in vitro has been adapted for enriching stem and progenitor cells from several cancer types. The mammosphere assay was originally developed by Dontu et al. [38] for enriching normal mammary stem cells in culture. Differentiated cells undergo anchorage-dependent programmed cell death (anoikis) when cultured under nonadherent conditions. In contrast, cells that have the capacity to self-renew can proliferate from single cells to form individual spherical colonies termed mammospheres. It was later demonstrated that breast tumor cells grown as mammospheres are relatively undifferentiated since they lack expression of both myoepithelial (CK14, CK18) and luminal cells (ESA) [14]. Mammospheres were shown to contain an increased SP fraction as well as an increased proportion of $CD44^+/CD24^-$ cells [14]. Mammospheres are enriched for CSCs as evidenced by the greatly increased tumor-initiation potential in vivo [39].

Finally, it has been reported that CSCs in breast cancers display low proteasome activity. This feature can be exploited to identify and track CSCs in vitro and in vivo [40]. In vitro, breast cancer cells grown as mammospheres exhibit decreased proteasome activity when compared to monolayer cells. In order to use this low proteasome activity to track CSCs, breast cancer cells can be engineered to stably express ZsGreen fused to the carboxyl-terminal degron of ornithine decarboxylase. When there is low 26S proteasome activity within a cell, an accumulation of the fluorescent fusion protein results. In vitro, ZsGreen-positive cells have an increased expression of CSC markers and a greater capacity to form mammospheres. Importantly, ZsGreen-positive cells demonstrate a ~100-fold greater ability to form tumors compared to ZsGreen-negative cells when injected into immunocompromised mice. This system can be used to detect the presence of ZsGreen-positive CSCs in tissue samples by immunohistochemical techniques

as well as in tumors using live in vivo imaging systems. Thus, this technique enables both in vitro and in vivo study of CSC populations.

Resistance to Cancer Therapy

Conventional therapies for managing locoregional and metastatic breast cancer involve combinations of surgery, chemotherapy, radiation, and hormonal therapy. Chemotherapy is very effective at debulking tumors, conferring some clinical benefits. Apart from initial response to therapy, up to one fourth of patients with early disease and almost all patients with metastatic disease will eventually relapse [41]. Similarly, although radiation therapy following lumpectomy in early stage breast cancer significantly reduces local recurrence, approximately 50 % of these patients experience a local recurrence. It is now believed that breast CSCs play an important role in the recurrence of tumors both locally and as distant metastases. Recent evidence indicates that this subset of cells is able to evade the effects of conventional cancer therapies. This innate resistance allows CSCs to survive treatment and thereby repopulate the primary tumor or spread as metastases following therapy. Strong evidence from both the laboratory and clinic supports this conclusion.

Chemotherapy Resistance

The resistance and enrichment of breast CSCs following chemotherapy has now been demonstrated both in vitro and in vivo [42]. The first studies to show this examined the $CD44^+/CD24^-/ESA^+$ population within several breast cancer cell lines (SUM159, SUM1315, and MDA-MB-231) [43]. Following 6 days of in vitro chemotherapy treatment with either Paclitaxel (taxol) or 5-fluorouracil, the CSC population was increased by 5- to 30-fold over the control-treated cells. Of the three cell lines, the MDA-MB-231 cells exhibited the greatest amount of cell death following the chemotherapy, but of the small fraction (1 %) of surviving cells approximately one third were $CD44^+/CD24^-/ESA^+$. Further studies by Ginestier et al. found similar results in vivo when breast cancer cells grown as xenografts in NOD/SCID mice were treated with chemotherapy [44]. Docetaxel treatment resulted in an increased percentage of $ALDEFLUOR^+$ CSCs in the SUM159 cell line as well as MC1, UM2, or UM3 primary xenografts compared to cells in the control mice.

There is also strong clinical evidence that breast CSCs are enriched following chemotherapy. The sphere-forming ability of cells derived from patients treated with neoadjuvant chemotherapy was shown to be increased 14-fold over cells from untreated patients [45]. Additionally, the chemotherapy-treated cells could be serially passaged as spheres for a minimum of 8–10 generations, while the untreated cells were sustainable only for 2–3 passages, demonstrating increased self-renewal following chemotherapy. While cells from the untreated patients contained around 10 % CSCs as determined by the $CD44^+/CD24^{-/low}$ phenotype, tumor cells from treated patients contained about 75 % CSCs. The most convincing evidence that CSCs are enriched by chemotherapy treatment comes from studies where paired specimens from patients before and after post-neoadjuvant treatment were compared. Chemotherapy induced a ~12-fold increase in the number of cells able to form mammospheres when compared to pretreated tumors. Moreover, a tenfold increase was seen in the percentage of $CD44^+/CD24^{-/low}$ cells in samples after chemotherapy [45]. Similar results from another clinical study showed enrichment of CSCs following 3 months of docetaxel or doxorubicin + cyclophosphamide. Importantly, this study demonstrated that chemotherapy treatment essentially doubled the tumor-initiation capacity of cells from patient tumors when reimplanted in immunocompromised mice [46]. Finally, overlapping gene expression signatures from mammospheres and $CD44^+/CD24^{-/low}$ cells are induced following docetaxel treatment in breast cancer patients [47]. Together, these provide strong evidence for the existence of a population of chemotherapy-resistant CSCs in breast tumors.

Potential mechanisms of resistance to chemotherapies in CSCs include increased expression of antiapoptotic proteins, increased drug efflux transporters, and increased efficiency of DNA repair [48]. Additionally, CSCs may be less sensitive to antimitotic drugs since they have a low proliferative index. Thus, therapies may eliminate the bulk population (non-CSCs) from tumors, while leaving the CSCs to potentially reseed the cancer.

Radiation Resistance

Similar to their resistance to chemotherapy, breast CSCs are also relatively resistant to radiation therapy. Ionizing radiation induces free radicals and reactive oxygen species (ROS) that can damage DNA, leading to cell death. Recurrence is fairly common in cancer patients following radiotherapy, and new research shows that this is potentially due to CSCs and their heightened ability to counteract ROS and DNA damage. Some of the earliest studies investigating the radiation response of CSCs were carried out in breast cancer [49]. Breast CSCs were first reported as radiation resistant in 2006 [15] and has since been confirmed by additional studies as well as subsequent studies demonstrating reactive radiation resistance in other cancer types [50–55]. Mammospheres grown from MCF-7 and MDA-MB-231 cell lines are more resistant to radiation than cells grown in monolayers [15]. Additionally, short courses of fractionated irradiation can increase the proportion of $CD44^+/CD24^{-/low}$ MCF-7 CSCs.

Breast CSCs produce less ROS and fewer double-strand DNA breaks following radiation therapy, suggesting there is an enhanced expression of free-radical scavengers in these cells [15]. Moreover, a strong anti-ROS gene signature was detected in CSCs using single cell RT-PCR. Pharmacological ROS scavengers were able to sensitize CSCs to radiation [53]. It was also proposed that the induction of the Notch pathway, a known stem cell regulator, helps mediate the radioresistance of breast CSCs. Notch can be activated by radiation in breast CSCs in a PI3K-dependent fashion. The activation of this pathway by radiation can thus regulate the process of self-renewal and increase the number of CSCs [15]. Further evidence suggests that radiotherapy can induce Wnt signaling, which in turn can confer radiation resistance as well as promote self-renewal of mammary cancer cells [50].

Together, this evidence indicates that breast CSCs possess cell properties that confer protection from radiation-induced damage and cell death. This CSC population therefore has potential to mediate local tumor recurrence following radiotherapy. Recent studies indicate that, in addition to targeting ROS scavengers, radiation resistance may be overcome by hyperthermia using nanoparticle technology. Hyperthermia therapy acts as a cancer treatment by direct cell killing and in addition can induce radiosensitization [56]. Hyperthermia increased the radiation sensitivity of breast CSCs in a process mediated by heat shock proteins [57].

Resistance to Endocrine Therapy

The sensitivity of some breast cancers to hormone manipulation was first described over 100 years ago. The development of Tamoxifen as the first antiestrogen therapy marked a milestone in breast cancer treatment. Since the 1970s, estrogen deprivation has been a mainstay of the treatment of breast cancers that express the estrogen receptor-a (ERa). Hormonal therapy for these patients was further improved with the introduction of aromatase inhibitors. Unfortunately most patients that initially respond to the endocrine therapy will ultimately become hormone resistant. It has been proposed that one possible mechanism for this resistance is mediated by a population of ER-CSCs within these tumors. Following antiestrogen treatment, remaining CSCs could form new tumors and metastases. The loss of ER expression during the progression of a malignancy from primary tumor to malignant relapse has been well documented; however, the theory of an antiestrogen-resistant CSC population has not yet been clinically verified.

The expression level of ER is highly predictive for the response to endocrine therapy. However, the high relapse rate in breast cancer patients following endocrine therapy strongly suggests that this line of treatment does not target CSCs likely due to their lack of ER expression. It has been shown that a rare population of cells can be isolated from luminal-like ER+T47D human breast tumor xenografts. This population is enriched in the tumorigenic fraction of these xenografts and the cells are ER- and $CD44^+$ [58]. Similarly, in human cell line models of acquired endocrine therapy resistance, ER expression is progressively decreased as resistance occurs [59]. This phenomenon is also observed in patients. In a clinical study using paired tissue samples, neoadjuvant endocrine therapy resulted in a decrease in ER gene expression in posttreatment tumors compared to pretreatment tumors [59]. Thus, the survival of an ER-, highly tumorigenic fraction of cells represents one potential mechanism for antiestrogen resistance to occur.

A second mechanism for how patients develop resistance to endocrine treatment is by an increase in growth factor signaling. Typically, when ER expression decreases in cell lines and patients during endocrine resistance, there is a reciprocal increase in epidermal growth factor receptor (EGFR)/HER2 expression. An increase in EGFR was first demonstrated using MCF-7 cells with acquired endocrine resistance to fulvestrant [60], and enhanced HER2 expression was later confirmed using the same cell line with resistance to Tamoxifen [61]. Importantly, targeting these signaling pathways could block growth of these hormone-resistant cells. Clinicians have in fact observed that inverse relationship between the expression of c-erbB-2 protein, a marker of poor prognosis in breast cancer, and sensitivity to antiestrogen treatment on relapse [62]. Similar to the increase in ER- stem-like cells that occurs concurrently with the development of acquired endocrine resistance in breast cancer, the increase in EGFR/HER2 signaling can potentially arise from the selection of CSCs. Recent studies have demonstrated the clinical benefit of adding an mTOR inhibitor to aromatase inhibitors [63]. When breast cancer cells are grown as mammospheres, they demonstrate an enhanced expression of HER2 levels [64]. Additionally, it has been demonstrated that HER2 is a key driver of the CSC population. HER2 overexpression is correlated with increased expression of stem cell-regulating genes [65]. The expression of HER2 itself is under the control of Notch 1, a key regulator of CSC maintenance [64]. Overexpression of HER2 in breast cancer cell lines results in an expansion of CSCs as determined by ALDH activity and increases the tumorigenicity of the cell population [65]. These studies further demonstrated that the effects of HER2 overexpression on breast CSCs can be inhibited by trastuzumab (Herceptin). Data from clinical trials shows that the treatment of breast cancer patients with lapatinib (a dual EGFR/HER2 inhibitor) can decrease the CSC population and mammosphere formation of cells from the posttreatment tumors [46]. The use of anti-HER2 therapy using trastuzumab in HER2-expressing breast cancers has been highly successful. In addition, there is now evidence from retrospective analyses suggesting that in the adjuvant setting, the clinical

benefits of HER2 blockade may also extend to breast cancers that are currently classified as HER2 negative [66]. This may be due to the classification of HER2– tumors that actually harbor a rare population of HER2+ CSCs. Together these studies indicate that CSC activity in breast cancers can be regulated by HER2 and suggest that the remarkable clinical efficacy of therapy of trastuzumab may be due to its ability to target CSCs.

