Stem Cell Biology and Regenerative Medicine

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Male Germline Stem Cells: Developmental and Regenerative Potential

Humana Press

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Foreword

The germ cell genome threads continuously from generation to generation via the sperm and egg. These gametes combine at fertilization to produce the totipotent zygote (one-cell embryo) that gives rise to each generation. Thus, the germ cell lineage is immortal and has extraordinary developmental and, perhaps, therapeutic potential. The germ cell field has generated several exciting discoveries and innovations during the past 15 years, which provide valuable insights into the mechanisms that regulate developmental potency and stem cell function. This monograph discusses the implications of these discoveries for understanding the fundamental biology of germline stem cells as well as their potential for human stem cell-based therapies.

The germ lineage exhibits an extraordinary breadth of developmental potentials during fetal, perinatal, and adult stages of life. Primordial germ cells (PGCs) first arise in the epiblast stage embryo and eventually migrate via the hindgut to colonize the gonads. Under normal, *in vivo* circumstances, these PGCs will give rise to oogenic or spermatogenic lineages. However, in some *in vivo* and *in vitro* situations, PGCs can adopt a pluripotent phenotype. Peter Donovan (Chap. 1) discusses the acquisition of pluripotency by PGCs and the implications for understanding the regulation of developmental potency, germ cell development, and testicular cancer. In males, PGCs that colonize the gonad become enclosed within pre-Sertoli cells of the seminiferous cords and give rise to pro-spermatogonia (aka: gonocytes). Pro-spermatogonia migrate to the seminiferous tubule basement membrane and establish the pool of spermatogonial stem cells (SSCs). SSCs are restricted to spermatogenic lineage development and balance self-renewing and differentiating divisions to maintain spermatogenesis. However, these adult tissue stem cells retain some developmental plasticity. Marco Seandel, Ilaria Falciatori, and Shahin Rafii (Chap. 2) describe conditions for converting spermatogonia from postnatal testes to pluripotent stem cells. They also discuss the potential for therapeutic application of testis-derived pluripotent stem cells compared to other pluripotent cell sources (e.g., embryonic stem cells and induced pluripotent stem cells). In converse, Sonya Schuh-Huerta and Renee Reijo Pera (Chap. 3) describe the conversion of pluripotent stem cells into the germ lineage. *In vivo* investigations of human germ cell development are inherently problematic. Therefore, pluripotent to germ cell conversion,

in vitro may provide a unique window for understanding the mechanisms of germ lineage commitment and development. In addition, pluripotent cells may one day be a source of *in vitro*-derived gametes (eggs or sperm) with potential application in the human fertility clinic.

The pool of spermatogonial stem cells (SSCs) in the postnatal testis gives rise to undifferentiated transit-amplifying progenitor spermatogonia followed by differentiating spermatogonia. Collectively these spermatogonial populations comprise the foundation of the spermatogenic lineage. Dirk de Rooij (Chap. 4) describes spermatogonial morphology and the kinetics of renewal and differentiation that maintain spermatogenesis throughout postpubertal life. Investigations of SSCs are complicated because there are no morphological and biochemical features that can distinguish these stem cells from other undifferentiated spermatogonia (except possibly whole mount analysis of clone size, described in Chap. 4). Jonathan Schmidt and Ralph Brinster (Chap. 5) describe SSC transplantation as a definitive bioassay for spermatogonial stem cells and SSC culture as a valuable *in vitro* tool for SSC expansion and mechanistic investigations. While transplantation is a valuable bioassay that has accelerated the pace of SSC research in the past two decades, it is technically challenging and has an inherent 2–3 month delay to analysis. Makoto Nagano and Jonathan Yeh (Chap. 6) have exploited the SSC culture system to establish a simpler and faster *in vitro* "cluster-forming activity (CFA) assay" to quantify SSC activity. While the CFA assay does not replace transplantation as a bioassay and does not recapitulate complete spermatogenesis, it can accelerate SSC research by providing a rapid and reliable readout during early experimental development. These experimental tools have enabled investigators to begin dissecting the mechanisms that regulate SSC renewal and differentiation. Olga Ocón-Grove and Jon Oatley (Chap. 7) explore the roles of extrinsic factors emanating from the local testicular microenvironment (niche) and the interplay between extrinsic and internal factors in the regulation of SSC fate decisions. Shosei Yoshida (Chap. 8) discusses the functional and anatomical features of the SSC niche and provides additional insights about molecular mechanisms regulating SSC behavior.

Treatments for some human diseases (e.g., cancer) can damage the spermatogonial stem cell pool and cause infertility. Gunapala Shetty, Gensheng Wang, and Marvin Meistrich (Chap. 9) describe the effects of gonadotoxic therapies in the testis, including the kinetics of germ cell demise and the potential for spermatogenic recovery. They also examine the effects of endocrine modulators on spermatogenic recovery and discuss potential clinical implications. Stefan Schlatt, Jose Rodriguez-Sosa and Ina Dobrinski (Chap. 10) describe ectopic xenografting of testis tissues from immature animals into immune-deficient mice as a means to recapitulate spermatogenesis and recover fertilization-competent sperm. This is an experimentally tractable approach to study spermatogenic lineage development in species that are less amenable to experimental manipulation, including humans. Grafting techniques may have application for preserving the fertility of prepubertal boys whose future fertility is at risk due to gonadotoxic treatments. In the final chapter, the editors of this volume, Brian Hermann and Kyle Orwig (Chap. 11) summarize progress applying SSC transplantation in a nonhuman primate model of male infertility and

considerations for translation to the human fertility clinic. Methods for isolating, preserving, and transplanting SSC in nonhuman primates and humans are described and may provide important preclinical insights.

The editors are grateful to the prominent researchers who have made important contributions to the germ cell field and provided chapters for this effort. We also thank the experts who reviewed chapters prior to publication.

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Part I
Germline Developmental Potential

Chapter 1
Pathways to Pluripotency: How Germ Cells Make Stem Cells

Peter J. Donovan

Abstract Recent studies have demonstrated that many types of differentiated cells can be reprogrammed to give rise to pluripotent stem cells. Reprogramming of differentiated cells is brought about by the forced expression or delivery of defined factors previously shown to be associated with pluripotent stem cells. But important questions about the safety, efficiency, and completeness of cellular reprogramming remain. Primordial germ cells (PGCs), specialized embryonic precursors of the gametes, can also give rise to pluripotent stem cells both *in vivo* and *in vitro*. Reprogramming of PGCs *in vitro* only requires the addition of exogenous growth factors. Moreover, genetic studies in both mice and humans have begun to elucidate the pathways responsible for reprogramming germ cells to the pluripotent state. Thus, these two situations, one *in vivo* and one *in vitro*, in which a PGC gives rise to a pluripotent stem cell provide important insights into the molecular mechanisms regulating the pluripotent state and could fill vital gaps in our knowledge about the successful reprogramming of other cell types.

Keywords Primordial germ cells • Embryonic germ cells • Testicular germ cell tumors • Pluripotent stem cells

1.1 Introduction

In all vertebrates and many invertebrates only a single lineage, the germ cell lineage, carries the genome on into the next generation. It does so by the creation of a single totipotent cell, the zygote, formed by the fusion of two germ cells or gametes, an egg and a sperm. The totipotent zygote gives rise to a so-called pluripotent groups of cells,

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the inner cell mass (ICM), that then produces all the structures of the embryo and subsequently the adult organism. In mammals, the totipotent zygote also gives rise to cells that will give rise to the extraembryonic lineages including the trophoblast that will support the development of the embryo in the postimplantation period. During development derivatives of the pluripotent ICM become more specialized or differentiated and typically lose the ability to give rise to a wide variety of differentiated derivatives. But the pluripotent ICM also gives rise to a new population of germ cells, specialized cells committed to giving rise to gametes, which can recapitulate the whole process over and over again. Thus, the germ cells go through a period of extreme differentiation in which they can truly be considered specialized cells. Indeed it is difficult to imagine two cells types, an egg and a sperm, that are more different from each other. Yet the unique properties of these two highly specialized cells types somehow carry the genome in a way that allows reprogramming of the genome in order that it can be utilized to re-create a totipotent zygote that can in turn create a new organism. In the normal lifecycle of most organisms there is no other cell type that has that ability.

Until relatively recently, it was thought that the genome of other specialized cells in the embryo and adult was maintained in such a way that did not allow it to be easily reprogrammed to give rise to either totipotent or pluripotent cells. But over the last several years important advances have been made in understanding the molecular mechanisms controlling developmental potency. Remarkably, studies carried out in the last few years have demonstrated that differentiated cells, thought to be restricted in their developmental potential, can be induced to return to a pluripotent state (Takahashi and Yamanaka 2006). In this way, specialized cells are turned into so-called induced pluripotent stem cells (iPSCs). These studies have implicated a number of key genes as being important in the "reprogramming" process. Included among those genes are key transcription factors already known to control developmental potency such as Oct4, Nanog, and Sox2. In addition, these studies identified the Kruppel-like factor-4 (Klf4) and Myc as also being important for reprogramming of differentiated cells back to a pluripotent state (Takahashi and Yamanaka 2006). But these remarkable studies have not been the first to demonstrate that specialized cells can be programmed into pluripotent stem cells. Previous studies have shown that germ cells can be reprogrammed into pluripotent stem cells both *in vivo* and *in vitro* (Matsui et al. 1992; Resnick et al. 1992; Stevens 1967a). Indeed pluripotent stem cells derived from germ cells were the first pluripotent stem cells to be described (Stevens 1967a). Unlike the reprogramming of differentiated somatic cells, which involves introduction of genes or proteins into cells, reprogramming of germ cells into pluripotent stem cells *in vitro* only requires the addition of growth factors to the cells (Matsui et al. 1992; Resnick et al. 1992). Importantly, the reprogramming of germ cells in this way provides an important insight into how reprogramming might be achieved more efficiently and how specific signaling pathways act to reprogram cells to a pluripotent state. Additionally, the analysis of how germ cells can give rise to pluripotent stem cells may provide important information about how normal germ cell development proceeds and how it sometimes can go wrong. Following is a review of the current knowledge of germ cell reprogramming and how studies of germ cell reprogramming might be used to develop methods for growth factor-mediated reprogramming of somatic cells.

1.2 Early Germ Cell Development

In mice up until about 6.25 days post coitus (dpc) there are no cells that can be distinguished as germ cells. At that time inductive signals, including bone morphogenetic proteins (BMPs), create a population of cells, termed primordial germ cells (PGCs), that begin to express what we know as germ cell markers (Saitou et al. 2003; Surani 2007) (Fig. 1.1). These markers include tissue non-specific alkaline phosphatase (TNAP or AlpL) and the C-Kit receptor tyrosine kinase (De 2000), both expressed on the cell surface of PGCs. While TNAP is either not required or redundant in germ cell development, the C-Kit receptor has a critical role in germline development (see below). An early event in the specification of the germline is the expression of Prdm1 (also known as Blimp1), a zinc finger containing, DNA-binding, transcriptional repressor. During early development in the pre-gastrulation embryo Prdm1 is expressed in a subpopulation of cells in the visceral endoderm overlying the proximal epiblast (Fig. 1.1). In the next stages of development, it is expressed in the anterior visceral endoderm and in the nascent PGCs. Prdm1 then remains expressed in developing PGCs up until the time at which they enter the developing gonads (Vincent et al. 2005; Ohinata et al. 2005). Evidence for the role of Prdm1 in PGC development comes from studies in mice lacking the gene. Loss of Prdm1 leads to failure of PGCs to form properly at the very earliest stages of germline development (Vincent et al. 2005; Ohinata et al. 2005). A few PGC-like cells form in Prdm1-deficient embryos but they fail to proliferate, migrate, or show the consistent down-regulation of homeobox genes observed during normal germ cell development. Importantly, these studies demonstrate that Prdm1 is required for formation of the germ cell lineage. It has been suggested that Prdm1 acts on differentiating pluripotent cells of the epiblast to suppress the somatic cell differentiation pathways including expression of Hox genes (which would be counterproductive to their effective differentiation into the germline) and to prevent newly formed PGCs from slipping back towards a pluripotent state (Ancelin et al. 2006; Kurimoto et al. 2008). Prdm1 is a member of a larger superfamily of proteins that contain an N-terminal PR/SET domain linked to C-terminal C2H2 zinc fingers (reviewed in Bikoff et al. 2009; John and Garrett-Sinha 2009). These proteins are known to mediate nuclear import and DNA binding. In other cell types Prdm1 blocks transcription at promoters of target genes known to regulate cell cycle progression such as Myc. In addition, Prdm1 is known to silence transcription factors in order to maintain the identity of those cells (reviewed in Bikoff et al. 2009; John and Garrett-Sinha 2009).

Interestingly, a second member of this family of factors, Prdm14, also plays a key role in PGC specification and development (Yamaji et al. 2008). Using a transgenic reporter line in which a fluorescent reporter is expressed from the Prdm14 upstream elements and using whole-mount immunofluorescence it has been shown that Prdm14 is co-expressed with Prdm1 in the few cells fated to become PGCs at the posterior of the early embryo (Fig. 1.1). Loss of Prdm14 in mice results both in loss of PGCs and in adult animals that are sterile. In Prdm14 mutant embryos it was found that PGC specification is defective from the earliest stages of the development of the lineage. Further studies on the role of Prdm14 in germ cell specification sug-

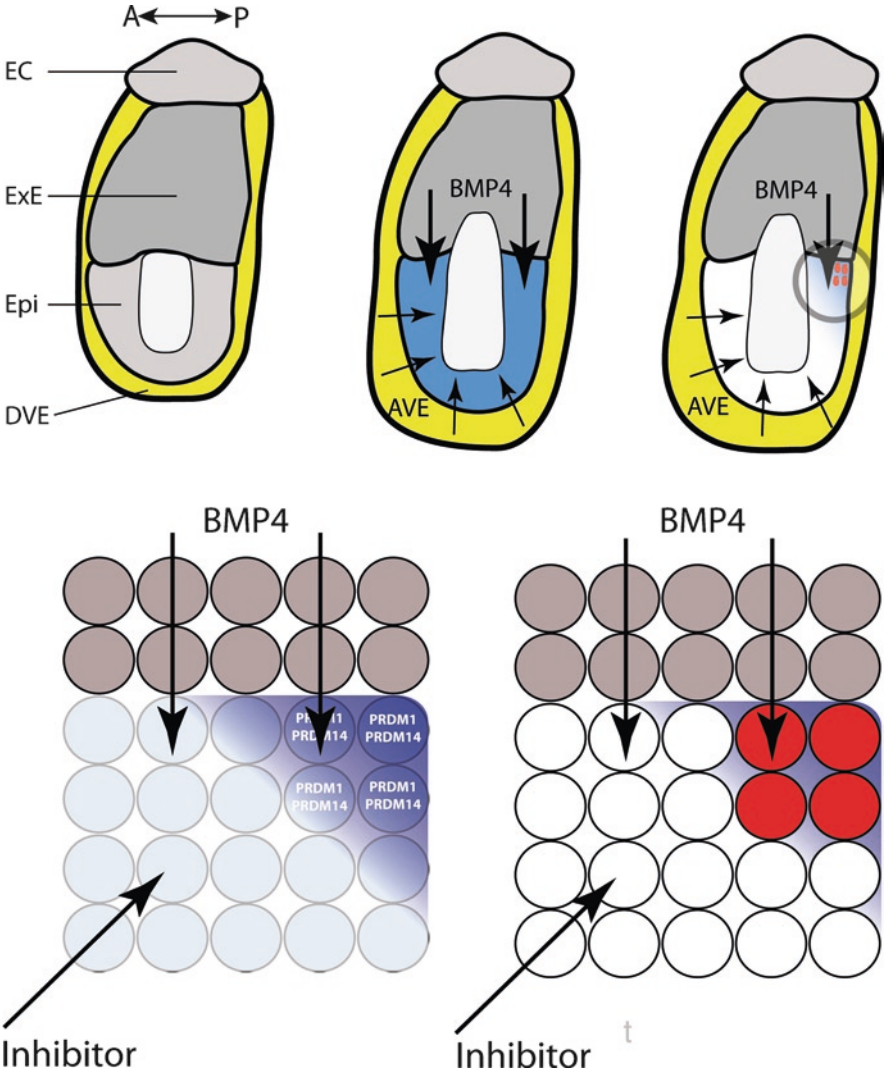

Fig. 1.1 Schematic representation of the specification of germ cells in the mouse embryo. Primordial germ cells form in the embryo between 5.0 and 6.25 dpc. In the early embryo (*upper panel*), the expression of Wnt3 makes the cells of the epiblast (*Epi*) responsive to BMP4 signals (blue = BMP4 responsiveness) emanating from the extraembryonic ectoderm (*ExE*) (*large arrows*). Inhibitory signals from the distal visceral endoderm (*DVE*) and later the anterior visceral endoderm (*AVE*) (*yellow*) indicated by *small arrows* restrict BMP4 signals from posteriorizing cells of the epiblast. Consequently, only cells at the posterior end of the epiblast receive sufficient BMP4 signaling to begin to express Prdm1 and Prdm14 and to form primordial germ cells (*red circles*). The circled area is shown in detail in the *lower panel* with the model of PGC specification. Ectoplacental cone (*EC*). Anterior posterior axis (*A*↔*P*) is indicated by the *arrows* above the embryo on the left

gest that roles for this protein may be to stimulate epigenetic reprogramming events that occur in germ cells and to up-regulate genes associated with pluripotency such as Sox2. On the other hand, Prdm14 does not appear to play a major role in the down-regulation of somatic genes in the forming PGCs (Yamaji et al. 2008).

Taken together these studies suggest that the Prdm proteins, Prdm1 and Prdm14, play a key role in the specification and subsequent development of the germ cell lineage. It has been proposed that Prdm1 may be responsible for repression of the somatic gene expression program (including Hox genes) in forming germ cells while Prdm14 is responsible for re-acquisition of pluripotency-associated genes and for the genome-wide epigenetic reprogramming that occurs in germ cells (Yamaji et al. 2008).

Once the PGCs have formed, morphogenetic movements of the embryo move them into the embryo proper so that by 9.5 dpc they lie within the epithelium of the hindgut (reviewed in Molyneaux and Wylie 2004) (Fig. 1.2). During the next few days of development they actively migrate from the hindgut through the hindgut mesentery towards the dorsal body wall where they colonize the developing embryonic gonads (Fig. 1.2). PGC colonization of the gonads is likely controlled by multiple mechanisms including substrate-mediated adhesion and chemotactic guidance (Molyneaux and Wylie 2004). During migration, interaction of PGCs with the extracellular matrix that comprises the substrate on which they migrate is likely mediated by multiple heterodimeric integrin receptors, including $\alpha 3$, $\alpha 6$, αv, and $\beta 1$ (Anderson et al. 1999). By 12.5 dpc the vast majority of PGCs have reached the embryonic gonad (Molyneaux and Wylie 2004). This period of germ cell development also sees the beginning of expression of genes that are unique to the germline such as the mouse vasa homolog, also known as DEAD box polypeptide 4 (Ddx4) (Fujiwara et al. 1994; Toyooka et al. 2000; Tanaka et al. 2000). During the period of migration into the gonads the germ cells will proliferate to establish the population of cells that will eventually form the gametes (Tam and Snow 1981). Signaling via the C-Kit receptor plays a key role in regulating PGC survival during this period. Mutations in C-Kit, encoded at the mouse *W* or *dominant white spotting* locus, result in reduction in number or complete loss of PGGs (see Besmer et al. 1993 for review). Once PGCs have colonized the gonad they undergo mitotic arrest at 13.5 dpc while female germ cells enter directly into meiosis in the embryonic gonad (Wylie 1999; De 2000; McLaren 2003).

1.3 How It All Goes Wrong

This normal progression of development goes awry when germ cells continue to proliferate in the developing gonad. In this situation small nests of proliferating PGCs are observed in the developing gonad at 15.5 dpc when the remainder of the germ cells have entered mitotic arrest (Stevens 1967b) (Fig. 1.2). These proliferating germ cells, now termed embryonal carcinoma (EC) cells, can differentiate and give rise to benign tumors termed teratomas, which first manifest themselves in the adult testis.

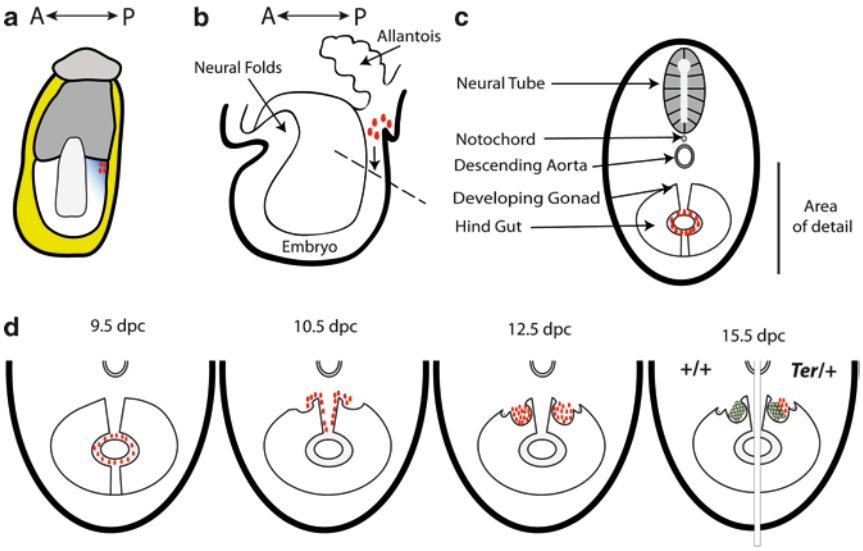

Fig. 1.2 Cartoon depicting primordial germ cell migration into the developing gonad and subsequent differentiation into gonocytes. *Upper panel* shows the major features of the embryo and the region depicted in detail in the *lower figure*. (**a**) Shows the position of PGCs (*red circles*) in the 6.5 dpc embryo. By 8.5 dpc (**b**), the embryo has enlarged, the anterior posterior axis (*A*↔*P*) is well defined and the germ cells begin to migrate into the embryo proper (*arrowed*) from their location near the allantois. The *dotted line* in (**b**) represents the area of section shown in (**c**). By the next day of development (**c**) the germ cells are found in the hindgut. *Lower panel* depicts sections through embryos at 9.5, 10.5, 12.5, and 15.5 dpc. At 9.5 dpc PGCs (depicted by the *red dots*) are localized within the hindgut, which is slung in the coelomic cavity suspended by both a dorsal and ventral mesentery. By the next day of development (10.5 dpc) the ventral mesentery has regressed and the PGCs have begun to migrate up the dorsal mesentery towards a thickened area of the dorsal body wall, the genital ridge or gonad anlagen, which is the structure that will form the gonad. Within the next two days of development, all of the PGCs will have reached the developing gonad. Once they have reached the gonad they will differentiate into gonocytes (*green circles*). In males at 15.5 dpc, the gonocytes will become incorporated into the developing testis cords. In animals that are susceptible to teratomas formation such as Ter/+ animals, small nests of proliferating cells (*red circles*) are seen within the developing gonad. These cells, termed embryonal carcinoma cells, retain the markers of PGCs such as TNAP and SSEA-1 and are also shared with other pluripotent stem cells

Teratomas are bizarre tumors that contain cell types representative of cells derived from all three primary germ layers present in the embryo. Because of their origin from germ cells these tumors may also be referred to as testicular germ cell tumors (TGCTs). Although teratomas can arise in female gonads their mechanism of development is different and quite distinct from those of TGCTs.

Importantly, studies of EC cells demonstrate that they can give rise to all the cell types present in the teratoma and, therefore, they could be defined as pluripotent. In defining EC cells as pluripotent Stevens compared them to the pluripotent cells of the early embryo. It was only later that it was discovered that the pluripotent cells of

the early embryo could also give rise to immortal pluripotent stem cells, termed embryonic stem (ES) cells. Remarkably, single EC cells introduced into mice can give rise to teratomas containing multiple cells types demonstrating at minimum that they have multipotent potential (Kleinsmith and Pierce, JR. 1964). In fact, EC cells represent the first pluripotent stem cell to be described. If EC cells fail to differentiate they give rise to malignant teratocarcinomas comprised solely of EC cells. Importantly, these studies of TGCTs determined that cells committed to entering the germline, and which might be considered very specialized cells, were capable of giving rise to a pluripotent stem cell (Stevens 1967a). Subsequent studies demonstrated that when PGCs were cultured *in vitro* they could give rise to another type of pluripotent stem cell termed an embryonic germ (EG) cells (Matsui et al. 1992; Resnick et al. 1992). Previous studies had shown that PGCs could be isolated from the embryo and cultured on feeder cells, but that the isolated cells proliferated and differentiated in culture in a manner that mirrored their normal pattern of differentiation *in vivo* (Donovan et al. 1986). In other words, their growth in culture seemed to follow the same timing, or developmental clock, as their development *in vivo*. The feeder cells on which the PGCs are grown are known to express many growth factors including, importantly, a ligand for the C-Kit receptor termed kit ligand (KL) that is essential for PGC survival (Dolci et al. 1991; Godin et al. 1991; Matsui et al. 1991). Furthermore, they express leukemia inhibitory factor (LIF), which can act together with KL to stimulate PGC proliferation (Cheng et al. 1994). But when fibroblast growth factor-2 (FGF2 or basic FGF) is added to the cultures, the PGCs proliferate for longer than they would normally and eventually form a population of cells that appear to be immortal (Matsui et al. 1992; Resnick et al. 1992). While PGCs normally grow for a short period of time in culture, PGCs grown in KL, LIF, and FGF2 can be subcultured and expanded indefinitely. In addition to being immortal these cells also are pluripotent. When they are introduced into blastocyst-stage embryos they incorporate into the embryo proper and give rise to chimeras containing donor cells that have contributed to the somatic and germ cell lineages. Importantly, chimeras derived from these cells transmit donor-derived DNA through the germline (Matsui et al. 1992; Stewart et al. 1994). To distinguish these cells from other pluripotent stem cells, such as EC cells and ES cells derived from the ICM of the pre-implantation embryo, as well as to distinguish them from PGCs, they have been termed EG cells (Resnick et al. 1992).

Therefore, both *in vivo* and *in vitro*, PGCs can give rise to immortal pluripotent stem cells. The exact relationship between EC cells and EG cells is uncertain but it is interesting to speculate that some of the same mechanisms that cause PGCs to give rise to EC cells *in vivo* might also be involved in the development of EG cells *in vitro* and some experimental data supports this idea (see below). One important clue as to the mechanism of EG cell derivation comes from analyses of the timing of when EG cells could be made from mouse embryonic PGCs. It was noted that after 12.5 dpc it was difficult, if not impossible, to derive EG cells (Matsui et al. 1992; Resnick et al. 1992). Many years earlier it had been noted that it was not possible to derive experimental TGCTs from PGCs after this time (Stevens 1966). Together these data suggest that some aspect of PGC differentiation that occurs at

this time makes the creation of pluripotent stem cells impossible (Matsui et al. 1992; Resnick et al. 1992; Stevens 1966). More specifically, this period in PGC development coincides with the cessation of mitosis in both the male and female germline, the erasure of imprints that occurs in the germline, and the onset of sexual differentiation in the somatic lineages. Unfortunately, to date the role that any of these mechanisms play in EG derivation remains unclear.

The derivation of EG cells from mice was soon followed by the derivation of the same cell type from humans and other species (Shamblott et al. 1998, 2001; Turnpenny et al. 2003). Like their murine counterparts, EG cell lines derived from human PGCs were also found to be pluripotent based on their ability to form teratomas containing cells from all three primary germ layers when injected into immune-deficient mice. Interestingly, human EG cells could be derived from embryos that had passed the point of sexual differentiation based on the observation of sex cords in male embryos (Shamblott et al. 1998; Turnpenny et al. 2003). Whether this implies a difference in mechanisms of PGC development between mice and humans remains unclear. Notably though, human EG cell lines have proven to be much more difficult to propagate than their murine counterparts and this has, unfortunately, limited their usefulness in regenerative medicine (Turnpenny et al. 2006).

1.4 Probing the Mechanisms of Pluripotency

The ability to make pluripotent stem cells from a source other than the ICM provided a new system with which to probe the mechanisms controlling developmental potency. Importantly, ES cells are derived from a population of cells that are already themselves pluripotent. Therefore, creation of ES cells may require alterations in cell cycle control or a clock that times cell division but not necessarily alterations in developmental potency. But both EC and EG cells are derived from a population of cells, PGCs, that are specialized. Functional analyses of PGC potency have been carried out in which genetically marked PGCs carrying specific coat color alleles or green fluorescent reporters were introduced into the blastocoel cavity of the pre-implantation embryo. This experiment has been carried out by several investigators and the donor PGCs did not give rise to coat color or germline chimeras (Donovan et al. unpublished observations; Stewart, personal communication; Papaioannou, personal communication; Durcova-Hills et al. 2006). Thus, the conclusion from these studies is that PGCs can be considered a specialized cell type that is highly restricted in developmental potency.

Interestingly, when PGCs are converted into EG cells not all PGCs give rise to EG cells. We estimated that between 8 and 23% of the cultured PGCs gave rise to EG cells (Resnick et al. 1992), suggesting heterogeneity in the PGC population. This conclusion is also supported by analysis of the morphology, cell surface antigen staining, and expression of the germ cell marker Ddx4 in PGCs isolated from the embryo (Durcova-Hills et al. 2006). Studies of PGCs have also revealed heterogeneity for α6

integrin and C-Kit in the developing embryo (Morita-Fujimura et al. 2009), indicating there is likely to be heterogeneity within the starting population of PGCs used to derive EG cells reflecting heterogeneity present *in vivo*. Cell sorting experiments also found PGCs expressing low or no α6 integrin were more able to give rise to EG cells (Matsui and Tokitake 2009). In addition, these studies examined evidence for the presence of a side population within the germ cell pool. When cells are stained with Hoechst 33342 dye and analyzed by flow cytometry using a UV laser it is possible to identify differently stained populations depending on the ability of the cells to discharge the Hoechst dye. Cells that express high levels of the ATP binding cassette reporter ABCG2 discharge the dye and therefore are less strongly stained by the dye. These cells constitute a distinct population of cells, the so-called side population. Interestingly, a large fraction of the PGC pool at 10.5 dpc can be defined as side population cells and moreover these cells showed an enhanced ability to give rise to EG cells (Matsui and Tokitake 2009). The implications of these results remain to be determined but the identification of some markers of PGC heterogeneity provides a powerful handle with which to determine the underlying molecular mechanisms.

The analysis of how PGCs can be converted into EG cells has been studied to some extent (Fig. 1.3). An important first question is how growth factors act on the PGCs in the first place. Compelling evidence based in part on genetic studies strongly suggests that KL acts directly on the PGCs via the C-Kit receptor tyrosine kinase (Dolci et al. 1991; Godin et al. 1991; Matsui et al. 1991). Similar but less compelling data suggest that FGF and LIF also act directly on the PGCs via specific receptors or receptor complexes (Resnick et al. 1998; Cheng et al. 1994; Takeuchi et al. 2005; Durcova-Hills et al. 2006). Several studies suggest that PGCs express FGF receptors during the period in which they are susceptible to conversion into EG cells (Resnick et al. 1998; Takeuchi et al. 2005; Durcova-Hills et al. 2006). However, the methods for culturing PGCs and inducing them to form EG cells involve the use of fibroblast feeder cells that themselves could respond to FGFs, and, in general, the isolated PGCs are contaminated with large numbers of embryonic somatic cells. So it remains formally possible that FGFs act indirectly to effect PGC conversion into EG cells. Derivation of EG cells from PGCs that have been separated from embryonic somatic cells by cell sorting rules out a major contribution by contaminating embryonic somatic cells in the process (Matsui and Tokitake 2009). Interesting studies by Durcova-Hills and colleagues have also shown that in the conditions used in their studies, FGFs produced by the feeder cells are unlikely to be important in EG derivation and that up-regulation of FGF2 within the PGCs themselves may be a key event (Durcova-Hills et al. 2006). Further, these studies suggest that FGFR3 activation within the PGC pool may be critical for conversion into EG cells as they observed up-regulated expression of FGFR3 within some PGCs (Durcova-Hills et al. 2006). In addition it was noted that conversion of PGCs into EG cells is associated with altered localization of the FGFR3 receptor from the cell surface to the nucleus. Taken together these studies suggest that FGFs act directly on PGCs to effect their conversion to EG cells and do so by activation of an FGF receptor, specifically FGFR3. More recent studies have shown that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, can replace FGF2 in the

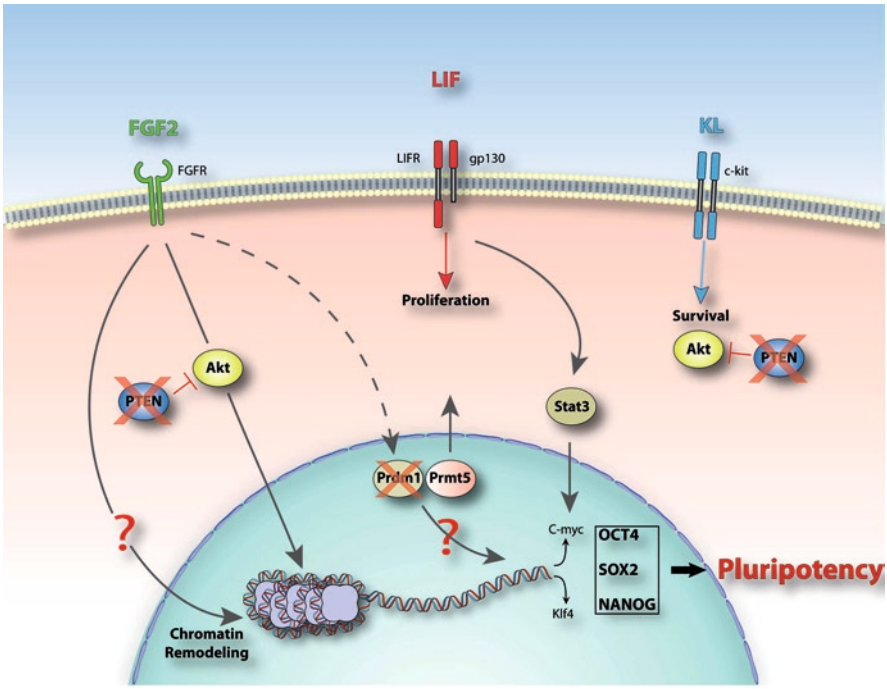

Fig. 1.3 Mechanism of growth factor action in the creation of embryonic germ cells from primordial germ cells. Kit ligand (*KL*) acting through the C-Kit receptor regulates primordial germ cell survival. Likely, that involves activation of the PI3 Kinase and AKT signaling pathway, which is counteracted by the activity of PTEN. Leukemia inhibitory factor (*LIF*), acting through the bipartite receptor comprised of gp130 and the LIF receptor (*LIFR*), also likely sends survival signals that together with signaling from the C-Kit receptor regulate PGC proliferation. One of the signaling molecules activated by LIF signaling is the signal transducer and activator of transcription-3 (*Stat3*), which in turn can regulate the levels of C-myc. The role of fibroblast growth factor (*FGF*) signaling in normal germ cell development is less clear. Nevertheless, activation of the FGF receptor (*FGFR*) in PGCs in culture can also activate AKT as well as leading to down-regulation of Prdm1 and later translocation of Prmt5 out of the PGC nucleus. These events are thought to lead to loss of PGC fate in developing PGCs. Together with Stat3-mediated up-regulation of C-myc, activation of the FGF signaling pathway leads to up-regulation of Kruppel-like factor 4 (*Klf4*), and transition of PGCs to a pluripotent state. FGF signaling may also have other effects on chromatin remodeling, which could also contribute to conversion of PGCs to EG cells

generation of EG cells, which raises interesting questions about the mode of action of FGF2 and the FGF signaling pathway in reprogramming of PGCs to pluripotency (Durcova-Hills et al. 2008).