Targeting Breast CSCs

There is now abundant evidence demonstrating that although most treatments are effective at targeting the bulk population of tumor cells, the CSCs are relatively resistant to these agents and may drive regrowth of tumors and metastases following therapy. This highlights the need for therapeutics that can directly target this cell population. There are at least three key ways proposed for targeting breast CSCs, including the inhibition of CSC self-renewal mechanisms, the inhibition of CSC resistance mechanisms, or blocking the effects of the CSC niche.

A full comprehension of breast CSC biology, including the pathways that regulate the cells, will be necessary to effectively develop CSC-targeted therapies. Recent studies have helped to elucidate the pathways maintaining the growth, survival, and self-renewal of CSCs. There are at least three primary signaling pathways, Notch, Wnt, and Hedgehog, demonstrated to regulate breast CSCs. New evidence also demonstrates roles of the tumor microenvironment as well as certain microRNAs in the regulation of CSCs.

Notch

Notch consists of an evolutionarily conserved signaling pathway which is initiated when a ligand from the Jagged or Delta families binds to one of the Notch receptors (Notch 1–4) on adjacent cells triggering a two-step receptor proteolysis event [67]. The first cleavage event is mediated by members of the ADAM metalloproteinase family, followed by the second proteolytic cleavage, mediated by γ-secretase. These proteolytic events result in the release of the Notch intracellular domain (NICD) from the plasma membrane and its transport to the nucleus, where it forms a DNA-binding complex with other coactivators. This stimulates the expression of target genes including *Hes*, *Hey*, *c-Myc*, *cyclin D1*, and *p21/Waf1*. NICD activity is further regulated by CDK8-dependent phosphorylation leading to its ubiquitination. Binding of Notch by the ligand Numb inhibits its activity through an endocytotic mechanism.

Notch activity has been extensively studied in breast CSCs, since the Notch signaling pathway has key functions in both normal breast development and in breast cancer development. In the mouse mammary gland, Notch signaling regulates the expansion of stem cells and their differentiation into luminal progenitor cells [68]. Normal human breast stem cells exhibit active Notch signaling, promoting their self-renewal as well as the differentiation of progenitor cells while having very little effect in fully differentiated epithelial cells [69]. A role for Notch signaling in breast cancer was demonstrated by the identification of retroviral insertions into Notch4 in mouse mammary cancers [70]. In tumors, expression of Notch receptors is upregulated in breast CSCs identified by expression of $CD44^{+}/CD24^{-}$ [67]. Furthermore, Notch4 activity is increased in breast CSCs, and inhibition of Notch4 signaling can reduce this population as well as completely block tumorigenesis [39]. Thus, Notch signaling represents an attractive therapeutic target in breast CSCs.

Both in vitro and in vivo studies have verified that targeting components of the Notch signaling pathway reduces the CSC population. A number of genetic and pharmacologic approaches to abrogate Notch signaling are under investigation. Notch receptors and ligands may be inhibited by selective means such as monoclonal antibodies or nonselective means such as soluble ligands or receptor decoys. Finally Notch signaling can be blocked by the inhibition of enzymes necessary for cleavage of receptors, such as γ-secretase inhibitors (GSIs) or ADAM inhibitors. One such GSI, DAPT, can significantly reduce the formation of mammospheres from pre-invasive ductal carcinoma in situ [71] and invasive breast cancer cell lines [39]. Other GSIs are currently being tested in early phase clinical trials for breast cancer [72].

It appears that not all Notch receptors are able to regulate breast stem cells equally. Notch1 activity is significantly lower in breast CSCs compared to more differentiated progenitor cells, and its specific inhibition has less effect on the CSC population than does Notch 4 inhibition [39]. This suggests that there is specificity of different Notch receptors in the regulation of breast stem and progenitor cells. If this is the case, then selective inhibition of Notch4 may be more efficacious than Notch1 inhibition and potentially less toxic than GSIs that inhibit all Notch receptors.

Hedgehog

Aberrant activation of the Hedgehog (Hh) pathway has been demonstrated in many different types of cancer and is another attractive target for CSC therapeutics. Hh has been demonstrated to signal through autocrine, juxtacrine, and paracrine mechanisms [73, 74]. Secreted Hh molecules (Sonic, Indian, or Desert) signal through the 12 span transmembrane Patched 1 (PTCH) receptor. In the absence of an Hh ligand, PTCH inhibits the activity of the 7-transmembrane receptor-like

protein, smoothened (SMO). The binding of Hh to PTCH thus relieves SMO inhibition, allowing SMO to transduce the Hh signal to the cytoplasm. The translocation of activated SMO to nonmotile cilia initiates a signaling cascade resulting in nuclear translocation of active GLI transcription family members and the induction of Hh target genes including GLI1 and PTCH1 [73, 75, 76].

Hh signaling is a critical regulator of normal stem cell maintenance, stimulating self-renewal and proliferation of stem cells in many types of tissues [77, 78]. Evidence for Hh signaling in CSCs was first demonstrated by deletion of SMO in BCR-ABL+ chronic myeloid leukemia (CML) stem cells. This deletion abrogates tumorigenicity and pharmacological inhibition of the Hh pathway can increase survival of mice transplanted with BCR-ABL leukemia cells [79]. Many members of the Hh pathway are highly expressed in $CD44^{+}CD24^{-}$ CSCs from breast cell lines, including PTCH1, Gli1, and Gli2. Furthermore, when cells are induced to differentiate, the expression of these genes is downregulated [80]. Exogenous Hh ligand can enhance the capacity to form mammospheres whereas inhibition of the pathway by cyclopamine (a naturally occurring plant alkaloid) blocks this effect [80]. The inhibition of Hh signaling with cyclopamine or an anti-Hh antibody can block growth of small-cell lung cancer cells and pancreatic cancer cells in vitro and in immunocompromised mice [81, 82], suggesting that targeting this pathway may also affect CSCs in malignancies other than breast cancer. Current clinical studies are combining an Hh inhibitor with a Notch inhibitor, based on evidence that these are both important regulators of CSC self-renewal and the two signaling networks interact with each other [72, 83].

Wnt

While agents targeting the Notch and Hh pathways have shown great promise in preclinical studies, the Wnt pathway has been more challenging to target. Nonetheless, many recent advances have been made in developing therapeutic agents to target this CSC regulatory pathway. Wnts are secreted glycoproteins that bind to cell surface receptors, activating signaling cascades [84]. As is the case for Notch and Hh, Wnt signaling pathways are highly conserved across species. Two pathways, canonical and non-canonical, make up the Wnt signaling cascade. Non-canonical Wnt pathways do not function through β-catenin, and have primarily been characterized in lower organisms [85]. Alternatively, canonical Wnt signaling is well studied in mammalian cells and is dependent on β-catenin activity. Canonical signaling is initiated when one of the 19 known Wnt ligands binds to co-receptors of the Frizzled (Fzd) and low-density lipoprotein receptor-related protein (LRP) families. This action initiates a cascade of events, which results in the disruption of the β-catenin destruction complex. Once stabilized, this pathway leads to the nuclear translocation of β-catenin where it forms a complex with members of the T cell factor/lymphoid-enhancer factor (TCF/LEF) family of transcription factors. Together the complex recruits cofactors which drive transcription of target genes including c-myc, cyclin D, and survivin [86–88].

Wnt signaling is misregulated in a vast range of human malignancies. The most notable and first described example of aberrant Wnt/β-catenin signaling is in colon cancer, where nearly 90 % of malignancies contain mutations of components of the Wnt pathway, resulting in abnormal β-catenin activation [89, 90].

In certain triple-negative breast cancer cell lines, high levels of Wnt leads to enhanced Wnt/β-catenin activity generating an autocrine feedback loop [91]. This signaling loop can be suppressed by the overexpression of endogenous inhibitors such as Dkk1, validating the potential to pharmacologically inhibit this network. A growing body of evidence suggests that Wnt signaling and β-catenin play an important role in CSC function [92, 93]. For example, colon CSCs can be isolated based on elevated β-catenin activity and possess greater tumorigenicity when implanted in immunocompromised mice compared to cells with low β-catenin signaling [94]. A key role for Wnt signaling in mammary CSCs has also been documented. In the MMTV-Wnt-1 and MMTV-β-catenin transgenic mouse breast cancer models, the mammary stem cell population is increased [95, 96]. Additionally, the conditional knockout of Apc, a component that regulates β-catenin degradation, in mammary cells induces the formation of mammary adenocarcinomas [97]. Finally, the Wnt/β-catenin network is a critical regulator of CSCs within breast cancer cell lines exhibiting overactive PI3K/AKT signaling. In MCF-7 and SUM159 breast cancer cells, knockdown of PTEN results in increased mammosphere formation as well as tumorigenicity in NOD/SCID mice [98]. This effect on CSC phenotype is mediated through Wnt/β-catenin via the phosphorylation of Gsk3β as well as phosphorylation of β-catenin facilitating its nuclear translocation.

Together, this evidence indicates the Wnt signaling cascade is another potential target for pharmacological intervention. The complexity of the Wnt signaling network provides multiple potential therapeutic targets. Several therapeutic strategies have been proposed including disrupting ligand–receptor interaction, inhibition of Wnt secretion, destabilization of β-catenin, and disruption of the β-catenin/TCF/coactivator complex. Directly targeting β-catenin activity is difficult since it does not possess enzymatic activity. However, a recent high-throughput screen for inhibitors of Wnt-/β-catenin-mediated transcription has identified a small molecule inhibitor of this pathway thought to function via β-catenin destabilization [99]. Several Wnt-targeting therapies are now under development or in early phase clinical

trials for cancer [100]. Although preliminary, the data show great potential for new compounds to target this pathway in breast CSCs.

CSC Microenvironment

Unlike in vitro cell culture, epithelial cancer cells within tumors exist among a myriad of other cells including fibroblasts, endothelial, and hematopoietic cells and may encounter environmental stressors that vary both spatially and temporally. Within the tumor complex, CSCs are able to communicate with and receive signals from these other cell types and from environmental factors. In fact, most of the CSC self-renewal pathways are activated by the CSC niche. For example, the Notch, Hedgehog, and Wnt signaling pathways all require cell-to-cell contact to activate canonical signaling. Not surprisingly, targeting the components of the CSC niche has long been proposed. In 1990, the use of antibodies against CD44 was shown to effectively block the growth of murine bone marrow progenitors in vitro by interfering with the interactions between the cells and the extracellular matrix [101]. Following this discovery, the targeting of CD44 in CSCs, including breast CSCs, has been an area of intense focus. One such therapy P245, a monoclonal antibody against CD44, was tested for efficacy on human breast cancer xenografts. This study showed promising results when the anti-CD44 therapy was combined with a cyclophosphamide treatment targeting both the bulk population and CSC populations within tumors [102].

Breast CSCs can interact with their microenvironment through cell–extracellular matrix communication via integrins [103]. A direct link between integrin signaling through focal adhesion kinase (FAK) and breast CSCs is likely since primary tumors from FAK knockout mice harbor a reduced proportion of CSCs [104]. Additional studies demonstrate that FAK is critical for mammary tumor formation and metastasis in mice, adding further evidence linking FAK signaling and CSC regulation. Thus, CD44 and integrin-related proteins that regulate CSCs serve as another alternative for therapeutic targeting.

Extrinsic factors within the tumor microenvironment can likewise regulate breast CSC activity and represent potential therapeutic targets. Several cytokines have been implicated in regulating breast CSCs such as stromal-derived factor-1, interleukin-6 (IL-6), and interleukin-8 (IL-8) [105–107]. Importantly, these environmental-derived regulators may play a role in breast cancer treatment resistance as was demonstrated in the case of IL-6 driving CSC-mediated trastuzumab resistance [108]. The cognate receptor for IL-8, CXCR1, is selectively expressed in ALDH+ cells in breast cancer cell lines, and treatment with IL-8 can expand CSC populations [109]. Further studies using human breast cancer xenograft mouse models have shown that blocking IL-8 signaling reduces CSC self-renewal. More importantly, the small molecule CXCR1 inhibitor repertaxin inhibits both tumor growth and metastases even though only <2 % of cells express CXCR1 [44]. Not only are CSCs regulated by autocrine production of cytokines, but other tumor cells within the CSC niche can secrete molecules promoting their activity via paracrine signaling [110]. This highlights the need for targeting these extrinsic mediators of CSC activity.

Hypoxia is a common feature of solid tumors and represents one of the hallmarks of the CSC microenvironment. It has long been known that tumor survival and progression are enhanced by the presence of hypoxia, and recent evidence demonstrate that this might be driven by CSCs. Low oxygen levels trigger changes in gene expression driven by hypoxia-inducible factor (HIF) proteins and their signaling pathways. HIFs can directly induce the expression of stem cell-regulating genes like Nanog, Oct4, and Sox2 [111, 112]. Additionally, HIFs can interact with and enhance other CSC regulatory pathways such as Notch and Wnt [113, 114]. Studies now demonstrate that hypoxia can increase the proportion of breast CSCs in vitro and CSC populations grow in response to hypoxia induced by antiangiogenic agents in breast cancer xenografts [115]. Targeting of HIFs pharmacologically has shown promise in reducing CSC populations in some hematopoietic cancers [116] and could potentially be used in combination with antiangiogenics in breast cancer. Several strategies for blocking HIFs are currently under development showing promising results in preclinical testing [117].

MicroRNA Signaling in CSCs

Regulation of CSCs by microRNAs (miRNAs) is a relatively new area of research focus. The ability of each miRNA to regulate the expression of up to 300 different genes [118] makes this class of small RNAs an attractive therapeutic target. This broad range of regulation by single miRNAs allows for a simple mechanism for the coordinate regulation of multiple CSC regulatory pathways. Many studies have highlighted the role of miRNAs in regulating embryonic stem cells [119–121], and evidence suggests a role for these miRNAs in the regulation of CSCs. When compared to differentiated cancer cells, expression of Let-7 miRNA family members is reduced in primary breast cancer cells enriched for mammosphere formation and tumorigenicity [45]. Induced expression of Let-7 reduces mammosphere-forming capacity while its repression in differentiated cells results in increased mammosphere formation. Moreover, forced expression of Let-7 can abrogate breast tumor formation in NOD/SCID mice [45].

Numerous miRNAs can be either upregulated or downregulated in CSCs when compared to more differentiated

tumor cells. $CD44^+/CD24^-$ breast CSCs from primary human tumors differentially express at least 37 miRNAs when compared to the non-tumorigenic cell population [122]. Three clusters of miRNAs are downregulated similarly in normal human breast stem cells and in breast CSCs, including miR-200c-141, miR-200b-200a-429, and miRNA-183-96-182 [122]. This strong overlap highlights the similarities in the regulation of normal and malignant stem cells in the breast. One of the numerous targets of miR-200c, BMI1, is a known regulator of both normal stem cells and CSCs [123, 124]. Similar to Let-7, induced expression of miR-200c in $CD44^+/CD24^-$ breast CSCs inhibits tumor formation in NOD/SCID mice.

Other Therapeutic Strategies

Many other CSC-targeting strategies are being investigated in the laboratory and in clinical trials for breast cancer as well as other malignancies [125, 126]. For example, studies have identified the NF-κB pathway as an attractive therapeutic target since it can cross talk with many of the other CSC-regulating pathways. Promising data show that three different inhibitors of the NF-κB pathway can preferentially inhibit mammosphere-forming capacity and proliferation of the SP of MCF-7 cells [127].

In the search of additional anti-CSC agents, researchers have turned to high-throughput screening techniques. However, this approach is difficult for identifying CSC-targeting compounds due to the relative rarity of CSCs as well as the difficulties performing numerous clonogenic assays to assess stem cell activity. To address these problems, a breast cell line engineered to induce properties of epithelial to mesenchymal transition (EMT) has been utilized to screen compounds that target CSC activity [128]. Using this method, salinomycin was found to selectively inhibit CSCs by inducing differentiation [129]. Other differentiation therapies are under investigation as well. Both vitamin A derivatives and BMPs have shown promise as agents inducing differentiation of CSCs [130–132].

Additional therapies under development include agents that inhibit ABC drug transporters, block heat shock protein 90, or target the EMT program in CSCs [126, 133, 134]. Other potential means for eradicating this cell population is through immunotherapy by targeting specific antigens expressed on CSCs. Although many breast CSC markers are known, the identification of which antigens are appropriate needs to be further investigated along with identifying the most effective way to stimulate an immune response using these antigens [135]. Recent studies have uncovered some exceptionally promising CSC-targeting candidates including metformin, a commonly utilized drug for diabetes therapy, which can selectively kill breast and other CSCs [136]. Finally, other treatments proposed for targeting breast CSCs include dietary compounds. The polyphenols curcumin and piperine have been shown to modulate self-renewal of breast CSCs in vitro by inhibition of the Wnt pathway [137]. Sulforaphane, a natural compound derived from broccoli, can decrease breast CSCs in vitro and eliminate this population in tumor xenograft models of breast cancer [138]. This compound has been reported to inhibit both Wnt and NF-kB signaling.

To effectively treat patients, standard cancer therapies will need to be used in combination with CSC-targeted therapies to provide an effective treatment strategy. Combining these strategies will allow the debulking of the proliferative cells in the tumor mass while preventing recurrence by eliminating the CSCs. Combination therapy would also prevent the potential for bulk tumor cells to dedifferentiate to a stem cell-like state that might be induced by the tumor microenvironment [139, 140].

Summary and Conclusions

Many advances have been made in the identification of breast CSCs utilizing markers such as $CD44^+CD24^-$ and $ALDH^+$. However, there are still no universal markers to isolate breast CSCs, perhaps due to the large heterogeneity observed between breast cancers. Functional assays, which themselves have limitations, are required to more conclusively identify CSCs.

CSCs are now recognized as mediators of breast cancer treatment resistance and as initiators of metastasis. The rapidly growing field of breast CSC research has significantly increased our understanding of their complex biology. These findings serve as a foundation to develop new therapies for targeting this cell population. With further investigation, CSC-targeted therapeutics have the potential to significantly improve patient outcome for breast cancer as well as for other types of cancer. Numerous agents targeting CSCs are now entering clinical trials, and these studies will determine the clinical utility of targeting this cell population.

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Nephroblastoma/Wilms Tumor: A Corrupted Cap Stem Cell Caricature

Michael D. Hall and Alan O. Perantoni

Introduction

The ability of a tumor to generate a tissue-specific cell lineage typical of its originating progenitor is arguably one of the most compelling evidences that tumors arise from transformed stem cells. This ability is well documented in the pediatric malignancy Wilms tumor (WT) or nephroblastoma, which is noted for its remarkable mimicry of metanephric development in producing a caricature of the differentiation process. Normally, development is driven by the reciprocal interactions between the inductor ureteric bud (UB), which branches to form the collecting duct network, and the metanephric mesenchyme (MM), which in part is induced by the bud to undergo mesenchymal–epithelial transition (MET) in generating the tubular epithelia of the nephron. Both the UB and MM arise from the intermediate mesoderm, which populates the urogenital tract. The MM originates in the nephrogenic cord, and these cells have the potential to develop into interstitial stroma and nephrogenic mesenchyme, which, in turn, is specified to form the epithelial structures of the nephron, including the podocytes of the glomeruli and the proximal and distal tubular epithelia. Blastemal cells of a nephroblastoma behave as metanephric mesenchyme or multipotent stem cells in that they have retained a capacity to differentiate along predicted lineages to yield primitive epithelial tubules and stroma while simultaneously populating the expanded blastema. Indeed the characteristic triphasic histologic phenotype of primitive tubular epithelial structures, stroma, and blastemal elements observed in the typical nephroblastoma and shown in Fig. 1 is consistent with a stem cell origin for these tumors. Furthermore, biochemical, molecular, and genetic analyses of the various cell populations in the nephroblastoma support this stem cell origin as will be described, but clearly tumor cells have retained some ability to differentiate, even if only to a neoplastic cell with characteristics of a more advanced cellular phenotype.

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Wilms Tumor: A Pediatric Problem

WT is predominantly a pediatric malignancy, arising with an incidence of nearly eight per million children between ages 0 and 14, although tumors may occur during fetal development as well or even in adulthood [1, 2]. The vast majority of tumors (more than 75 %), however, are diagnosed by age 5. Its relatively high incidence (6 % of all childhood neoplasms), makes it the most common form of childhood kidney cancer and the third most common cancer in children under age 5 after acute lymphoid leukemia and neuroblastoma [3]. While the majority of adult neoplasms show signs of widespread genomic instability, WTs contain a limited number of chromosomal abnormalities and even fewer of these have been demonstrated to associate with the disease or patient prognosis. These include trisomy 6, 8, 12, or 18, and partial gain of 1q. Chromosome losses occur less frequently and involve chromosomes 11, 16, or 22, while loss of heterozygosity has been reported for 1p, 1q, 11q, 16q, or 22q [4]. In fact, the limited but common association between chromosomal abnormalities and disease leads us rather optimistically to the belief that understanding the pathogenetic process and genetic mechanism(s) responsible for WT should be attainable. However, the genetic regions that have been implicated thus far are rather large and will require further resolution in order to identify candidate genes associated with WT pathogenesis. This is complicated by the fact that WT is predominantly a sporadic and genetically heterogeneous disease and that epigenetic mechanisms are also in play.

The vast majority of Wilms tumors occur unilaterally but 5–10 % arise bilaterally. Additionally, their appearance is most often sporadic, although a familial predisposition is

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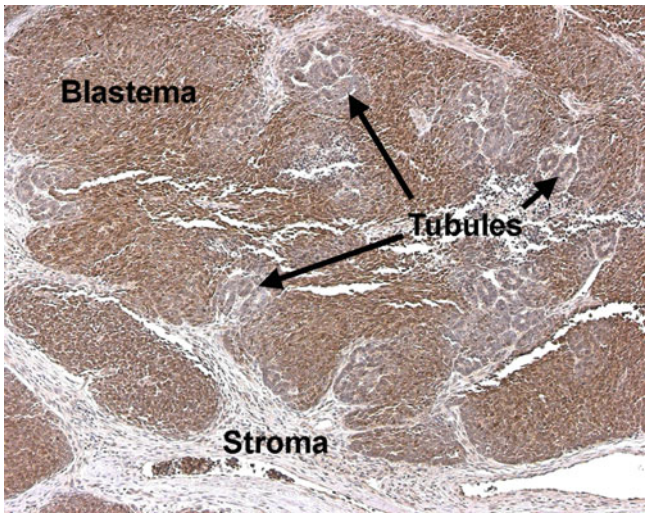

Fig. 1 Wilms tumor with favorable histology. Wilms tumors typically contain blastemal, stromal, and epithelial/primitive tubular elements but with variable proportions of each

observed in 1–2 % of WT cases [5], and this manifests as an autosomal-dominant trait with incomplete penetrance. Since familial tumors are more often bilateral and exhibit an earlier onset, there may be a cancer predisposition involving a germline mutation, which accelerates tumorigenesis. In one case, a familial predisposition locus has been mapped to chromosome 17q12–q21. The appearance of WTs in this family shows incomplete penetrance (~30 %), with no evidence for loss of heterozygosity in tumors [6, 7]. The genes responsible for this predisposition as well as the predisposing locus in chromosome 19q13.3–q13.4 in other families [8] have yet to be determined. Although Wilms tumors arise to a limited extent in association with other congenital anomalies, the vast majority of familial tumors are not associated with any apparent phenotype other than the tumor itself.