Of course a key question is how signaling pathways such as the FGF pathway, or those affected by TSA treatment, cause conversion of PGCs into EG cells. One of the important pathways downstream of FGF receptors is a pathway including the phosphoinositide-3 kinase (PI3K), which produces the second messenger, phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)*P*3) from PtdIns(4,5)*P*2 (reviewed

in Turner and Grose 2010). This molecule then transmits the signal via molecules such as the serine/threonine kinase AKT. Signaling via this pathway sends anti-apoptotic signals that allow cells to survive and, with other appropriate signals, to proliferate. The activity of PI3K is attenuated by the phosphatase and tensin (PTEN) homolog, which is a phosphatase that dephosphorylates the messenger and therefore antagonizes the activity of PI3K (see Courtney et al. 2010 for review). Because of the important role of the PI3K/AKT signaling pathway downstream of many receptor tyrosine kinases, examining the role of AKT in PGC growth and EG development is an important goal. Nakano and colleagues produced mice in which they could conditionally activate AKT in specific lineages. When AKT was activated in PGCs it dramatically augmented the production of EG cells (Kimura et al. 2008) (Fig. 1.3). In addition these studies found that activation of AKT in this manner could partially substitute for FGF-2 (Kimura et al. 2008). Interestingly, one of the actions of AKT signaling might be to suppress p53 activity by stabilizing Mdm2, a key regulator of the p53 protein. The role of p53 in induction of pluripotency is still being explored, but these studies perhaps provide a clue as to how FGF signaling might lead to the formation of pluripotent cells from PGCs.

Other important clues as to the molecular mechanisms that drive cells into the pluripotent state have come from studies by Yamanaka and colleagues in which they were able to reprogram fibroblasts into a pluripotent state to create so-called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). These studies demonstrated that forced expression of four genes in mouse fibroblasts could convert them into iPSCs. These genes include the POU domain transcription factor Oct3 (POU5f1), the Sry-HMG-box related factor Sox2, the Kruppel-like factor Klf4, and the Myc proto-oncogene. Subsequent studies have refined our knowledge of the factors required for somatic cell reprogramming. Studies on human cells also identified the Lin28 gene as capable of reprogramming fibroblasts in conjunction with Oct4, Sox2, and Nanog (Yu et al. 2007), and other studies demonstrated that Myc is not required for iPSC generation (Nakagawa et al. 2008). Exclusion of Myc from the transduction cocktail still allows iPSC generation albeit at reduced efficiency (Nakagawa et al. 2008). Nevertheless, these studies indicate some of the key pathways required for reprogramming cells to pluripotency and, therefore, what factors might be involved in reprogramming PGCs to a pluripotent state. Analysis of EG cell derivation reveals the role that some of these factors play in this process. Of course PGCs, like ES cells and iPSCs, express Oct4, Sox2, and Nanog (Scholer et al. 1990; Chambers et al. 2003; Avilion et al. 2003; Yamaji et al. 2008) and conditional knockout studies in mice demonstrate that Oct4 and Nanog have important functions in PGCs (Kehler et al. 2004; Yamaguchi et al. 2009). Therefore, converting PGCs into pluripotent cells likely does not require the up-regulation, or the level of up-regulation, of those factors as it does in the conversion of fibroblasts or other somatic cells to pluripotency.

Because of the role of Prdm1 in normal germ cell development from pluripotent cells of the early embryo, an interesting question concerns the role of Prdm1 in the process of conversion of PGCs back into pluripotent stem cells. It has been suggested that one role of Prdm1 in normal PGC development is to block reversion of nascent

PGCs to a pluripotent state. Studies of EG derivation from PGCs demonstrate that down-regulation of Prdm1 may indeed be an important first step in the formation of pluripotent cells. When PGCs are isolated from the embryo and placed into culture they express both Oct4 and Prdm1. After exposure of PGCs to FGF, Prdm1 is rapidly down-regulated in some of the PGCs within 24 h (Fig. 1.3). Later in the culture period, Prmt5, a protein that acts in a complex with Prdm1, translocates from the nucleus to the cytoplasm. While the role of Prmt5 in the regulation of pluripotency is unclear, some of the targets of Prdm1 are known and include Myc and Klf4, two of the factors required for the reprogramming on fibroblasts to iPSCs. Examination of Myc and Klf4 expression during EG formation reveals that both genes are up-regulated following exposure of PGCs to FGF. Thus one key role of Prdm1 in response to FGF might be to cause up-regulation of two of the key genes required for cellular reprogramming. Interestingly, these studies also suggest that up-regulation of Myc could also be brought about by activation of the signal transducer and transcriptional activator-3 (STAT-3), which is a direct target of the LIF signaling pathway. Taken together these data suggest a key series of events must occur in order to convert PGCs to EG cells. Down-regulation of Prdm1 must occur in order to relieve repression that maintains the germ cell fate. Together with activation of STAT3 via the LIF signaling pathway this leads to up-regulation of a set of genes, including Myc and Klf4, required for establishment of the pluripotent stem cell state (Durcova-Hills et al. 2008). One of the other genes involved in the specification of the germline is Prdm14 (Yamaji et al. 2008). Interestingly, PGCs isolated from Prdm14$^{-/-}$ embryos seem unable to form EG cells (Yamaji et al. 2008). Thus, unlike Prdm1, whose down-regulation may be required for EG formation, loss of Prdm14 seems to inhibit the formation of these pluripotent stem cells. Although both proteins have been proposed to have repressive activities, clarification of their precise function will likely shed light on these results. One proposed role of Prdm14 in normal PGC development is to up-regulate Sox2 in nascent PGCs (Yamaji et al. 2008). Therefore, the inability of Prdm14$^{-/-}$ PGCs to be able to give rise to EG cells may be due to the fact that, unlike normal PGCs, they may have low levels of Sox2 and therefore may be resistant to reprogramming.

It seems likely that conversion of PGCs to the pluripotent state might also require the down-regulation of many other genes involved in germ cell development. Some of these genes have been identified by differential screening of PGC and pluripotent stem cell-derived cDNA libraries and include genes such as CREB-binding protein (CBP), a transcriptional co-repressor/histone acetyltransferase, which has been found to play an important role in PGC development (Elliott et al. 2007). One of the key questions is how FGF signaling leads to PGC conversion via down-regulation of Prdm1 and activation or repression of other genes. An important clue comes from the proposed mode of action of FGFs, which can act to modify chromatin and allow access of transcription factors to promoter regions. Presumably this action leads to transcriptional and epigenetic changes that cause PGCs to convert into pluripotent EG cells. The finding that TSA can replace FGF2 in the cocktail of growth factors used to convert PGCs to EG cells also suggests that chromatin modification plays a key role in converting PGCs to EG cells because of the ability of TSA to act as a HDAC inhibitor (Durcova-Hills et al. 2008). Indeed these

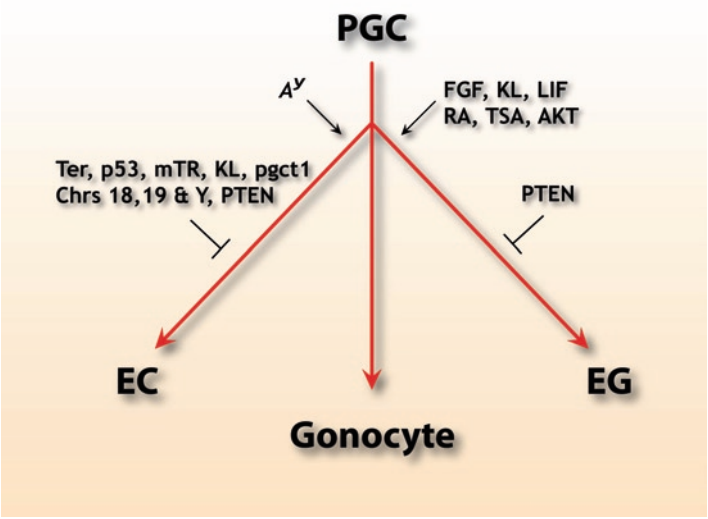

Fig. 1.4 Genetic pathways regulating the formation of embryonic germ (*EG*) cells and embryonal carcinoma (*EC*) cells from primordial germ cells. During normal development PGCs will differentiate in the developing gonad and give rise to gonocytes. *In vivo* PGCs can also give rise to EC cells, the stem cells of testicular germ cell tumors (*TGCT*). In mice, some of the genetic pathways regulating that process are known and include the *Ter*, *pgct1*, *p53*, and *mTR* genes. In addition, genes on chromosomes 18 and 19 and the Y chromosome can affect the incidence of TGCT. Similarly, *in vitro* PGCs can give rise to another type of pluripotent stem cell, an EG cell. Some of the growth factors that cause PGCs to turn into EG cells are also known and include FGF2, KL, and LIF. Retinoic acid (*RA*) and Trichostatin A (*TSA*) can also affect the ability of PGCs to give rise to EG cells, as can overexpression of the AKT kinase. Loss of the PTEN tumor suppressor can affect the ability of PGCs to give rise to both EC and EG cells, indicating that there are at least some shared pathways to pluripotency

studies found that TSA accelerated the process of conversion of PGCs to EG cells possibly because TSA may make the chromatin more accessible to transcription factors by allowing histones to become more acetylated (Durcova-Hills and Surani 2008). Studies on EG cell derivation provide an important insight into the mechanisms regulating pluripotency and the acquisition of the stem cell state (summarized in Fig. 1.4). The derivation of EG cells from PGCs also provides an important experimental system with which to investigate the pathways regulating pluripotency.

1.5 Lessons from Testicular Cancer

While studies of the molecular mechanisms guiding the formation of EG cells is a relatively recent development, genetic studies carried out over several decades in both mice and humans have provided some insights into the molecular mechanisms

regulating the formation of testicular tumors and, therefore, of the formation of pluripotent EC cells (reviewed in Matin and Nadeau 2005). Therefore, these studies provide valuable information about the mechanisms by which pluripotency is regulated. What is still somewhat controversial is whether the cell type of origin in humans is the same as that in mice and, therefore, whether data from human genetic studies is relevant to our understanding of the control of pluripotency. Nevertheless, studies using inbred strains of mice have identified several genes involved in the formation of teratomas or teratocarcinomas. Most mouse strains have a very low incidence of testicular germ cell tumors. Importantly, Stevens identified a strain of mice that showed an increased incidence of testicular teratocarcinomas, the 129Sv/J strain, which had a TGCT incidence of about 5% (Stevens and Hummel 1957). Further he demonstrated that the cell type of origin of these tumors were PGCs (Stevens 1967a). In the normal course of PGC development in males, PGCs enter the developing gonad and, at about the same time that the somatic cells show the first signs of differentiation, PGCs begin to enter mitotic arrest and form gonocytes. These cells will remain arrested in mitosis until after birth, at which time they will resume mitosis and give rise to spermatogonia. In mouse strains that are susceptible to TGCT, small groups of PGCs in the developing gonads continue to proliferate after the time at which they would normally have entered mitotic arrest. These small nests of proliferating PGCs give rise to EC cells. After birth these EC cells differentiate into differentiated cells representative of those found in the embryo and adult forming a benign tumor called a teratoma. These studies therefore identified PGCs as the stem or progenitor cell of these tumors. Having identified a mouse strain with susceptibility to developing TGCT it was possible to introduce gene mutations onto that strain background and therefore analyze the effect on the incidence of TGCT and, consequently, to identify genes that influence that process both negatively and positively. These genes include the C-Kit receptor tyrosine kinase, the Kit-ligand (KL), and Agouti (Fig. 1.4). In addition, Stevens subsequently identified a locus termed *Ter* (for *Teratocarcinoma*) that arose spontaneously in the 129Sv/J strain that is a powerful modifier of TGCT (Stevens 1973). Modern techniques of genome analysis have allowed identification of the role of many of these genes as well as the identification of other genes and chromosomal regions conferring susceptibility to TGCT. One of the loci identified as being a modifier of TGCT was the *Steel* (*Sl*) locus on mouse chromosome 10. Subsequent cloning of this locus demonstrated that the *Sl* locus encodes a transmembrane growth factor that can be cleaved to give rise to a soluble growth factor. Both transmembrane and soluble factors act as ligands for the C-Kit receptor tyrosine kinase and are termed Kit-ligand. Both KL and its receptor have been shown to play a key role in PGC development and have been proposed to play a critical role in regulating PGC survival. Many mutations at the Sl locus and the W locus encoding the C-Kit receptor cause dramatic reductions in PGC numbers and can cause reduced fertility or complete sterility. Introduction of different *Sl* alleles onto the 129/SvJ background has allowed further dissection of the role of KL in TGCT. Multiple mutants have been described at the *Sl* locus and include intragenic mutations, complete deletions, and mutations in the regulatory elements that leave the coding regions intact. Some *Sl* alleles, such as *Sl* and Sl^{J}, which delete the entire

KL gene, influence TGCT incidence. The Sl^{d} mutation, which represents an intragenic deletion that results in production of only a soluble form of the ligand, has no effect on TGCT incidence. Because the *Sl* and Sl^{j} alleles are large deletions that delete more than just the KL gene it was possible that the effect on these mutations on TGCT was not due to deletion of the KL gene but rather to mutation of another gene. Subsequent studies utilizing the *Sl grizzle-belly* (Sl^{gb}) allele, which deletes only the KL gene, demonstrated that indeed the effect of *Sl* mutations on increased TGCT incidence is due to loss of the transmembrane form of the KL gene (Heaney et al. 2008). These studies also point to a paradoxical aspect of TGCT. Mutations that affect TGCT incidence can also have a negative impact on germ cell numbers. This seems counterintuitive since typically tumor development is associated with increased rather than decreased survival and proliferation of cells.

One of the most interesting modifiers of TGCT incidence is the *Ter* gene, which was identified as a spontaneous mutation in the 129/SVJ strain of mice (Stevens 1973). Mice carrying the *Ter* mutation have a dramatically increased incidence of TGCT by comparison with the background 129SvJ strain (Fig. 1.4). Noguchi and Noguchi (1985) noted that 94% of Ter/Ter 129SvJ males had teratomas (75% bilateral), while that number was only 17% in hemizygotes (Noguchi and Noguchi 1985). Cloning of the *Ter* gene revealed it encodes a mouse ortholog of the zebrafish *dead end* (*Dnd*) gene (Youngren et al. 2005). The *Dnd* gene encodes a protein most closely related to the apobec complementation factor (ACF), which is part of the editosome that controls the editing of gene transcripts (Matin and Nadeau 2005). It is thought that editing arose as an innate defense against DNA and RNA viruses. But editing also modulates mRNA stability and translation as well as the occurrence of alternative transcripts of nuclear genes. So how might mutations in the *Dnd* gene affect the incidence of TGCT? That remains a key question. A number of transcripts that are known to be edited and include apolipoprotein B, a glutamate receptor, a cyclooxygenase, and an immunoglobulin, but it has been pointed out that they seem unlikely to be involved in PGC development or the development of TGCT (Matin and Nadeau 2005). Interestingly, when the *Ter* mutation is introduced onto other strain backgrounds it does not confer susceptibility to TGCT, but rather decreases PGC numbers: a phenotype that seems to be a prerequisite for the development of TGCT. As described above this phenotype of PGC loss is also seen in *Sl* mutants, which also increase the susceptibility to TGCT. Interestingly, when $Dnd1^{Ter/Ter}$ mutant mice were crossed onto a Bax-null background the loss of PGCs seen in $Dnd1^{Ter/Ter}$ mice was partially rescued. Bax, a Bcl-2-associated X protein, is a pro-apoptotic protein that promotes apoptosis by competing with Bcl-2 itself. The effect of loss of Bax on PGC death suggests that during normal development Bax may play a role in PGC loss and may protect animals from testicular tumor formation on certain genetic backgrounds (Cook et al. 2009). These data provide further support for the link between PGC death and the development of EC cells and subsequently teratomas. Clearly there is much more to know on that subject.

The most powerful negative modifier of TGCT is the agouti-yellow (A^{y}) deletion since it is the only locus found to decrease the incidence of tumorigenesis in 129Sv/J mice (Noguchi and Stevens 1982; Heaney and Nadeau 2008) (Fig. 1.4).

The A^y mutation induces the ectopic expression of *agouti* as well as deleting the *Raly* and *Eif2s2* genes. All of these changes could affect the incidence of TGCT. But genetic studies in which the *agouti* gene product was expressed ectopically in mice and others in which the expression of *Raly* was reduced in mice had no effect on TGCT incidence, suggesting that the *Eif2s2* gene was responsible for the decreased incidence of TGCT in A^y mice (Heaney et al. 2009). Indeed deletion of the *Eif2s2*, which encodes the beta subunit of the translation initiation factor eIF2, caused a twofold decrease in TGCT incidence in mice (Heaney et al. 2009). Interestingly it was found that reduced expression of Eif2s2 decreased the number of aberrantly proliferating PGCs in susceptible embryos at 16.5 dpc, suggesting that this could be one mechanism by which the *Eif2s2* gene could influence TGCT incidence (Heaney et al. 2009). Yet reduction in Eif2s2 also was associated with impaired spermatogenesis, implying that some aspect of germ cell differentiation may also be affected (Heaney et al. 2009). How could loss of Eif2s2 influence TGCT incidence? The Eif2s2 protein plays a role in regulating translation efficiency, a process that has been associated with tumorigenesis. It has long been noted that several oncogenes and cell cycle regulators (such as Myc, p27, and Cyclin D) have complex 5′-UTR structures. Consequently, in order to maintain the expression of such genes, cells require highly efficient translation machinery, which in turn can also suppress apoptosis. It has been proposed in other systems that increased translation rates might promote tumor progression by supporting proliferation, suppressing apoptosis, and promoting pluripotency (reviewed in Heaney et al. 2009). Heaney et al. suggest a mechanism by which loss of Eif2s2 could negatively influence TGCT formation. They propose the proliferation and differentiation events involved in germ cell development and the derivation of EC cells from PGCs could be sensitive to gene dosage. Therefore, reduced availability of Eif2s2 could attenuate the ability of aberrantly dividing PGCs to attain the self-renewal capacity or pluripotency required to transit to a pluripotent stem cell state (Heaney et al. 2009). This intriguing idea about the role of translation in generating pluripotent stem cells is also supported by the identification of the *Ter* locus as encoding *Dnd*, a gene with homology to a component of the RNA editing complex (see above). The Dnd protein blocks microRNA (miRNA) access to 3′-UTRs of transcripts involved in both PGC development such as Nanos1 and in cell cycle progression such as Cdkn1b. Piwi or Argonaute proteins associated with miRNAs in the RNA-induced silencing complex (miRISC) bind to 3′-UTRs or target transcripts. This complex inhibits translation by binding to the 5′ cap of mRNAs and blocking assembly of the cap-binding complex. Thus, the proposed roles of Dnd and Eif2s2 suggest that the regulation of translation by regulation of the 3′-UTR and 5′ cap of mRNAs is important in PGC development and the progression to the pluripotent state. This idea is confirmed to some extent by the observation that *Dnd* and *Eif2s2* interact to modulate the incidence of TGCT (Lam et al. 2007).

Several other genes or genetic loci have been identified as modifiers of TGCT, including the tumor suppressors p53 (Harvey et al. 1993) and PTEN (*p*hosphatase and *t*ensin homolog) (Kimura et al. 2003), as well as the pgct1 locus (Muller et al. 2000) and telomerase (Rudolph et al. 1999) (Fig. 1.4). The pgct1 locus is located

on chromosome 13 in a region syntenic to a portion of human chromosome 5q that has been associated with susceptibility to TGCT in humans (Muller et al. 2000). But further information about the pgct1 is so far lacking. Similarly, while mice carrying a targeted disruption of the gene encoding the essential RNA component of the telomerase holoenzyme (mTR) provide evidence for a role for telomerase in the etiology of TGCT, there is little understanding of how loss of mTR leads to TGCT (Rudolph et al. 1999). Loss of p53 is one of the most common events in human cancer, and mice lacking p53 develop multiple tumor types but mostly lymphomas (Harvey et al. 1993). However, when the p53 mutation was introduced onto the 129/SV background, tumors developed more quickly and the spectrum of tumors was altered. While these mice still developed lymphomas, about half of the 129/Sv p53-deficient males developed testicular tumors with a phenotype of teratocarcinomas. By comparison, only about 10% of control animals with a mixed genetic background developed this type of tumor (Harvey et al. 1993). Therefore, loss of p53 increased tumor incidence on the 129/Sv background but also altered the type of tumor from teratomas to teratocarcinomas. Whether the loss of p53 stimulates development of testicular tumors or promotes the growth of the tumors once they have formed is unclear. The recent discovery that down-regulation of p53 can greatly accelerate the generation of iPSCs from fibroblasts indicates that p53 could play a role in development of pluripotent EC cells from PGCs (Hanna et al. 2009; Zhao et al. 2008). Therefore, further studies on the mechanism by which loss of p53 stimulates the development of TGCT in the 129/Sv strain are clearly warranted.

Another major tumor suppressor is the PTEN gene, which is mutated at high frequency in a large numbers of human cancers. In order to determine the role of the PTEN gene in PGCs, Nakano and colleagues carried out targeted deletion of PTEN in PGCs using both a floxed allele of PTEN and mice expressing the Cre recombinase from the TNAP gene, which is expressed in PGCs (Kimura et al. 2003). These animals allowed deletion of the PTEN gene in PGCs during embryogenesis. Examination of *Pten*^{flox}/+:*TNAP/Cre*$^{+}$ male mice at birth revealed that, remarkably, all of the animals had developed bilateral testicular tumors each with multiple foci. When PGCs were examined in the *Pten*^{flox}/+:*TNAP/Cre*$^{+}$ embryos it was found that they had increased proliferation, exactly the phenotype described by Stevens in his original description of TGCT in mice (Kimura et al. 2003). In wild-type mice only 3% of PGCs were found to be proliferating at 13.5 dpc. By 15.5 dpc no proliferating PGCs could be detected. In mice in which PTEN was deleted in germ cells, the number of proliferating PGCs at 13.5 dpc was similar to that seen in wildtype embryos. But at the later stages of development, 14.5 and 15.5 dpc, a significant number of mitotic figures were identified in PTEN-deficient PGCs. Importantly, these animals develop TGCT. Thus, loss of PTEN causes susceptibility to TGCT (Fig. 1.4). These studies also examined the ability of PGCs in which the PTEN gene had been floxed to give rise to EG cells *in vitro*. Such cells were found to have in increased ability to give rise to EG cells. Thus, loss of PTEN makes PGCs susceptible to giving rise to pluripotent stem cells both *in vivo* and *in vitro*. These data suggest that at least some of the mechanisms controlling the transition of PGCs into these two pluripotent states are shared. Interestingly, these studies

also suggested that loss of PTEN did more than just stimulate PGC proliferation, but in addition had an effect on the differentiation of PGCs (Kimura et al. 2003). One of the key roles of the PTEN protein is to regulate the activity of the AKT kinase. Indeed, in both PGCs and testicular tumors in *Pten*^{flox}/+:*TNAP/Cre*$^{+}$ mice, high levels of AKT were observed.

In order to further our understanding of the genetic causes of TGCT, Nadeau, Matin, and colleagues have also utilized chromosome substitution strains (CSS) (Matin et al. 1999). These CSS strains are produced by intercrossing specific inbred strains of mice and transferring a single full-length chromosome from a donor strain to a second host strain by repeated backcrossing. Such strains, each carrying a single full-length chromosome from a donor strain, allow for identification of genetic loci involved in a specific trait (such as TGCT) via quantitative trait locus (QTL) analysis. Using such panels Nadeau and colleagues have identified several chromosomes that influence TGCT incidence in mice including chromosomes 18 and 19 and the Y chromosome (Matin et al. 1999; Anderson et al. 2009a, b) (Fig. 1.4). While these studies deserve more attention, in the interests of space they will not be discussed further here, but without doubt these studies will continue to contribute to our knowledge of TGCT and the generation of pluripotent stem cells from PGCs.

1.6 Parallel Pathways to Pluripotency

The ability of PGCs to give rise to pluripotent stem cells in two different situations, one *in vivo* and one *in vitro*, provides a great opportunity in which to compare different routes to the same end. Several pieces of evidence suggest that some of the molecular mechanisms that play a role in the two processes are shared. The ability of PGCs to give rise to both experimentally induced teratomas or to EG cells ends at the time at which they cease proliferation in the embryo at 12.5 dpc, suggesting that some aspect of PGC differentiation limits both processes. Further, conditional knockout of the PTEN gene reveals that this gene plays an important role in both EG derivation and TGCT formation. Future studies aimed at determining whether the mechanisms regulating EG formation also could affect the incidence of TGCT could have a significant impact on our understanding of the etiology of testicular cancer. Conversely, determination of the role of TGCT susceptibility genes in the process of EG cell formation could reveal important new information about the control of developmental potency. Ultimately the reward could be a better understanding of how normal germ cell development proceeds and why it sometimes goes wrong.

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Chapter 2
Pluripotent Stem Cells from the Postnatal Testis: Unlocking the Potential of Spermatogonial Stem Cells

Marco Seandel, Ilaria Falciatori, and Shahin Rafii

Abstract While embryonic stem (ES) cells are well known to give rise to tissues comprising all three germ layers, only recently was it shown that cells from the postnatal testis could produce embryonic-like stem cells in culture. The latter, arising *in vitro* from spermatogonia, can undertake most, if not all, the functions of ES cells. This chapter explores the potential predisposing factors for postnatal germ cells to become pluripotent, including expression of pluripotency-associated genes and epigenetic factors. The major published studies describing the production of ES-like cells from mice and human tissues are reviewed. Finally, we assess the data demonstrating functionality of the differentiated derivatives of ES-like cells. The possible uses of testis-derived stem cells for the study of pluripotency and for regenerative applications is also discussed in comparison to other approaches using ES cells and induced pluripotent stem (ips) cells.

Keywords Spermatogonia • Adult stem cells • Pluripotent stem cells • Testis • Cell transplantation

2.1 Introduction

More than one hundred years ago, it was recognized that testicular cells in adult men could give rise to outgrowths comprised of endoderm, mesoderm, and ectoderm, now well known as teratomas (Young 2005). Then, in the 1960s, Leroy

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Stevens made the startling observation that even transplantation of gonadal tissue out of the embryonic microenvironment and into the adult testis was sufficient to induce teratomas in mice of the proper genetic background (Stevens 1964). In the 1990s, a series of experimental conditions were established to efficiently obtain pluripotent clones, known as embryonic germ (EG) cells, by simply transferring murine primordial germ cells (PGCs) during a precise developmental window from the gonadal niche to an *in vitro* milieu defined by specific growth factors and feeder cells, as reviewed elsewhere in this volume (Matsui et al. 1992). These observations, in conjunction with the fact that the solitary task of the germline is to transmit the genetic and epigenetic information required for embryogenesis, all pointed to the possibility that postnatal germ cells could be predisposed to pluripotency. Data supporting this hypothesis has now been published by multiple groups of investigators, following a landmark study from T. Shinohara's laboratory in 2004 (Kanatsu-Shinohara et al. 2004).

In this chapter, we first introduce the mammalian spermatogonial stem cell (SSC), the cell type from which pluripotent stem cells are believed to arise, and discuss the technology that has facilitated investigation of this phenomenon. The unique properties of SSCs are highlighted in comparison to somatic cells and embryonic stem (ES) cells. We then address the factors that may predispose SSCs to pluripotency and review the studies in which murine and human germ cells have been observed to become pluripotent spontaneously *in vitro*, a phenomenon that is not observed with somatic cells in culture. Finally, we discuss the implications of the most recent findings related to male germline stem cells and we compare the properties of the germline-derived pluripotent cells with those of pluripotent cells generated from somatic cells through the delivery of exogenous pluripotency factors [induced pluripotent stem (iPS) cells].

2.2 The Putative Precursors: Spermatogonial Stem Cells (SSCs)

The SSC, responsible for maintaining near life-long spermatogenesis in mammals, is contained within the population of undifferentiated spermatogonia, along the basement membrane of the seminiferous tubule, but represents only about 0.03% of germ cells in mice (Tegelenbosch and de Rooij 1993). While morphologic criteria were previously used to define these stem cells, the advent of technology to transplant and later to expand them in culture has allowed a series of investigations into the molecular features that define SSCs, as reviewed elsewhere in this volume (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al. 2003). The notion that postnatal testicular cells are predisposed to pluripotency remained untestable prior to the advent of technology to accurately identify and propagate SSCs. In 2003, the Shinohara group described a set of culture conditions that allowed long-term culture of SSCs, by employment of mouse embryonic fibroblast (MEF) feeder cells, in conjunction with a rich culture medium supplemented with several recombinant

growth factors, including glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) (Kanatsu-Shinohara et al. 2003). As opposed to immunoselection, only a negative selection was required to remove the majority of somatic cells via binding to gelatin. However, the efficiency of deriving long-term SSC lines from adult mouse testis was only 20–50% in these culture conditions (Kanatsu-Shinohara et al. 2004; Ogawa et al. 2004). This could be due to a relative decline in the number of functional stem cells in older animals or in the self-renewal capacity of such cells. Nonetheless, multiple studies have subsequently confirmed that the SSCs could be passaged over many generations and retain the ability to restore fertility in animals with deficient spermatogenesis (Kanatsu-Shinohara et al. 2003, 2005a, b; Ryu et al. 2005; Kubota et al. 2004a, b). However, it has been estimated that only 1–2% of cultured SSCs exhibit testicular repopulation capacity (Kanatsu-Shinohara et al. 2005b). Conversely, recent data suggest that differentiating germ cells can display plasticity, potentially reverting back to the stem cell phenotype *in vitro* or *in vivo* (Nakagawa et al. 2007; Barroca et al. 2009). As our understanding of the nature of SSCs has evolved, the tools to study them have become increasingly sophisticated, revealing a number of unique properties as discussed below.

2.3 Molecular Features that Could Predispose SSCs to Pluripotency

It is reasonable to suppose that some of the same characteristics of male germ cells that facilitate initiation of embryogenesis at the time of fertilization could also play a role in spontaneous cellular reprogramming that would lead to formation of pluripotent stem cells *in vitro*. But what are these special molecular characteristics? Both in terms of gene expression and chromatin structure, SSCs have been found to share certain features (but also notable differences) with pluripotent stem cells. In the sections below, we first review the current understanding of the normal expression levels of the core pluripotency genes (particularly *Oct4, Nanog*, and *Sox2*) in the testis and in cultured SSCs then examine data describing the unique state of chromatin and its modifications in the germ lineage (see Fig. 2.1).