Specific constitutional syndromes have been identified which present with an increased risk for WT. For example, aniridia, a rare malformation of the iris, is a disorder of haploinsufficiency caused by intragenic mutations in one copy of *PAX6*. However, about 5 % of aniridia patients are also at risk for WT and show a WAGR (Wilms tumor, aniridia, genitourinary anomalies, and mental retardation) syndrome. High resolution deletion analysis of chromosome 11p13, which contains the *PAX6* gene, has revealed that aniridia patients with WT also bear deletions in the Wilms tumor suppressor gene *WT1*, which resides within 650 kb of *PAX6* [9]. The Denys–Drash syndrome [DDS] (WT, genitourinary anomalies, early-onset renal failure, and predisposition to germ cell tumors) also predisposes patients to WT (~3 %) [10, 11]. In this case, germline mutations of chromosome 11p13 have been identified and specifically affect the zinc-finger domain of *WT1* [12], inhibiting its ability to interact with DNA [13]. The urogenital pathology associated with this syndrome is believed to be mediated by the production of a dominant-negative form of WT1, which then competes with the normal protein.

WT1: A Predisposition Gene for Wilms Tumor

WT1 was the first mutated gene to be linked with susceptibility to Wilms tumorigenesis. Following identification of chromosomal deletions in 11p13 of WAGR patients, a gene encoding this transcription factor was subsequently cloned and implicated in Wilms pathogenesis [14]. The so-called Wilms tumor suppressor gene *WT1* encodes a DNA-binding protein with four C-terminal zinc fingers. It is localized predominantly to the nucleus and can either suppress or activate transcription depending upon its target and/or interactive partner (reviewed in [15]). During metanephric development, *WT1* expression is up-regulated in induced metanephric mesenchyme, and expression remains high in condensates and subsequently in newly formed epithelia [16]. With nephron segmentation, however, expression becomes restricted to podocytes, where it remains into adulthood. In the mouse, *WT1* null homozygotes display a severe renal phenotype, showing no metanephric development since the mesenchyme undergoes apoptosis [17]. Thus, *WT1* plays a critical role in renal progenitor/stem cell maintenance and differentiation. Recently, *WNT4* was shown to be a direct target of WT1 transcriptional regulation [18]. This secreted patterning molecule is essential for MET in metanephric mesenchyme during nephron formation [19]. Thus, WT1 may regulate not only progenitor maintenance but also the conversion of progenitors to nephronic epithelia through the stimulation of *WNT4* expression. WNT4-induced MET is driven by a calcium-dependent Wnt signaling mechanism and not by canonical/β-catenin-dependent Wnt signaling [20, 21]. Moreover, WT1 regulates the expression of WT1-induced inhibitor of *DISHEVELLED* (*WID*, also known as *CXXC5*), which inhibits canonical Wnt signaling through a direct interaction with DISHEVELLED [22]. In the absence of *WT1*, one could envision a mechanism whereby canonical Wnt signaling proceeds unrestrained in the absence of *WID*, while MET is inhibited due to an attenuation of WNT4 expression, resulting in the accumulation of renal progenitors. While the existence of such a mechanism is speculative at this time, recent studies of WNT signaling indicate that the β-catenin- and calcium-dependent WNT mechanisms can be antagonistic during development [20, 23]. Moreover, the predicted consequences of *WT1* loss as a suppressor of canonical Wnt signaling are consistent with the observed histology of the tumors, i.e., an expansion of the blastemal/progenitor populations with nuclear localization of β-catenin.

Mutations in the *WT1* Locus from Wilms Tumors

The detection and distribution of *WT1*-inactivating mutations in a number of studies have implicated the *WT1* locus in the pathogenesis of a significant portion of WTs [24–26]. The vast majority of tumors from patients with the WAGR syndrome contain a germline loss of chromosomal locus 11p13 causing deletion of a constitutional *WT1* allele. Under these circumstances, the second allele frequently contains a somatic point mutation [27]. Thus, loss of both alleles, i.e., loss of heterozygosity, may be necessary for WT development, suggesting that WT1 functions as a tumor suppressor in these neoplasms. As mentioned above, WT formation in patients with DDS is associated with specific point mutations in exons 8 and 9 of *WT1*, which involve the zinc-finger DNA-binding domain of the encoded protein. Most often the mutation results in an Arg residue replacement for Trp in codon 394 within the third zinc finger [28]. Of more than 50 cases examined, about 50 % contain missense mutations in this codon or the adjacent codon Asp396. While it is thought that these mutant proteins behave as dominant-negative factors, the remaining wild-type allele is apparently also lost in those tumors [12], so tumor formation could simply be dependent upon the loss of normal WT1 function. A third nephropathy associated with *WT1* mutations called the Frasier syndrome resembles DDS in that germline alterations involving intronic point mutations interfere with splicing between zinc fingers 3 and 4 of *WT1*; however, these alterations do not result in WTs, so mutations in this case are not sufficient for renal tumorigenesis, although patients with this syndrome are predisposed to gonadal tumors. In any case, the study of mutations in *WT1* from WAGR and DDS does suggest that it is functioning as a classical tumor suppressor in Wilms.

Familial predispositions to WT are autosomal-dominant [29], and tumor formation with no associated congenital abnormalities is often the only indication of a predisposition. In the rare familial form of WT, *WT1* mutations are infrequent. In one study, only 1 of 26 tumors exhibited a mutation: in this case a germline deletion passed from parent to child [25]. Such observations suggest the involvement of other genes (perhaps downstream of *WT1*) either individually or in combination with *WT1* in tumorigenesis [30].

WT1 mutations are considerably more prevalent in sporadic tumors. Of more than 600 neoplasms now analyzed, roughly 10 % carry *WT1* mutations. These have included somatic inactivating deletions, insertions, or missense mutations. The bilateral tumors examined in these studies all contain germline mutations, while unilateral tumors generally carry somatic *WT1* alterations [25]. Mutations have been detected not only in tumors but also in the putative preneoplastic lesion, i.e., nephrogenic rest, which will be discussed later [31]. Its presence in these early lesions suggests that a *WT1* deficiency may lead to the development of nephrogenic rests and enhance the likelihood of neoplastic conversion. In some WTs, germline mutations in *WT1* are detectable in one allele, but the remaining allele appears normal, which suggests that other genes may participate in the silencing of the normal allele. Finally, a large fraction of Wilms tumors have no *WT1* mutations and, in fact, express high levels of normal functional WT1 protein, showing clearly that other mechanisms can drive tumor formation. In this regard, abnormalities implicating chromosomes or chromosomal loci previously described may be responsible [32–36].

Beckwith-Wiedemann Syndrome Involves a Second Locus WT2 in Chromosome 11 and Imprinted Genes in This Locus

Beckwith-Wiedemann syndrome (BWS) is a congenital growth disorder characterized by umbilical hernia, organomegaly, and predisposition to malignancies including Wilms tumor, rhabdomyosarcoma, and hepatoblastoma. It affects some 1 in 14,000 live births and has been linked to the WT2 chromosomal locus 11p15.5, through an examination of familial cases of BWS. Familial cases account for approximately 15 % of BWS patients, and the tumor risk is about 7.5 % in children under 8 years of age. The majority (60 %) of those tumors are WTs. The remainder includes hepatoblastomas, rhabdomyosarcomas, and neuroblastomas. Overall, approximately 80 % of BWS cases are associated with alterations in 11p15.5, which comprises two domains of imprinted genes [37, 38]; however, only one of those domains predisposes to WTs. The first domain encompasses fetal-specific *Insulin-like Growth Factor-2* (*IGF2*) and *H19*, a putative tumor suppressor gene, which encodes a noncoding RNA that regulates *IGF2* expression. Normally, *H19* is expressed only from the maternal chromosome; whereas, *IGF2* is transcribed from the paternal chromosome, and both are reciprocally regulated by Imprinting Center 1 (IC1), a differentially methylated region (DMR) upstream of *H19* (Fig. 2a). The absence of IC1 methylation on the maternal chromosome allows transcriptional repressor and zinc-finger protein, CCCTC-binding Factor (CTCF), to bind to DNA, blocking access of the *IGF2* promoter to downstream enhancers and instead permitting *H19* expression. Conversely, methylation of the paternal chromosome prevents CTCF binding, thus allowing access by enhancers to the *IGF2* promoter and causing repression of *H19* [39–41]. Loss of imprinting (LOI) for *IGF2*, which results in its overexpression, has been demonstrated in about half of all WTs [42, 43] and includes, but is not limited to, BWS patients [38]. With LOI for *IGF2*, aberrant methylation of the maternal chromosome (i.e.,

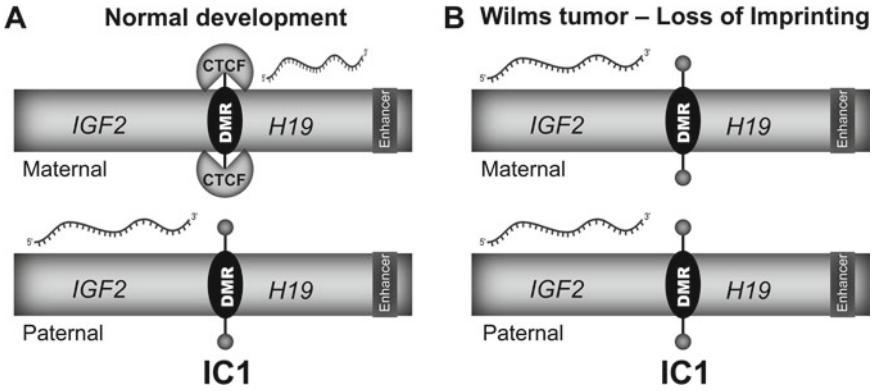

Fig. 2 Loss of imprinting at Imprinting Center 1 (IC1) for *IGF2* expression in Wilms tumor. (**a**) Normally, *IGF2* is expressed only from the paternal allele, and maternal expression is suppressed due to the binding of CCCTC-binding factor (CTCF) to the *H19* differentially methylated region (DMR). CTCF blocks access by the *IGF2* promoter to downstream enhancers. Instead, the enhancers are used to promote transcription from the *H19* locus. (**b**) With loss of imprinting in tumors, the maternal DMR becomes hypermethylated, preventing CTCF from binding the DMR and resulting in *IGF2* expression from both maternal and paternal alleles

methylation of IC1) occurs, which, in turn, leads to repression of *H19* and expression of *IGF2* from both paternal and maternal chromosomes (Fig. 2b). Approximately, 5 % of BWS patients have alterations that cause methylation of maternal IC1 and of these, more than 25 % develop tumors, most of which are WTs [44]. Presumably the overexpression of *IGF2* contributes to organ enlargement, expansion of the progenitor population, and subsequent tumor formation. This hypothesis is supported in mouse models in which *IGF2* levels have been manipulated. By removing controls for imprinting and allowing both parental copies of *IGF2* to be expressed, organomegaly occurs; however, other characteristics of BWS are not observed [39, 45, 46], suggesting the involvement of other sequences in the deleted locus.

A second imprinting domain has been implicated in BWS pathogenesis and involves *CDKN1C* (*Cyclin-Dependent Kinase Inhibitor 1C* or *p57KIP2*) and *KCNQ10T1*. *CDKN1C* is a tumor suppressor which negatively regulates cell proliferation at the G1 cell cycle phase. Its expression is regulated by the paternal gene *KCNQ10T1*, which, like *H19*, encodes a noncoding RNA. The 5′ end of *KCNQ10T1* also functions as the Imprinting Center 2 (IC2) site [47, 48]. IC2 is normally methylated on the maternal chromosome, which silences *KCNQ10T1* transcription and allows expression of *CDKN1C*. In BWS cases involving IC2, loss of methylation negatively regulates *CDKN1C*. Although LOI at IC2 accounts for about half of all defects associated with BWS, it yields no predisposition to WT. Mutations have been reported in *CDNK1C*; however, this also does not predispose to WT, nor do mice with ablated *Cdnk1c* develop nephroblastomas. However, null homozygotes for this gene have numerous characteristics of BWS, including renal dysplasia and adrenal cortical hyperplasia, but without the organomegaly associated with IGF2 overexpression [49].

Only 4 % of BWS patients eventually develop WTs [50, 51]; however, in a small number of patients, normal tissues show a mosaic pattern of 11p15 LOH with somatic duplication of the paternal loci and loss of imprinted maternal sequences [52]. The percentage of cells with LOH exceeded 75 % in several normal tissues, suggesting that these cells undergo selection during tissue differentiation and maintenance. Since tumors also arise from cells with LOH, it is conceivable that these abnormalities provide a growth advantage during tumorigenesis. This is further supported by the observation that the imprinted genes are also perturbed in Wilms preneoplastic lesions [53].

Nephroblastoma: A Stem Cell Tumor?

As described above, the triphasic histological phenotype of WT is consistent with the concept that these neoplasms arise from a multipotent progenitor in the metanephric blastema. This hypothesis is further supported by genomic microarray analyses, which reveal that WTs express predominantly genes associated with the early development of metanephric mesenchyme. Comparative analysis of WTs [54] with rat embryonic kidneys [55] revealed greater concordance with early metanephric development as opposed to later stages. Moreover, a smaller set of genes was overexpressed in WTs relative to normal embryonic kidney, and this group was enriched with several transcription factors essential for survival, proliferation, or early development, such as *EYA1*, *PAX2*, *HOXA11*, *SIX1*, or *SALL2*. A further technical refinement using laser-capture microdissection to isolate tumor subpopulations has also demonstrated that the resident blastemal cells in WTs molecularly resemble the earliest stages of nephron formation [56]. In addition, markers for the cap cells, the condensate over the tip of the inductor ureteric bud and progenitor for nephronic epithelia, are highly expressed in the blastemal component of WTs. SIX2 and CITED1 both delineate this small population of nephronic stem cells (Fig. 3a)

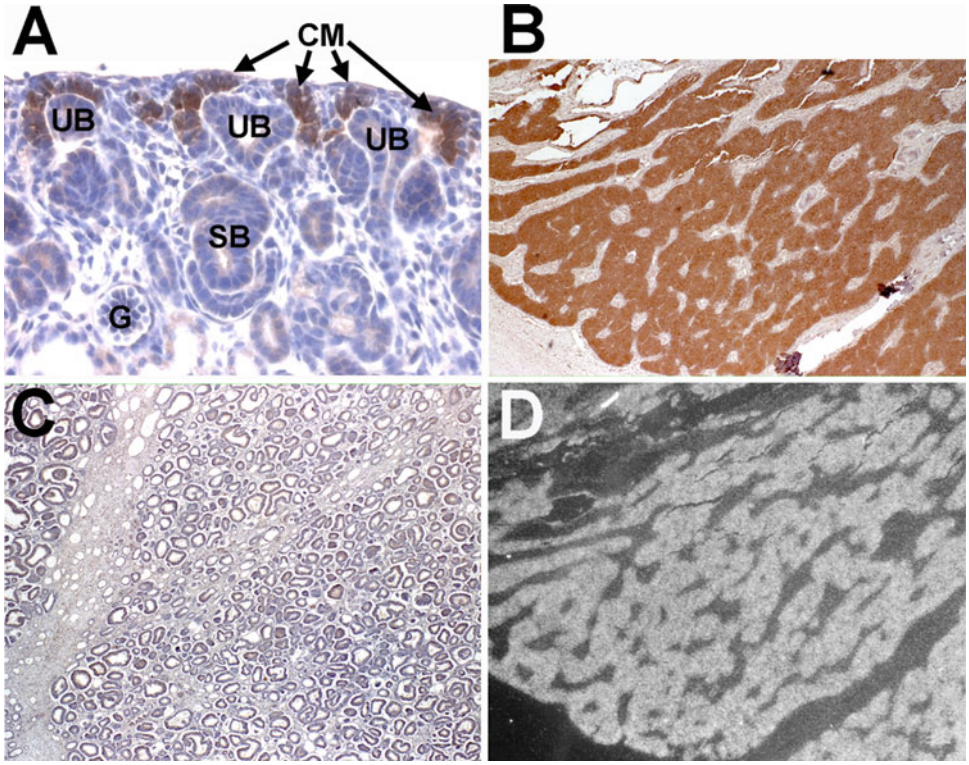

Fig. 3 Wilms tumor blastemal cells express markers of condensed cap mesenchyme. (**a**) The transcriptional co-activator CITED1 is normally expressed in a restricted population of cells that overlay the inductor ureteric bud (UB). Cell lineage analysis shows that these cells are not only incorporated into all segments of nephronic epithelia but also self-renew to maintain the progenitor/stem cell population for nephron elongation. The blastemal population in Wilms tumor expresses CITED1 protein (**b**) and transcript (**d**), but the primitive epithelial tubules in the tumor do not produce CITED1 (**c**). This suggests that the tumor's blastemal cells are derived from the cap progenitors. *CM* cap mesenchyme, *G* glomerulus, *SB* S-shaped body (stage of nephron development), *UB* ureteric bud

and are well expressed in WT blastemal cells (Fig. 3b–d) [57–59]. Since in normal kidney development, the *SIX2*-expressing population is self-renewing and provides progenitors for the nephron [60], it is likely that cells in this population are the origin of the transformed progenitors in WTs.

In addition to their utility in defining markers for specific cell populations in nephrogenesis and tumorigenesis, microarray studies have also provided insight into potential signaling mechanisms involved in development and tumor pathogenesis. Comparative studies of metanephric blastemal cells and the similar population in WTs has delineated a small set of differentially regulated genes associated with specific cell signaling mechanisms [56]. For example, *PHOSPHOLIPASE C-gamma-2* (*PLCG2*) is deficient in most WTs, and we now know that metanephric mesenchyme is dependent upon calcium signaling for conversion to the epithelia of the nephron [20, 21]. Hypothetically, a deficiency in *PLCG2* might inhibit this morphogenesis, resulting in the accumulation of blastemal cells. Furthermore, upregulated genes in WTs include members of the FRIZZLED family, the receptors for WNT ligands, and implicate the WNT signaling pathway in the disease process [61]. In efforts to better define a WT stem cell progenitor, Dekel et al. [62], passaged WTs in immunodeficient mice to select for a more aggressive and less heterogeneous population. The resulting xenografts differentially expressed several critical early metanephric progenitor genes, such as *LIM1*, *SIX2*, and *WT1*, as well as WNT pathway genes and the cell adhesion molecule *NCAM* [63]. They also overexpress genes associated with stem cell maintenance, such as Polycomb genes *EZH2*, a histone methyltransferase that represses cell cycle inhibitors p16/p14, and *BMI-1*, a ring finger oncogene that represses p16 and p19. These Polycomb family members have been implicated in stem cell self-renewal and tumor development in other systems, so their detection in this model is not surprising. Their role in the biology of these tissues, however, remains unclear.

While WTs generally exhibit a triphasic histological phenotype, there is a continuum in the quantitative representation of each subpopulation within different tumors. Thus, tumors may appear predominantly blastemal, epithelial, or stromal. While the blastemal and epithelial elements resemble the structures observed in the differentiating metanephros, stromal-predominant tumors can undergo heterologous differentiation to yield cartilage, adipose tissue, striated smooth muscle, and even bone, suggesting that cells of metanephric mesenchyme retain this plasticity, that tumor cells are derived from an even more primitive stem cell population,

e.g., a mesenchymal stem cell, or that plasticity is acquired with transformation. Recent lineage studies of FOXD1-positive stromal cells in the kidney have demonstrated that these progenitors are responsible for forming not only the interstitial mesenchyme in the kidney but also the pericytes, associated with the vasculature, and smooth muscle [64]. Thus, some of this differentiation can be attributed to a putative renal progenitor. However, there is some evidence to suggest that these renal stromal progenitors are derived at least in part from the paraxial mesoderm, and not just from the intermediate mesoderm [65]. Moreover, cultured, predominantly stromal WTs express markers consistent with cultured mesenchymal stem cells [66]. A paraxial mesodermal origin for these tumor cells would help explain the greater differentiation potential of this population, although the assumption here is that all of the stromal cells are derived from the tumor, but they may instead be in part recruited from adjacent tissues by the tumor.

Intermediate Filaments and Extracellular Matrix Components

Patterns of intermediate filament expression are also useful in analyzing the nature of the histologic components of WT. Blastemal cells either in WT or in the metanephros express vimentin, while primitive normal and neoplastic epithelia show staining for cytokeratins and stromal tissues for vimentin [67]. Integrin and extracellular matrix transitions have been implicated in metanephric differentiation, and patterns observed in WTs resemble the expression profiles of normal tissue components [68–70]. Specifically, integrins α2, α3, and α6 as well as laminin and type IV collagen are all elevated in the epithelial component of WTs and normal primitive tubules, while α1, α4, and α5 with fibronectin are detected in stromal populations. Blastemal elements in WTs, however, are deficient for expression of most ECM protein examined but do stain for α3 and α6 integrins and show variable levels of fibronectin. Neural cell adhesion molecule (NCAM) and the transcription factor MYCN have also proven useful as markers of both neoplastic and nonneoplastic renal blastema [71–74], and as mentioned above, NCAM may identify a stem cell population in WTs [63].

Each WT Cell Component Carries the Same Genetic Polymorphism

That the various populations are indeed derived from tumor tissues and not just recruited from normal surrounding structures has been demonstrated through microdissection of the various tissue elements. In all tumors analyzed with LOH for the *WT1* locus, each histologic element contained the identical polymorphism [75], indicating a clonal origin for each tumor component despite the histological differences. Thus, stromal elements, including muscle, show the same loss of *WT1* as the blastemal populations, suggesting that all heterologous elements are neoplastic.

Epigenetic Changes Are a Common Feature of Wilms Tumors

As already described, epigenetic changes in chromatin are commonly observed in WTs. Notably, *IGF2* expression from the paternal allele is replaced with biallelic expression in BWS due to hypermethylation of the *H19* DMR, i.e., LOI, and this results in more than a twofold increase in serum IGF2 levels. In addition, recent advances in chromatin analysis now permit the mapping of promoters, transcripts, or Polycomb-binding repression sites based upon the state of histone H3 methylation. The presence of bivalent domains, i.e., DNA regions with both promoter- and Polycomb-specific trimethylated-histone binding sites, is believed to identify a silent gene that is primed for expression. It is thought that transient silencing of genes in this manner may leave them vulnerable to aberrant hypermethylation and result in heritable silencing and cancer [76]. Using chromatin analysis in comparing WTs, embryonic stem cells (ESCs), and normal kidney, studies of whole-genome profiles have revealed striking similarities between gene expression programs in WTs and ESCs [58]. Thus, WT cells are primed to maintain stem cell/blastemal markers, such as *SIX2*, *EYA1*, and *OSR1*, and to proliferate. More importantly, there is a subset of genes, which is normally expressed in epithelial cells but which in tumor cells is maintained in a "bivalent" or poised state, suggesting that these cells are inhibited in their ability to convert to an epithelium. Moreover, genes associated with early stromal, e.g., *FOXD1*, or epithelial, e.g., *LHX1*, are also bivalent, suggesting that the WT blastemal progenitor precedes the divergence of these two differentiation pathways. Whether these bivalent genes are also hypermethylated and silenced in tumor cells remains to be determined.