Oct4 is a homeobox transcription factor that is crucial for pluripotency in embryonic stem cells (Nichols et al. 1998; Niwa et al. 2000). Oct4 is part of a core network of molecules, including Sox2 and Nanog, that both autoregulate and co-regulate downstream factors that maintain self-renewal and block differentiation (Boyer et al. 2005). Studies revealing the expression of Oct4 in the postnatal testis have relied both on immunological methods and genetic reporter systems with varying results, though no study has documented levels in postnatal germ cells comparable to those observed in ES cells. Pesce et al. (1998) found diffuse Oct4 protein by immunohistochemistry in spermatogonia up to 7 days postnatally but in adult animals only a subset of spermatogonia (type A) were positive (Pesce et al. 1998). However, in a report using transgenic mice that expressed GFP under control of an 18 kilobase

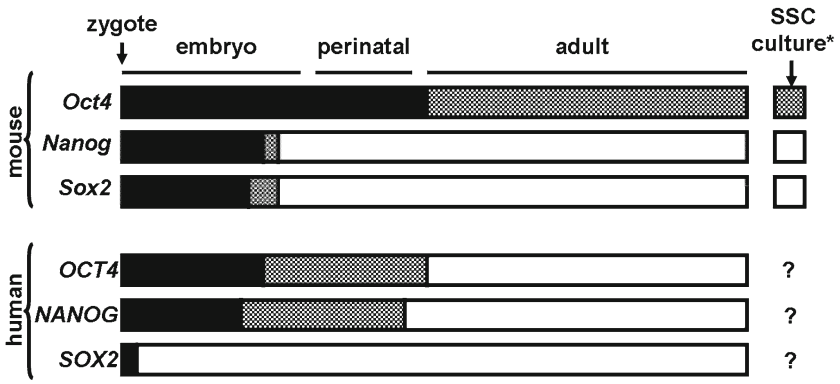

Fig. 2.1 Expression of core pluripotency-associated genes *OCT4, SOX2*, and *NANOG* in the testis. Relative levels are denoted by *black bars* (high), *gray bars* (down-regulated but detectable), and *white bars* (absent) for mouse and human testis *in vivo*. *Boxes* at right denote expression in long-term SSC culture. This data represents a summary of findings from multiple studies (Kanatsu-Shinohara et al. 2004, 2005b; Pesce et al. 1998; Yoshimizu et al. 1999; Ohbo et al. 2003; Ohmura et al. 2004; Tadokoro et al. 2002; Buaas et al. 2004; Tokuda et al. 2007; Looijenga et al. 2003; Rajpert-De et al. 2004; Seandel et al. 2007; Dann et al. 2008; Imamura et al. 2006; Avilion et al. 2003; Western et al. 2005; Perrett et al. 2008; de Jong et al. 2008; Shi et al. 2006; Chambers et al. 2003; Yamaguchi et al. 2005; Hoei-Hansen et al. 2005; Yeom et al. 1996) (see Addendum). *Question marks* indicate absence of published data

Oct4 gene fragment containing both the distal enhancer and the epiblast-specific proximal enhancer, the investigators were unable to directly visualize GFP expression more than 10 days after birth (Yoshimizu et al. 1999). Using similar *Oct4/GFP* transgenic reporter mice, Ohbo et al. (2003) identified *Oct4*-expressing spermatogonia (containing the majority of stem cell activity) in mice up to 14.5 days postnatally, after which point expression decreased (Ohbo et al. 2003). A subsequent study revealed *Oct4* expression by RT-PCR in the Ep-CAM$^+$ fraction of adult Oct4/GFP$^+$ cells (Ohmura et al. 2004). However, detection of endogenous Oct4 protein in histologic sections of the adult testis required significant amplification in the latter study, due to low levels of antigen compared to what was seen in the spermatogonia of younger animals. Nonetheless, Tadokoro et al. (2002) confirmed Oct4 protein expression in a substantial subpopulation of adult undifferentiated spermatogonia in progeny-deficient strains of mice and proposed that Oct4 expression is reversible in SSCs, depending on microenvironmental conditions, although the functional significance of this result was not clear. Buaas et al. (2004) demonstrated co-expression of Oct4 and Plzf, a marker of undifferentiated spermatogonia, in adult mouse testes, while Tokuda et al. (2007) found co-expression of Oct4 and Cdh1 in adults (Buaas et al. 2004; Tokuda et al. 2007). Taken together, these studies suggest that a subset of murine spermatogonia maintain Oct4 expression into adulthood, albeit at relatively low levels under normal (i.e., nonpathological) conditions.

In the human testis, though fewer data are available, OCT4 expression in the male germline appears rapidly down regulated after ~20 weeks of gestation (Looijenga et al. 2003; Rajpert-De et al. 2004). A small number of OCT4-positive cells are

detectable at 3–4 months postnatally but these normally disappear thereafter (Rajpert-De et al. 2004).

A similar picture has emerged for *Oct4* expression in cultured SSCs. Multiple studies confirmed expression by RT-PCR in neonatal SSCs (Kanatsu-Shinohara et al. 2004, 2005b). However, a quantitative analysis of neonatal SSCs later demonstrated sharply lower Oct4 expression by either mRNA or protein, compared to ES cells (Imamura et al. 2006). Not surprisingly, the same pattern was seen in adult SSCs in long-term culture (Seandel et al. 2007). Notably, this heterogeneous and relatively low magnitude of Oct4 expression has recently been found to be functionally important in self-renewal and survival of cultured SSCs (Dann et al. 2008).

A second major pluripotency gene studied in the male germ line is *Sox2* (SRY [sex determining region Y] – box 2, which, like *Oct4* is expressed in the germline) (Avilion et al. 2003). In mice, germline expression of *Sox2* is lost by E15.5 (Western et al. 2005). In humans, *SOX2* mRNA was detected in adult testis in two studies (Gure et al. 2000; Schmitz et al. 2007). However, using more rigorous methods SOX2 was later shown to be absent even in human PGCs and also absent in adult testis by both message and protein (Perrett et al. 2008; de Jong et al. 2008). In cultured neonatal murine SSCs, no Sox2 protein was detectable despite significant transcript levels (Imamura et al. 2006; Shi et al. 2006). In contrast, Seandel et al. (2007) found that adult SSCs in culture did not even express *Sox2* message (Seandel et al. 2007). Therefore, in both mice and human functional SOX2 protein is not likely to be present beyond an early developmental window, and this decline in expression maybe paralleled in cultured SSC lines derived from mice of increasing age.

The third canonical pluripotency-associated transcription factor is Nanog, another homeodomain-containing protein strongly expressed in ES cells (Chambers et al. 2003). Beyond the mouse blastocyst stage, *Nanog* expression is present in the male germ lineage, and the protein is detectable through E16.5, at which time it is largely down-regulated coincident with mitotic arrest (Chambers et al. 2003; Yamaguchi et al. 2005). No Nanog protein was detected in the adult mouse testis (Hoei-Hansen et al. 2005). Similarly, in the human testis, NANOG protein is present through 19 weeks of gestation, but the rare positive cells that remain at 3–4 months postnatally are completely absent by childhood and also in adults (Hoei-Hansen et al. 2005). Perhaps not surprisingly cultured murine SSCs from neonatal or adult stages do not express Nanog (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007).

As mentioned above, OCT4, SOX2, and NANOG are thought to form a core regulatory network in ES cells (Boyer et al. 2005). Moreover, both OCT4 and SOX2 are key transcription factors in the cocktail of genes used to generate iPS cells starting from somatic cells, with OCT4 being the most critical of the two (Takahashi and Yamanaka 2006; Kim et al. 2009c). However, based on the studies described above, these proteins, with the exception of OCT4, are not present either in postnatal germ cells or in cultured SSCs (Fig. 2.1). Therefore, it is unlikely that a resident subpopulation of ES-like cells in the postnatal testis could give rise to pluripotent stem cells *in vitro*. Furthermore, based on their absence in the precursor population, it seems that neither SOX2 nor NANOG-driven signals are likely to be the most proximal mediators in the signaling pathway leading to the conversion of

SSCs into pluripotent stem cells. Of note, however, other pluripotency associated genes have been found to be expressed in the adult testis. For example, *Lin28*, previously associated with regulation of let-7 precursor microRNA processing, was found to induce pluripotency of somatic cells (when introduced ectopically in conjunction with *Oct4*, *Sox2*, and *Nanog*) (Viswanathan et al. 2008; Yu et al. 2007). Recently, *Lin28* was found to be expressed in adult undifferentiated spermatogonia (Zheng et al. 2009). It is not known whether *Lin28* can constitute an upstream signal leading to expression of other core pluripotency genes.

2.4 Epigenetic Factors that Could Predispose to Pluripotency

The state of chromatin in the development of the male germline represents a key distinguishing feature from somatic cell types and one that is crucial for reproductive success. Deficiencies of genes that drive chromatin modifications such as DNA methylation can result in male sterility (Kaneda et al. 2004). Likewise, the unique chromatin state could also represent a predisposing factor for premature acquisition of pluripotency. Murine PGCs, unlike somatic lineages, undergo erasure of recently acquired DNA methylation in both imprinted and nonimprinted loci around the time of entry into the gonads by about E12.5 (Hajkova et al. 2002). Subsequently, male imprinting patterns become reestablished during the remainder of the prenatal period and into early postnatal life (Davis et al. 1999; Li et al. 2004; Schaefer et al. 2007; Oakes et al. 2007). However, Farthing et al. (2008) recently found unexpected similarities in the global promoter methylation status between ES cells, EG cells, and sperm, suggesting that male germline cells could activate transcription of pluripotency-associated genes more easily than somatic cells (Farthing et al. 2008). ES cells have recently been shown to exhibit characteristic sets of histone methyl marks, linked to their pluripotent status (Bernstein et al. 2006). The histone methylation profile of postnatal SSCs is poorly characterized but a distinctive pattern of perinuclear histone H3 lysine 9 and H4 lysine 20 tri-methylation was recently described on postnatal undifferentiated spermatogonia, although the patterns at specific loci were not examined (Payne and Braun 2006). Recently, the acquisition of pluripotency in mouse PGCs at E8.5 was linked to DNA demethylation, with subsequent loss of pluripotency following histone replacement after E11.5 (Hajkova et al. 2008).

Since pluripotency is acquired *in vitro*, the chromatin status of cultured SSCs could affect the stability of lineage commitment and predispose the cells to pluripotency. The Shinohara Laboratory found that neonatal SSCs bear the expected androgenetic pattern of methylation at imprinted genes, which was stable in long-term culture (Kanatsu-Shinohara et al. 2004, 2005b). When pluripotency-associated genes were examined specifically, both sperm and cultured SSCs exhibited relative hypomethylation of regulatory regions in a number of such genes, although this was not the case for specific key genes, such as *Sox2*, and did not necessarily correlate with the presence of the corresponding protein (Imamura et al. 2006). The authors

concluded that, for certain key pluripotency genes, post-transcriptional mechanisms could be very important in controlling the phenotype. Thus, the extent to which the preexisting epigenetic profile of germ cells contributes to the observed acquisition of pluripotency is a matter of speculation.

2.5 Culture-Induced Pluripotency in Mice

While multiple laboratories have demonstrated the acquisition of pluripotency by germ cells *in vitro*, the procedures and conditions used are remarkably different (see Figs. 2.2 and 2.3 and Table 2.1). In 2004, it was discovered that SSCs derived from the neonatal testis could reproducibly give rise to pluripotent embryonic-like stem cells within 4–7 weeks of initiation of the stem cell culture in ~20% of experiments but at a very low rate relative to the number of cells plated (Kanatsu-Shinohara et al. 2004). These ES-like cells not only physically resembled ES cells but also bore a similar gene expression profile, marked by the presence of mRNA for *Nanog*, *Rex1*, *Utf1*, *Esg1*, and *Cripto*, among others. When wild-type testes

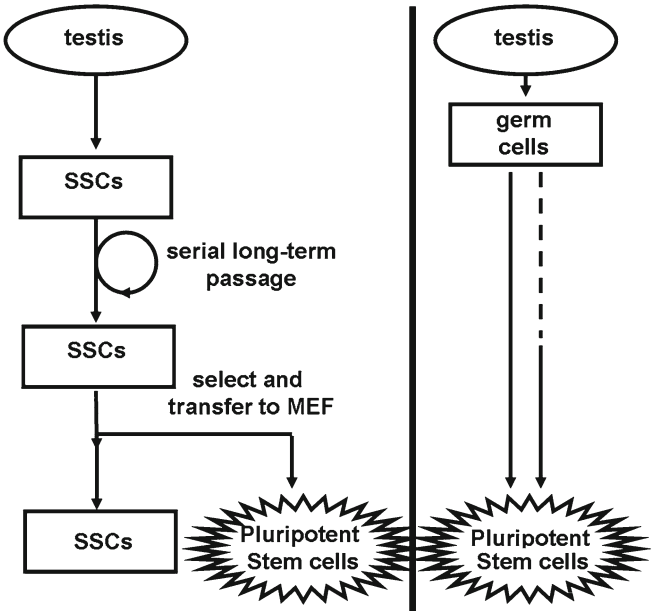

Fig. 2.2 Variations in experimental approaches for obtaining pluripotent stem cells from postnatal testis. *Left panel*: Some laboratories have employed long-term culture of SSCs prior to obtaining pluripotent stem cells that were then separated from the parental cells by selection (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007; Ko et al. 2009; Yu et al. 2000). *Right panel*: Other groups have employed short-term culture without parallel propagation of the precursor (parental) cell population (Guan et al. 2006; Ko et al. 2009; Mizrak et al. 2010)

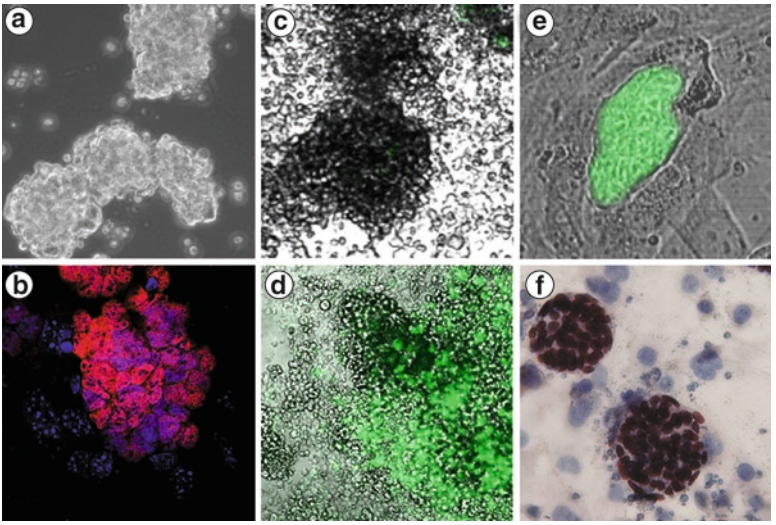

Fig. 2.3 Culture-induced up-regulation of OCT4 in spermatogonial stem and progenitor cells coincident with appearance of ES-like colonies. SSCs were derived from adult wild-type or OCT4-GFP reporter mice. (**a**) Phase contrast appearance of routine SSC cultures. (**b**) Specific nuclear labeling of SSCs using anti-PLZF antibody (*red*). (**c**) Fluorescence microscopy for OCT4-GFP reporter in routine SSC cultures. (**d**) Spontaneous up-regulation of OCT4-GFP (*green*) in long-term culture of SSCs. (**e**) Uniform OCT4-GFP expression (*green*) in ES-like colonies mechanically transferred to MEF feeder cells. (**f**) Immunohistochemistry demonstrating uniform endogenous OCT4 protein expression (*brown*) in ES-like cells derived from SSC cultures. Counterstain in (**b**) and (**f**) is *blue* (Seandel et al. unpublished data)

(3–8 weeks old) were the source of SSCs, no ES-like cells appeared, although the parental adult SSC lines could be derived in only 20% of experiments. Adult-derived *P53* knockout SSC, which could be derived at higher rate, also gave rise to ES-like cells. The investigators provided substantial evidence that the ES-like cells were not only distinct from the parental SSCs but that the ES-like cells could undertake most if not all of the functions of ES cells, including long-term self-renewal in culture, multi-lineage differentiation and formation of chimeric animals, including germline transmission.

A major distinction from the parental SSCs was that the ES-like cells formed teratomas in both subcutaneous teratoma assays and upon injection into the seminiferous tubules, indicating that the novel ES-like phenotype was stable and that the cells could not simply revert back to the SSC phenotype upon placement back into the normal SSC niche (see Table 2.1) (Kanatsu-Shinohara et al. 2004). This was in contrast to the parental SSCs that did not form teratomas at all, consistent with our own experience (Seandel et al. unpublished data). The authors proposed that a predisposition to pluripotency could be a general property of SSCs but that the somatic cells *in vivo* may help to suppress such aberrant cell phenotypes, in order to prevent teratoma formation in the normal testis. Furthermore, the mechanism of conversion of SSCs into ES-like cells appeared to be different from that in which

Table 2.1 Selected studies of germ cell-derived pluripotent stem cells

Parameter	Kanatsu-Shinohara et al. (2004)	Guan et al. (2006)	Seandel et al. (2007)	Izadyar et al. (2008)	Ko et al. (2009)	Conrad et al. (2008)	Kossack et al. (2009)
Age/species of donor	Neonatal/ mouse	Adult/ mouse	Adult/ mouse	Neonatal-adult/ mouse	Adult/ mouse	Adult/human	Adult/ human
Designation for pluripotent cell lines	mGS	maGSC	MASC	mGC	gPS	haGSC	hMGSC
Precursor population kept in long-term culture	Yes	No	Yes	No	Yes	No	No
Precursor population restores spermatogenesis[a]	Yes	Yes	Yes	No (c-kit+)	Yes	N/A	N/A
Precursor population forms teratoma	No	N/A	No	N/A	No	N/A	N/A
Pluripotent stem cells differentiate *in vitro*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pluripotent stem cells form teratoma	Yes	Yes	Yes	No	Yes	Yes	No[c]
Precursor population forms chimeric blastocysts	No	Yes	No[b]	N/A	N/A	N/A	N/A
Pluripotent stem cells form chimeric blastocysts	Yes	N/A	Yes	Yes	Yes	N/A	N/A

[a] In spermatogonial stem cell-type transplantation assay

[b] Seandel et al. unpublished data

[c] Additionally, studies by Golestaneh et al. (2009) and Mizrak et al. (2010) did not yield large, complex teratomas from the ES-like cells. *N/A* not applicable

PGCs convert into EG cells, since the latter set of culture conditions were not successful for conversion of SSCs. These conclusions raised the intriguing question of whether SSCs should generally be considered capable of "dedifferentiation" and therefore inherently multipotent or alternatively whether a small primitive subpopulation of germ cells in the postnatal testis could be responsible for the production of ES-like cells in culture. To answer this, genetic marking was used to demonstrate that single SSC clones could give rise both to continually self-renewing SSC cultures and to ES-like cells (Kanatsu-Shinohara et al. 2008a). Of note, the partial androgenetic pattern of imprinting in SSCs was invoked as an indication that epigenetic instability in culture could contribute to the change in fate of SSCs, upon conversion into ES-like cells (Kanatsu-Shinohara et al. 2004).

Several studies subsequently confirmed the general concept that pluripotent stem cells could be derived from the testis and specifically found that even the adult testis harbors cells with such capability. Guan et al. (2006) found that, after a very brief period of culture, the Stra8^{+} population of adult testicular cells was able to generate ES-like cells (Guan et al. 2006). However, the published nomenclature provided did not unequivocally distinguish the identity of the parental population from the ES-like derivatives. Interestingly, the authors found that even the parental SSCs could directly contribute to chimerism in blastocyst injection assays, although no SSC transplantation data were shown to establish the purity and functionality of the starting population of germ cells, which were precultured for 1 week prior to initiation of ES-like induction culture conditions. The authors suggested that the presence of somatic factors such as GDNF could serve to inhibit plasticity both *in vitro* and *in vivo*. This hypothesis is in contrast with the results obtained by another group (Huang et al. 2009). The latter reported the derivation of alkaline phosphatase-positive pluripotent cells from unselected neonatal testicular cell suspensions in short-term culture (1 week), but they proposed that the testicular somatic cells present in the culture, specifically the Leydig cells, are responsible for the production of Igf1 which, through Akt signaling, maintains pluripotency of SSCs. In this study, there is no distinction between SSC and pluripotent cells, but rather they were proposed to be the same cell type able to contribute both to spermatogenesis after transplantation in busulfan-treated testes and to chimera formation upon blastocyst injection. Notably, these cells did not form teratomas when transplanted into the testis, whereas they did form teratomas when transplanted subcutaneously in NOD-SCID mice. Another group reported the development of pluripotent stem cells during short-term culture of neonatal and adult testicular cells from *Oct4/GFP*-reporter mice (Izadyar et al. 2008). This report also did not distinguish between the different populations of germline-derived cells in question (i.e., SSCs and ES-like cells) in the culture system, a similar semantic and experimental issue as in the Guan et al. (2006) study (Izadyar et al. 2008). In fact, both populations appeared to coexist (based on heterogeneous morphology of colonies in the images provided). Perhaps not surprisingly, the authors found a remarkably similar expression profile and imprinting pattern between germ cells before and after culture. This could be due to dilution of ES-like cells by a majority of SSC-like cells in the culture (which could also explain the absence of teratoma formation), although this issue was not specifically addressed.

Seandel et al. (2007) developed a means to derive adult SSC lines from mice up to 11 months of age with overall >90% efficiency, using feeders comprised of mitotically inactivated primary testicular stromal cells. We found that the novel G-protein coupled receptor Gpr125 was expressed in a population of cultured germ cells that contained SSC activity based on transplantation assays. These experiments were performed using engineered mice (and SSCs derived from such mice) in which *lacZ* was placed within the endogenous *Gpr125* locus (*Gpr125-lacZ*), representing an extremely sensitive and specific reporter system. After about 3 months following initiation of SSC cultures even without preselection of cells from the testes of Gpr125-lacZ mice (or from other strains), morphologically distinct colonies comprised of ES-like cells [referred to as multipotent adult spermatogonial-derived stem cells (MASCs)] appeared spontaneously (Fig. 2.3). These colonies were selected and transferred into ES culture conditions for long-term propagation, establishing new colonies that closely resembled mouse ES cells when plated upon inactivated MEFs. These adult-derived ES-like cells expressed Oct4 and Nanog protein and readily differentiated into derivatives of all three germ layers *in vitro*, including contractile cardiogenic tissue. Similarly, the ES-like cells produced tri-lineage teratomas in immunodeficient mice (Seandel et al. 2007), including foci of de novo germ cell differentiation (Seandel et al. unpublished data).

As more stringent evidence of pluripotency, the ES-like cells that had been cloned at the single cell level were found to be competent at forming chimeric embryos, with contributions to multiple organ systems. Of note, despite obtaining live-born chimeric mice from ES-like cells, we have observed generally low contributions of the adult ES-like cells in chimeras and a reproducible congenital abnormality comprised of hyperplasia and abnormal chondrogenesis of the anterior rib cage (Falciatori et al. unpublished data). This phenotype, reminiscent of androgenetic embryos, would be consistent with the partial androgenetic imprinting profile previously described for ES-like cells (Kanatsu-Shinohara et al. 2004; Mann et al. 1990). Also of great interest, the gene expression profile of the adult-derived ES-like cells was not identical to that of ES cells. Among the pluripotency genes markedly lower in the ES-like cells were *Rex1, Esg1,* and *Gdf3*, while *Nanog* was also lower but still expressed at absolutely high levels. Certain lineage commitment markers were present at substantially higher levels in ES-like cells than in bona fide ES cells, including the mesodermal gene *brachyury*.

More recently, the ability of adult SSCs in culture to produce pluripotent stem cells was confirmed by Ko et al. (2009). These authors not only demonstrated the ability of authentic SSC clones to acquire pluripotency (including a germline contribution) but also showed that the initial plating density of SSCs was crucial for efficient conversion (estimated at about 0.01% of cells). Furthermore, this study also compared methylation patterns at imprinted genes to demonstrate the origin of the pluripotent stem cells from spermatogonia. The latter analysis showed that the ES-like cells exhibited an androgenetic imprinting pattern at the differentially methylated region of the H19 gene, similar to what was seen in spermatogonia but dissimilar to the somatic pattern. These data argued against a possible origin from a somatic stem cell. As with the prior studies, the authors demonstrated that derivatives

of the pluripotent stem cells were functional in various assays. Together, these results highlight the conclusion that the adult spermatogonial-derived ES-like cells should not be considered equivalent to ES cells, despite their common ability to form functional tissues, since differences were found in both gene expression by Seandel et al. (2007) and in imprinting status by Ko et al. (2009), respectively.

2.6 Culture-Induced Pluripotency in Humans

Recent provocative studies have found evidence for pluripotent stem cells derived from the adult human testis (Conrad et al. 2008; Kossack et al. 2009; Golestaneh et al. 2009; Mizrak et al. 2010). Conrad et al. (2008) employed the following steps to obtain highly enriched germ cells from fresh tissue: 4 days of culture of mixed enzymatically dispersed testicular cells, followed by immunoselection using alpha6 integrin, and differential matrix selection for collagen-non-binding and laminin-binding cells. It is quite remarkable that within 4 days of culture the selected germ cells activated expression of OCT4 protein in the nucleus and cytoplasm, even though no OCT4 is found subsequent to embryonic stages in the human testis (Rajpert-De et al. 2004; Conrad et al. 2008). After several additional weeks of incubation, cultures were obtained containing fibroblast-like monolayers that surrounded discrete multilayered colonies of cells with ES-like properties. Subsequent culture in the presence of LIF resulted in generation of new colonies of such stem cells, although it is not clear whether these represented de novo conversion from the precursor spermatogonial cells.

The ES-like cells expressed *OCT4, SOX2,* and *NANOG*, demonstrated by RT-PCR, while both OCT4 and NANOG protein were present by immunofluorescence, flow cytometry, and Western blot analysis. Microarray expression profiling detected expression of all three of these pluripotency genes not only in the ES-like stem cells but interestingly also in the precursor spermatogonial cells, implying that transcriptional activation also of *SOX2* and *NANOG* (in addition to *OCT4* and other pluripotency associated genes such as *REX1*) must take place during the initial four-day culture period. While this study actually detected *SOX2* by RT-PCR in normal adult testis, neither *SOX2* nor *NANOG* were found expressed after embryonic stages by other investigators (Perrett et al. 2008; de Jong et al. 2008; Hoei-Hansen et al. 2005; Conrad et al. 2008). The reason for these discrepancies between laboratories is unclear but may be due to technical differences. While the global expression profile of the human ES-like cells was generally similar to ES cells, the retention of a germline signature could be seen in high levels of expression of *DAZL* and *POU6F1*. To demonstrate pluripotency, the authors formed teratomas from independent ES-like cultures from eight normal samples in 23 of 32 attempts, using cells with normal karyotype. These data portray a relatively robust system for obtaining pluripotent stem cells.

Kossack et al. (2009) reported the derivation of two ES-like cell lines from testicular biopsy-derived cells (Kossack et al. 2009). These lines were obtained

after a 1-week culture of mixed testicular cells followed by a manual selection and transfer of putative stem cell colonies onto MEF feeders. While these ES-like cells lacked *NANOG* expression, they did express *OCT4* and *SOX2*. In addition, the ES-like cells exhibited high levels of telomerase and normal karyotype. The ES-like cells exhibit an intermediate pattern of methylation at both imprinted and non-imprinted loci, similar, in general, to that described by (Kossack et al. 2009; Conrad et al. (2008)). Although functional during *in vitro* differentiation assays, the ES-like cells did not form teratomas in immunocompromised mice. This contrasts to a third report in which ES-like cells derived from biopsy-sized quantities of human testicular tissue from organ donors were able to give rise to small teratomas (Golestaneh et al. 2009). In this study, total testicular cell suspensions were plated in defined human ES cell culture medium in order to generate ES-like colonies within 1 week after testicular cell isolation. Another report also describes the generation of pluripotent cells from testicular biopsies but with slower kinetics (3–8 weeks) (Mizrak et al. 2010). When transplanted into immunodeficient mice, these cultured testicular cells were also able to give rise to tissues containing some differentiated human cells but not complex teratomas (which were produced, in contrast, using either hESC or iPS cells). Since neither of these latter studies used preselection of germ cells, the exact origin of the pluripotent cells derived is unclear.

Thus, the aforementioned pluripotent cell lines derived from postnatal testis share certain important properties, including expression of pluripotency genes, trilineage differentiation ability, and variations on the expected androgenetic methylation profile at imprinted genes, in conjunction with intermediate levels of methylation at other important loci (Table 2.1). The functionality of human pluripotent cell lines is generally somewhat more difficult to assess due to technical and ethical limitations. Nonetheless, data initially generated using the murine system appear to be relevant for the human testis. Unfortunately, different approaches were used to generate the pluripotent lines in each study, precluding their direct comparison (see Fig. 2.2). For example, multiple different culture media were used for induction conditions, in the presence or absence of fetal bovine serum, GDNF, bFGF, EGF, and LIF, with somewhat conflicting results about their respective effects (de Rooij and Mizrak 2008). Furthermore, the timeframe for conversion of unipotent germ cells into pluripotent stem cells is also quite variable. These observations suggest that more than one mechanism could theoretically be responsible. It should be noted, however, that spontaneous teratomas occur only very rarely in males, with an incidence of <1 in ~11,000 in wild-type laboratory mice and <1 in ~16,000 in the human testis (Krausz and Looijenga 2008; Stevens and Mackensen 1961). In comparison, the experimental rate of emergence of pluripotent cells observed in the aforementioned studies is much higher in aggregate and no such similar process is known to occur in somatic cells without experimental delivery of pluripotency factors. Nonetheless, it is difficult to formally rule out the possibility that a spontaneous genetic change, as opposed to an epigenetic or culture-induced phenotypic change, contributed the observed results. While the mechanism of conversion will be extremely important to determine and may have relevance for acquisition of pluripotency by other cell types, the crucial criteria in

considering such cells for eventual clinical implementation is their functionality and safety, as discussed below.

2.7 Potential Applications of Germline-Derived Pluripotent Cells: A Comparison with ES Cells and iPS Cells

Pluripotent stem cells derived from the adult testis could serve two distinct and important functions in the future. First, this alternative stem cell type provides a useful research tool in parallel with ES cells and iPS cells to probe the mechanisms by which pluripotency is acquired and maintained. Second, patients affected by a plethora of pathological conditions (including heart failure, neurodegenerative diseases, diabetes, etc.) could theoretically benefit from an alternative source of self-renewing pluripotent stem cells. These cells could be expanded and differentiated *in vitro* into the desired cell type before being transplanted to the patient. ES cells represent the paradigm of such a stem cell. However, the use of ES cells in clinical practice is hindered by two major problems. First, ethical concerns arise from the fact that the derivation of human ES cells requires the destruction of human embryos. Moreover, it is impossible to derive patient-specific ES cells and therefore immunological rejection is a major issue for their use in regenerative medicine. If pluripotent stem cells could be derived directly from adult tissues, both issues could be solved.

It has already been discussed in this chapter that pluripotent ES-like stem cells can be spontaneously derived from the adult testis, perhaps from SSCs. Theoretically, similar cells could be derived directly from a male patient, serving as a tool for regenerative therapy without ethical or immunological concerns. We and others demonstrated that upon *in vitro* differentiation ES-like cells can originate different cell types, as contractile cardiomyocytes, endothelial cells, neural cells, pancreatic cells, and others (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007; Guan et al. 2006; Izadyar et al. 2008; Conrad et al. 2008; Kossack et al. 2009). We also found that the endothelial cells formed by the ES-like cells participate in the generation of blood vessels that are connected with the host circulation and therefore are functional (Seandel et al. 2007).

More recently, two research groups have independently demonstrated that murine pluripotent stem cells derived from either adult or neonatal SSCs can be efficiently differentiated into cardiomyocytes using the protocols already available for ES cells. In the first study, pluripotent cells derived from mouse neonatal SSCs were compared to other pluripotent cells (ES and EG cells) in terms of their ability to differentiate into cardiomyocytes and endothelium (Baba et al. 2007). Notably, ES-like cells and EG cells were actually more efficient than ES cells in generating cardiomyocytes, suggesting that germ cell-derived pluripotent cells might be somewhat biased toward mesodermal differentiation. This is consistent with the fact that, even in the undifferentiated state, ES-like cells may express higher levels of mesodermal markers than ES cells (Seandel et al.

unpublished data; Seandel et al. 2007). The ability of ES-like cells to differentiate into functional cardiomyocytes exhibiting spontaneous action potentials, as well as electromechanical coupling between cells, have been confirmed by another study (Guan et al. 2007). Moreover, when undifferentiated ES-like cells were transplanted directly into the hearts of normal mice they proliferated in the site of engraftment, gradually lost their pluripotency, and differentiated into vascular endothelial and smooth muscle cells. Unfortunately, no evidence of differentiation into cardiomyocytes was found (Guan et al. 2007). This could be due to the fact that the ES-like cells were implanted into normal hearts and failed to be efficiently recruited to the cardiomyocyte fate, as occurs in infarcted hearts (Singla et al. 2006). The generation of cardiomyocytes could also be improved by pre-differentiating the cells *in vitro* before transplantation (Zeineddine et al. 2005). It is quite interesting that the transplantation of undifferentiated ES-like cells did not result in tumor formation, since the ability to form teratomas is an intrinsic property of pluripotent cells (and a key criteria for demonstrating pluripotency) (Damjanov and Solter 1974; Evans and Kaufman 1981; Wobus and Boheler 2005). However, it has been reported previously that intramyocardial transplantation of undifferentiated ES cells in both mice and rats did not result in tumor formation either (Singla et al. 2006; Min et al. 2002). More recently, it has been found that the formation of teratomas in the heart depends on the number of undifferentiated ES cell transplanted (Behfar et al. 2007).