Precursor Lesions in Wilms Tumor Pathogenesis

In humans, metanephric development is completed around 36 weeks of gestation, so the presence of metanephric mesenchyme postnatally is abnormal. In a retrospective study of over 1,000 necropsies of newborns, almost 1 in 100 carried persistent blastemal cells in the kidney; 100 times the incidence of Wilms tumors. Furthermore, more than 40 % of Wilms tumor patients with unilateral disease and 100 % with

bilateral disease have readily demonstrable preneoplastic lesions. The term nephrogenic rest has been applied to these WT precursors and includes all lesions from dormant to maturing, hyperplastic, or neoplastic. Nephroblastomatosis then is used to describe a condition characterized by multiple or diffuse nephrogenic rests. Beckwith has characterized these lesions based upon their distribution in the renal lobe, which also reflects their differentiation status [77, 78]. Since differentiation occurs along a proximal–distal axis with nephron layering from medulla to cortex, the most medullary structures are produced first and those distributed cortically occur at the termination of organogenesis. Lesions are therefore classified either as perilobar (PLNR), which are distributed at the periphery of the renal lobe, or intralobar (ILNR), which arise within the lobe and deep within the renal parenchyma [78]. Moreover, ILNRs tend to be more heterogeneous histologically, displaying elements of cartilage, fat, and bone, and therefore may originate in an earlier progenitor. WTs associated with ILNR also have a high rate of mutation in both *WT1* and *CTNNB1*. Morphologically, the PLNRs appear as peripheral spherical blastemal foci but are more epithelial in character in later lesions. These are commonly observed in BWS patients. Kidneys often contain several, well-defined lesions, although their margins may also be diffuse. On the other hand, ILNRs are predominantly stromal but with some blastemal and epithelial populations. These occur randomly within the renal lobe as single foci with irregular margins. In the 40 % of unilateral Wilms tumors with nephrogenic rests, the incidence of either PLNR or ILNR is roughly equivalent. In bilateral disease, however, tumors with PLNR vastly outnumber those with ILNR by 3 to 1. Similarly, in WT patients with BWS, PLNR also predominates. Conversely, in cases of Wilms tumor associated with WAGR or DDSs, the primary lesion is ILNR, which arises in most tumors evaluated (84 % and 91 %, respectively), while PLNR is observed in less than 20 % of cases.

Beckwith's descriptive studies suggest a varied outcome to the presence of nephrogenic rests [77]. Clearly all rests do not progress to Wilms tumors as only 1 in 100 patients with rests develops neoplastic disease. In fact, the rests may remain dormant for years, mature and sclerose, become cystic, regress, or form hyperplastic nodules. These outcomes are not unlike those described in tumor progression involving preneoplastic lesions in other tissues, e.g., the liver or skin. However, a clear association between rests and tumors has not been established as for other tissues. One might predict from Knudson's model that the rest would carry the first "hit," e.g., a mutation in one allele of *WT1*, with the second alteration arising during neoplastic conversion. Thus far, this has not been demonstrated. In the case of *IGF2*, constitutively high levels of expression have been reported for both nephrogenic rests and Wilms tumors and even epithelial structures occasionally showed sustained expression [79]. On the other hand, *WT1* mutations have been observed in nephrogenic rests but a progression has not [31]. This may reflect the complexity of nephroblastoma development in that all of the genes involved have not been identified. This is supported by genetic studies linking high frequencies of PLNR with either trisomy 13, which is associated with hyperplastic nephromegaly and Wilms tumor [80], or trisomy 18, which also predisposes to Wilms tumor in surviving patients [81, 82].

Genes Associated with Wilms Tumorigenesis

While thus far a limited number of genes have been implicated in the etiology of WT, namely *WT1* and *IGF2*, the variety of alterations associated with their involvement have greatly complicated our understanding of WT pathogenesis. Both genetic and epigenetic alterations in 11p13 (*WT1*) and 11p15.5 (*IGF2* domain) have been reported. Moreover, many tumors contain alterations at multiple loci. In a comprehensive examination of 35 sporadic WTs, more than 80 % contained single or multiple genetic or epigenetic changes in *WT1* or *WT2* [83]. For the gene *WT1*, loss of expression was not always accompanied with a demonstrable genetic alteration, indicative of epigenetic causation. Indeed, LOI at 11p13 and involving *WT1* has been described [84]. Conversely, LOI for *IGF2* is not the only mechanism associated with dysregulated *IGF2* expression. Notably, constitutional paternal uniparental disomy, which causes paternal *IGF2* duplication [85], or microdeletion/microinsertion in the *IGF2*/*H19* (IC1) imprinting center are also associated with the disease [86]. These mutations are demonstrable in lymphocytes from patients and occur either in the germline or very early in development due to their wide somatic cell distribution. Moreover, their early appearance suggests a predisposing or initiating role in tumorigenesis. Similarly for *WT1*, mutations observed in WTs may also be detected in nephrogenic rests, the putative preneoplastic lesion, but not in the germline [31]. This suggests that inactivation occurs as an early event in tumorigenesis. As previously mentioned, however, *WT1* mutations alone are not sufficient for tumorigenesis, and this has been confirmed in a mouse model [87], which will be discussed later. Therefore, it is not surprising that *WT1* mutations are often associated with alterations involving other genes, most notably in *IGF2*, as already mentioned, or *CTNNB1*, as will be discussed. These pathways interact to regulate growth or progenitor maintenance at some level. For example, the *IGF2* promoter contains multiple binding sites for WT1, which represses the expression of *IGF2* [88]. Derepression of *IGF2* following *WT1* deletion then results in the increased availability of this important oncogenic growth factor.

Wnts and β-Catenin/CTNNB1 in Metanephric Development

Assuming that WTs originate from metanephric mesenchymal progenitors, then understanding the signaling mechanisms that drive normal development may provide substantive clues to the factors responsible for the dysregulation that occurs in tumorigenesis. Accordingly, some attention has been focused on characterizing the mechanisms that mediate the maintenance of these progenitors and signaling factors that direct their MET. WNTs play critical roles in stem cell maintenance and renewal, differentiation and morphogenesis, and tumorigenesis, and several are expressed during metanephric development. Typically a WNT interacts with a Frizzled membrane receptor to indirectly stabilize a secondary messenger β-catenin through inactivation of a GSK-3β-mediated ubiquitination pathway responsible for β-catenin degradation. Accumulated β-catenin can then translocate to the nucleus for transcriptional activation of Wnt-dependent targets, such as cell cycle regulator *CYCLIN D1* [89] or *C-MYC* [90], via canonical (CTNNB1-dependent) cooperation with a member of the T-cell factor (TCF) family of DNA-binding proteins. Although both Wnt4 [19] and β-catenin [91] are essential for nephron formation, an examination of the role of canonical Wnt signaling in MET has revealed that it may be critically important for MM progenitor maintenance and proliferation but not for its differentiation or morphogenesis. Notably, constitutive activation of *CTNNB1* in MM expands the clusters of condensed metanephric mesenchymal cells, but these clusters fail to epithelialize [91, 92]. On the other hand, Wnt4, which induces MET in mesenchymal progenitors, activates a calcium-dependent signaling mechanism without stimulating canonical Wnt signaling [20], and a calcium ionophore triggers MET in these cells. Calcium signaling in response to a Wnt is initiated through a G-protein-coupled receptor, which activates phospholipase C (PLC). PLC then generates active phosphoinositide (PIP) secondary messengers which stimulate the release of calcium stores from the endoplasmic reticulum. The released calcium initiates a signaling cascade involving calmodulin (CaM) and the phosphatase calcineurin, and calcineurin then activates members of the NFAT family of transcription factors through direct dephosphorylation [20, 21]. It is NFAT transcriptional activation that is currently thought to mediate MET. The above-described signaling cascade is operative in MM progenitors and responsible for their differentiation (Fig. 4). A similar mechanism was recently described in the specification of mouse ESCs. Calcineurin-NFAT signaling is essential for the conversion of pluripotent self-renewing stem cells to an early lineage commitment [93]. Furthermore, inhibition of this signaling sustained the self-renewing stem cell population long term. It is tempting to propose a similar role for NFAT signaling in metanephric mesenchymal progenitors.

Dysregulated Calcium Signaling in WTs

There is ample evidence in WTs to suggest that calcium signaling is disrupted in tumor cells, although its precise role in the disease process has not been established. The expression of *STIM1*, a key activator of store-operated calcium entry, is deficient in a small sampling of passaged WTs [94]. Also, *PAX2*, an early metanephric progenitor marker, is commonly expressed in WTs [54], and *Calcineurin A-Binding Protein* (*CnABP*) functions as a target downstream of *PAX2* [95]. CnABP interacts with calcineurin to prevent its dephosphorylation of NFAT, thus attenuating calcium-dependent transcriptional activation. It is overexpressed in more than 70 % of WTs and, when transfected into kidney cells, it stimulates proliferation and migration, suggesting that it plays a significant role in WT pathogenesis. On the other hand, *CACNA1E*, a calcium channel gene, has been implicated by comparative genomic hybridization studies as a microamplification in about 8 % of WT patients, and its appearance correlates with WT relapse [96]. Its expression upregulated the proliferation-associated genes, *EGR* and *FOS*, although its effect on calcium signaling in WTs has not been established. Regardless, the potential role of calcium in tumor suppression is intriguing and further supported by studies in other tissues, which document the antagonistic relationship between calcium and canonical Wnt signaling [23]. Indeed, calcium-dependent NFAT activation suppresses canonical Wnt signaling in Xenopus embryos [97], and this suppression is mediated at least in part by the binding of NFAT to Dishevelled, which otherwise would be recruited to the β-catenin transcriptional complex [98]. In so doing, NFAT sequesters Dishevelled from the transcriptional activation of canonical Wnt target genes (Fig. 4) and thus interferes with proliferation in tissue progenitors.

A Role for β-Catenin/*CTNNB1* in WTs

CTNNB1, through the canonical Wnt pathway, can behave as an oncogene in a variety of tumors [99, 100], and mutations in this gene represent dominant lesions, requiring single changes for dysregulation. In an analysis of multiple WTs, 15 % contained mutations in a domain of β-catenin targeted by GSK-3β for phosphorylation, resulting in stabilization of the protein [101, 102]. Moreover, nuclear localization of β-catenin in subpopulations of cells in the majority of WTs has been reported [103, 104]. Mutations are observed in sporadic and Denys–Drash- or WAGR-associated neoplasms. Furthermore, WTs exhibiting mutations in *CTNNB1* consistently also carry *WT1* mutations [102]. As already mentioned, WT1 induces the expression of *WID*, an inhibitor of canonical WNT signaling through its interaction with DISHEVELLED (Fig. 4). However, in tumorigenesis,

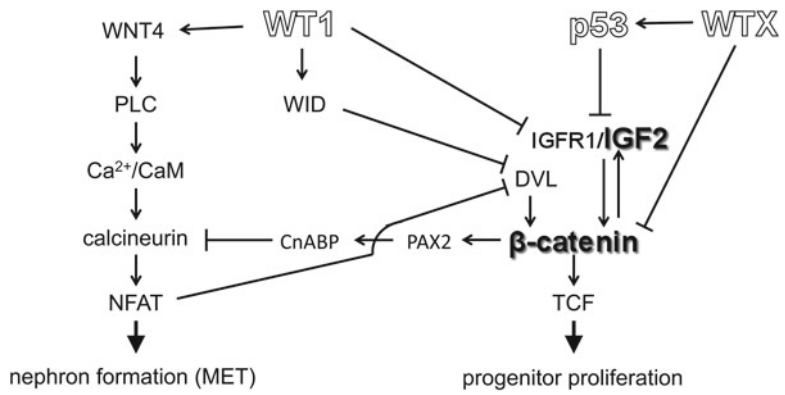

Fig. 4 Hypothetical model for the role of Wilms tumor-associated genes in regulating the balance between differentiation/morphogenesis and proliferation. Tumor suppressors, WT1, WTX, and p53, all negatively regulate signaling by IGF or β-catenin, which function as oncogenes in Wilms tumor development. Dysregulation of any of these factors might shift the balance in the metanephros towards expansion of the self-renewing blastemal population observed in these neoplasms. *Ca^{2+}/CaM* calcium/calmodulin sensor, *CnABP* calcineurin A-binding protein, *DVL* DISHEVELLED, *PLC* phospholipase C, *WID* WT1 inhibitor of DISHEVELLED

mutations in CTNNB1 were detected only in tumors and not in normal somatic tissue or nephrogenic rests, suggesting that it functions primarily in tumor progression [105].

WTX/FAM123B/APC Membrane Recruitment 1 (AMER1) Tumor Suppressor

The *WTX* locus and its association with WTs was first revealed using high resolution comparative genomic hybridization arrays with DNA from sporadic Wilms tumors [106]. This approach identified deletions in chromosome Xq11.1 in about a third of tumors examined and led to the discovery of the *WTX* gene. The encoded protein is expressed in two isoforms, the larger of which contains three APC-binding sites and is localized to the plasma membrane due to the presence of two PIP binding domains at the proteins C-terminus. The smaller alternatively spliced form, which lacks the PIP domains, translocates to the nucleus where it interacts with the β-CATENIN degradation complex. Functional studies describe a rather enigmatic protein with conflicting roles. WTX interacts with several proteins in the β-CATENIN destruction complex, including AXIN1 and APC, and negatively regulates WNT/β-CATENIN signaling by promoting the ubiquitination of β-CATENIN (Fig. 4) and thus facilitating its subsequent proteasomal degradation [107]. Conversely, WTX inhibits the ubiquitination of transcription factor NRF2, which mediates cellular antioxidant responses, by binding to its ubiquitin ligase adapter KEAP1 [108]. Furthermore, the smaller WTX isoform interacts with WT1 and up-regulates *WT1* expression (Fig. 4), presumably enhancing its tumor suppressor function by negatively regulating the canonical WNT pathway. To further complicate matters, it recruits the AXIN/GSK3/CK1 complex to the plasma membrane in order to promote phosphorylation of LRP6, the FRIZZLED coreceptor and coactivator of canonical WNT signaling. The result is activation of canonical WNT signaling, which seemingly opposes its role as a suppressor in the β-CATENIN destruction complex [109]. In fact, AXIN behaves similarly in that, in the absence of a WNT ligand, it promotes β-CATENIN degradation; however, in the presence of a ligand, axin also stimulates canonical WNT signaling through phosphorylation of LRP6 [110].

During development, *WTX* is expressed in metanephric mesenchyme in cap mesenchyme and primitive tubular structures [111]. Germline ablation of *WTX* in the mouse manifests as malformations principally in tissues of mesodermal origin, namely, the bones and kidneys, which exhibit dramatic overgrowth [112]. The kidneys in particular contain massively expanded caps of mesenchyme, the putative origin of WTs. *WTX* loss results in neonatal lethality; however, conditional ablation in the bones yields defects consistent with the human X-linked disorder, osteopathia striata with cranial sclerosis (OSCS), which is associated with germline mutations in *WTX* [113]. The lack of predisposition to WT in OSCS patients suggests that these mutations are not sufficient to initiate tumorigenesis, and this is supported by studies which have demonstrated heterogeneity for mutations within the confines of the same tumor [114].

TP53 in Anaplastic WTs

Although *TP53* is one of the most commonly mutated genes in human cancer, mutations in this gene are detected in only about 5 % of all WTs. On the other hand, nearly 75 % of

anaplastic WTs, which have a propensity for relapse, contain inactivating mutations in *TP53* [115, 116], which then serve as a prognostic marker for these aggressive neoplasms. In an examination of disease relapse, an initial primary WT showing favorable histology presented as anaplastic upon relapse and molecularly was homozygous for *TP53* deletion, suggesting that the loss of the suppressor was indeed responsible for the change [117]. More recently, a variety of genomic alterations involving loci that are independent of *TP53* have been demonstrated for this category of WTs, including a gain of *MYCN* [118]. Loss of *TP53* is associated primarily with the later stages of tumorigenesis, i.e., progression, relapse, and metastasis [119, 120]. The molecular basis for *TP53* involvement in WT development may be related to its critical role in regulating *IGF-1R* expression [121]. Wild-type TP53 suppresses *IGF-1R* transcription, while mutant forms of *TP53* enhance expression [122]. Moreover, TP53 suppresses expression of the IGF-1R ligand *IGF-2* [123] and stimulates expression of *IGF-Binding Protein 3* (*IGFBP3*) [124], which inhibits IGF signaling. Thus, TP53 functions at several levels to negatively regulate IGF signaling (Fig. 4), which, as mentioned previously, is a frequent participant in the pathogenesis of WTs. Similarly, WT1 suppresses *IGF-1R* levels and IGF-induced proliferation through interaction with the IGF-1R promoter [125], and now WTX has also been reported to regulate *TP53* activity (Fig. 4). In this case, however, WTX enhances TP53 acetylation by CBP/p300, which is required for TP53-driven cell cycle arrest and induction of apoptosis [126]. Presumably WTX further suppresses *IGF-1R* and its ligands indirectly through its effect on TP53, but this remains to be determined. Regardless, all of the tumor suppressors associated with WTs can inhibit cell growth by a TP53-mediated process and loss of any of them could result in increased IGF signaling.

Signal Transducers and Activators of Transcription Activation in Metanephric and Nephroblastoma Development

Members of the *STAT* family, especially *STATs 3* and *5*, play key roles in the pathogenesis of a wide variety of both hematologic and solid neoplasms [127]. Furthermore, recent studies in invertebrates and mammals indicate that signal transducers and activators of transcription (STAT) signaling is required for maintenance and renewal of stem cell populations [128, 129]. In the developing metanephric rudiment, STATs 1, 3, and 5 are highly expressed and also tyrosine and serine phosphorylated (transcriptionally activated) [130]. Surprisingly, STAT1 was found to stimulate proliferation and inhibit the differentiation of metanephric mesenchymal progenitors. An investigation of STAT activation in WTs revealed that STAT1 is activated but only by serine phosphorylation and that serine phosphorylation contributed to the tumorigenic phenotype [131]. The role of activated STAT1 was attributed to up-regulation of expression of the survival factors *MCL1* and *HSP27* and proliferation factor *CUX1*/*CDP*. These same proteins are highly expressed in primary WTs. Activation of STAT1 is mediated by protein kinase CK2, a serine-threonine kinase commonly expressed in many types of cancer, but the mechanism responsible for CK2 activation in tumors is ill understood. Furthermore, at what point STAT activation becomes relevant to the cancer process in WTs remains undefined.

Other Predispositions to WT

In addition to BWS, WAGR, and DDSs, other developmental disorders carry a small but significant risk for nephroblastoma. These can be divided into syndromes involving organ/tissue overgrowth, such as BWS, and those not associated with tissue overgrowth, such as WAGR. Among the former category is Perlman syndrome, which is characterized by fetal gigantism, renal dysplasia, and a predisposition for WT. The responsible locus has been mapped and determined to involve *DIS3L2*, an exonuclease with tumor suppressor activity [132]. Ablation of the gene causes mitotic abnormalities, and partial deletions in this gene are detected in 30 % of sporadic WTs. Sotos syndrome manifests as a cerebral gigantism. Sotos is caused by mutations in *Nuclear Receptor SET Domain-containing Protein* (*NSD1*) [133, 134]. NSD1 encodes a methyltransferase that modifies a variety of proteins, but prominent on the list of substrates is lysine residue 36 on histone 3 (H3K36) [135]. This event is generally associated with transcription of active euchromatin. Finally, the Simpson-Golabi-Behmel syndrome is characterized by macroglossia and renal and skeletal abnormalities. In this case, the molecular basis of the disorder has been traced to the X-linked heparin sulfate proteoglycan *Glypican 3* (*GPC3*) [136]. GPC3 plays a significant role in growth control, and loss of expression in mice causes tissue overgrowth due in part to hyperactivation of HEDGEHOG signaling [137].

Of the nonovergrowth-associated WT predisposing syndromes, Alagille syndrome is characterized by congenital cardiopathy, facial dysmorphy, vertebral defects, bile duct paucity, and renal abnormalities. Mutations in *JAGGED1*, a ligand for the Notch pathway, have been detected in the majority of Alagille patients [138]. Bloom Syndrome is also included in this category but exhibits quite different characteristics. BS patients experience immunodeficiency, hypogonadism, growth retardation, sensitivity to sunlight, and predisposition to a variety of cancers. The etiology of the syndrome has been linked to mutations in a DNA helicase from the RecQ family, which affects DNA repair and results in chromosomal fragility [139, 140]. Finally, Li-Fraumeni

Syndrome dramatically increases cancer susceptibility as a result either of the loss of *TP53* or CHECKPOINT KINASE 2 (*CHK2*), which regulates TP53 activity [141, 142].

All of the syndromes described in this section implicate distinct genetic loci in their respective pathologies. How this reflects on WT pathogenesis, however, is unclear. Whether each of these putative suppressor genes is capable directly of suppressing WT development or whether they act indirectly through a mechanism described previously has not been determined. In this regard, it will be critical to establish their impact on those major factors already implicated directly in WTs.

Modeling Nephroblastoma in Animals

Despite the described molecular heterogeneity of WT tumor, current treatment modalities for patients afflicted with the disease are often remarkably effective at achieving eradication of detectable neoplasms. Roughly 10 % of patients, however, do not achieve successful remission, and while many do, these individuals become more susceptible to secondary cancers like leukemia or may suffer a recurrence of the original disease. As such, there is still a great need for the establishment of solid preclinical animal models in which the disease can be evaluated for new therapies.

Non-laboratory Animals

In animals, neoplasms bearing resemblance to WT are labeled nephroblastoma, and they have been observed in a great variety of species as a spontaneous tumor. In the Japanese eel, *Anguilla japonica*, a report of wild-caught and farm-raised specimens revealed a large percentage of animals with encapsulated nephroblastoma that contained a significant blastemal component [143]. Interestingly, a follow-up study demonstrated that the eel homolog of WT1 (EWT1) is pathologically involved in a large proportion (>44 %) of the tumors, suggesting evolutionary conservation of function [144]. While anecdotal evidence exists for spontaneous nephroblastoma in other fish like Koi, *Cyprinus carpio* [145], there are as yet no substantial reports in the established and widely used zebrafish model. Intriguingly, several chemical carcinogens, most notably *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), have been reported to induce nephroblastoma in rainbow trout, *Oncorhynchus mykiss* when administered at various developmental stages [146, 147].

Nephroblastomas arise spontaneously in chickens [148] and can be induced to reliably form tumors at 8–10 weeks of age after injection of newborn chicks with myeloblastosis-associated virus [149]. Other evidence for spontaneous tumors exists for higher vertebrates such as swine, where nephroblastoma is the most common tumor reported by slaughterhouses and arises as both prevalent unilateral (80 %) and bilateral (20 %) cases [150]. In exceedingly rare cases, tumors have been reported in cattle [151, 152], and there is a small body of evidence for nephroblastoma in other domestic and nondomestic animals like dogs [153, 154], horses [155], sheep [156], guanaco [157], foxes [158], and meerkat [159].

Laboratory Animals

Considering their broad value as a model for carcinogenesis, it is not surprising that there is much more detailed information concerning nephroblastoma in laboratory rodents. Of great curiosity, there exists an apparent inability of hamsters or of the mouse, regardless of genetic strain, to develop nephroblastoma under either spontaneous or chemically induced circumstances. While mice do naturally or inducibly develop renal tumors, they are always of the adult-type adenomas and adenocarcinomas irrespective of developmental age at carcinogenic challenge [160]. In fact, in a mouse model of the Aniridia-Wilms Tumor deletion syndrome where the congruous human susceptibility genes are deleted, the mice develop exquisite aniridia, but despite the loss of the *Wt1* locus, no nephroblastomas develop [161]. Curiously, both rats and rabbits are susceptible to the development of nephroblastoma, whether spontaneously or following chemical induction [162]. These nephroblastomas exhibit the classic triphasic histological features of WT and bear resemblance to the human disease.

Utilizing a transplacental delivery method to expose embryos to a direct-acting alkylating agent such as *N*-nitrosethylurea (ENU), nephroblastomas can be reliably induced in rabbits and in laboratory rats. In the rat, it is noteworthy that both spontaneous and induced tumors are dependent upon the strain. Where tumors have been described for the Sprague–Dawley [163, 164], Noble [165], Wistar [166], F344 rats are resistant to nephroblastoma induction by chemical carcinogen but, as for Noble strain, they do develop tumors that histologically resemble mesoblastic nephroma, being stromal in nature and localized to the outer cortex of the organ [167]. Where Sprague–Dawley rats are considered, a sub-strain (Upj:TUC(SD)spf.nb) which exhibits a heritable 6–15-fold (female and male, respectively) increased incidence of spontaneous nephroblastoma carrying a blastemal component, suggests the emergence of a potential model for heritable WT study [168]. In these rats, spontaneous preneoplastic lesions have been observed and described as intralobar nephroblastomatosis, which resembled human ILNR [169]. Concordant with a subset of human tumors, MNU-induced nephroblastomas in the parent Sprague–Dawley strain display a point mutation in the *Wt1* gene, implicating it pathologically in the progression of chemically induced rodent disease [170].