Using conditions already established for ES cells, germ cell-derived ES-like cells have been induced towards neural differentiation (Glaser et al. 2008). Similar to ES cells, they formed different kinds of neurons (GABAergic, glutamatergic, serotonergic, and TH-positive) and glial cells (astrocytes and oligodendrocytes). During the differentiation process multipotent neural stem cells were formed that could be propagated as stem cells for many passages and also differentiate into both neurons and glia. The neurons derived from ES-like cells showed action potentials and were organized in functional synaptic networks. Interestingly, oligodendrocytes derived from ES-like cells were able to home and form myelin in slices of central nervous system tissue of myelin-deficient rats, suggesting that ES-like cells could be useful to treat demyelinating disorders (Glaser et al. 2008). More recently, ES-like cells have been differentiated in hepatocytes at a level comparable to ES cells. However, the amount of hepatocytes generated with the protocols described from both ES and ES-like cells is still too little to be considered useful in clinical setting (Loya et al. 2009).

The studies above demonstrate that mouse ES-like cells can be differentiated *in vitro* using the same protocols already established for mouse ES cells. Although still very preliminary in this regard, some of these studies tried to evaluate both the ability of ES-like cells to improve pathological conditions and their safety after transplantation (Guan et al. 2007; Glaser et al. 2008). It appears likely that further studies with the same rationale will support the ability of transplanted ES-like cells to rescue disease models in a manner comparable to that of ES cells.

In addition, it has also been reported that is possible to correct a genetic defect in mouse ES-like cells by using a human artificial chromosome (HAC). The delivery

of a HAC has been used to restore P53 expression and function in P53 null ES-like cells. These cells retained the ability to differentiate along several lineages both *in vitro* and *in vivo*. This report constitutes the first indication that ES-like cells can be used for gene therapy (Kazuki et al. 2008).

The recent demonstration that pluripotent stem cells can also be derived from human testis demonstrates the feasibility of using a testis biopsy to derive patient-specific ES-like cells for the use in regenerative medicine (Conrad et al. 2008; Kossack et al. 2009). These studies showed that human ES-like cells can be differentiated *in vitro* into myogenic, osteogenic, pancreatic, and neural cells via spontaneous differentiation or using the protocols currently available for human ES cells. The developmental plasticity of the human ES-like cells was generally comparable to that of human ES cells, which were actually *less* efficient than ES-like cells in forming differentiated pancreatic cells (Conrad et al. 2008).

Perhaps the most important consideration in the use of pluripotent cells in clinic is safety. Teratomas are benign tumors that contain self-renewing stem cells, as well as their differentiated progeny, and can be induced experimentally through injection of pluripotent stem cells in different sites. The ES-like cells, as a pluripotent stem cell type, are generally able to form teratomas, with certain exceptions (see Table 2.1). Therefore, it is possible that teratomas could arise even after therapeutic transplantation. Since only the undifferentiated stem cells produce teratomas, one way to circumvent this problem, already exploited for ES cells, is to efficiently differentiate the pluripotent cells before transplantation (Blum and Benvenisty 2008). There is no a priori reason to believe that the risk of teratoma formation associated with the ES-like cells would be higher than the risk associated with ES cells.

The pioneering work of S. Yamanaka's group introduced a promising alternative to obtain pluripotent cells from adult tissue (Takahashi and Yamanaka 2006). Using viral transduction of pluripotency-related genes (*Oct4, Sox2, Myc, Klf4*) differentiated cells were reprogrammed into iPS cells. The latter approach has been since exploited by numerous research groups, and it is now possible to produce both mouse and human iPS cells (Okita et al. 2007; Takahashi et al. 2007; Park et al. 2008). The safety of the first generation of iPS cells for clinical use has been a major concern. Besides the previously discussed risk of teratoma formation, which is intrinsic to any pluripotent cell, iPS cells also have an increased risk of oncogenic transformation due to multiple stable integrations of the oncogenes typically used to trigger the reprogramming process (like *Myc* and *Klf4*) and that can be reactivated over time (Okita et al. 2007). Therefore, while terminally differentiated cells produced from ES cells or ES-like cells should be safe in theory, those derived from iPS cells still bear a high risk of oncogenic transformation. Recently, different strategies have been applied to reduce the oncogenic potential of iPS cells, for example, by reduction of the number of exogenous genes necessary for obtaining the reprogramming. This can be achieved by using somatic cells that endogenously express some of reprogramming factors, like neural stem cells or by substituting some of the reprogramming factors with small molecules (Kim et al. 2009b, c; Shi et al. 2008). Additionally, nonintegrating means of gene delivery have been

exploited (Okita et al. 2008; Stadtfeld et al. 2008). The most promising strategy toward the clinical application of iPS cells is the direct delivery of the reprogramming factors as recombinant proteins instead of DNA, which has been demonstrated very recently to be feasible in both mouse and humans and which may decrease the possibility of oncogenic transformation of these iPS cells (Kim et al. 2009a; Zhou et al. 2009). Nevertheless, germline-derived stem pluripotent stem cells, iPS cells and ES-like cells should all be regarded as good model systems to study the reprogramming process involved in the reacquisition of pluripotency. The comparison between these systems can shed light on the underlying mechanisms of pluripotency and on why germline cells are able to be reprogrammed to pluripotency simply by the culture environment whereas somatic cells require the delivery of cocktails of pluripotency-related factors.

2.8 Conclusions

The spontaneous conversion of unipotent germ cells into pluripotent ES-like cells constitutes an ideal model with which to unravel the mechanisms necessary to switch a lineage-committed cell into a pluripotent cell. While the pluripotent stem cells derived from conversion of SSCs produced in different laboratories all share several major characteristics with ES cells, including their ability to self-renew and differentiate into many cell types, there are potentially important and revealing distinctions (Kanatsu-Shinohara et al. 2008b). Elucidation of these differences could reveal the answer to the intriguing question of whether more than one set of signals can lead to the pluripotent phenotype. Functional data indicate that ES-like stem cells derived from the testis represent at least a good research tool for probing the acquisition of pluripotency and perhaps even a viable alternative to ES cells and iPS cells for *in vitro* production of tissues for use in regenerative applications. However, extensive work needs to be done to improve the efficiency of production of ES-like cells, as well as to demonstrate their safety, before they can be implemented in clinical trials.

One might hypothesize that the reason why SSCs can convert to pluripotent cells spontaneously is that they are the only adult cells that maintain expression of the most important pluripotency factor OCT4. In this scenario their conversion to pluripotent cells would just simply follow the same processes that are involved in the conversion of somatic cells in iPS cells. In fact, it has already been demonstrated that somatic cells that express endogenously some of the pluripotency factors (like neural stem cells) can be reprogrammed by the expression of only the missing ones (Kim et al. 2009b). However, at the present stage it cannot be excluded that the set of molecular changes that take place to convert a SSC to a pluripotent cell could be different from those necessary to convert a somatic cell to a pluripotent stem cell. We believe that the comparison of the two conversion processes at the molecular level would shed light on the basic requirements for pluripotency as well as on how many possible pathways exist to regain pluripotency after differentiation.

2.9 Addendum

Since this chapter was originally prepared, two recent publications merit brief mention. First, Kuijk et al. (2010) have demonstrated that NANOG is present at the level of mRNA and protein in the adult testis of various species, particularly in meiotic and post-meiotic germ cells but also at lower levels in spermatogonia (Kuijk et al. 2010). Second, using an experimental approach, Ko et al. (2010) have strongly questioned the results of Conrad et al. (2008) and suggested that the cells produced in the latter study were not actually pluripotent but rather more similar to fibroblasts (Conrad et al. 2008; Ko et al. 2010).

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Chapter 3
Making Germ Cells from Human Embryonic Stem Cells

Sonya M. Schuh-Huerta and Renee A. Reijo Pera

Abstract Human embryonic stem cells are proliferating, self-renewing cells that have the great potential of differentiating into diverse cell types *in vivo* and *in vitro*. Investigations on human embryonic stem cells have allowed us to probe critical early stages of development, including the mechanisms of pluripotency, lineage specification, the formation and differentiation of specific cell and tissue types, and the underlying molecular and genetic mechanisms. In addition to the three main tissue lineages, embryonic stem cells can also give rise to the germ cell lineage, which produces the male or female gametes. With the difficulties of studying early human germ cell development *in vivo*, stem cells can provide a unique model and window into human germ cell differentiation. Further, as infertility is quite common in humans, most often due to defects in sperm and egg quantity or quality, embryonic stem cells and the recently discovered induced pluripotent stem cells might one day provide clinical applications for the treatment of infertility and reproductive disorders. Thus, stem cells have the great potential to revolutionize regenerative and reproductive medicine and numerous cutting-edge investigations and techniques are underway. This chapter summarizes our current understanding of the earliest events of human germ cell formation and gamete differentiation both *in vivo* and *in vitro* and the genetic requirements of this process as resolved from both human and animal studies. We also review the current literature on the formation of germ cells from embryonic stem cells and, finally, discuss needed future improvements and clinical implications of this work.

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Keywords Germ cells • Gametes • Sperm • Oocytes • Deleted in AZoospermia • Human embryonic stem cells • Induced pluripotent stem cells

3.1 Introduction

The study of early human development has been difficult due to obvious technical, biological, and ethical constraints, and much of what we know has come from studies of animal models. However, current scientific advances in the fields of stem cell and reproductive biology and genetics have led to an improved understanding of some of the genes, molecular events, and signaling pathways of early human development. Over the past decade, with limited studies on human embryos and numerous investigations using human embryonic stem cells (hESCs), we are beginning to make great strides in understanding early human development and disease. Human embryonic stem (ES) cells have generated much excitement as an unlimited source of cells that have the potential of differentiating into all cell types. More recently, a variety of human somatic cell types have been reprogrammed using specific factors *in vitro* to generate induced pluripotent stem cells (iPSCs), capable of giving rise to all tissue lineages (Kim et al. 2009; Park et al. 2008; Takahashi et al. 2007; Yu et al. 2007, 2009). Of great interest is the observation that human ES and iPS cells can also give rise to the germ cell lineage, which produces the male or female gametes or sex cells. This is the focus of this chapter.

Human ES cell lines currently in existence were originally derived from donated early stage human embryos (Thomson et al. 1998). The inner cell mass of the blastocyst-stage human embryo, shown at several early stages in Fig. 3.1, develops into the embryonic epiblast and hypoblast. The epiblast gives rise to the three somatic embryonic lineages, including the endoderm, mesoderm, and ectoderm, in addition to the germ cell lineage, while the hypoblast will form the extraembryonic endoderm. Embryonic stem cells are obtained from the inner cell mass of the embryo (Fig. 3.1d5, *white arrow*) and are cultured and propagated *in vitro*. ES cells are an unlimited source of self-renewing, proliferating cells as well as pluripotent, since they have the potential to give rise to all somatic cell types (Fig. 3.1). Excitingly, recent investigations have demonstrated that both mouse and human ES cells can also give rise to germ cells. Remarkably, mature germ cells with all of the hallmarks of sperm were recently differentiated from mouse ES cells (mESCs) *in vitro* and used to fertilize eggs, resulting in the first live births of viable offspring in mice (Nayernia et al. 2006). Several studies have revealed convincing evidence that human ES cells might also be able to generate mature germ cells and that hESCs may be used as an *in vitro* human genome-based model system to study germ cell formation and gametogenesis.

Embryonic stem cells have been differentiated into germ cells *in vitro* using several successful culture conditions, including the following: differentiation of ES cells in suspension into embryoid bodies (EBs), which mimic the early developing embryo containing multiple tissue lineages, then subsequent isolation of EB-derived

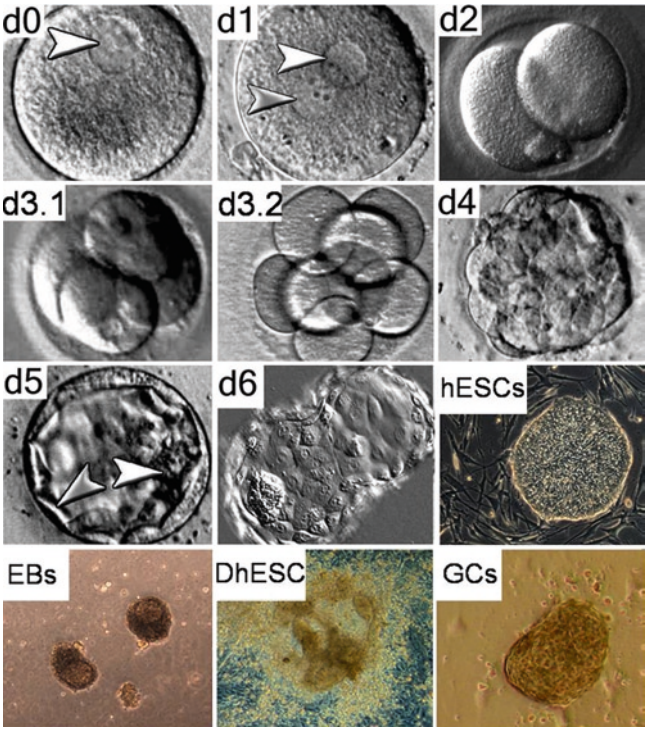

Fig. 3.1 Early human embryonic development and germ cell differentiation from human embryonic stem cells. Shown is a series of microscopic images taken of: (*d0, day 0*) the primary oocyte with an intact germinal vesicle (*arrow*); (*d1*) one-cell embryo, commonly referred to as a zygote, on day 1 as evidenced by the appearance of two pronuclei containing the male and female set of chromosomes (*arrows*); (*d2*) two-cell embryo on day 2; (*d3.1*) four-cell embryo on day 3; (*d3.2*) eight-cell embryo at the end of day 3; (*d4*) morula on day 4; (*d5*) the blastocyst showing the inner cell mass (*white arrow*) on day 5; (*d6*) hatching of the blastocyst from the zona pellucida on day 6; (*hESCs*) a human ES cell colony cultured on mouse embryonic fibroblast feeder cells for 4 days; (*EBs*) human ES cells spontaneously differentiating in suspension as embryoid bodies for 2 days; (*DhESC*) differentiating human ES cells in adherent culture on Matrigel for 5 days; (*GCs*) a germ cell colony after isolation of germ cells from EBs and subsequent culture in defined medium on mouse embryonic fibroblast cells for 14 days (*d0*, *d1*, *d3.1*, and *d3.2* adapted from Dobson et al. 2004; *d2*, *d4*, *d5*, and *d6* from R. Reijo Pera; *hESCs*, *EBs*, and *DhESC* from S. Schuh-Huerta; *GCs* image from K. Kee)

germ cells and further culturing with various defined factors or somatic cell types; and adherent differentiation of ES cells cultured on Matrigel, and subsequent isolation and culturing of putative germ cells (Fig. 3.1). In most cases germ cells are identified and isolated by using either integrated genetic constructs fused with fluorescent proteins that serve as germ cell reporters and/or germ cell-specific cell surface markers and cell sorting techniques. This relatively new field of germline stem cell biology has the potential to uncover the as yet to be identified unique genetic requirements, signaling pathways, and developmental programs of the human

gametes. The ability to derive and differentiate human male and female germ cells *in vitro* holds great promise for not only better understanding early gamete development and disease, but also may have important clinical implications for treating reproductive disorders. As infertility affects about 15% of reproductive-aged couples (Hull 1985), most commonly due to quantitative and qualitative defects in sperm and egg production, human ES and iPS cells might one day provide clinical applications for the treatment of infertility. Further, a human egg-like cell generated from hESCs or iPSCs *in vitro* has the potential for use in somatic cell nuclear transfer (SCNT). Thus, embryonic and pluripotent stem cells have the great potential to revolutionize regenerative and reproductive medicine.

3.2 The Formation and Differentiation of Gametes *In Vivo*

3.2.1 Germ Plasm Versus Inductive Signaling

The formation and differentiation of the gametes, sperm and eggs, are arguably two of the most important events for sexually reproducing species. In animals, the specification and formation of the primordial germ cells (PGCs), the founding cells of the germ cell lineage, occur by one of two distinct mechanisms: a germ plasm mode of inheritance or inductive signaling (Santos and Lehmann 2004; Strome and Lehmann 2007). In most organisms including invertebrate species such as flies and worms, and non-mammalian vertebrates such as frogs and fish, the germ cells arise through the inheritance of the germ plasm. This microscopically distinct, specialized cytoplasm is a maternally deposited collection of specific RNAs, RNA-binding proteins, and ribosomes that are set aside early in embryonic development from the somatic cells that form the rest of the embryo. The germ plasm is segregated within the zygote and the subsequent primordial germ cells are prevented from differentiating into somatic cells by repression of the global transcriptional and translational machinery (Santos and Lehmann 2004; Strome and Lehmann 2007). Elegant studies in the early twentieth century demonstrated that the germ cell lineage is established by the assembly, segregation, and deposition of this microscopically detectable germ plasm from the oocyte to the cells that are destined to become the germ cells. Later work demonstrated that this germ plasm is sufficient for the formation and maintenance of early germ cell populations and that several specific proteins, RNAs, and genes are involved in germ plasm assembly, germ cell formation, identity, and migration (Santos and Lehmann 2004; Zhou and King 2004).

In contrast, in mice and probably all mammals, the germ cells arise later in embryonic development through the process of inductive signaling, whereby specific signals are secreted by neighboring cells that serve to induce the formation of the germ cells. Although a germ plasm is not detectable in the mammalian oocyte, recent evidence indicates that many genes that encode homologs of germ plasm components are conserved and expressed in germ cells in mammals. These germ plasm homologs include *DAZ* (*Deleted in AZoospermia*), *DAZL* (*DAZ-Like*),

BOULE, *NANOS*, *PUMILIO* (*PUM*), and *VASA* genes, and functional data indicates that these genes are required for the establishment, maintenance, and differentiation of germ cells in various organisms (Eberhart et al. 1996; Houston et al. 1998; Houston and King 2000; Johnson et al. 2001; Karashima et al. 2000; Lin and Page 2005; Maegawa et al. 1999; Reijo et al. 1995; Ruggiu et al. 1997; Tsuda et al. 2003). Notably, in both germ plasm inheritance in non-mammalian species and inductive signaling in mammals, the germ cells are maintained by mechanisms that prevent them from differentiating into somatic cells. Indeed, the observation that in many animals the PGCs arise outside of the embryo proper during the time that the somatic cell program is being established may have functional significance in normal germ cell formation.

3.2.2 The Morphological Features of Gamete Development

From limited human embryological studies and studies in animal models focused on germ cell development we have gained some understanding of the complex temporal and spatial sequences of early germ cell development and gamete differentiation *in vivo*. The formation of the PGCs is an early event during human development. The PGCs arise in the proximal epiblast immediately outside of the extra-embryonic ectoderm. Within the first 2–3 weeks of embryonic life, after gastrulation of the embryo, the germ cell lineage begins as a small population of PGCs located just outside of the embryo in the developing yolk sac (Larsen 1997; McKay et al. 1953; Moore and Persaud 1998; Motta et al. 1997). During the first 5 weeks of gestation, the PGCs begin to migrate from the yolk sac into the developing embryo (Park et al. 2009). At this time the genital ridges begin developing from the intermediate mesoderm, which ultimately forms all the somatic cells of the gonad. The PGCs migrate along the hindgut to the genital ridges, undergoing massive proliferation along their journey.

Once PGCs arrive in the nascent gonads, between 5 and 6 weeks of gestation, the PGCs continue to proliferate, remaining connected by intercellular cytoplasmic bridges (Motta et al. 1997; Heyn et al. 1998). In the female fetus, within the ovaries these PGCs, termed gonocytes, begin oogenesis, differentiating into some 5–7 million oogonia. Each oogonium then enters meiosis and develops within the supportive surrounding granulosa and thecal cell layers and connective tissue that make up the follicle. The development of oocytes is then halted at meiotic prophase I during the 12th week of gestation (Motta et al. 1997). Subsequent follicle loss by atresia or cell death begins, resulting in approximately one million follicles by the time of birth. By the time of sexual maturation or puberty, roughly 500,000 follicles remain within the ovaries. At this time oocytes are either recruited to resume meiosis, mature, and are ovulated, or they continue to undergo atresia. With no oogonial stem cells, over time the oocyte population is depleted until approximately 1,000 or less remain, menstrual cycling ceases, and menopause ensues. In fact, the total oocyte pool or "ovarian reserve" can be correlated with reproductive potential and

reproductive events including ovarian aging, the decline and eventual loss of fertility, and menopause (Moore and Persaud 1998; Bancsi et al. 2002; de Boer et al. 2002; Faddy et al. 1992; Hansen et al. 2008; Nichols et al. 2006; Richardson et al. 1987; te Velde et al. 1998; Whelan et al. 1990). Despite recent work in mice suggesting the existence of oogonial stem cells (Johnson et al. 2004, 2005), it is thought that without this population of developmentally arrested oocytes the ovarian reserve becomes depleted.

In contrast to that of the female, male germ cell development and spermatogenesis proceeds along quite a different course. The male PGCs form gonocytes, which colonize the basement membrane of the seminiferous cords of the testes, which later form the seminiferous tubules. The gonocytes differentiate into intermediate spermatogonia and prespermatogonia, which are the precursors of the diploid spermatogonia of the adult (Gaskell et al. 2004). Similar to other mammals, the gonocytes and later, spermatogonia, remain connected by cytoplasmic bridges and are enclosed within the supportive Sertoli cells lining the seminiferous tubules, isolating the germ cells within their own compartment. The Sertoli cells are also in close proximity to the interstitial hormone-secreting Leydig cells. Both the Sertoli cells and spermatogonia are mitotically active during the pre-pubertal period in primates. Notably, the spermatogonia do not enter meiosis during fetal development, but rather await the endocrine signals of puberty to initiate spermatogenesis, proliferate into clonal expansions of spermatogonia, undergo meiosis, become terminally differentiated, and produce mature haploid spermatozoa capable of fertilization (McLaren 2000; Yanagamachi 1994). Importantly, a reserve of spermatogonial stem cells located at the periphery of the seminiferous tubules remains throughout the male's entire life, allowing the continual production of sperm (Larsen 1997; Yanagamachi 1994; Adams and McLaren 2002; McLaren and Southee 1997).

The development of sperm and their movement in the testis occurs progressively from the periphery to the lumen of the tubules, and occurs in waves along the tubules. In the final stage of spermatogenesis spermatids undergo the process of spermiogenesis where they grow a tail or flagellum, develop an acrosomal vesicle, condense and repackage their chromatin, and shed most of their cytoplasm. This process gives rise to fully developed sperm that are shed from the lumen, leave the testis, and enter the epididymis for final maturation and storage. Mature sperm are highly polarized, terminally differentiated cells incapable of transcription and protein synthesis. The specialized structural and cellular features of the spermatozoa reflect its unique physiological role: to ensure the delivery of the male's genetic material to the egg. The specialized sperm cell has the unique ability to find, fuse with, and activate the egg. In the human, the entire process of spermatogenesis and maturation takes approximately 77 days. A man in his reproductive prime produces up to 240 million sperm per day, up to 450 million per ejaculate, and nearly 3,000 per heart beat [(Schuh 2007; Sutovsky and Manandhar 2006); J. Amory, personal communication].

Although most sperm have the same basic morphology, including a head and flagellum, and essential cellular components, there is variation in size and structure between different species (Bedford and Cross 1998; Eddy and O'Brien 1994).

Generally, sperm range from ~30 to 250 μm long, depending on the species (Baccetti and Afzelius 1976). However, there are striking exceptions, including the giant 6-cm-long sperm of the tiny fruit fly *Drosophila bifurca* (Pitnick et al. 1995) and the tail-less ameboid sperm of the round worm *Caenorhabditis elegans* (Kubagawa et al. 2006). One interesting paradox is that some of the smallest animals have some of the largest sperm, and the largest animals have some of the smallest sperm (whales with ~30–60 μm long sperm). These differences may be due to evolutionary pressures shaped by mating behavior, occurrence of inter-male sperm competition, and the shape and size of the female reproductive tract (Schuh 2007; Bjork and Pitnick 2006; Parker et al. 1972; Shuster and Wade 2003).

While there are great size differences, mammals show less variation in sperm structure than other animals. Although sperm are uniform in size and shape within most species, there is some variability in sperm morphology, especially the head, in human sperm (Eddy and O'Brien 1994). There is also great variation in the numbers of sperm that are produced both between species and among members of the same species. Notably, there is variability in the quantity of sperm produced by different men and by the same man over time. The reference values for normal sperm parameters of seminal fluid are >50% motile sperm, >20 million sperm per ml, and >40% normal morphology (WHO 1999). Decreases in any of these can synergistically decrease male fertility. A main cause of infertility in men is the production of reduced numbers of sperm, and sperm with defects in motility [J. Amory, personal communication; (Guzick et al. 2001; Turner 2003)]. It is estimated that about 75% of infertile men are either oligospermic (have few sperm; <20 million per ml) or have a high percentage of immotile sperm (asthenozoospermia) (Baker et al. 1986).

3.2.3 The Molecular Requirements of Germ Cell Development

Although we have an increasing understanding of the morphological sequences of human germ cell, and subsequent sperm and egg development *in vivo*, we have yet to elucidate the many genes, proteins, and molecular events that underlie this important process. We also have gained some insight about many of the genes and signaling pathways that function in mature human gametes. However, despite its importance to understanding reproductive health and fertility, what little we know about the genetics and molecular events of early human germ cells has been mostly extrapolated from studies in mice. It is likely that human germ cells are specified and formed through the action of sequentially expressed genes similar to that of mice and other model organisms. Thus, the generation of knockout and transgenic mice has been integral in elucidating some of the key genetic pathways of early germ cell development.

In mice, with a total gestation of roughly 20 days, specification of PGCs occurs relatively late in embryonic development. After implantation of the blastocyst, which occurs on embryonic day 5 (E5), the PGCs arise around E7.2 by inductive

signaling of a group of cells located in the proximal epiblast at the base of the allantois (Santos and Lehmann 2004; Hayashi et al. 2007; McLaren 2003). These PGCs express specific mRNA and protein markers including the cell membrane protein tissue nonspecific alkaline phosphatase (TNAP), Oct4 (or Pou5f1, POU-domain class-5 transcription factor 1), and Stella (Dppa3), markers also expressed in human ES cells (Clark et al. 2004b; Niwa et al. 2000; Saitou et al. 2002; Scholer et al. 1990). Recent work has found that specific bone morphogenetic proteins (BMPs) are released by the extraembryonic ectoderm and visceral endoderm to induce the neighboring cells of the proximal epiblast to adopt this primordial germ cell fate (Santos and Lehmann 2004; Hayashi et al. 2007). Specifically, null alleles of *Bmp4*, *Bmp7, Bmp8b*, *Bmp2*, the TGF-β type I receptor *Alk2*, and the BMP signaling transduction molecules *Smad1*, *Smad4*, and *Smad5* all result in greatly reduced or absent primordial germ cells (Chang and Matzuk 2001; Chu et al. 2004; de Sousa Lopes et al. 2004; Tremblay et al. 2001; Winnier et al. 1995; Ying and Zhao 2001; Ying et al. 2000; Zhao et al. 1996, 2001). Most notably, these findings emphasize the important role of BMP signaling in early germ cell development and maintenance.

Signaling by the transcriptional repressor, Blimp1/Prdm1 is also critical in the specification and maintenance of the early primordial germ cell precursors. Animals lacking normal *Blimp1/Prdm1* genes have reduced numbers of PGCs and misexpression of several somatic genes in their germ cells (Hayashi et al. 2007; Chuva de Sousa Lopes and Roelen 2008; Ohinata et al. 2005). *Blimp1* expression is first detected at E5.5 in a few proximal-posterior epiblast cells, which are the lineage-restricted PGC precursor cells. *Blimp1* represses the somatic program in these cells and promotes their progression toward the germ cell fate. Other studies, using neutralizing antibodies during specification to block E-cadherin and hence cell-to-cell-contact, found a reduction in the number of founder PGCs, emphasizing the importance of intercellular interactions among these initial PGCs (Okamura et al. 2003). Formation of the initial cohort of approximately 40 identifiable founder PGCs, expressing the germ cell marker *Stella*, occurs at E7.25 (Hayashi et al. 2007; Saitou et al. 2002; Ohinata et al. 2005).

During the time that the PGCs migrate into the embryo and along the hindgut to the genital ridges near the mesonephros, an entirely different developmental program occurs and other distinct molecular events are critical. It is during this migration, from ~E7.25 to E10.5, that massive proliferation and reprogramming of the PGCs occurs, including erasure of genomic methylation at both imprinted (sex-specific) and non-imprinted loci as well as changes in histone modifications and chromatin structure. Subsequent colonization and proliferation of the PGCs, now termed gonocytes, in the gonad occurs around E10.5. The gonia then become developmentally arrested at E15.5 (McLaren 2000, 2003; Hayashi et al. 2007). Several molecules, especially those that are involved in the transduction of extracellular signals and interaction with the surrounding somatic environment are critical during the process of migration and colonization. Mouse knockout models lacking the genes for the *Kit* (kit oncogene) ligand, *ckit* receptor, *Sdf1* ligand, the chemokine receptor *Cxcr4*, and the *β1 integrin* receptor all have great reductions in the number of migrating PGCs before reaching the gonads at E9.5 (Anderson et al. 1999;

Ara et al. 2003; Besmer et al. 1993; Koshimizu et al. 1992; Molyneaux et al. 2003; Zou et al. 1998). Further, knockout mice with null alleles of the RNA-binding proteins, *Tial1* and *Nanos3*, have normal numbers of specified PGCs, but no germ cells that survive the migration and colonize the gonads. Thus, these knockout animals are both male and female infertile (Tsuda et al. 2003; Beck et al. 1998; Suzuki et al. 2008). Another gene involved in the process of PGC migration is *Oct4*, the classic marker of pluripotency that is critical for the establishment of the embryonic inner cell mass and the generation of embryonic stem cell lines (Thomson et al. 1998). A conditional mouse knockout lacking *Oct4* exclusively in the germ cell lineage was created using the *Cre Recombinase* strategy to circumvent the importance of *Oct4* in the blastocyst. These germ cell-specific *Oct4* null animals have a reduced number of PGCs that reach the gonads and diminished fertility or complete infertility (Kehler et al. 2004). Several of the above mentioned genes function as translational repressors (*Tial1* and *Nanos3*) and transcription factors (*Oct4*), but exactly how they regulate PGC survival, migration, reprogramming, and germ cell identity has yet to be elucidated.

Although we have gained important information about the candidate genes and signaling pathways of germ cell development from the mouse model, it has become clear that there are many unique aspects to human germ cell development, especially pertaining to the genetic requirements. Several of the genes expressed and functional in human germ cells are distinct from that of model organisms, including mice and other mammals. Many genes located on the human X and Y sex chromosomes have homologs that are expressed in different doses or, in some cases, are altogether absent in mice (see Sect. 3.3) (Reijo et al. 1995, 1996; Skaletsky et al. 2003; Vogt et al. 1996; Zinn et al. 1993). Women require two X chromosomes for oocyte development, while female mice are fertile with a single X chromosome (Zinn et al. 1993; Davison et al. 1999). Further, meiotic chromosome missegregation occurs much more frequently in human germ cells (5–20%) than in model organisms (Hunt 1998, 2006; Hunt and Hassold 2008). The human genome also contains rapidly evolving autosomal genes that are uniquely expressed in human germ cells, but differ greatly in sequence and timing of expression between even closely related species (Clark et al. 2004b; Saitou et al. 2002; Hendry et al. 2000; Swanson and Vacquier 2002). These observations demonstrate the need for further studies on human germ cell development, and the establishment of an *in vitro* human genome-based system to study this process.

3.3 The DAZ Gene Family

3.3.1 The Y Chromosome DAZ Genes

The most well-characterized genes to date definitively involved in human germ cell development and linked to infertility are the *DAZ* genes. The *DAZ* gene cluster, comprised of four nearly identical duplicated genes in tandem, were discovered in a screen for genes on the Y chromosome that cause azoospermia or the lack of

sperm production in men (Reijo et al. 1995, 1996; Saxena et al. 1996, 2000). Reijo et al. (1995) found that 10–15% of men with azoospermia or severe oligospermia [fewer than 20 million sperm per ml of ejaculate; WHO (1999)] had specific deletions of the long arm of the Y chromosome. Specifically, these deletions fell within the so-called Azoospermia Factor (AZF) region, and encompassed the newly discovered gene, termed *Deleted in AZoospermia* or *DAZ* (Reijo et al. 1995, 1996; Saxena et al. 1996). The most common phenotype associated with these deletions was the Sertoli Cell Only Syndrome, in which men are infertile, lack sperm, and only Sertoli cells can be identified in testicular biopsies (Fig. 3.2A). The *DAZ* genes are expressed exclusively in testis, in gonocytes of the fetus and several stages of developing sperm of the adult male (Reijo et al. 2000). As shown in Fig. 3.2B the *DAZ* genes contain a series of 8–18 *DAZ* repeat elements, an RNA-recognition motif, and presumably function as RNA-binding proteins in sperm. It was subsequently discovered that only humans and our closest hominid relatives, the great apes, including bonobos, chimpanzees, gorillas, and orangutans, as well as the Old World monkeys including macaques, have the Y chromosomal *DAZ genes* (Tung et al. 2006a; Yen et al. 1996).

3.3.2 *DAZ Family Genes Are Evolutionarily Conserved and Function in Gametogenesis*

Homologs of *DAZ* have been identified on human autosomal chromosomes, as well as in numerous animal species. The human *DAZ* gene family now comprises at least three members including: the Y chromosomal *DAZ*; *DAZL* (*DAZ-Like*), the autosomal "father" of *DAZ*; and the more recently discovered *BOULE*, the "grandfather" or ancestral gene of *DAZ* (Fig. 3.2B) (Saxena et al. 1996; Xu et al. 2001). *DAZL* is 95% identical to *DAZ* in protein sequence, and maps to chromosome 3, while the ancestor gene, *BOULE* maps to chromosome 2 (Fig. 3.2B). Interestingly, the genomes of different animals possess different complements of *DAZ* family genes. Thus, invertebrates such as flies and worms contain only a single *DAZ* homolog *boule*, while vertebrates, other than catarrhine primates (Old World monkeys and hominids) possess *Boule* and *Dazl* genes. As the *DAZ* gene cluster on the Y chromosome most recently arose during primate evolution within the last 30–40 million years, catarrhine primates are the only animals to possess *BOULE*, *DAZL*, and the *DAZ* genes (Yen et al. 1996; Xu et al. 2001).

As discovered from recent studies of animal models, these closely related RNA-binding proteins are universally expressed in the germ cell lineage and function in female and/or male germ cell development and gametogenesis. Members of the *DAZ* gene family are required for fertility in diverse organisms, but differ in null phenotypes and expression patterns. In the fruit fly *Drosophila*, disruption of the *DAZ* homolog, *Boule*, causes male meiotic arrest and halted spermatogenesis (Eberhart et al. 1996). In the nematode worm *Caenorhabditis elegans*, disruption of *Boule* causes meiotic arrest in oogenesis only (Karashima et al. 2000). In the frog

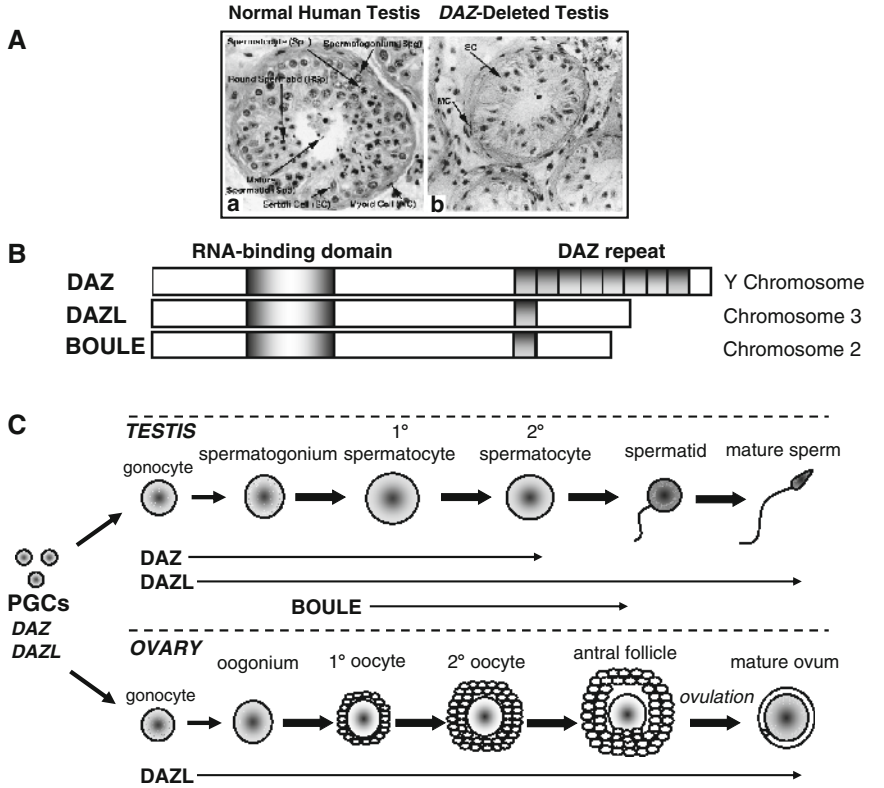

Fig. 3.2 Genes of the *DAZ* family encode RNA-binding proteins that are involved in human germ cell development and fertility. (**A**) Photomicrographs of hematoxylin and eosin stained cross-sections of seminiferous tubules obtained from testicular biopsies from of: (**a**) a normal fertile man showing normal spermatogenesis, and (**b**) a man with deletions of the *DAZ* genes showing a complete loss of developing sperm or Sertoli cell-only syndrome (*Spg* spermatogonia, *Spc* spermatocyte, *RSp* round spermatid, *Spd* mature spermatid, *MC* myoid cell, *SC* Sertoli cell). (**B**) Schematic representation of the human *DAZ* gene family including the *DAZ*, *DAZL*, and *BOULE* genes, depicting their conserved RNA-binding domains and *DAZ* repeat elements. (**C**) A chronological diagram of human gamete development from PGCs to mature sperm and oocytes, indicating the sequence of expression of the three *DAZ* genes; genes are indicated in bold under the different stages, with temporal gene expression indicated by *arrows* extending to the *right*

Xenopus, and the zebrafish *Danio*, the autosomal *DAZ* homolog *Dazl* encodes components of the germ plasm (Houston and King 2000; Houston et al. 1998; Maegawa et al. 1999; Mita and Yamashita 2000). In the frog *Dazl* is required for embryonic germ cell production and migration (Houston and King 2000) and in the zebrafish *Dazl* is expressed in primordial germ cells and developing gametes of the testis and ovary (Maegawa et al. 1999). In mice, disruption of the *Dazl* gene results in a loss of germ cells and complete absence of mature sperm and eggs and infertility in both males and females (Lin and Page 2005; Ruggiu et al. 1997; Lin et al. 2008; Saunders et al. 2003). Most evidence indicates that the sperm and eggs of

Dazl null mice cannot progress past meiotic prophase, with germ cell loss occurring in the prenatal female and postnatal male. However, the specific function of *Dazl* and exactly when the defect occurs during germ cell development in *Dazl* null mice has yet to be fully elucidated; work is underway to determine the functional role of *Dazl* in both *in vivo* and *in vitro* studies in mice (see Sect. 3.5).

In humans, all three *DAZ* family members are expressed in either fetal germ cells and/or various stages of gamete development and maturation (Fig 3.2C) (Reijo et al. 2000; Xu et al. 2001; Dorfman et al. 1999). *DAZL* is expressed in prenatal primordial germ cells and adult oocytes and follicles of the human female (Figs. 3.2C and 3.3A a and b) (Dorfman et al. 1999; Brekhman et al. 2000; Cauffman et al. 2005). *DAZ* and *DAZL* are expressed in prenatal germline stem cells, and spermatogonia, spermatocytes, and spermatids of the adult man (Figs. 3.2C and 3.3A e and f) (Reijo et al. 2000; Xu et al. 2001). *BOULE* is expressed later in development in the cytoplasm of pachytene spermatocytes, persists through meiosis, and decreases in early spermatids (Fig. 3.2C) (Xu et al. 2001). Interestingly, *DAZ* and *DAZL* transit from the nucleus of spermatogonia to the cytoplasm of spermatocytes during meiosis. Unlike *DAZ*, *DAZL* is further expressed in developing spermatids and even mature spermatozoa (Fig. 3.2C) (Reijo et al. 2000). It is likely that the *DAZ* genes have distinct, yet overlapping spatio-temporal functions as RNA-binding proteins in the establishment of spermatogonial stem cell and oogonial populations and gametogenesis.

If a gene impacts the number of germ cells a person is born with or the total gamete "pool" available, that gene should be expressed in the gametes and variable in the human population. Interestingly, Tung et al. (2006b) demonstrated that the *DAZL* gene is variable and associated with the age of menopause, an indirect measurement of the rate of oocyte/follicular depletion in women, as well as sperm counts in men. Variation in the *DAZL* gene was analyzed by direct genetic sequencing in 93 women with *Premature Ovarian Failure* (POF; menopause before the age of 40), 324 women with early (before age 46) or normal (after age 46) menopause, and 102 infertile men ages 22–51. Ninety-five sequence variants of *DAZL* were identified, most of which were single nucleotide polymorphisms or SNPs, where a single nucleotide differs between members of a species and typically exists as one of two alleles. Twelve of these *DAZL* SNPs were investigated for their association with POF. Four of these common SNPs were significantly associated with the age of menopause in the POF group, while six were significantly associated with total sperm count and total motile sperm count among the infertile male group. Haplotypes or combinations of associated SNPs of the *DAZL* gene were even more strongly correlated with reproductive parameters in men and women. Several other investigations on genetic variants of *DAZL* and reproductive parameters in men and women have yielded contradictory findings, likely due to variation in study population demographics, ethnic differences, sample sizes, and the polymorphisms analyzed among the studies (Becherini et al. 2004; Nuti and Krausz 2008; Teng et al. 2006; Thangaraj et al. 2006; Tschanter et al. 2004; Zerbetto et al. 2008).

The reported variants in the *DAZL* gene and their association with gamete parameters and infertility and/or age of ovarian failure further indicate a possible role

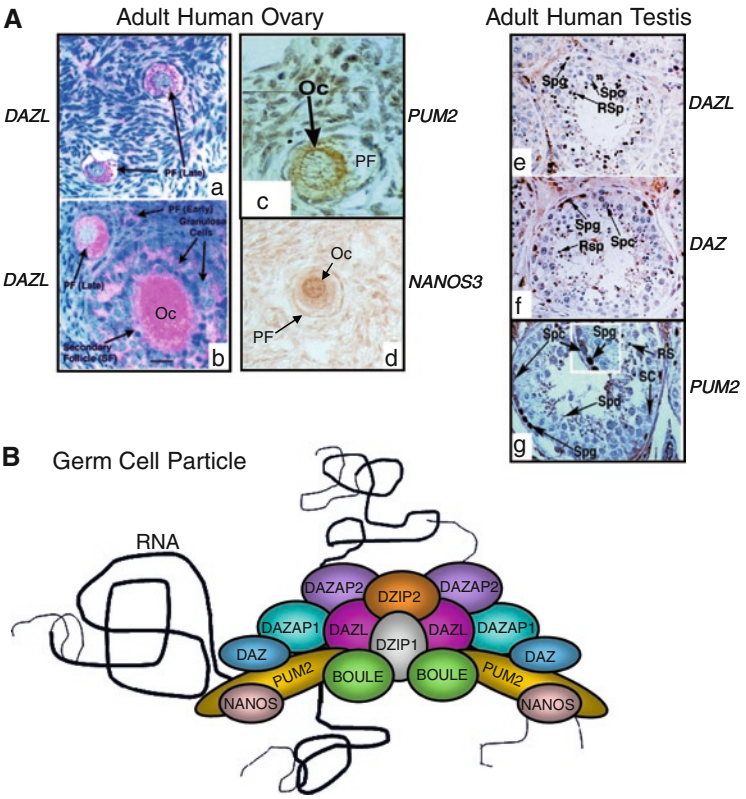

Fig. 3.3 *DAZ* gene family members and DAZ-interacting protein genes are expressed and function in human germ cells. (**A**) Immunohistochemical staining using specific antibodies or antisera to detect the protein expression of: (**a**) and (**b**) *DAZL*, (**c**) *PUM2*, and (**d**) *NANOS3* is limited to the oocyte (Oc), primary follicle (PF), secondary follicle (SF), antral follicle (not shown), and granulosa cells of the ovary of the adult woman; (**e**) *DAZL*, (**f**) *DAZ*, and (**g**) *PUM2* is limited to the spermatogonia (Spg), spermatocytes (Spc), spermatids (Spd), and round spermatids (RS/Rsp) of the testis of the adult man; note expression of these genes begins in the primordial germ cells of the female and male fetus. (**B**) Diagram of *DAZ* gene family proteins, DAZ-Interacting proteins, other associating proteins, and RNAs present in human germ cells. These molecules may interact in a complex that functions as a "germ cell particle" and contains many homologs of germ plasm components that function in germ cell development in diverse organisms (magnification ×200; bar = 10 μm; (**a**) and (**b**) adapted from Dorfman et al. 1999; (**c**) and (**g**) adapted from Moore et al. 2003; (**d**) provided by V. Angeles)

for this gene in the establishment of the germ cell population in humans. With great advances in genome-wide microarray technologies, it will be important to assess how common genetic polymorphisms across many genes and chromosomes are associated with follicle number in women and sperm counts in men. Rather than relying on retrospective indirect measurements of follicle depletion such as age at menopause/ovarian failure, we can now use transvaginal ultrasound technology to measure a woman's antral follicle count (AFC). Antral follicles are one of the final

stages of follicular development. One antral follicle gives rise to the large dominant follicle, which contains the oocyte that will be ovulated. AFC is correlated with oocyte number and may be an accurate reflection of the number of remaining oocytes in a woman's ovaries and a noninvasive window into the oocyte reserve (Giacobbe et al. 2004; Kwee et al. 2007; Morris et al. 2002). Ongoing studies are focusing on the determination of the genes and environmental factors that may impact the oocyte pool as assessed by AFC in a large group of women of several racial/ethnic groups (Schuh-Huerta et al., unpublished).

Pertaining to the molecular functions of the *DAZ* genes, it is interesting to note that DAZ and DAZL associate with RNAs (Fox et al. 2005; Venables and Eperon 1999), ribosomes (Tsui et al. 2000b), and several other RNA-binding proteins including, Pumilio-2 (PUM2), BOULE, NANOS, DAZ-Interacting Protein 1 (DZIP1), and DAZ-Associated Protein-1 and -2 (DAZAP1 and DAZAP2) (Fox et al. 2007; Moore et al. 2003, 2004; Tsui et al. 2000a; Urano et al. 2005). Several of these proteins are also required for fertility and germ cell development in diverse organisms (Eberhart et al. 1996; Houston and King 2000; Johnson et al. 2001; Karashima et al. 2000; Lin and Page 2005; Maegawa et al. 1999; Reijo et al. 1995; Ruggiu et al. 1997; Tsuda et al. 2003). Homologs of PUM2 and NANOS proteins act as translational repressors in germ cells in several species and are required for germ cell migration and proliferation. Notably, when these genes are knocked out in worms, flies, and mice the phenotype is a reduced number of germ cells in the gonads and complete infertility (Tsuda et al. 2003; Forbes and Lehmann 1998; Subramaniam and Seydoux 1999). It has been discovered that along with *DAZL* and associated proteins, *PUM2* and *NANOS3* are expressed at various stages of oocyte and follicular development in the woman and spermatogenesis in the man (Fig. 3.3A) (Moore et al. 2003). In fact, many of the proteins that associate with DAZ/DAZL are homologs of germ plasm components found in invertebrates and non-mammalian vertebrates. We propose that these molecules may interact in a complex or "germ cell particle" that is evolutionarily conserved and functions in germ cell development in diverse organisms from worms to humans (Fig. 3.3B). *DAZL*, along with several interacting proteins, may function in this germ cell complex to regulate RNA stability and translation of key proteins in early germ cells, eggs, and sperm. Early repression by this complex may ultimately determine quantitative and qualitative characteristics of the germ cell population. The lack of scientific tools to investigate this hypothesis *in vivo* as well as better understand the various developmental programs of the germ cells, necessitate studies using human embryonic stem cells *in vitro*.

3.4 The Generation of Germ Cells from ES Cells *In Vitro*

Accumulating recent work indicates that human ES cells can be used as a human genome-based model system to generate germ cells and more mature gametes and to study the molecular events of germ cell development and differentiation *in vitro*.

The following sections will summarize the current literature, outlining mouse and human ES cell investigations and their findings, in a chronological perspective leading up to the current status of this field and an examination of what the future holds.

3.4.1 Similarities Between ES Cells and Germ Cells

There are numerous similarities between embryonic stem cells and primordial germ cells, including morphological, genetic, and developmental features. Primordial germ cells have large nuclei with dispersed chromatin, prominent nucleoli, and very little cytoplasm or a high nucleus-to-cytoplasm ratio (Motta et al. 1997). Interestingly, human ES cells share these same ultrastructural cellular characteristics (Thomson et al. 1998; Sathananthan et al. 2002).

Both PGCs and ES cells also have great self-renewal capabilities and potential for pluripotency. Although PGCs are not pluripotent *in vivo*, remarkably when they are removed from the gonad and cultured *in vitro* they generate pluripotent embryonic germ cell (EGC) lines with nearly identical potential and morphology to hESCs (see Chap. 1 of this volume) (Donovan and De Miguel 2003; Shamblott et al. 1998). Exciting recent work has successfully used germline stem cell lines derived from the neonatal ovary to produce offspring in mice (Zou et al. 2009). Furthermore, recent studies in mice and humans have shown that adult spermatogonial stem cells (SSCs) can be cultured *in vitro* and that a subpopulation has the ability to be "reprogrammed" to a state of multipotency or pluripotency (see Chap. 2 and Part II of this volume). Although it was once thought that only fetal PGCs have the potential for pluripotency and giving rise to germ cell lines, these adult mouse multipotent germline stem cells (mGSCs) obtained from the testis can spontaneously differentiate into derivatives of all three primary germ layers and the germ line and contribute to chimeras (Brinster 2002; Guan et al. 2006, 2009; Kanatsu-Shinohara et al. 2004; Seandel et al. 2007). Notably, human adult germline stem cells derived from testicular biopsies have been propagated and differentiated into the three germ layers (Conrad et al. 2008; Kossack et al. 2009; Payne and Braun 2008). Some current studies in our laboratory are focused on expanding spermatogonia from small testicular biopsies, optimizing the conditions required to obtain hESC-like cells, and comparing the differentiation potential of these hESC-like cells with other pluripotent cell lines (Clavijo and Reijo Pera, unpublished).

Of great interest is the observation that both PGCs and hESCs express a unique combination of genes, referred to as stem/germ cell genes, that are exclusive to cells of the preimplantation embryo, inner cell mass, ooyctes, and/or human embryonic carcinoma cells (hECCs) (Clark et al. 2004b; Bortvin et al. 2003). Many proteins expressed in early germ cells are also expressed in mouse and human ES cells including, the transcription factor *OCT4* (Niwa et al. 2000), *PUM2* (Moore et al. 2003), and *NANOS1* (Clark et al. 2004a; Jaruzelska et al. 2003). Human and mouse PGCs and ES cells also display strong cell surface staining for TNAP (Gaskell et al. 2004). Further, three additional human genes, *NANOG* (the human homolog of

mouse *Nanog*), *STELLAR* (*Stella-Related*, or *Germ and embryonic stem cell enriched protein STELLA*), and *GDF3* (*Growth Differentiation Factor 3*) were cloned, and similar to *OCT4* their expression was exclusive to both undifferentiated hESCs and human germ cells. Upon differentiation of hESCs the expression of all three genes decreased, while the reduction in *NANOG* activity specifically led to a loss of hESC pluripotency (Clark et al. 2004b). Underscoring the importance of the proteins in common to both ES cells and germ cells is the observation that mutant phenotypes are often associated with both ES cell maintenance (or early embryonic development) and germ cell development (i.e., *Oct4* and *Tial1*) (Beck et al. 1998; Kehler et al. 2004).

The early differentiation of human and mouse germ cells is marked by formation of distinct translational protein complexes, which form a "germ cell particle." This germ cell particle includes homodimers and heterodimers of *DAZ* gene family members as well as many germ plasm homologs, such as the *PUM* and *NANOS* genes (see Fig. 3.3B) (Fox et al. 2007; Moore et al. 2003, 2004). Although human ES cells and germ cells express several of these same critical proteins, the spatial and temporal patterns of protein–protein interactions and signaling complexes apparently differ between germ cells and ES cells as resolved by Fluorescence Resonance Energy Transfer (FRET) (Fox et al. 2007).

In light of much recent work it has become evident that in addition to similar ultrastructural and developmental characteristics the genes expressed in ES cells and early germ cells form a largely overlapping set, suggesting that these cell types may be closely related or identical or may share common regulatory pathways (Clark et al. 2004a, b; Fox et al. 2007; Moore et al. 2003, 2004).

3.4.2 Can Human ES Cells Give Rise to Germ Cells?

There are clear morphological, developmental, and genetic similarities between undifferentiated germ cells and embryonic stem cells. Many investigations have shown that mouse and human ES cells can be differentiated into multiple tissue lineages *in vitro*. Therefore, it was logical to test whether human ES cells could also give rise to the germ cell lineage. Our laboratory first investigated whether human ES cell lines, like those of mice, were capable of forming germ cells *in vitro*. Clark et al. (2004a) used quantitative polymerase chain reaction (PCR) to examine the transcriptional profiles of three pluripotent human ES cell lines, HSF-6 and H9 (karyotype 46; XX), and HSF-1 (karyotype 46; XY) before and after differentiation.

Undifferentiated cultures of human ES cells were maintained on irradiated mouse embryonic fibroblast (MEF) cells, as human ES cells grow best when in contact with fibroblast cells of either mouse or human origin. All cultures were grown at 37°C with 5% CO_2 in a standard medium containing recombinant human basic fibroblast growth factor (bFGF). Similar to previous work, *OCT4* was expressed at high levels in all undifferentiated ES cell lines tested. Other genes examined included those previously reported in undifferentiated human ES cells,

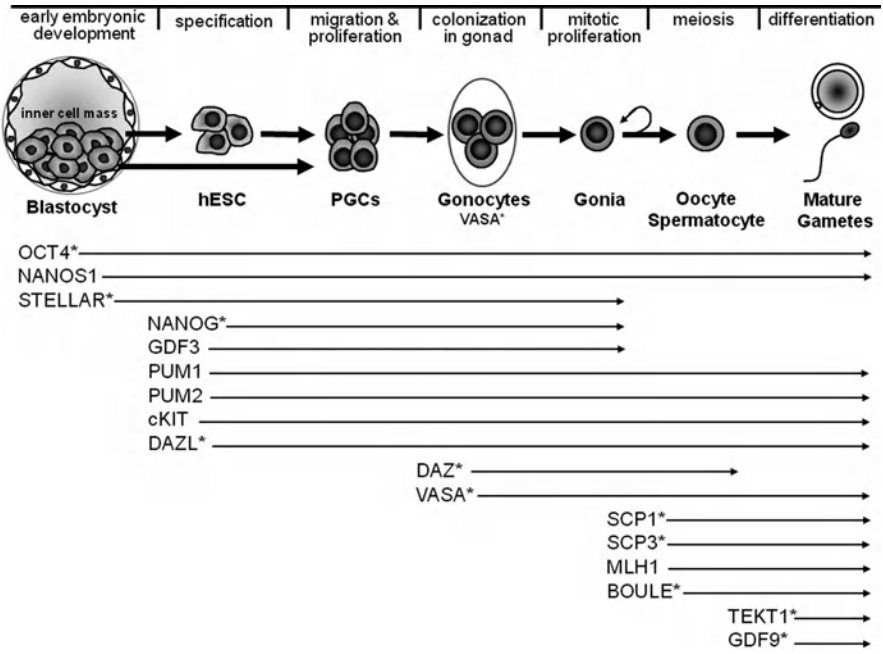

Fig. 3.4 Diagrammatic representation of the different stages of human germ cell development during human ES cell differentiation resolved from both *in vivo* and *in vitro* studies. Shown are the expression patterns of genes that define undifferentiated ES cells and each subsequent stage of germ cell development with each gene name on the left and its temporal expression indicated by the *arrows* extending to the *right*. All of the genes are enriched in germ cells relative to somatic cells, and those that are only expressed in the germ cells following the blastocyst stage embryo *in vivo* are indicated by an *asterisk*. Note that *Gdf9* is an adult oocyte-specific marker in mouse, and *TEKT1* is an adult spermatid-specific marker in human and mouse

those that were germ cell-specific (not expressed in somatic lineages), and those that were germ cell-enriched (highly expressed in germ cells with limited expression in somatic cells). The genes *GDF3* and *NANOG* were expressed in undifferentiated ES cells. Surprisingly however, as depicted in Fig. 3.4, undifferentiated human ES cell colonies also expressed RNA and protein for the germ cell-specific genes *DAZL* and *STELLAR*, as well as the germ cell-enriched genes *cKIT* (a marker of premeiotic migrating germ cells and pre-meiotic germ cells of the postnatal testis), and *NANOS1*, *PUM1*, and *PUM2* (markers specific to premeiotic primordial germ cells of the fetal gonads) (Clark et al. 2004a).

The presence of this early germ cell program in undifferentiated hESCs indicates that either: hESCs originate from PGC precursors in the inner cell mass; hESCs and PGCs share common genetic programs of pluripotency, self-renewal, and survival; and/or that a subpopulation of hESCs spontaneously differentiates into the germ cell lineage. In support of the hypothesis that hESCs *in vitro* and the inner cell mass *in vivo* are not identical cell populations it was discovered that the transcriptional profiles of these cell types were different. Cells of the inner cell

mass expressed *STELLAR*, *OCT4*, and *NANOS1*, but unlike ES cells, did not express the germ-cell specific marker *DAZL* and the somatic marker *NCAM1* (*Neural Cell Adhesion Molecule 1*) (Clark et al. 2004a). These results indicate that in the absence of the somatic cell environment of the developing embryo, a subset of the inner cell mass population embarks on the germ cell lineage pathway.

In these same investigations, when human ES cells were differentiated *in vitro* there was a shift in RNA and protein expression from that of immature/premeiotic germ cells to those of mature germ cells and gametes. Methods for spontaneous differentiation included taking undifferentiated hESCs and pooling them into a starting homogeneous population before distributing into individual wells of an ultralow-attachment plate, which facilitates the growth of embryoid bodies in suspension (without bFGF). Human ES cells maintained in these culture conditions for up to 21 days differentiated into embryoid bodies, which expressed several markers of later germ cell differentiation including, the gonocyte marker *VASA*.

Because VASA-expressing putative germ cells were detected in EBs the expression of other germ cell markers were also assayed. The meiotic markers *Synaptonemal Complex Protein 1* and *3* (*SCP1* or *SYCP1* and *SCP3* or *SYCP3*) and *BOULE*, and the post-meiotic gamete markers *Growth Differentiation Factor 9* (*GDF9*) and *Tektin1* (*TEKT1*), were expressed at day 14 of EB differentiation (Clark et al. 2004a). VASA-expressing cells occurred in clusters of cells at the edges and throughout small sections of EBs. STELLAR and DAZL expression on the other hand, was more predominant in cells of EBs, occurring in clusters, lining the edges, and throughout EB sections. It was also noted that with increasing differentiation of EBs, expression of mRNA markers of the somatic lineages also increased, including *NCAM1* (differentiated ectoderm), *Alphafetoprotein* (*AFP*; differentiated mesoderm), and *Tyrosine Kinase Receptor* (*KDR*; differentiated endoderm). As expected, somatic cell differentiation occurred in subpopulations of cells in parallel with germ cell differentiation. Taken together, this work was the first to clearly demonstrate that a subset of human ES cells can differentiate toward the germ cell lineage and produce cells with markers characteristic of gonocytes (Fig. 3.4). Isolation of these ES cell-derived germ cells, extended differentiation, characterization at the single-cell level, and subsequent functional assays were the next steps of these pioneering investigations.

3.4.3 Generation of Mouse Germ Cells and Gametes In Vitro

Several previous studies have shown that a cocktail of soluble growth factors, including kit ligand (KL), leukemia inhibitory factor (LIF), stroma-derived factor 1 (SDF1), BMP4, and bFGF, and compounds including *N*-acetyl-L-cysteine, forskolin, and retinoic acid (RA), are able to sustain the survival and self-renewal of mouse germ cells, after being removed from their somatic cell support in the gonads and cultured *in vitro*. The culture conditions used minimized apoptosis (programmed cell death) in germ cells, enhanced their proliferation, and allowed

many of them to progress through meiotic prophase I (Bowles et al. 2006; Farini et al. 2005; Koshimizu et al. 1995, 1996). Based on these observations, several groups have attempted to drive mouse ES cells towards the germ cell lineage and produce mature gametes *in vitro*. These pioneering investigations in mice have clearly demonstrated that mouse ES cells can give rise to the germ cell lineage, postmeiotic germ cells, and more mature gametes (Nayernia et al. 2006; Geijsen et al. 2004; Hubner et al. 2003; Qing et al. 2007; Toyooka et al. 2003). Mouse ES cells have been differentiated *in vitro* into EBs and subsequently into male sperm-like and female egg-like cells using various defined media, conditions, and germ cell-specific markers or genetic reporters. Cells with transcriptional profiles, protein expression, and morphologies similar to that of sperm and oocytes, have now been derived *in vitro*.

In 2003, it was first demonstrated that oocyte-like cells could be generated by spontaneous differentiation of mouse ESCs in adherent cultures (Hubner et al. 2003). Germ cell formation was identified by using a reporter construct containing the germ cell-specific *Oct4* promoter (*Oct4*Δ*PE*) to drive the expression of Green Fluorescent Protein (GFP) in the germ cells exclusively. GFP-positive cells were found associated with follicular-like structures that expressed high levels of known oocyte- and germ cell-specific genes and exhibited profiles of steroidogenesis similar to that of the somatic cells of the gonad. Apparently, the cells resembling oocytes were able to "recruit" adjacent ES cells, which differentiated in parallel into gonadal somatic-type cells. When these follicular-like structures were cultured further *in vitro*, the oocytes underwent spontaneous parthenogenesis into blastocyst-like structures, which then degenerated. A second study also identified oocyte- and follicular-like cells by co-culturing the differentiating mouse EBs with medium that had been conditioned by testicular cultures from newborn male mice (Lacham-Kaplan et al. 2006). These mature germ cells resembling oocytes were identified by germ cell-specific marker analysis and morphological characteristics. Presumably, certain secreted factors and hormones from this testis-conditioned medium induced female gamete differentiation.

In other studies attempting to differentiate sperm from ES cells, Toyooka et al. (2003) used a germ cell-specific reporter construct containing the *mouse VASA homolog* (*mvh*) promoter and *GFP*. Mvh is specific for differentiating mouse germ cells from the late migration stage to the postmeiotic stage (Toyooka et al. 2000). The putative male germ cells were isolated from EBs that had been co-cultured with isolated fetal mouse gonadal cells. After these ES-derived GFP-expressing cells were isolated and transplanted into the mouse testis, remarkably, they underwent meiosis and differentiated into cells resembling elongated spermatids (Toyooka et al. 2003). Another study used the stage-specific embryonic antigen 1 (SSEA1), a marker of pluripotency, as a cell surface marker in the isolation of mouse PGCs from EBs cultured in RA (Geijsen et al. 2004). Genomic reprogramming was examined in these cells and it was found that these mature male germ cells had the expected erasure of methylation at the sex-specific imprinted *Igf2* (*Insulin-like growth factor 2*) and the *H19* locus. Further, these cells underwent meiosis and formation of a haploid genome and when injected into oocytes they restored the

somatic diploid chromosome complement and induced development to the blastocyst stage (Geijsen et al. 2004).

While these studies were innovative and the findings impressive, significant unanswered questions remain regarding the functionality of these *in vitro*-derived "gametes." However, in 2006 Nayernia and co-workers were the first to generate what resembled spermatids *in vitro* that were indeed capable of fertilization. Notably, the full functional ability of these gametes was shown by using them in intracytoplasmic sperm injection (ICSI) to fertilize normal mouse oocytes and generate live viable offspring (Nayernia et al. 2006). In these pioneering studies, the male gametes were isolated by using two germ cell-specific fusion genes, *Stra8-eGFP* (*stimulated by retinoic acid gene 8* with *enhanced GFP*) and *Prm1-DsRed* (*protamine 1* with *Red Fluorescent Protein*). Mouse ES cells expressing the *Stra8* fluorescent reporter were induced with RA, cultured without RA for 2 months, then transfected with the *Prm1* fluorescent reporter. Using this strategy two transgenic cell lines were established, that subsequently formed EB-like structures and expressed *mvh*. After subsequent induction with RA for 72 h the cell lines produced dsRed-positive cells that arose from the GFP-positive cells. When these spermatid-like cells containing structures resembling immature flagella were released into the supernatant medium, they were reportedly motile as observed with phase contrast microscopy. These cells fertilized oocytes, supported development to embryos, and of the 65 embryos transferred into the oviducts of pseudopregnant females, 12 animals were born. Of these, six of the seven animals generated from these ES cell-derived sperm developed into adult mice. It was noted that there was a relatively low fertilization rate; of 210 oocytes microinjected only 65 oocytes developed to two-cell embryos. This may have been due to ICSI techniques or the heterogeneity and quality of the *in vitro*-derived male gametes.

3.4.4 Generation and Isolation of Human Germ Cells In Vitro

Although great progress has been made in the generation of mature gametes from mouse ES cells, comparable studies in the human have been more challenging. However, recent investigations, outlined below, have provided accumulating evidence and valuable tools to isolate, quantitate, and enhance the maturation and meiotic progression of human germ cells *in vitro*.

Determination of the essential growth factors and signaling molecules is important for studies attempting to generate germ cells from human ES cells. It is critical to establish whether factors known to be required for mouse PGC development both *in vivo* and *in vitro* might also induce the differentiation of human germ cells *in vitro*. Previous work indicates a central role of BMPs in germ cell specification and maintenance in the mouse. As outlined in Sect. 3.2 of this chapter, Bmp4, Bmp7, and Bmp8b play critical roles in the initial stages of germ cell development. Additional studies have found that when mouse epiblast explants were cultured with BMP4 and BMP8b *in vitro* they formed PGCs (Ying et al. 2001). Further,

Toyooka et al. (2003) found that co-culturing of BMP-producing cells, such as isolated fetal gonadal cells, increased the number of PGCs formed from mouse ES cells *in vitro*.