Induction of nephroblastoma in the Noble rat strain is exquisitely dependent upon both chemical carcinogen and developmental stage at the time of challenge. For instance, in these rats ENU induces nephroblastoma with an incidence rate of nearly 50 % after a single transplacental dose at embryonic day 18. When administered neonatally, however, the resulting renal tumors are almost exclusively mesenchymal [171]. For comparison, dimethylnitrosamine, a carcinogenic agent relying on host metabolism for alkylating activity, causes only renal mesenchymal tumors in neonatal Noble rats, and never a nephroblastoma despite any earlier embryonic exposure [172]. When crosses between Noble and F344 strains are made, the susceptibility to ENU-induced tumors is inherited as an incomplete dominant trait that is independent of mutations in the predicted WT-associated genes *Wt1* and *Wtx* or *Ctnnb1*, but demonstrates elevated expression of Wnt and Notch pathway genes, connecting these pathways in the pathogenesis of nephroblastoma [173].

Recently and importantly, several advances in modeling WT in mice have been made. In one report, a transgenic mouse wherein K-RAS and *Ctnnb1* were synergistically overexpressed formed renal tumors, which had histologically similar features to those of epithelial WTs [174]. A major shortcoming of this mouse, perhaps, is the lack of a more classical triphasic histology that resembles the broader human disease. A more significant advance to the field is the generation of a mouse in which *Wt1* has been ablated in a mosaic fashion in the metanephros while *Igf2* is constitutively activated [87]. These mice develop an arrest in metanephric mesenchyme differentiation coincident with an elevated phosphorylation of downstream MAPK signal effectors that results in a triphasic neoplasm and thereby truly mimic a subset of WTs. This mouse model is quite imaginative in its design as it mimics human *Wt1* deletion and concomitant *Igf2* up-regulation—a phenomenon that can occur simply by the inactivation of the *Wt1* gene on chromosome 11. In the mouse, these genes are located on different chromosomes where loss of heterozygosity at the *Wt1* locus does not result in the simultaneous genetic rearrangement affecting *Igf2*, and, as such, the mice do not develop nephroblastoma. This new model's creativity, mimicking synteny, has also added to the field through the discovery and confirmation of the involvement of MAPK signaling in a subset of human WTs, opening a door for new study.

In Vitro Models of Wilms Tumor

A continuing effort to develop a reliable and renewable source of WT and/or nephroblastoma tissue for use in routine molecular and biochemical analyses has proven to be challenging and largely unsuccessful. The avenues pursued have included spontaneous and induced immortalization of cultured tumor cells, heterotransplantation of both spontaneous and chemically induced nephroblastomas, short-term primary culture of dissociated tumors or tissues transformed by transduction of a virus, and optimization of conditions for the culture of normal, intact metanephric mesenchyme tissue. To date, these practices have failed to produce a universally accepted standard in vitro model with which to reliably study nephroblastoma. Aside from a dwindling number of inadequately characterized cell lines, most culture models of WT rely on the scarce availability of fresh primary human or rodent tumors, or those tumors which have been maintained by in vivo serial transplantation in immunocompromised mice.

Cell Lines and Primary Culture

A major historical hurdle for the maintenance of WT in vitro has been their seemingly limited life span in the dish. Typical new cultures grow, with diminishing prowess, for a period of 10–15 passages before succumbing to growth arrest at the cell crisis phase—a phenomenon likely associated with the cessation of proliferation or death of the blastemal component that drive the tumors. In certain rare instances, however, cell lines have been established that appear to have overcome this crisis stage and are apparently immortalized. One such line is designated SK-NEP-1, and was derived from a malignant pleural effusion which was cultured in RPMI-1640 supplemented with 10 % fetal bovine serum [175]. These cells resemble metanephric blastema and coexpress TGF-α and EGFR [176], form tubule-like epithelial structures when grown in a matrix and are growth suppressed when ectopic human chromosome 11 is forcibly expressed [177]. Further studies utilizing this line demonstrate its effectiveness in modulation of heterotransplant aggressiveness [178] and they were subjected to microarray analysis in the search for prognostic markers of WT [179]. Regrettably, recent genetic profiling of the cells have reclassified them as likely derivative Ewing's sarcoma cells, since they express the classic fusion protein EWS-Fli1, decreasing their usefulness as a model for Wilms study [180]. A rat nephroblastoma cell line, ENU-T-1, was isolated from a chemically induced tumor that had previously been maintained as a serial xenotransplant [181]. These cells have demonstrated the ability to form tumors in nude mice and display discrete clonal histological subtypes, suggesting the tumor seed cells have retained some level of multipotency [182]. The most frequently studied cell line is the G401 line, though they also suffer from a questionable origin and are believed to be most representative of a renal rhabdoid tumor following further characterization [183]. A newer and potentially more robust cell line, WiT49, is derived from a first-generation xenograft of a WT lung metastasis and has demonstrated expression of *WT1* with

Table 1 Other established cell lines useful for the study of WT

Cell line	Species of origin	Histological pattern	References
CCG-99-11	Human	Blastemal	Kim [197]
HFWT	Human	Undescribed/anaplastic	Ishiwata [198]
RM1	Human	Epithelial	Haber [199]
WT-CLS1	Human	Undescribed	http://www.cell-lines-services.de

overexpression of *IGF2* and a classic mutation of *p53* [184]. These cells have been shown to propagate a tumor in nude mice when transplanted orthotopically; however, the resultant tumors lack an appreciable blastemal component and are as such predominantly epithelial and stromal in nature [185]. Culturing conditions for the generation of WT lines are steadily improving; however, as longer-lived cultures have been described arising from WT1-mutant tumors [66] and with variable success from hTERT immortalization of primary WTs of multiple histological and molecular description [186]. Still, however, the literature is populated with data arising from other cell lines with limited application (see Table 1).

A slightly altered approach to the problem of culturing defined WT cells is the use of cells arising from metanephric mesenchyme, the cells of origin for the neoplasms. HEK293 cells which were isolated from human embryonic kidney and immortalized by adenovirus 5 transduction [187] are robust models for the mechanistic study of kidney signal transduction. Though not without a level of public controversy as they represent a member of the sometimes taboo "embryonic stem cell" group, these cells retain characteristics of metanephric mesenchyme and demonstrate similar biological properties to previously mentioned cells in an analysis of WT cell survival [131]. A major drawback to the use of HEK293 is their inability to generate a triphasic tumor, despite their striking resemblance to the cell of tumor origin. Finally, and similarly to the HEK293 story, short-term culture of intact primary metanephric mesenchyme cells from rat and mouse are being used to study normal kidney-inductive signaling [20, 130] and these results may logically be extended to the development of nephroblastomas.

WT as a Xenotransplant

Another method for the propagation of relevant WT material is the use of xenotransplantation, i.e., serial passaging of tumors in immunocompromised or syngeneic rodents. This method has proven quite efficient in several instances where nephroblastomas are serially transplanted in syngeneic hosts and human WTs are maintained in immune-deficient mice [69, 165, 168, 188]. The limitations with this method are the obvious need for costly host animals and concerns about changes in tumor histiotype over successive transplantation. These primary tumors can generate a metastatic and more aggressive disease [190], which may be useful for the study of advanced-stage WT. Also of note, xenotransplantation drives the selection of blastemal elements in human disease, while apparently optimizing conditions for epithelial outgrowth of rat nephroblastomas.

An adaptation of this method of propagation is to grow select xenotransplants in tissue culture, leaning on the supposition that these tumor cells have somehow gained a distinct growth and survival advantage and may stand up to the challenges of life in the dish. Extensive efforts in this area have been undertaken with dissociated WT xenotransplants in Balb/c nude mice, where the cells are grown in defined media, which can select for either blastemal [191], stromal [192], or epithelial components [193]. To achieve outgrowth of the epithelial population of cells however, requires direct culture of primary tumor tissue in a complex milieu. In a supportive study of the direct outgrowth from primary WT samples, a cellular heterogeneity encompassing all three major cellular types was observed and these cells exhibited a concordant molecular heterogeneity [194]. Finally, cell clones have been established from serially transplanted rat nephroblastomas, which give rise to tumors of varied histological appearance, suggesting maintenance of the multipotent component despite a lengthy term in vivo [195]. Unfortunately, there have been no recent demonstrative advances in the application of this heterotransplant-to-tissue-culture model method.

Immortalizing WT Cells by Viral Transduction

Immortalization with SV-40 transforming genes has also been attempted in an effort to support the sustained growth and characteristics of primary tumor cells in vitro. Though this method potentially introduces confounding circumstances for downstream interpretation of model data, it may be useful as a frontline approach if the findings can be subsequently verified in other models of tumorigenesis. Sadly, SV-40 large T antigen transduction provides WT cells with the ability to grow in vitro for 35+ passages, but does not truly immortalize the cells as they inevitably undergo crisis and do not recover [196]. Again, pointing to the limitations of this system, the cells behave in only a partially tumorigenic fashion, demonstrating anchorage-independence but

failing to seed tumors as a xenograft. To date, and despite technological advances in immortalization methodology, i.e., viral delivery systems for various pro-growth genes like hTERT, and Epstein-Barr viral transduction among others, only the hTERT model has been employed to sustain WT growth in vitro, and as mentioned was met with variable and modest success.

Conclusion

Nephroblastoma or WT provides a compelling paradigm of a tumor originating in a defined multipotent stem cell population. This has been aptly demonstrated histologically and confirmed molecularly and biochemically. The expression patterns of most blastemal markers resemble the profiles observed for stem cells of putative preneoplastic lesions, i.e., the nephrogenic rests, and of the WTs themselves. Most striking, however, is the genetic evidence that despite morphologic differences, each tissue component, whether primitive epithelia or stroma, is derived from the same stem cell clone in any given WT. Mutations associated with tumorigenesis in genes such as *WT1*, *WTX*, *IGF2*, and *CTNNB1*, which are relevant and in some cases critical to normal blastemal differentiation, provide strong evidence that WT is indeed a disease of differentiation and that an understanding of the events responsible for the accumulation of these blastemal cells may eventually lead to therapies that could reregulate and commit cells to a nonneoplastic and differentiated phenotype, which is of course the ultimate goal of stem cell research for any tumor.

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About the Editor

Dr. Sell is a board certified pathologist and immunologist. He began research in 1957 while a medical student at the University of Pittsburgh, in the Department of Pathology, under Frank J. Dixon. After a pathology residency at Massachusetts Hospital, and postdoctoral fellowships at NIH and Birmingham England he returned to the University of Pittsburgh for 5 years before moving to the University of California at San Diego. After 12 years at UCSD, Dr. Sell took the position as Chair of Pathology and Laboratory Medicine at the University of Texas (UT) at Houston before moving to Albany Medical College. As of February 2003 he has been a senior scientist at Wadsworth Center, a Professor at University at Albany School of Public Health and an Adjunct Professor at Albany College of Pharmacy. Dr. Sell received the Rudolph L. K. Virchow Award of the Italian Medical Magazine LeadershipMedica.com for his concept of cancer stem cells; he was named Legacy Laureate of the University of Pittsburgh for his work on alpha-fetoprotein as a marker for liver cancer; and in 2007 received the Abbott Award of the International Society for Oncodevelopmental Biology and Medicine for recognizing the role of stem cells in carcinogenesis. He has published over 400 papers and 12 books, including 6 editions of Immunology, Immunopathology, and Immunity. His paper with Barry Pierce (Lab. Invest. 70:6–21, 1994.) was selected as an AACR scientific landmark.

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