Similar to these studies in mice, our laboratory sought to develop methods to more efficiently differentiate germ cells from human ES cells. Toward this goal, Kee et al. (2006) differentiated human ES cells in the presence of human recombinant BMP4, BMP7, and BMP8b and assayed the differentiation of germ cells by genetic and immunohistochemical analyses. Human ES cells were differentiated into embryoid bodies using the standard differentiation medium either with or without various concentrations of human recombinant BMPs (1–100 ng/ml). The addition of BMP4, at 10 and 100 ng/ml, led to an approximate threefold increase in the expression of the germ cell-specific markers *VASA* and *SCP3* in embryoid bodies. BMP7 and BMP8b showed synergistic effects on germ cell induction when used in combination with BMP4. EBs differentiated in the presence of all three factors had a 16-fold increase in *VASA* expression relative to undifferentiated hESCs. Furthermore, the addition of BMPs to differentiating hESCs also increased the percentage of cells in EBs that stained positively for VASA protein, with a fivefold increase above that of EBs spontaneously differentiated in the absence of BMPs. Although the effects of BMPs on the total putative germ cell population were modest, the effects were significant and reproducible (Kee et al. 2006). These experiments suggested that BMPs promote the differentiation of germ cells from human ES cells.

A few subsequent studies demonstrated the derivation of germ cells from human ES cells. Tilgner et al. (2008) developed a protocol that promoted the differentiation of PGC-like cells from embryoid bodies using a simple fluorescence-activated cell sorting (FACS) strategy. The isolated putative germ cells had greatly increased expression of *VASA*, removal of parental imprints, and chromatin modifications indicative of PGCs. In another study it was reported that small changes to human ES cell growth conditions, including selecting for smaller ES cell colonies (20–50 cells/colony) and decreasing the number of culture feeding cycles, rapidly induced cells that were comparable to migratory PGCs (Bucay et al. 2009). Interestingly, they found that expression of the chemokine receptor, CXCR4, allowed the purification of this PGC-like population by FACS analysis. Upon differentiation, these CXCR4-expressing cells increased their RNA and/or protein expression of germ cell-specific markers including *DAZL*, *PRDM1* (*PR domain containing 1*, aka *Blimp1*), *STELLAR*, *NANOG*, *TRA-1-60*, *SSEA-4*, *VASA*, and *ACROSIN*. They also identified Sertoli-like cells associated with these developing PGC-like cells. These Sertoli-like cells showed expression of FSHR (follicle stimulating hormone receptor) and SOX9 (SRY-sex determining region Y-box 9), an autosomal gene necessary for Sertoli cell development. Further, electron microscopy demonstrated morphological and ultrastructural characteristics reminiscent of Sertoli cells, including a highly invaginated nucleus and prominent nucleolar complexes (Bucay et al. 2009). It is interesting to note that these male germ- and Sertoli-like cells were obtained from XX chromosome-bearing female ES cell lines (H9 and HSF-6).

Germ cells differentiated in these studies generally did not develop beyond the primordial germ cell stage and did not enter meiosis. Further, there has been a lack of available cell surface markers or fluorescent reporters to isolate reproducibly the nascent human germ cell population and promote their maturation. Therefore, Kee and co-workers sought to develop a system that would allow the isolation and propagation of human germ cells differentiated from pluripotent human ES cells or iPSCs (induced pluripotent stem cells). A germ cell-specific VASA-GFP reporter system was constructed to enrich for germ cells from the complex population of differentiated cell types derived from hESCs (Fig. 3.5) (Kee et al. 2009). In this lentiviral vector pLVGV, enhanced GFP (eGFP) is flanked by 2.5 Kb of 5′ promoter upstream sequence and 1 Kb downstream 3′ untranslated region (UTR) of human *VASA* inserted into the p2k7 lentiviral vector (Fig 3.5a).

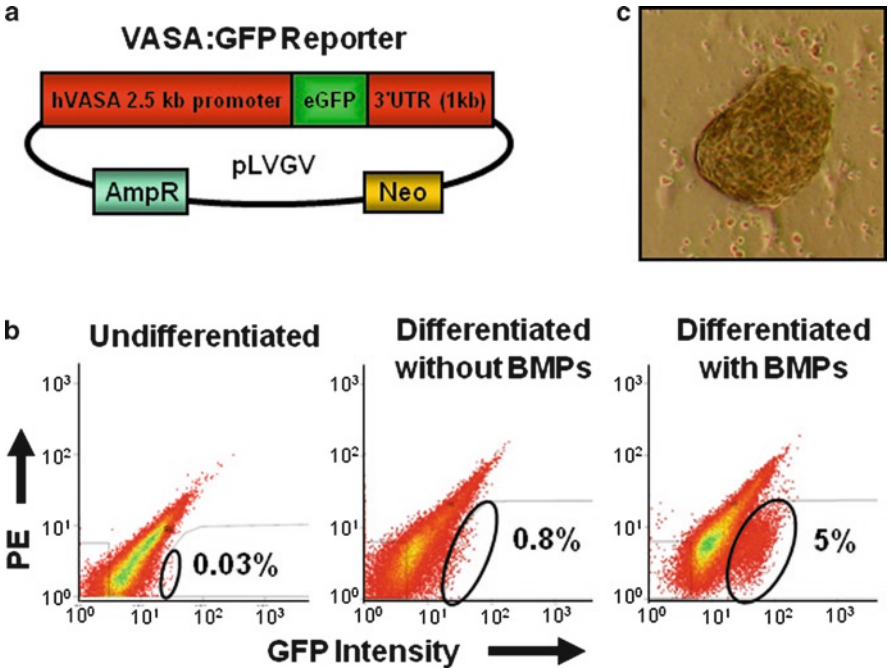

Fig. 3.5 VASA-GFP reporter system allows the isolation and characterization of germ cells differentiated from human ES cells. (**a**) Simplified map showing the human VASA-GFP lentiviral reporter vector, pLVGV. eGFP is flanked by 2.5 Kb upstream promoter sequence and 1 Kb downstream sequence of human VASA and inserted into the p2k7 lentiviral vector. (**b**) The percentage of GFP-positive cells obtained by FACS analysis in three representative H9 ES cell populations infected with the VASA-GFP reporter, including undifferentiated cells, spontaneously differentiated cells, and cells differentiated with defined medium containing BMPs. The percentage of GFP-expressing cells are circled. Note that the incubation with BMPs greatly improves the percentage of GFP-positive cells isolated in comparison to those spontaneously differentiated. (**c**) Phase contrast image of a representative germ cell colony showing tightly packed morphology after being isolated *via* FACS and replated and propagated on inactivated MEFs for 7 days (magnification ×40; data provided by K. Kee)

Both female (XX-bearing) and male (XY-bearing) undifferentiated hESC lines were stably transduced with this reporter and subsequently cultured in adherent differentiating conditions. As noted, previous studies found that spontaneous human germ cell differentiation in EBs was inefficient and comprised a relatively low percentage of the overall cell populations. Therefore, adherent differentiation of ES cells cultured on Matrigel in the presence of BMPs was selected for these studies. Conditioned medium (bFGF-containing medium collected after overnight incubation on irradiated MEFs) was used to maintain the undifferentiated cells when drug selection was required for lentiviral transduction. For hESC differentiation, cells were incubated in differentiation medium supplemented with 50 ng/ml recombinant BMP4, BMP7, and BMP8b, cultured for 0, 7, or 14 days, and analyzed by FACS.

BMPs reproducibly increased the number of PGCs formed, which displayed the expression patterns of mRNAs and proteins characteristic of germ cells. Following the addition of BMPs for 14 days, the percentage of cells that were positive for GFP increased to ~5% of the total differentiated cell population, compared to ~0.8% of cells differentiated in the absence of BMPs (Fig. 3.5b). The germ cell-specific markers *DAZL*, *VASA*, and *STELLAR* were highly enriched in the GFP-positive population, as was expression of the germ cell genes *PRDM1* (*Blimp1*) and *NANOG*, and the meiotic gene, *SCP3*, which is essential for synaptonemal complex formation in meiosis. In addition, due to the importance of erasure of methylation at imprinted loci in early germ cell development, the methylation status of imprinted loci as well as global DNA methylation levels in GFP-positive and GFP-negative cell populations was examined. Bisulfite genomic sequencing and 5-methyl-cytosine nuclear staining indicated that the VASA-GFP germ cell population was generally hypomethylated and likely undergoing global imprinting erasure.

To further investigate the properties of these isolated GFP-positive germ cells and their ability to propagate *in vitro*, their alkaline phosphatase (AP) activity, a marker for pluripotency, was tested. After isolation of germ cells by FACS, GFP-positive cells were replated onto MEFs and examined for morphological characteristics and AP activity. After 7 days the GFP-positive cells gave rise to tight colonies of cells resembling those of embryonic germ cells (Fig. 3.5c), while no colony was found in the replated GFP-negative cells. Further, the VASA-GFP-expressing germ cells showed intense AP activity, similar to that seen in EGCs. Therefore, the isolated cells had all the hallmarks of developing human male and female germ cells and could be propagated *in vitro* (Kee et al. 2009). Moreover, this VASA-GFP reporter system mediated the efficient isolation of the human PGC population and provided a mechanism to propagate and characterize developing germ cells in order to probe their morphological changes, epigenetic reprogramming, and genetic requirements.

In addition to using human ES cells to generate PGCs *in vitro*, current work is focused at deriving PGCs from iPS cells. Two studies using specific germ cell reporter systems have already demonstrated that germ cells can be derived from iPS cells (Park et al. 2009; Panula et al., unpublished). Further, several iPSC lines are being established from fibroblasts of normal men and women and females with

premature ovarian failure and Turner's syndrome. These studies will interrogate the molecular mechanisms of reproductive disorders and the genetic requirements of germ cell development (Dominguez and Reijo Pera, unpublished). The differentiation of germ cells from a variety of iPS cell lines will provide an *in vitro* cell-based system to address fundamental questions of normal and aberrant early human development.

3.5 Genetic Requirements of Making Germ Cells

3.5.1 Key Germ Cell Genes

The germ cell-specific *DAZ* genes are expressed in diverse organisms including humans and may play integral roles in human PGC development and gametogenesis. However, functional proof of the role of *DAZ* family genes in humans has not been directly tested due to several factors: difficulties of studying early human germ cell development, the unique combinations of *DAZ* genes present in the human genome in comparison to model organisms, and deletions of the human Y chromosome *DAZ* genes often encompassing neighboring genes. Therefore, in order to ascertain the role of the *DAZ* gene family in human PGC formation and gametogenesis, in recent work we used the VASA-GFP reporter system along with gene silencing and overexpression technologies in differentiating human ES cells (Kee et al. 2009). To silence or overexpress *DAZL*, *DAZ*, and *BOULE* alone or in combination, specific shRNA (short hairpin RNA) and overexpression vectors were employed. The shRNAs targeting the three genes were constructed using the Block-iT inducible H1 lentiviral system (Fig. 3.6). All shRNAs were first introduced into pENTR/H1/TO vectors then transferred into pLenti4/Block-iT-Dest destination vectors, which were used to transduce hESCs on Matrigel. The overexpression vectors were constructed by inserting the EF1α promoter, and *DAZL*, *DAZ*, or *BOULE* genes into the $p2k7_{blas}$ vectors (Fig. 3.6). The specificity and efficiency of the silencing and overexpression vectors were tested in 293T cells prior to experiments with ES cells.

After transduction of hESCs carrying the VASA-GFP reporter with individual shRNAs it was discovered that the silencing of *DAZL* had significant effects on PGC formation—a 50% reduction in the number of GFP-positive PGCs generated in both XX (H9) and XY (HSF-1) cell lines. In other experiments, silencing of *BOULE* reduced the GFP-positive population slightly in the XX line, but not in the XY line, while silencing of *DAZ* was virtually ineffective in reducing the number of GFP-positive cells differentiated from either cell line. Surprisingly, *BOULE* overexpression increased the VASA-GFP population to nearly 12%, compared to 4% in control cells in the XX line, but was ineffective in the XY line. As overexpression of *DAZL* and *BOULE* increased both VASA protein expression and the quantity of VASA-GFP-positive cells produced, it is likely that these genes are involved in early human PGC formation and development (Kee et al. 2009).

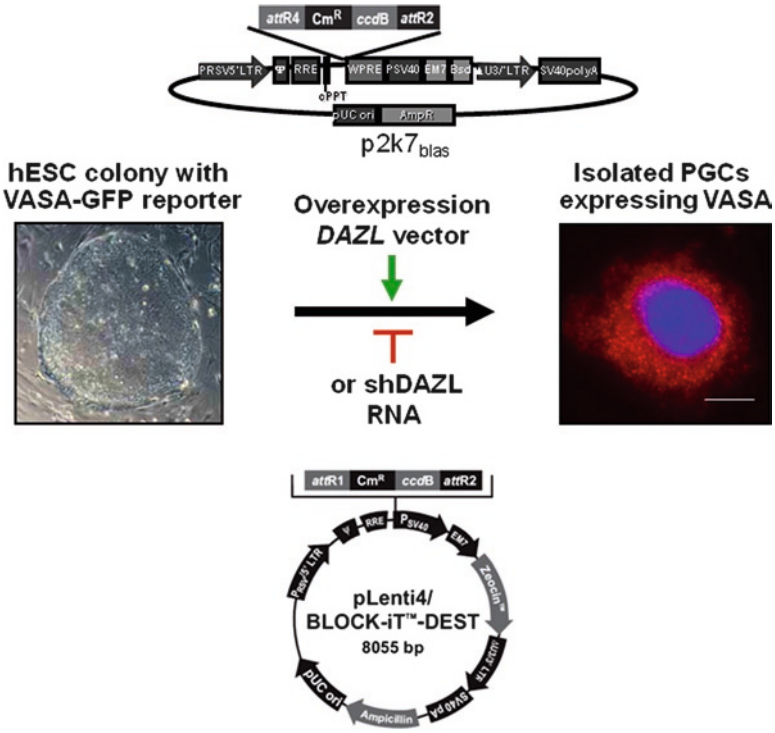

Fig. 3.6 Diagram of the methods of using overexpression vectors or silencing shRNA vectors targeted to *DAZL*, *DAZ*, or *BOULE* to increase or decrease their expression, respectively, in differentiating human ES cells. *Above* is the simplified map showing the p2k7 inducible overexpression vector and *below* is the simplified map showing the lentiviral inducible vector pLenti4/ Block-iT DEST used to introduce the shRNAs into human ES cells. Subsequent analysis of VASA-GFP+ germ cells via FACS reveals the effects of overexpressing or ablating these genes on the number of germ cells that differentiate *in vitro* (magnification ×50, bar = 10 μm; images provided by K. Kee)

To determine the role of these genes in later stages of germ cell development and meiosis, various combinations of *DAZ*, *DAZL*, and *BOULE* were overexpressed in differentiating hESCs. The expression of key meiotic genes, formation of meiotic synaptonemal complexes (SC), and production of haploid gametes were subsequently examined at 7 and 14 days of differentiation. Overexpression of *DAZL* alone or all three genes together led to an increase in mRNA of the meiotic marker *SCP3* in the female and male lines, respectively. Moreover, meiotic spreads and SCP3 staining showed that a subset of differentiating germ cells that overexpressed the three *DAZ* genes underwent extensive SC formation. Significant numbers of cells had nuclei with SC formation at the leptotene, zygotene, pachytene, and diplotene stages of meiotic prophase I. Overexpression of *BOULE* alone promoted extensive SC formation in the female line, while overexpression of *DAZ* alone led to increased SC formation in the male line. Due to the known association of the

DAZ gene with human male infertility, it is of note that overexpression of *DAZ* in the male line resulted in the greatest numbers of cells (more than 20%) reaching the meiotic leptotene stage at day 7 and 14.

Finally, it was determined whether haploid gametes were produced in the male ES cell line overexpressing all three genes. *ACROSIN* and *TEKT1*, markers of late spermatogenesis and mature sperm, were expressed at high levels by day 14. Additionally, FACS analysis to sort cells by DNA content using human semen as a positive control showed that approximately 2% of ES-derived germ cells overexpressing *DAZ*, *DAZL*, and *BOULE* were haploid (1N). Of these cells, many stained positively for the sperm marker ACROSIN. The majority of cells in the 1N population indeed had a haploid chromosome complement as determined by fluorescence in situ hybridization (FISH). It was noted that these 1N cells were spherical or elliptical in morphology, lacked flagella, and were closest in resemblance to round spermatids. Notably, with the overexpression of the three genes, BMPs were not necessary to induce meiotic progression, suggesting that *DAZ*, *DAZL*, and *BOULE* are intrinsic factors involved in this process and may function downstream of the BMP signaling pathway. Taken together, these results highlight the roles of the *DAZ* family genes in human germ cell development and that each of the three genes may have distinct roles in this process and sex-specific differences. *DAZL* may play a greater role in human primordial germ cell formation, whereas *DAZ* and *BOULE* may modulate later stages of gametogenesis including meiotic progression and development of haploid gametes.

Other investigations in our laboratory have interrogated the genetic requirements of *Dazl* in mouse germ cell development both *in vitro* and *in vivo* (Haston and Reijo Pera, unpublished). A double transgenic cross was used to produce mice and mESC lines with genetic ablation of *Dazl* (Ruggiu et al. 1997), which also contained a transgene expressing GFP from a germ cell-specific *Oct4* promotor, Δ*PE-Oct4-GFP* (Palmieri et al. 1994), described above in Sect. 3.4. This germ cell-specific ΔPE-Oct4-GFP was used to determine the effects of knocking out *Dazl* on the development of germ cells produced both *in vivo* in mouse embryos and *in vitro* from mouse ES cells. FACS analysis was used to isolate germ cells and quantitatively characterize the loss of germ cells in *Dazl* null embryonic gonads and mESC lines. The isolated Oct4-GFP-positive cells were utilized to determine gene expression and imprinting status of these *Dazl* null germ cells. It was found that *Dazl* null animals have significantly reduced primordial germ cell numbers during embryonic development. Further, these isolated GFP-expressing PGCs show sex-specific aberrant gene expression of pre-meiotic and meiotic germ cell markers and failure to erase methylation at imprinted loci and reestablish sex-specific methylation patterns, and they are unable to generate pluripotent EG cell lines. Moreover, the mESCs generated from these mice lacking the *Dazl* gene have a reduced number of GFP-positive germ cells produced *in vitro* and additional defects in gene expression and methylation patterns (Haston and Reijo Pera, unpublished). Therefore, the *Dazl* gene likely plays a key role in the development of germ cells in both mice and man.

3.5.2 Definitive Proof of Mature Germ Cell Formation

Currently, more stringent criteria and assessments are needed for determining whether an ES cell-derived germ cell truly is a functional mature germ cell, sperm or oocyte. Ultimately, the real proof of concept and gold-standard for mature germ cell identity is that the gamete-like cell can be transplanted *in vivo* to generate mature viable oocytes or sperm and/or ultimately be used to fertilize and generate offspring. Cell surface markers and mRNA and protein expression profiles characteristic of the germ cell lineage have been used in numerous ES cell studies in mouse and human. Formation of mature sperm from mouse ESCs has been relatively successful, as demonstrated by genetic, meiotic, and morphological characterizations and transplantation studies. Although "oocyte-like" cells have been differentiated from mouse ES cells by several groups, their ultimate functionality or ability to generate offspring has not been demonstrated.

Previous reports and observations from our laboratory have indicated that these oocyte-like cells are produced in limited quantity and are unable to progress through meiotic prophase I. Rigorous studies are needed to promote these germ cells to undergo meiosis and acquire the potential to differentiate into mature oocytes. To this end, our laboratory has aimed to differentiate mature and functional female oocytes from mouse ES cells by using transplantation strategies. Due to the critical importance of the ovarian somatic cell niche for primordial germ cell commitment to an oocyte developmental program, meiotic progression, and follicular maturation, Nicholas et al. (2009) developed an ovarian tissue co-culture and transplantation system to achieve functional maturation of ESC-derived oocytes (Fig. 3.7a). Using the ΔPE-Oct4-GFP germ cell-specific reporter and *Dazl*-null mESC lines it was demonstrated that germ cells formed after 3 weeks of spontaneous EB differentiation and were subsequently isolated via FACS. GFP-positive putative germ cells were sorted, and 100,000 were co-aggregated with dissociated wild-type newborn ovarian tissue, and transplanted under the kidney capsule of ovariectomized recipient female mice. The grafts were harvested 3 weeks later, and the oocytes derived from ΔPE-Oct4-GFP ESCs were confirmed by GFP expression using immunohistochemistry. Excitingly, 23 ESC-derived oocytes were identified in sections of the transplanted ovarian tissue. Many oocytes were contained within follicles, with some reaching the primary follicle stage. These results demonstrate that this *in vitro* gonadal co-culture and transplantation system may be an effective way to promote meiotic progression, maturation, and functional potential of ES-derived oocytes (Fig. 3.7a) (Nicholas et al. 2009). This work is now being translated to the human, with the goals of generating mature human oocytes and sperm from human ES and iPS cells using similar gonadal transplantation strategies.

As illustrated above, mouse and human ES cells can differentiate to the germ cell lineage and produce cells with characteristics of mature germ cells. However, in the majority of studies to date, rigorous analysis of genomic remodeling or methylation status, and assessment of meiosis and meiotic checkpoints, recombination and

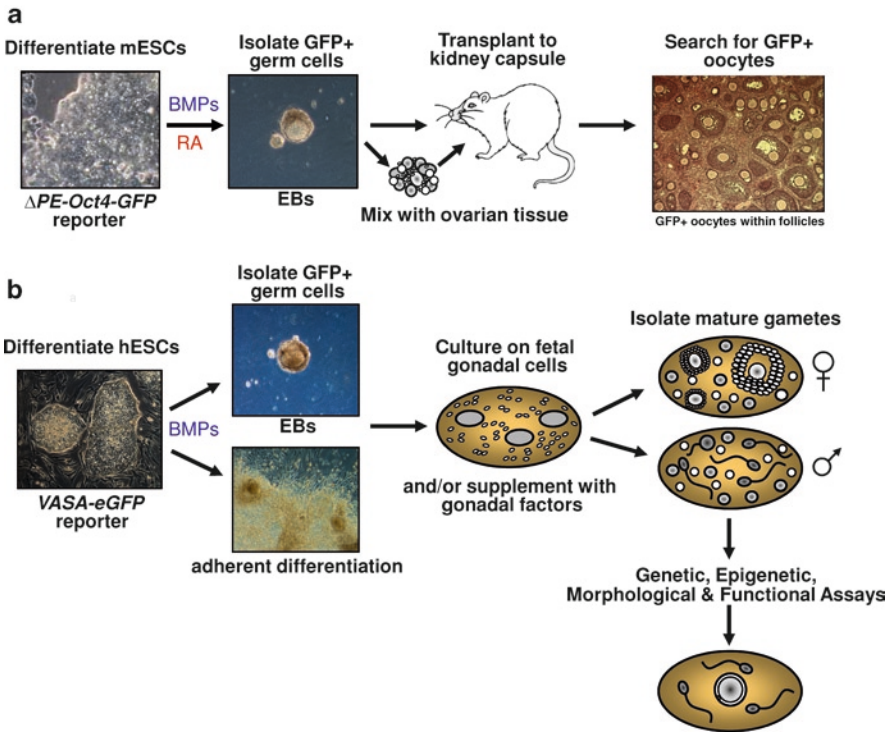

Fig. 3.7 Several methods used to obtain mature germ cells from ES cells. (**a**) This diagram shows the differentiation of mouse ES cells that contain the ΔPE-Oct4-eGFP germ cell reporter and subsequent isolation of Oct4-GFP+ cells from embryoid bodies by FACS analysis. Oct4-GFP+ cells are then mixed with wild-type fetal mouse ovary tissue, transplanted into the kidney capsule of live mice, and then the re-aggregated tissue is examined at various time-points for GFP-expressing oocytes. (**b**) This illustration shows the differentiation of human ES cells containing the VASA-GFP reporter, subsequent isolation of GFP+ germ cells from differentiating embryoid bodies or adherent cultures, and culturing of germ cells in conditions that promote the formation of normal mature gametes (images provided by A.M. Schuh-Huerta, S.M. Schuh-Huerta, and C. Nicholas)

formation of a haploid genome, have not been completed to assess germ cell quality, fidelity, and functionality. The need for more stringent assessments and criteria is highlighted by the fact that only one group to date has successfully used ESC-derived gametes for *in vitro* fertilization to generate viable offspring (Nayernia et al. 2006). In fact, although this is a remarkable accomplishment, these ES cell-derived offspring had growth defects (were either smaller or larger than controls), shorter life spans, abnormal methylation patterns resulting from disturbed male germline-specific methylation imprints, and phenotypic abnormalities. These observations highlight the need for improved methods, analysis, and screening of germ cells derived in culture.

3.6 The Future: Improved Methods and Clinical Implications

In future studies it will be necessary to carefully dissect and characterize *in vitro*-derived germ cells at the single-cell level. Careful examinations and comparisons can then be made with single ES-derived gametes and mature human sperm and eggs. Using advanced microarray technologies for the analysis of genetic variants and cellular gene expression will be highly useful in dissecting the protein and genetic make-up of ES-derived germ cells. In addition, improved imaging techniques might also allow stringent comparative analysis between the morphology and ultrastructure of germ cells and gametes derived both *in vivo* and *in vitro*. Most importantly, careful analysis of successful meiosis, genomic methylation, chromatin status, nuclear architecture, and chromosome ploidy of ES cell-derived sperm and eggs will be paramount to assess quality and functionality, especially with respect to human gametes.

3.6.1 Improving Culture Conditions for Enhanced Gamete Formation

The process of germ cell formation, differentiation, and maturation in the fetal and adult gonad consists of a series of complex molecular and cellular events. The developing germ cells and gametes are in constant contact with the somatic support cells and hormone/secretory cells of the gonad. In fact, the close physical connections and intercellular signaling are apparent in both sperm with Sertoli cells, and oocytes with granulosa and theca cells of the follicle. Therefore, it is logical to speculate that the generation of more mature gametes from ES cells in culture will require certain key elements of the somatic gonadal niche. Recapitulating the *in vivo* factors and cellular environment will likely be crucial for the development and further maturation of germ cells *in vitro*. Indeed, the most successful investigations have used co-culture gonadal cell systems, gonadal transplantation strategies, and/or signaling factors known to exist within the gonad (Fig. 3.7a). One recent study used the co-culturing of human fetal gonadal stromal cells with human embryonic or induced pluripotent stem cells to greatly enhance the formation of germ cells (Park et al. 2009). Using ovarian and testicular cells/aggregates or using cell lines developed from fetal gonadal somatic cells may provide an effective system to enhance the development and maturation of normal functional human gametes *in vitro* (Fig 3.7b).

3.6.2 Clinical Implications and Outlook

Development of functional mature gametes is a key event for sexually reproducing species. Establishment of an *in vitro* cell-based system, like that of human ES cells,

to study germ cell development has great benefits. Using this system we can more easily probe the early genetic and epigenetic events of gamete development and uncover the genetic requirements, signaling pathways, and molecular programs that are unique to the human germ cells. Now that numerous investigations in the mouse and human have generated germ cells and even more mature gametes from ES cells, the road has been paved for achieving the formation of bona fide eggs and sperm that are fully functional and capable of generating healthy viable offspring.

The creation of gametes *in vitro* would provide several potential scientific and clinical applications. Establishment of this system could provide a novel and faster approach for the generation of transgenic and knockout mice. Additionally, as the oocyte is the only cell type that is capable of complete genomic reprogramming to generate a totipotent embryo, human ES cells might also serve as an *in vitro* method of generating an oocyte-like cell capable of nuclear reprogramming. Oocytes generated in culture could provide an excellent alternative reprogramming cell for SCNT. As eggs produced *in vivo* are limited in number and difficult to obtain, oocytes generated *in vitro* might be advantageous for SCNT and might have great regenerative and therapeutic applications.

As infertility is remarkably common in humans relative to other species and often results from problems with gamete quantity or quality, the ES cell-germ cell model system might also provide a greater understanding of various reproductive disorders and pathologies resulting from aberrant germ cell development. Moreover, as the exact cause of infertility is often unknown, developments and insight gained from the study of gametes *in vitro* could promote the creation of genetic screens and assays for the diagnosis of infertility. Other important potential applications of germline stem cell biology include using iPS cells to generate gametes *in vitro* as an alternative to hESC-derived germ cells. This possibility is exciting as it would allow the reprogramming of an adult cell type, such as a skin cell or fibroblast, into a pluripotent cell that could subsequently be differentiated into a mature male or female gamete. An iPS cell-derived gamete would be specific to the individual from whom it was obtained. For individuals who are unable to conceive naturally and either make little or no gametes or have failed with assisted reproductive technologies, this might one day present a way to generate functional gametes that are genetically unique to the individual. Importantly, a mechanism to create mature gametes "in the dish" by the use of embryonic or pluripotent stem cells holds great promise for the conservation of endangered species and may one day have clinical applications for the treatment of human infertility.

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Part II
Spermatogonial Stem Cells

Chapter 4
Morphometric Description of Spermatogonial Stem Cells and Expansion of Their Clonal Derivatives

Dirk G. De Rooij

Abstract Spermatogonial stem cells (SSCs or A_s spermatogonia) in rodents and rams are single cells that can self-renew or form a pair (A_{pr}) that will continue to differentiate and form chains of A_{al} spermatogonia. A_{al} spermatogonia differentiate into A1 spermatogonia that after six divisions produce spermatocytes via A2, A3, A4, In, and B spermatogonia. The cell cycle times of each of the generations of A1-B spermatogonia are similar and are about 14% of the duration of the epithelial cycle. In contrast, the cell cycle times of the $A_{s,pr,al}$ spermatogonia are highly variable, the minimal cell cycle time being about 30% longer than that of the A1-B spermatogonia. During the epithelial cycle the $A_{s,pr,al}$ spermatogonia start to proliferate at about stage X and the A_{pr} and A_{al} spermatogonia stop dividing around stage II, while the SSCs continue to proliferate until stage VI. There is a feedback regulation between the numbers of A1-B spermatogonia and the length of the proliferative period of the $A_{s,pr,al}$ spermatogonia. When the number of A1-B spermatogonia is low, the $A_{s,pr,al}$ spermatogonia continue to proliferate longer. The $A_{s,pr,al}$ spermatogonia in different epithelial areas produce variable numbers of A1 spermatogonia but always more than needed, subsequently the surplus of A2–A4 will enter apoptosis, ensuring an even distribution of germ cells from In spermatogonia onwards.

Keywords Spermatogenesis • Spermatogonial stem cells • Spermatogonia • Cell kinetics • Differentiation • Self-renewal

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4.1 Outline

This review first deals with the various types of spermatogonia that can be distinguished and the scheme of spermatogonial multiplication and stem cell renewal in rodents and the ram, in which this process has been studied in most detail. Then the morphological characteristics of the spermatogonial cell types are described, including their presence and behavior during the epithelial cycle and how these cells can best be studied at the cellular level. In the next section, the numbers of the $A_{s,pr,al}$ spermatogonia during the course of the epithelial cycle and the number of stem cells per testis are discussed. Subsequently, the extensive cell-kinetic studies using ^{3}H-thymidine in the rat and Chinese hamster are summarized. Detailed data on the cell cycle characteristics of all types of spermatogonia in these species are known. The spermatogonial stem cells that are supposed to be single cells are discussed. However, theoretical and experimental considerations seem to indicate that some of the pairs may still have stem cell properties that can split in singles or new pairs again at division. Next, the proliferative activity of the $A_{s,pr,al}$ spermatogonia during the course of the epithelial cycle is described, as well as the growth fraction data that can be calculated for the various cell types. The duration of the period of high proliferative activity of the $A_{s,pr,al}$ spermatogonia during the epithelial cycle depends on the numbers of differentiating type of spermatogonia present, in a kind of a feedback regulation loop. This regulation on the cellular level is also described. The final section describes how the density of germ cells is regulated by apoptosis of the surplus of A1–A4 spermatogonia produced by the $A_{s,pr,al}$ spermatogonia.

4.2 Introduction

Up until about 1994, when the spermatogonial stem cell (SSC) transplantation assay was published (Brinster and Avarbock 1994; Brinster and Zimmermann 1994), most research on spermatogonia was carried out at the cellular level in testis sections and whole mounts of seminiferous tubules. Studies on SSCs could not be conducted otherwise than by way of cell counts and labeling experiments and by studying recovery after cell loss. Now virtually all research on SSCs and its direct progeny is carried out *in vitro* and on populations of SSCs purified directly or indirectly, using membrane markers for SSCs, under the guidance of the transplantation assay. A host of factors have now been detected that play a role in regulating SSC behavior and spermatogonial differentiation (Oatley and Brinster 2008; Aponte et al. 2005). Now the new data, largely obtained *in vitro*, can be compared with the old knowledge obtained at the cellular level in testis tissue. To facilitate this comparison this review summarizes the morphological and morphometrical data obtained before the molecular and culture era, starting at about 1994. After all, the final goal still is to understand the regulation of the spermatogonial compartment in the normal *in vivo* situation.

4.3 Scheme of Spermatogonial Multiplication and Stem Cell Renewal

In rodents and the ram, the SSCs have been proposed to be single spermatogonia, the so-called A_s spermatogonia (de Rooij 1973; Huckins 1971c; Lok et al. 1982; Oakberg 1971). When an A_s spermatogonium divides, its daughter cells can either migrate away from each other and become two new stem cells, or they can stay together connected by an intercellular bridge, and form so-called A_{paired} (A_{pr}) spermatogonia (Fig. 4.1). In the normal epithelium, the formation of a pair is the first step on the differentiation pathway to spermatozoa. The pairs divide further into chains of four, eight, and often 16 $A_{aligned}$ (A_{al}) spermatogonia. In stages VII/VIII of the epithelial cycle, almost all of the A_{al} spermatogonia differentiate into A_1 spermatogonia (Schrans-Stassen et al. 1999) that after six divisions produce spermatocytes via, in mouse and rat, A_2, A_3, A_4, In, and B spermatogonia. At present it is still a matter of debate whether all A_s spermatogonia should be considered to be real stem cells and whether A_{pr} and A_{al} spermatogonia can "de-differentiate" and regain stem properties (Nakagawa et al. 2007).

In the normal epithelium exclusively clones composed of 2^n A_{al} spermatogonia are seen (Huckins 1971c; Lok et al. 1982). However, after administration of busulfan or irradiation odd clones, for example, composed of three or five cells are also seen (van Keulen and de Rooij 1973; Van Beek et al. 1984). The cause for this phenomenon likely is apoptosis of individual cells in A_{al} clones. After low doses of irradiation or administration of a low dose of busulfan not always whole clones enter apoptosis but individual cells or only some of the members of a clone can enter apoptosis (Hamer et al. 2003). Such an event will cause the breaking up of the clone and the formation of an odd numbered clone. It is not known whether after cell loss, single cells from clones of A_{pr} and A_{al} spermatogonia, which become detached in this way, will behave like stem cells again.

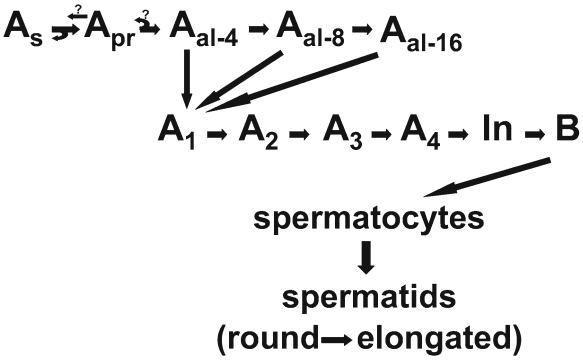

Fig. 4.1 Scheme of spermatogonial multiplication and stem cell renewal in rodents and the ram. The question marks indicate the uncertainty about whether or not A_{pr} and A_{al} spermatogonia already are too different from A_s spermatogonia to function as stem cells

All spermatogonia in mammals, including the SSCs, are situated on the basal membrane of the seminiferous tubules. After division all daughter cells of spermatogonia will remain in the same two-dimensional space with the basal membrane on one side and the junctions between Sertoli cells, forming the blood–testis barrier, on the other side (Russell et al. 1990). At their last division, spermatogonia produce spermatocytes that go through a G1 and S phase and then start the prophase of the first meiotic division. After the start of the meiotic prophase the spermatocytes move through the blood–testis barrier and leave the basal membrane (Russell et al. 1990).

4.4 Morphological Characteristics of $A_{s, pr, al}$ Spermatogonia

The $A_{s,pr,al}$ spermatogonia cannot be discerned from each other in testis sections because the morphological differences between these cell types, if any, are too small. However, as first described by Clermont and Bustos-Obregon (1968), it is also possible to study spermatogonia in whole mounts of seminiferous tubules. This gives the advantage that one always sees all morphological details of the nucleus of each spermatogonium, enabling one to make use of the nuclear changes that take place during the cell cycle. Importantly, in whole mounts one can also observe the topographical arrangement of the cells in singles, pairs and chains consisting of up to 16 A spermatogonia (Fig. 4.2). By definition, cells with a similar morphology, the nuclei of which are not farther apart than 25 μm, are supposed to belong to the same clone of interconnected cells (Huckins 1971c). The validity of this criterion has been confirmed in a study in the Chinese hamster in which internuclear distances were measured between cells of the same clone. The great majority of the cells had an internuclear distance between 10 and 20 μm (Lok et al. 1982). Tokuda et al. immunohistochemically stained $A_{s,pr,al}$ spermatogonia for the membrane marker CDH-1 (Tokuda et al. 2007). It was found that these cells often had very fine cell processes, longer than 20 μm. However, the authors concluded that these processes likely did not represent intercellular bridges as they did not always connect cells.

On the tubule basal membrane, besides $A_{s,pr,al}$ spermatogonia, there also are A1 through A4 and Intermediate (In) and B spermatogonia. In and B spermatogonia show heterochromatin in their nuclei while A spermatogonia do not, making it easy to distinguish these cells. In sections, $A_{s,pr,al}$ spermatogonia can only be discerned from A1–4 spermatogonia morphologically when plastic embedding and a particular fixation and staining procedure are employed (Chiarini-Garcia et al. 2001, 2003). In whole mounts these two categories of spermatogonia can be distinguished by the fact that A1–4 spermatogonia have a larger internuclear distance, as these cells are spread out over the basal membrane, starting from A1 spermatogonia in epithelial stages VII/VIII (Fig. 4.2e). In contrast, A_{pr} and A_{al} spermatogonia form pairs and chains the composing cells of which remain close together.

Spermatogenesis is arranged in such a way that, along the seminiferous tubules, the stages of the epithelial cycle follow each other (Russell et al. 1990),

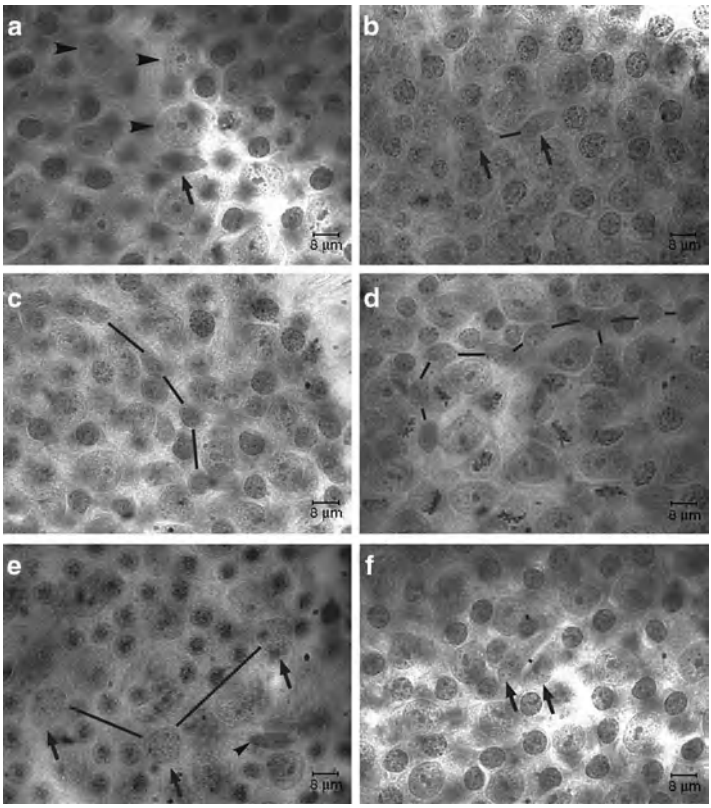

Fig. 4.2 Photographs of the cells on the basal membrane of whole mounts of seminiferous tubules of a Chinese hamster, stained with hematoxylin staining the nuclei of the germ cells and Sertoli cells. Sertoli cell nuclei can be recognized by their big nucleolus and the clumps of chromatin attached to them at either side. A few are indicated in (**a**) (*arrowheads*). As the tubule is a three-dimensional structure, not all nuclei in an area will be in focus as they can be at a slightly different level from the basal membrane. (**a**) A_s spermatogonium (*arrow*). (**b**) A_{pr} spermatogonia (*arrows*). A *line* has been drawn between the cells of the pair. (**c**) A chain of four A_{al} spermatogonia. (**d**) A chain of nine A_{al} spermatogonia that are part of a chain that continues beyond the area of the photograph, consisting of in total 16 cells. (**e**) Three A1 spermatogonia (*arrows*) with representative internuclear distances that are larger than those between the cells belonging to a clone of A_{pr} or A_{al} spermatogonia. In this area there is also an A_s spermatogonium (*arrowhead*). (**f**) Two A spermatogonia close together that technically form a pair as their internuclear distance is less than 25 µm. However, one of the nuclei is bigger than the other and has an elongated shape while the other has a more oval nucleus. This likely is a false pair, i.e., two A_s spermatogonia that have stayed together. Bar = 8 µm

a phenomenon called the wave (Perey et al. 1961). When one follows the wave of spermatogenesis along a seminiferous tubule, the stages of the epithelial cycle pass by and also one can follow the subsequent phases of the cell cycle of the A1, A2, A3, A4, In, and B spermatogonia (Fig. 4.3; Lok and de Rooij 1983a). For example, when a certain area contains A3 spermatogonia in G1 phase of the

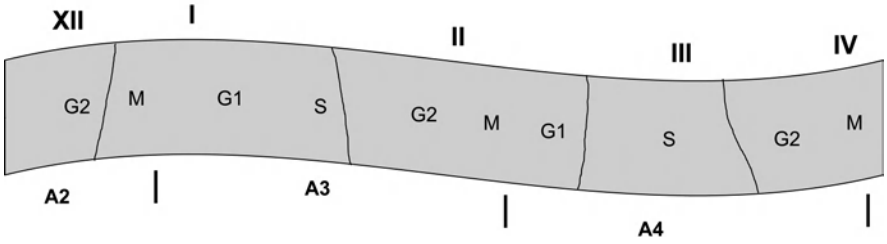

Fig. 4.3 Schematic of a seminiferous tubule showing the wave of spermatogenesis through which epithelial stages (*roman numerals*) follow each other sequentially. The differentiating type of spermatogonia (A2, A3, A4 are indicated) also follow each other and go through the phases of the cell cycle in an epithelial stage related order. G1, G2, S, M – phases of the cell cycle

cell cycle, somewhat further along the tubule one can see these cells become bigger as they carry out S phase and still further they will be in G2 phase and show some heterochromatin at the nuclear membrane. Subsequently, the cells enter mitosis and A4 spermatogonia are formed. These A4 spermatogonia are small at first as they are in G1 phase and then again further along the tubule, they will become bigger and so on. This synchronization originates not only from the intercellular bridges between cells from the same clone but also from a synchronization of the cell cycle progress of all clones in the same area (Lok and de Rooij 1983a). Hence, following the A1-B spermatogonial generations along the length of a tubule, one encounters relatively extended fields in which all spermatogonia from an A1-B spermatogonial cell type are in late G2 phase of the cell cycle or are in mitosis. In contrast, the clones of $A_{s,pr,al}$ spermatogonia always proliferate at random, and separate clones in a tubule area do not cycle synchronously with each other and do not follow the behavior of the differentiating type spermatogonia. These phenomena can be used to easily discern differentiating type A spermatogonia from $A_{s,pr,al}$ spermatogonia (de Rooij 1973; Lok et al. 1982; Tegelenbosch and de Rooij 1993).

Hence, $A_{s,pr,al}$ spermatogonia can best studied in those areas in which the generations of differentiating type A spermatogonia are synchronously in G2 phase of the cell cycle or in mitosis (Fig. 4.3). In these areas the type of differentiating type A spermatogonia present are big and show some heterochromatin or are in mitosis and the $A_{s,pr,al}$ spermatogonia stand out from the differentiating A spermatogonia as they generally have much smaller nuclei because they are not in G2/M. The cells composing the occasional clones of $A_{s,pr,al}$ spermatogonia that also happen to be in G2/M are much closer to each other than the differentiating type A spermatogonia. An additional advantage of this procedure is that when one studies $A_{s,pr,al}$ spermatogonia in areas in which the subsequent generations of A1, A2, A3, A4, In, and B spermatogonia are in G2/M, one studies these cells at regular intervals of time because the cell cycle time of the A1-B spermatogonia is always similar (see below). Cell counts are usually made using the numbers of Sertoli cells present in the same area as a reference, and numbers are then usually expressed per, for example, 1,000 Sertoli cells.

Recently, it became clear that there is an additional level of organization in the seminiferous epithelium. The $A_{s,pr,al}$ spermatogonia appeared to be preferentially localized in those parts of the basal membrane near the stretches of interstitial tissue and or blood vessels (Chiarini-Garcia et al. 2001, 2003; Yoshida et al. 2007; de Rooij 2009). Such an area of preferred localization is called a niche and is found in many other tissues too. The data discussed in this chapter do not directly relate to the presence of niches and the niche will be discussed in detail elsewhere in this book.

4.5 Numbers of $A_{s,pr,al}$ Spermatogonia During the Epithelial Cycle

Detailed counts of $A_{s,pr,al}$ spermatogonia throughout (most of) the epithelial cycle have been performed in rats, mice, Chinese hamsters, and the ram (de Rooij 1973; Huckins 1971c; Lok et al. 1982; Oakberg 1971; Tegelenbosch and de Rooij 1993). In some strains of mice, the density of the A_s, A_{pr}, and A_{al} spermatogonial clones is too high to tell the clones apart in all instances (Tegelenbosch and de Rooij 1993). In such cases, the clones are close together and the differences in cell cycle phase and, with that, the morphology of the cells in the neighboring clones, are not always large enough to unequivocally tell the clones apart and to determine clonal sizes.

The cell count results always show that the numbers of A_s and of A_{pr} spermatogonia do not fluctuate very much during the epithelial cycle while there is a steep increase in the numbers of A_{al} spermatogonia from stage X to about stage IV, after which the increase diminishes (Fig. 4.4). What happens is that the A_s spermatogonia proliferate and form clones of A_{pr} spermatogonia as well as renew themselves in such a way that the A_s spermatogonial numbers remain more or less similar. At the same time, while new A_{pr} spermatogonia are formed by stem cells, already existing A_{pr} spermatogonia divide into chains of four A_{al} spermatogonia. The balance between the loss of A_{pr} spermatogonia because of the formation of A_{al} spermatogonia and the replenishment of A_{pr} spermatogonia by differentiating stem cell divisions is such that the numbers of A_{pr} spermatogonia remain about constant. Finally, a steep increase in A_{al} spermatogonial numbers is observed because new chains of four keep being formed by A_{pr} spermatogonia while already existing chains of four and eight A_{al} spermatogonia divide on to become longer chains. However, chains of 32 A_{al} spermatogonia are very rare. Apparently, chains of 16 are less likely to proliferate any further.

A complete quantification of the spermatogonial compartment has been carried out in C3H/101 F1 hybrid mice. In this strain of mice, there are about 35,000 stem cells per testis and 1.3 and 10.6%, respectively, of all spermatogonia and $A_{s,pr,al}$ spermatogonia in the testis are stem cells (Tegelenbosch and de Rooij 1993). However, these numbers vary with species and strains. For example, in mouse strains the total numbers of $A_{s,pr,al}$ spermatogonia in epithelial stage VIII varies from

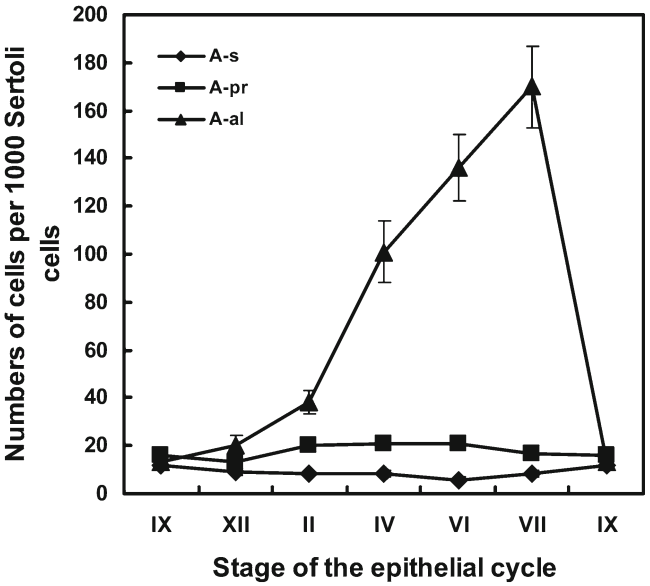

Fig. 4.4 Numbers of A_s, A_{pr}, and A_{al} spermatogonia throughout the stages of the cycle of the seminiferous epithelium in the Chinese hamster. Data are from Lok et al. (1982)

26 in 101xC3H mice to 60 per 1,000 Sertoli cells in C3H mice (Tegelenbosch and de Rooij 1993; Huckins and Oakberg 1978).

4.6 Cell Cycle Characteristics of $A_{s,pr,al}$ Spermatogonia

As first shown by Huckins and Kopriwa, it is possible to carry out autoradiography on whole mounts of seminiferous tubules (Huckins and Kopriwa 1969). Using this technique after ^{3}H-thymidine administration, cell cycle times have been established of all types of spermatogonia in the rat (Huckins 1971a, d) and the Chinese hamster (Lok and de Rooij 1983a; Lok et al. 1983). Again advantage has been taken of the wave of spermatogenesis and the synchronous behavior of the A1-B spermatogonia. Shortly after injection of the ^{3}H-thymidine all A1-B spermatogonia in S-phase incorporate this precursor and become labeled. Observing whole mounts of autoradiographs of seminiferous tubules, one can see sharply defined tubule areas in which cells of one of the generations of A1-B spermatogonia are all labeled, meaning that in that specific area the various clones of differentiating spermatogonia synchronously traverse the S phase. These areas with a labeled generation of A1-B spermatogonia are interspaced by large areas in which these cells are unlabeled as they had been in G1, G2, or M phase at the time of the injection of ^{3}H-thymidine. In the latter areas the labeled spermatogonia are exclusively clones of $A_{s,pr,al}$ spermatogonia

that, as mentioned above, are not synchronous with the A1-B spermatogonia. In these areas the cell cycle properties of the $A_{s,pr,al}$ spermatogonia can be studied, using the labeled mitoses method. In this method label incorporated during the S phase of cells is followed with time to determine how long it takes for the labeled cells to go through mitosis, traverse the next cell cycle, and carry out a second mitosis. From the curve of labeled mitoses of a particular spermatogonial cell type, the duration of each phase of the cell cycle can be calculated (Lok and de Rooij 1983a; Lok et al. 1983; Huckins 1971a, d).

Labeled mitosis studies in mice, rats, and Chinese hamsters have shown that the generations of A2-B spermatogonia have similar, species-specific, total cell cycle times. In mice, the cell cycle time of the differentiating type spermatogonia is 28.5–34 h (Fabrikant 1979; Monesi 1962), in rats it is 42 h (Huckins 1971a; Hilscher et al. 1969), and in the Chinese hamster 60 h (Lok and de Rooij 1983a). Interestingly, within one species the cell cycle times of the generations of differentiating spermatogonia are similar and also the second and subsequent peaks of the labeled mitoses of these spermatogonia reach 100% again, meaning that there is hardly any variation in cell cycle times and that at each division all cells divide. This is quite extraordinary because in other types of cells there generally is much variation in the duration of the G1 phase and the second peak of the labeled mitosis curve rarely reaches high levels. Furthermore, there seems to be a fixed relation between the duration of the cycle of the seminiferous epithelium and the cell cycle time of the differentiating type spermatogonia. In the mouse, rat, and Chinese hamster the cell cycle time of A1-B spermatogonia is about 14% of the duration of the epithelial cycle (Lok and de Rooij 1983a; Huckins 1971a; Fabrikant 1979; Monesi 1962; Hilscher et al. 1969).

$A_{s,pr,al}$ spermatogonia have a considerably longer cell cycle time than A1-B spermatogonia. In the rat, the cell cycle time of the A_s, A_{pr}, and A_{al} spermatogonia is 56 h compared to 42 h for the differentiating spermatogonia (Huckins 1971a, d). In the Chinese hamster the minimal cell cycle time is also similar for A_s, A_{pr}, and A_{al} spermatogonia and is about 90 h compared to 60 h for differentiating type spermatogonia (Lok and de Rooij 1983a; Lok et al. 1983). Interestingly, in both rats and Chinese hamsters, the second peak of the labeled mitoses curve for these spermatogonia does not come close to 100%. This means that, in sharp contrast to differentiating type spermatogonia, the duration of the G1 phase of $A_{s,pr,al}$ spermatogonia varies greatly. This variation in cell cycle time is caused by the fact that not all of these cells directly continue cycling after a division and stay in G1 phase for a variable period of time. This large variation in the duration of the G1 phase strongly suggests that exit from this phase of the cell cycle depends on regulatory mechanisms.

Finally, in the labeling studies attempts have also been made to find out whether there are spermatogonia that retain the ^{3}H-thymidine label for a very long time, i.e., weeks or months. During proliferation of (stem) cells, at each division the amount of ^{3}H-thymidine per cell halves and, after a number of divisions, the amount of radioactivity will get below the detection level. In several tissues, cells have been found that retain label for a relatively long time. These label retaining cells (LRCs) are generally supposed to be the stem cells of the tissue as stem cells proliferate

more slowly than differentiating cells. Huckins carried out an autoradiographic study on whole mounts of seminiferous tubules of rats that were given ^{3}H-thymidine and studied the presence of labeled spermatogonia at longer times after injection (Huckins 1971b). She reported the existence of LRCs and proposed that the LRCs, a subpopulation of the A_s spermatogonia, were the real stem cells and that the short-cycling A_s spermatogonia were inclined to differentiate. However, Lok et al. carried out a comparable but more extensive study in the Chinese hamster and did not find evidence for the presence of LRCs in this species (Lok et al. 1984).

In conclusion, differentiating type spermatogonia have a strictly determined cell cycle while A_s, A_{pr}, and A_{al} spermatogonia have a rather variable cell cycle time indicating that the proliferation of the latter cells is subject to regulation. The minimal cell cycle time of A_s, A_{pr}, and A_{al} spermatogonia is longer than that of differentiating type spermatogonia. In rats and Chinese hamsters the duration of the minimal cell cycle of the $A_{s,pr,al}$ spermatogonia is about one third longer than that of the differentiating type spermatogonia. It is tempting to speculate that the cell cycle time of the $A_{s,pr,al}$ spermatogonia in mice will then be between 38 and 45 h, depending on the strain.

4.7 The Occurrence of "False" Pairs of Spermatogonia

As already indicated by Huckins, when A_s spermatogonia carry out a division and two new A_s spermatogonia are formed in a self-renewing division, the daughter cells will need time to migrate away from each other (Huckins 1971c, d). As long as these cells are within 25 μm from each other, they will be counted as A_{pr} spermatogonia. These cells have been called a "false" pair (Fig. 4.2f). As not much is known about the speed at which the daughter stem cells migrate away from each other, the number of false pairs cannot be determined.

In addition to false pairs occurring because of the time required to migrate away from each other, the decision of daughter cells to stay together as a pair or to migrate away from each other and become new stem cells may not have to take place directly after division. In the ^{3}H-thymidine incorporation studies in the Chinese hamster, an imbalance was found between the numbers of labeled metaphases of A_s and of A_{pr} spermatogonia. There were 100 metaphases of A_s against 175 of A_{pr} spermatogonia, while in steady state kinetics these numbers should be similar (Lok et al. 1983). Also, more labeled interphase A_{pr} than A_s spermatogonia were found (Lok and de Rooij 1983b). It was speculated that at division some A_{pr} spermatogonia lose their bridge and split into new pairs or even into A_s spermatogonia. In this respect, it is interesting that GFRA1, one of the receptors for GDNF that stimulates SSC self-renewal, is expressed by most A_s and A_{pr} spermatogonia, and gradually becomes less expressed in A_{al} spermatogonia when chain length increases (Tokuda et al. 2007; Hofmann et al. 2005; Meng et al. 2000). This suggests that some A_{pr} spermatogonia may be still be similar to A_s spermatogonia at the molecular level.

Clearly, the nature of the A_{pr} spermatogonia with respect to differentiation status and self-renewing capacity needs to be studied in more detail. Unfortunately, progress

will depend on *in vivo* studies because purification for GFRA1 positive cells will render a mixture of original A_s and of A_{pr} spermatogonia split into single cells during the purification procedure. However, this information will be necessary to enable a full understanding of the regulation stem cell renewal and spermatogonial multiplication.

4.8 Proliferative Activity of A_s, A_{pr}, and A_{al} Spermatogonia During the Epithelial Cycle

The proliferative activity of the $A_{s,pr,al}$ spermatogonia is not constant during the cycle of the seminiferous epithelium but follows a certain pattern. For the Chinese hamster the ^{3}H-thymidine labeling index has been determined for $A_{s,pr,al}$ spermatogonia at multiple moments during the epithelial cycle (Lok and de Rooij 1983b). The proliferative activity of these cells is lowest, but not zero, in stages VII–IX (Fig. 4.5). At about stage X, $A_{s,pr,al}$ spermatogonia start to proliferate, suggesting that the stimulus for this to occur is similar for all three types of spermatogonia. Remarkably, the labeling index of both A_s and A_{pr} spermatogonia does not get higher than about 10%, while this parameter reaches almost 25% for A_{al} spermatogonia. Knowing the cell cycle times and the ^{3}H-thymidine labeling index of the cells, it is possible to calculate the growth fraction of the $A_{s,pr,al}$ spermatogonia. In their period of active proliferation, during epithelial stages X to early VII, on the average about 55% of the A_s spermatogonia are in active cell cycle. For the A_{pr} and A_{al} spermatogonia, during stage X to stage IV the growth fraction data are 66 and 80%, respectively (Lok and de Rooij 1983b). The proliferative activity of

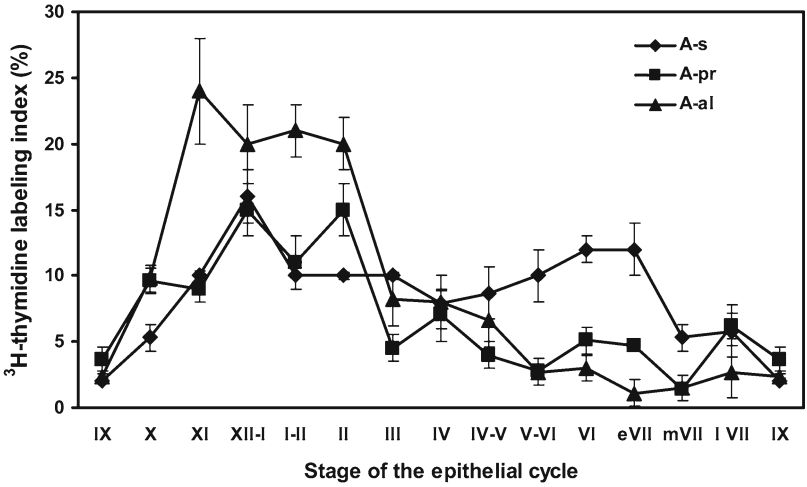

Fig. 4.5 ^{3}H-thymidine labeling index of A_s, A_{pr}, and A_{al} spermatogonia throughout the stages of the cycle of the seminiferous epithelium in the Chinese hamster. Data are from Lok et al. (1984). eVII, mVII – early and mid stage VII, respectively; l VII – late stage VII

the A_s spermatogonia decreases at about stage VI and that of the $A_{pr,al}$ spermatogonia at about stage III.

The higher growth fraction of the A_{al} spermatogonia during the active period may be related to the fact that the cells composing the chains are connected by intercellular bridges. Factors stimulating cells to cross the G1/S barrier induced in one cell of a chain may well diffuse to other cells and initiate cell cycle progress in the whole chain. Then the more cells present in a chain, the larger the chance of cell cycle initiation.

While in the Chinese hamster $A_{s,pr,al}$ spermatogonia start to actively proliferate at about the same epithelial stage, they do not decrease their proliferative activity simultaneously. The A_s spermatogonia keep proliferating considerably longer than A_{pr} and A_{al} spermatogonia (Lok and de Rooij 1983b). A similar phenomenon was seen in the rat (Huckins 1971d).

4.9 Regulation of the Proliferative Activity of A_s, A_{pr}, and A_{al} Spermatogonia at the Cellular Level

Which mechanisms determine the stages of the epithelial cycle at which inhibition and stimulation of $A_{s,pr,al}$ spermatogonial proliferation take place? Several pieces of evidence indicate the nature of the cellular cause for the inhibition of the proliferation of A_{pr} and A_{al} spermatogonia from stage II onwards.

First, in the 1970s experiments were carried out to detect a so-called chalone for the regulation of spermatogonial proliferation. Chalones are supposed to be factors secreted by the differentiated cells in a tissue that inhibit the proliferation of the preceding stem cells and early amplifying cell types. The general idea is that, in this way, an equilibrium can be formed between the numbers of cells needed by a tissue and cell production (Iversen 1973). When the number of differentiated cells is low, little chalone is produced and proliferation of progenitor cells will increase because these cells are no longer inhibited and vice versa. With one exception (Cunningham and Huckins 1979), testicular extracts have been found to inhibit the proliferation of early spermatogonial cell types (Clermont and Mauger 1974; Irons and Clermont 1979; de Rooij 1980; Thumann and Bustos-Obregon 1978, 1982). Of course, this may also have been caused by an effect of the testicular extracts via the somatic component of the testis. However, in the mouse, injection of extracts from mouse testes from which the spermatogonia have been removed by way of administration of busulfan, fails to produce a diminution of the formation of differentiating spermatogonia making this possibility unlikely (de Rooij 1980). Hence, these results suggest an inhibitory action of differentiating type spermatogonia on $A_{s,pr,al}$ spermatogonial proliferation. No reports have been published as yet describing a successful purification of the inhibiting factor.

Second, more direct evidence for a role of differentiating type spermatogonia in the proliferation of $A_{s,pr,al}$ spermatogonia came from an experiment in which Chinese hamsters were given cytosine 1-β-D-arabino-furanoside (Ara-C), which

kills cells in S phase at the time of administration (de Rooij et al. 1985). This drug also kills spermatogonia in S phase and in epithelial stage VIII it will kill all A1 spermatogonia in a particular area because these cells synchronously go through S phase in stage VIII. As a result of the disappearance of the A1 spermatogonia from tubule areas in stage VIII at the time of ARA-c administration, particular stretches of the seminiferous tubules will not contain any differentiating type spermatogonia during the ensuing epithelial cycle. In contrast, very few $A_{s,pr,al}$ spermatogonia go through S phase in stage VIII. As a result of this, the areas emptied from A1 spermatogonia will have a virtually normal complement of $A_{s,pr,al}$ spermatogonia. By following these particular areas with time after administration of Ara-C, one can study the proliferative behavior of the $A_{s,pr,al}$ spermatogonia in a situation in which they are not surrounded by differentiating spermatogonia. Interestingly, in such a situation the proliferative activity of the cells is not inhibited around stage II and very much larger numbers of A1 spermatogonia are produced (Fig. 4.6) (de Rooij et al. 1985). Previously, in the mouse it was found that when more than about 50% of the spermatogonia are killed by administration of busulfan, there is an enhanced proliferation of $A_{s,pr,al}$ spermatogonia, findings compatible with these results (van Keulen and de Rooij 1974). Together, these results confirm and expand the notion that came forward from the chalone work, i.e., that differentiating spermatogonia

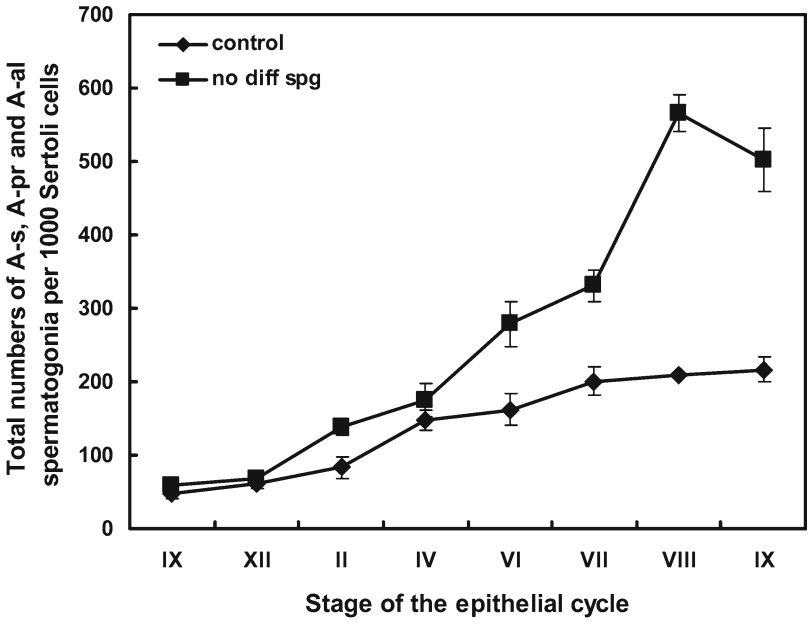

Fig. 4.6 Comparison between the numbers of cells produced by A_s, A_{pr}, and A_{al} spermatogonia during the cycle of the seminiferous epithelium in an area from which the generation of differentiating spermatogonia has been removed and cell production in the normal epithelium of the Chinese hamster. More cells are produced when no differentiating spermatogonia are present indicating that these cells inhibit $A_{s,pr,al}$ spermatogonial proliferation. Data from de Rooij et al. (1985)

have an inhibiting effect on the proliferation of earlier spermatogonial cell types. A corollary of this is that, apparently, the lengthy period of quiescence of the A_{al} spermatogonia that normally lasts from about stage II up until stage VII/VIII is not required for a proper differentiation of these cells into A1 spermatogonia.

The next question then is, how are the A1 spermatogonia in stage VIII and the $A_{s,pr,al}$ spermatogonia around stage X stimulated to proliferate again? The proliferative activity of spermatogonia being inhibited by the density of these cells, the most obvious reason for a resumption of spermatogonial proliferation would be a decrease in spermatogonial density. Two events may be of importance here. First, in stage VI the B spermatogonia give rise to the preleptotene spermatocytes and possibly these cells no longer inhibit spermatogonial proliferation. In stage VIII, spermatocytes enter premeiotic S phase and thereafter meiotic prophase. However, as at about the same time the A1 spermatogonia also enter S phase, these events are too late for being the stimulus for A1 proliferation. Also, all of these events are too early to constitute the stimulus for the proliferation of the $A_{s,pr,al}$ spermatogonia. Second, in stage VIII the A1 spermatogonia that previously were chains of A_{al} spermatogonia, lying relatively close together in or near the SSC niche, move out of the niches to the space left by the leptotene spermatocytes that moved to the adluminal compartment (Yoshida et al. 2007). The departure of the A1 spermatogonia may lower the spermatogonial density in the niches; because of this, $A_{s,pr,al}$ spermatogonia are no longer inhibited to proliferate and start to do so from about stage X onwards. Clearly, these speculations will need further studies.

4.10 Regulation of Spermatogonial Numbers

A final question is whether or not there are regulatory mechanisms that ensure a more or less constant production of spermatogonia and spermatocytes throughout the seminiferous epithelium. To answer this question, first the $A_{s,pr,al}$ clonal content has been determined in a number of large areas of tubule basal membrane of the Chinese hamster (de Rooij and Janssen 1987). Surprisingly, there are large differences in the density of these cells between different areas. When a regulatory mechanism exists to ensure an even production of A1 spermatogonia in all areas it will try to increase the numbers of $A_{s,pr,al}$ spermatogonia in areas where their density is low. The way to do that is to increase the numbers of stem cells in these areas. However, the ratios between the numbers of A_s spermatogonia and the numbers of clones of $A_{pr,al}$ spermatogonia in high and in low density areas are not significantly different. Apparently, the chance of self-renewal of A_s spermatogonia remains similar whether they are surrounded by high numbers of clones of $A_{s,pr,al}$ spermatogonia or not. As a result, in some areas very low numbers of A1 spermatogonia and in others very many are formed. Up to fivefold differences have been found (de Rooij and Janssen 1987).

Despite the large differences in the numbers of A1 spermatogonia in different areas, the numbers of In and B spermatogonia and preleptotene spermatocytes are

similar everywhere, in tubules in the appropriate stages (de Rooij and Lok 1987). Hence, in between A1 spermatogonia in stage VIII and In spermatogonia in stage III, the differences in differentiating spermatogonial density are eliminated. As can be concluded from cell kinetic studies using ^{3}H-thymidine labeling, at each division all differentiating spermatogonia go through division (Lok and de Rooij 1983a). Therefore, differences in cell density cannot be evened out by letting cells in high density areas skip a division. The most likely explanation is that in the normal situation the stem cells always produce too many differentiating spermatogonia and that the surplus of cells produced is eliminated by way of apoptosis in between A1 and In spermatogonia. In high density areas relatively many A2–A4 spermatogonia will enter apoptosis and only few when the density is low. In the end, differentiating spermatogonial density is the same everywhere. Apparently, the well-known phenomenon of spermatogonial apoptosis in the normal testis is just the way germ cell density is regulated and probably has nothing to do with a selection for the best germ cells, as has often been suggested.

4.11 Conclusion

The spermatogonial lineage in rodents and rams has been described in great detail at the morphological level. All subsequent types of germ cells are known, spermatogonial numbers have been determined, and their cell cycle behavior has been documented in detail. Furthermore, a scheme of spermatogonial multiplication and stem cell renewal has been devised on which most investigators agree, although some details are still a matter of debate. It remains to be unequivocally clarified whether or not A_{pr} and A_{al} spermatogonia can still split into A_{s}, A_{pr}, or smaller chains of A_{al} spermatogonia and whether some sort of de-differentiation can take place. An interesting point for further studies will be to find the factors responsible for the inhibition of $A_{s,pr,al}$ spermatogonial proliferation and to see how the various growth factors found to enhance $A_{s,pr,al}$ spermatogonial proliferation function with respect to the epithelial cycle. Is there a changing pattern in the secretion of growth factors by Sertoli cells during the epithelial cycle? It will be a challenging task to fit in the many data that are presently generated on purified spermatogonia/SSCs into an understanding of how spermatogonial multiplication and stem cell renewal is regulated in the normal *in vivo* situation, which is even more complex because of the presence of the epithelial cycle, something that cannot be simulated *in vitro*.

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Chapter 5
Spermatogonial Stem Cell Transplantation and Culture

Jonathan A. Schmidt and Ralph L. Brinster

Abstract The study of spermatogonial stem cells (SSCs) has been hampered by the lack of techniques to identify, quantify, and maintain these cells both *in vitro* and *in vivo*. Although markers to definitively identify a specific cell as a SSC are still unknown, techniques to maintain SSCs *in vitro* and to quantify the number of SSCs in a specific cell population have been developed. SSC transplantation is a technique in which a population of cells is transplanted into the testis of a recipient. Presence of SSCs within the transplanted cells can later be validated by the production of donor-derived colonies of spermatogenesis within the recipient testis. This technique is extremely valuable for the quantitative analysis of the effect of experimental treatments on SSC numbers as well as the generation of donor-derived and transgenic spermatozoa for biotechnological applications. *In vitro* SSC culture has allowed for a platform to study mechanisms regulating SSC function outside of the testis, and when combined with SSC transplantation, serves as a powerful tool to study these cells. The development of SSC transplantation and culture techniques has revolutionized the study of these important cells; however, care must be taken when interpreting data using these techniques due to the absence of a definitive SSC marker. Nevertheless, many recent advances using these techniques, including SSC transplantation in livestock and companion animals and the elucidation of the roles of growth factors in SSC function, have significantly advanced the field of SSC biology.

Keywords Spermatogonial stem cell • Culture • Transplantation • Germ cell • Testis

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5.1 Introduction

There are no known molecular or phenotypic markers that definitively distinguish a spermatogonial stem cell (SSC) from its daughter cells committed to differentiate into sperm. Thus, it is impossible to state that an individual spermatogenic cell is a SSC. Because the SSCs lack definitive markers, the study of this important cell has been limited. Furthermore, lack of an *in vitro* culture system and a reliable assay to quantify SSC activity exacerbated the inability to conduct reliable experiments examining the SSC. The absence of these techniques was further hampered by the extreme rarity of the SSC in the adult mouse testis, which was estimated to be present at a concentration of 1 in 3,000 testis cells (Tegelenbosch and de Rooij 1993). However, over the last 15 years our laboratory and others have developed and continuously refined techniques to quantify SSC activity (SSC transplantation) and to maintain the SSC *in vitro* (SSC culture), and when combined, SSC transplantation and culture provide a powerful tool to identify mechanisms regulating SSC function. The development of these techniques has allowed for many important discoveries pertaining to the male germline, and translation of these techniques to other species will open many doors for novel methods of reproductive management and are currently the next frontier in the study of SSC biology.

5.2 Spermatogonial Stem Cell Transplantation

5.2.1 History

The existence of a germline stem cell population that resided in the testis and that was responsible for continued fertility throughout the life of a male was first postulated by Huckins and Clermont in 1968. However, direct study of the properties of the spermatogonial stem cell was not feasible until a functional transplantation assay was developed in 1994. In this work, Brinster and Zimmermann (1994) and Brinster and Avarbock (1994) demonstrated that when placed in the seminiferous tubules of infertile recipients, donor spermatogonial stem cells were able to migrate from the lumen through the tight junctions to the basement membrane and initiate complete donor cell-derived spermatogenesis. Furthermore, donor spermatozoa were fully functional and could generate normal offspring through natural mating (Fig. 5.1).

5.2.2 Implications

In addition to the basic study of male reproduction and stem cell biology, the implications for SSC transplantation are far-reaching. From a clinical standpoint, the future extension of SSC transplantation to humans would allow for the preservation

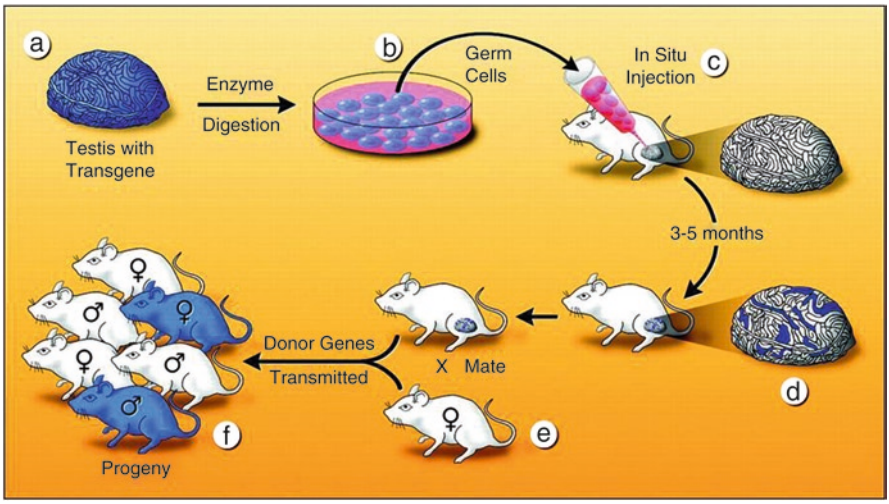

Fig. 5.1 Procedure for transplantation of testis cells. **(a)** Donor testes carrying a transgene are digested resulting in a single cell suspension. **(b)** These cells can be enriched for SSC using methods such as differential plating, MACS, and FACS, and can be cultured using appropriate conditions. **(c)** Testis cells are injected into the testis of a recipient animal that is immunologically compatible to the donor and that has little to no endogenous spermatogenesis due to natural mutation of experimental treatment. **(d)** During the subsequent 2–5 months, donor SSCs colonize the recipient testis and initiate donor-derived spermatogenesis. If the donor was transgenic for a colorimetric marker such as LacZ or GFP, testes can be removed and examined using appropriate techniques to quantify colonies of spermatogenesis, each of which is derived from a single SSC. **(e)** The recipient male can also be mated with a fertile female and produce donor derived progeny. **(f)** Donor-derived progeny can be identified using appropriate techniques. It is possible that recipient-derived progeny are also produced owing to endogenous spermatogenesis. This image was used with permission from Brinster (2002) © American Association for the Advancement of Science

of the germline and reinitiation of spermatogenesis in prepubertal individuals undergoing chemotherapy. SSC transplantation would also be very useful in agriculture and for the conservation of endangered species. Thus, preservation of the germline of valuable animals and transplantation of these cells into less valuable recipients resulting in donor-derived spermatogenesis could increase the quality of agricultural products and contribute to the preservation of endangered species. Of particular interest from a human clinical, agricultural, and biotechnological viewpoint is the potential to generate transgenic individuals by manipulation of the male germline. This technique would be useful for gene therapy of the male germline to correct mutations that affect spermatogenesis in infertile individuals or to remove deleterious genes from the gene pool in animals and potentially humans. Additionally, transgenic animals could be generated that express economically valuable proteins in meat or milk. Indeed, the groundwork for many of these applications has been established in rodents and attempts have been made to extend these techniques to other more economically valuable species.

5.2.3 Clonality and Quantification

An interesting and useful characteristic of colonization of recipient seminiferous tubules by donor SSCs is that when transplanted at the right concentration, distinct colonies of donor-derived spermatogenesis are formed. Colonies of spermatogenesis can be easily identified if the donor recipient is transgenic for a marker such as β-galactosidase (LacZ) or green fluorescent protein (GFP). When SSCs are transplanted into recipient testes at low concentrations, each colony is theoretically derived from a single SSC and quantification of colonies is directly related to the number of stem cells in the initial population (Dobrinski et al. 1999b; Nagano et al. 1999; Kanatsu-Shinohara et al. 2006). This relationship allows for the quantification of SSCs in populations of cells from various experiments and has been used to identify molecules useful for SSC enrichment.

5.2.4 Species Specificity

5.2.4.1 Rodent

The classical species that has been used to study the SSC is the mouse. Transplantation of mouse SSCs into infertile mouse recipients results in robust spermatogenesis that can lead to the production of donor-derived offspring through natural mating of the recipient or intracytoplasmic sperm injection (Brinster and Avarbock 1994). However, because the SSCs reside in the basal compartment of the seminiferous tubule, which is on the blood side of the blood–testis barrier, immune-compatible donors and recipients must be used. Alternatively, animals with naturally or artificially depressed immune systems can also be used for recipients.

The rat SSC has also received much attention in the field. Like in mice, transplantation of rat SSCs into an immunologically compatible recipient results in the production of fully functional spermatozoa (Ryu et al. 2003). Additionally, xenotransplantation of rat SSCs into mouse recipients also results in complete spermatogenesis (Clouthier et al. 1996). When rat SSCs are transplanted into mouse recipient testes, the pattern of germ cell development (cycle of the seminiferous epithelium) follows that of the rat, indicating that the mechanisms and timing of germ cell development are intrinsic to the germ cell and not directed by the somatic environment (Franca et al. 1998).

5.2.4.2 Non-rodents

SSCs from every species examined have the ability to colonize the basement membrane of the mouse seminiferous tubule after xenotransplantation; however, only rodent SSCs are able to undergo complete spermatogenesis, indicating that species specific mechanisms regulating SSC differentiation exist (Clouthier et al. 1996; Dobrinski et al. 1999a, 2000; Nagano et al. 2001, 2002; Oatley et al. 2004).

Transplantation of SSCs between individuals in non-rodent species is complicated because of the difficulty in generating recipients devoid of endogenous spermatogenesis, technical limitations on the delivery of SSCs to the seminiferous tubules, and absence of inbred lines of animals that provide for immunologically compatible donor–recipient combinations. Nevertheless, complete donor-derived spermatogenesis following transplantation in species such as goats (Honaramooz et al. 2003), dogs (Kim et al. 2008), and pigs (Honaramooz et al. 2002; Mikkola et al. 2006) has been reported. Furthermore, it has been demonstrated that ultrasound guided intratesticular transfer is the most appropriate method for transplantation into bovine, monkey, and human testes (Schlatt et al. 1999). Recently, reports have described successful SSC colonization in sheep (Rodriguez-Sosa et al. 2006, 2009) and cattle (Herrid et al. 2006).

SSCs from human and primate testes colonize mouse seminiferous tubules, but do not differentiate (Nagano et al. 2001, 2002). When primate testis cells are transplanted into primate recipient testes, it has been suggested that donor cell colonization occurs based on an increase in testis weight in some recipients (Schlatt et al. 2002).

5.2.5 Procedures and Considerations

5.2.5.1 Cell Labeling and Enrichment

The ability to visually identify donor colonies after SSC transplantation is dramatically improved by the use of transgenic donor cells with a visible reporter gene. Common transgenic animals that have been used for SSC transplantation include those carrying genes either for Lac Z or GFP. Additionally, animals that are transgenic for other fluorescent markers (such as DS red) can also be useful for SSC identification post transplantation (Fig. 5.2). Donor-derived spermatogenesis can also be identified using microsatellite markers, cell membrane dyes, or by genotyping spermatozoa or offspring.

Isolation of populations of cells enriched for the SSC is important for efficient generation of donor-derived spermatogenesis post-transplantation. Many methods have been reported that enrich for the SSC. These methods include cell isolation from immature or cryptorchid males (Shinohara et al. 2000a), Percoll centrifugation, differential plating (Shinohara et al. 2000b), and selection using fluorescence- or magnetic-activated cell sorting (FACS or MACS) based on expressed specific cell surface markers or fluorescence driven by germline-specific promoters (Shinohara et al. 1999; Kubota et al. 2003, 2004a, b). The most efficient methods are those that employ selection using cell surface markers such as Thy-1 (5–30-fold) and CD9 (7-fold) in the mouse (Kubota et al. 2004a; Kanatsu-Shinohara et al. 2004) and Tacstd1/Epcam in the rat [11-fold; Schmidt et al. 2008, unpublished; and Ryu et al. (2004)]. When deciding which marker should be used, care must be taken to assure that putative markers do indeed enrich for the SSC. Furthermore, even with selection, age of the donor and species can have a significant effect on degree

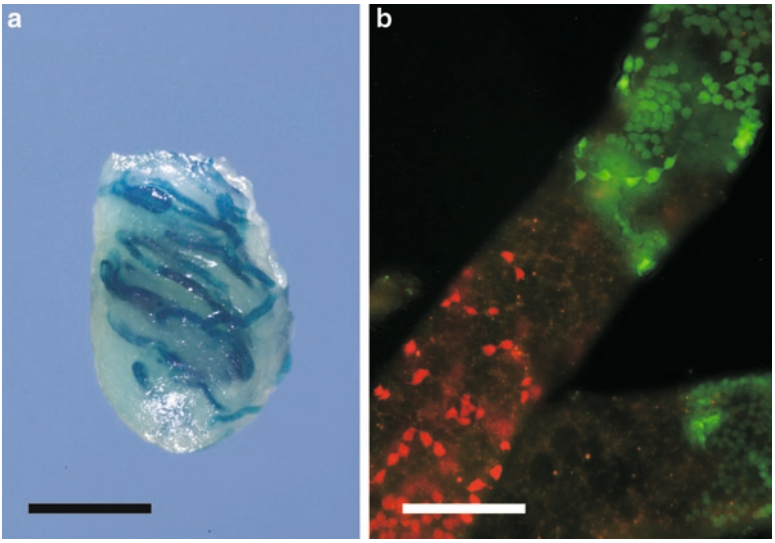

Fig. 5.2 Examples of recipient testes transplanted with various transgenic donor cells. (**a**) Immunodeficient nude mouse recipient transplanted with LacZ positive rat cells. Blue colonies from donor-derived stem cells are clearly visible. Scale bar = 2 mm. (**b**) Immunocompatible recipient transplanted with both GFP (*green*) and DS-Red (*red*) mouse donor cells. Individual spermatogonia can be seen on the basement membrane of the recipient seminiferous tubules. Scale bar = 140 μm

of enrichment (Ebata et al. 2005). For example, Gfra1 has been considered a putative marker for the SSC population. Selection of Gfra1 positive cells from neonatal mouse pups has been reported to enrich for SSCs approximately 1.8–2.5-fold; however, selection of Gfra1 positive cells from adults actually depletes the positive population to only 13% of the nonselected population (Hofmann et al. 2005a, b; Buageaw et al. 2005). Interestingly, when applied to rat pups, we have observed an over 60-fold increase in SSCs in the Gfra1 positive population (Schmidt et al. 2008, unpublished).

5.2.5.2 Homing Efficiency

The ability of a specific stem cell to home to a particular niche is a hallmark of adult stem cell transplantation techniques. Little research has examined this phenomenon outside of the hematopoietic system, owing in part to an inability to study directly the stem cell in the niche. In the hematopoietic stem cell system, homing is a rapid process, thought to take less than 2 days, that is dependent on a variety of components including chemokines, cell adhesion, and extracellular matrix molecules (Lapidot et al. 2005). The ability of the SSC to home to the SSC niche is essential for transplanted SSCs to colonize and undergo spermatogenesis in the recipient testis. For the recipient Sertoli cell to recognize and initiate homing of the SSC to

the niche is a remarkable phenomenon. The mechanisms involved presumably are mediated by the expression of specific molecules on the surface of the SSC and the interaction of these molecules with Sertoli cells within the seminiferous epithelium. The efficiency of SSC homing to the niche has been estimated to be approximately 5–10% in adult mice (Shinohara et al. 2001; Ogawa et al. 2003). Past experiments have suggested several possible modifiers of SSC homing. For example, colonization of donor cells in pup compared to adult testes is 9- to 10-fold greater, and each colony in the pup testis is approximately four times longer (Shinohara et al. 2001). Also, rat donor SSCs form two to three times longer colonies than mouse SSCs in nude mouse recipients (Orwig et al. 2002). Thus, both the microenvironment (niche) as well as the donor cells are likely to influence homing. It is possible that the presence of endogenous SSCs and the treatments used to remove these cells for successful donor SSC colonization could influence donor SSC homing. It is very difficult to study these effects; however, some experiments do give indirect insight into the effects of these treatments on SSC homing. The presence of endogenous SSCs and spermatogenesis in the seminiferous tubules decreases the colonization ability of donor SSCs, and the W mouse pup, which is devoid of endogenous spermatogenesis, has been regarded as the best recipient for mouse SSC transplantation (Brinster et al. 2003). Lack of colonization in testes with endogenous SSCs is possibly due to occupation of available niches by the endogenous SSCs and the presence of differentiating germ cells in the seminiferous epithelium, however, an effect of the resident SSC on the ability of a donor SSC to home to the niche cannot be ruled out. Studying the effects of SSC ablation techniques on homing and colonization would be very difficult due to the necessity to remove endogenous spermatogenesis for efficient colonization and quantification. However, examination of these techniques (chemotoxic drugs and irradiation) using naturally sterile recipients would allow for the evaluation of any direct effects of SSC ablation treatment on homing.

A recent publication describes experiments designed to identify molecules that may be important for homing of the SSC to the niche (Kanatsu-Shinohara et al. 2008b). The role of the β1-integrin surface antigen was examined, probably because it has been shown to be present on SSCs and is known to bind to laminin, a common cell matrix/basement membrane molecule found in the seminiferous epithelium (Shinohara et al. 1999). In this work β1-integrin was experimentally knocked-out of a cultured SSC population using a cre-lox system. The removal of the gene from SSCs also resulted in the expression of the lacZ gene, thereby providing a colorimetric marker for donor SSCs. Following transplantation, the authors demonstrated that at 3 months, there were fewer colonies in the animals transplanted with β1-integrin knockout cells than in control cells, and indicated that β1-integrin was important for binding of the SSC in the niche. In addition, defects in spermatogenesis within the knockout colonies were found. Therefore, β1-integrin appears to be important for both maintenance of the SSC within the niche and germ cell differentiation. As previously suggested, β1-integrin, in conjunction with α6-integrin, is a likely essential binding molecule for SSCs, and this binding is important in maintaining the SSC within the niche as well as for maintenance of

spermatogenesis (Shinohara et al. 1999; Kanatsu-Shinohara et al. 2008b). Additionally, because of the role of β1-integrin in homing of transplanted SSCs to the recipient niche, it is possible that it may also have a role in the endogenous homing of gonocytes to the basement membrane during testis development. It will be interesting to learn if β1-integrin is important for function of stem cells in other tissues, because of its wide distribution, or play a unique role in spermatogenesis. The mechanism of SSC homing is of great importance and the above experiments provide guidance in understanding this phenomenon, which has proven difficult to study and quantify in all stem cell systems.

5.2.5.3 Recipient Preparation and Injection

Successful SSC transplantation is directly dependent on the recipient's ability to initiate and maintain donor-derived spermatogenesis. The best recipients are those that are able to maintain spermatogenesis and are devoid of endogenous spermatogenesis. Thus, efficient recipient preparation is essential for successful donor-derived spermatogenesis (Brinster et al. 2003). Recipients can either be naturally sterile or generated by experimental means. The most useful natural mutant for SSC transplantation is the W mouse (Brinster and Zimmermann 1994). These mice have defects of various severities in the c-kit receptor. The interaction between the c-kit receptor and its cognate ligand kit (stem cell factor) is essential for migration of the PGC to the genital ridge and later, differentiation of spermatogonia in the adult testis. Even though the testes of W animals contain a greatly reduced number of SSCs, they serve as excellent recipients for SSC transplantation because the endogenous germ cells cannot differentiate. Treatment with busulfan and irradiation are two experimental means to destroy endogenous spermatogenesis (Brinster and Avarbock 1994). Busulfan is a cytotoxic drug used for chemotherapy that destroys SSCs. Irradiation also selectively destroys actively dividing cells and is useful for non-rodent species in which the level of busulfan needed to eliminate spermatogenesis is toxic (Withers et al. 1974; Meistrich et al. 1978; Van Beek et al. 1990; Zhang et al. 2006). Neither busulfan nor irradiation completely eliminates endogenous spermatogenesis, so transplantation of labeled donor cells is still desirable in order to readily differentiate donor-derived from endogenous spermatogenesis.

The optimal route of cell injection varies among species, but include microinjection of cells into efferent ducts, the rete testis, or seminiferous tubules (Ogawa et al. 1997) (Fig. 5.3). The efferent ducts connect the rete testis to the caput epididymis. In rodents the efferent ducts are outside of the testis and are easily injected using a microinjector. However, in non-rodent species, the efferent ducts are not readily available for injection. In addition, the rete testis in non-rodent species lies inside the testis and consist of many interconnected tubules. Thus, the most efficient means of cell injection in this instance is to use ultrasound guided injection of the rete testis (Honaramooz et al. 2002; Schlatt et al. 1999). Alternatively, cells can be directly injected into the seminiferous tubules. However, direct injection of cells

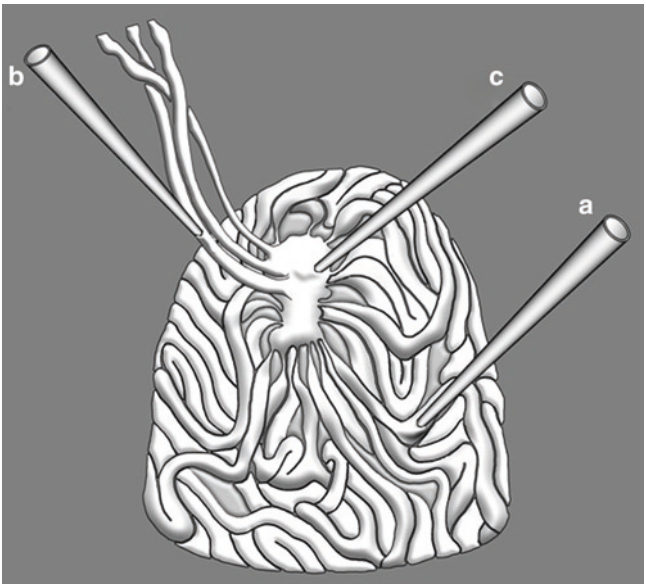

Fig. 5.3 Methods of microinjection of testis cells into a donor testis. **(a)** Cells can be injected directly into the seminiferous tubules. **(b)** Cells can be injected into the efferent ducts or **(c)** the rete testis. Cells that are injected into either the efferent ducts or the rete testis migrate to the rete testis and colonize the seminiferous tubules

into seminiferous tubules is inefficient; because of the cell suspension must flow to the rete to fill tubules other than the injected one.

5.3 Spermatogonial Stem Cell Culture

5.3.1 History

Considerable effort has been directed towards the development of a culture system for the *in vitro* maintenance and amplification of spermatogonial stem cells. Original experiments were conducted shortly after the development of the SSC transplantation technique; however, SSCs survived only about 4 months *in vitro*, the results were highly variable, and increases in SSC numbers were not observed. This was believed to result from competition by contaminating somatic cells and the lack of factors within the culture environment that are necessary for SSC survival and self-renewal. Since the development of a long-term culture system, considerable progress has been made defining the molecular mechanisms of SSC self-renewal. It is important to note early in this discussion that SSC cultures are NOT cultures of pure stem cells and experiments must be designed with this in mind. SSC cultures are ENRICHED for SSCs, and estimates indicate that approximately 1

in 10 or 1 in 15 (6.6–10%) germ cells in our mouse SSC culture are true SSCs (Kubota et al. 2004b). Others have reported that the true concentration of SSCs in culture can be as low as 0.02% (Kanatsu-Shinohara et al. 2005b). The remaining cells within the culture are considered to be daughter cells that are committed to differentiation.

5.3.2 Implications

The ability to culture the SSC has many important implications for human health, animal management, and basic stem cell research. Culture of the human SSC would allow for amplification of SSC numbers prior to transplantation into testes of individuals that underwent childhood chemotherapy or irradiation. Boys that would need to undergo either of these procedures are often infertile as adults, but if SSCs could be isolated from testis biopsies before cytotoxic treatment, they could be amplified *in vitro* and stored until an appropriate time for SSC transplantation, thereby restoring fertility. Similar procedures could be utilized for the amplification of SSCs obtained from valuable livestock or endangered species. Culture of SSCs from genetically valuable individuals, followed by transplantation or xenotransplantation to inferior or less-endangered recipients could perpetuate genetic material indefinitely; thereby conferring a biological immortality to the male. Cultured SSCs have been demonstrated to be readily used to generate transgenic mice (Nagano et al. 2000), rats (Ryu et al. 2007), and goats (Honaramooz et al. 2008). The ability to introduce foreign genes into the SSCs of livestock, followed by transplantation and ultimately the production of transgenic offspring, would allow for the production of economically valuable compounds that could be secreted in milk or produced in meat. These techniques might also be utilized for gene therapy to correct mutations present in an individual's germline. Because many stem cell systems in the body may share regulatory mechanisms, SSCs could serve as a model to understand the fate decisions of self-renewal vs. differentiation in other tissues dependent on stem cells for maintenance. Thus, culture of SSCs could serve as a valuable tool to identify these mechanisms in other adult stem cell populations. Finally, culture of SSCs will serve as a foundation for experiments to develop a system for *in vitro* spermatogenesis. The production of spermatozoa *in vitro* would revolutionize assisted reproductive techniques and allow for spermatozoa production without SSC transplantation.

5.3.3 Short- Versus Long-Term Culture

SSCs can be cultured for various periods of time. The first report of a long-term SSC culture system (Nagano et al. 1998) demonstrated that SSCs could be maintained, but did not actively proliferate, over a 4-month period on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) mouse embryonic fibroblast feeder layers.

In subsequent experiments, a positive role of glial cell line-derived neurotrophic factor (GDNF) for SSC maintenance *in vitro* was demonstrated (Nagano et al. 2003). Several groups have now reported the development of long-term SSC culture systems using techniques to enrich for germ cells, thereby removing contaminating cells, and including cocktails of growth factors in the growth media. In 2003, it was demonstrated that gonocytes isolated from day 0 mouse pups could be maintained and proliferate over a 5-month period (Kanatsu-Shinohara et al. 2003). These experiments resulted in a long-term culture system that supported SSC self-renewal in DBA-derived strains of mice, but not other mouse strains. However, the culture medium included serum and proprietary components; therefore, it was difficult to determine the critical growth factors required for SSC self-renewal. In 2004, a procedure to maintain the SSCs of many mouse strains *in vitro* in a defined culture medium containing GDNF, GDNF family receptor alpha 1 (GFRα1), and basic fibroblast growth factor (bFGF) was reported (Kubota et al. 2004a). This work unequivocally proved that GDNF was the essential growth factor responsible for self-renewal of mouse SSCs. Subsequently, others reported a more complex serum-free medium containing proprietary ingredients was also able to maintain SSC function and support SSC self-renewal of DBA-derived strains of mice (Kanatsu-Shinohara et al. 2005a). In these culture systems, germ cells, including the SSC, form clump-like structures *in vitro* that resemble colonies of ES cells in culture (Fig. 5.4). Mouse SSC clumps appear more three-dimensional than rat clumps.

Since the discovery of GDNF as the main regulator of SSC self-renewal, many experiments have been conducted evaluating mechanisms of SSC function using long-term culture systems. For example, gene expression in these cultured cells using stable or transient transfection of transgenes or siRNA has been evaluated (Ogawa et al. 2003; Oatley et al. 2006; Dann et al. 2008; Schmidt et al. 2008, unpublished). Using SSC cultures treated with siRNA in combination with SSC transplantation, it was demonstrated that *Bcl6b*, *Etv5*, and *Lhx1* are important for mouse SSC self-renewal (Oatley et al. 2006). Recently, both *Bcl6b* and *Etv5* were found important for rat SSC self-renewal (Schmidt et al. 2008, unpublished). With a similar experimental approach and treatment of cultured cells with inhibitors of

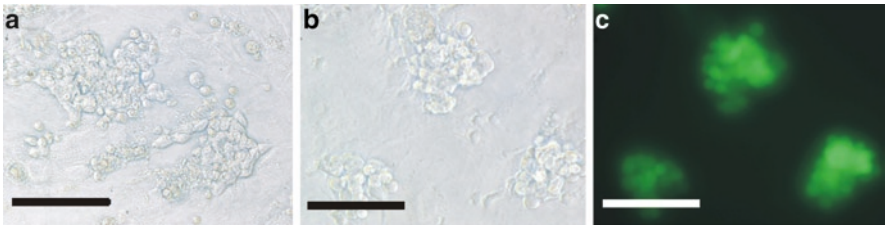

Fig. 5.4 Microscopic images of cultured germ cells. (**a**) EpCam positive rat germ cells enriched for SSCs growing as clumps. (**b**) Thy1 positive mouse germ cells enriched for SSCs growing as clumps. (**c**) The clumps in (*B*) were isolated from a GFP-positive individual and fluoresce when stimulated with UV light. Scale bars = 100 μm

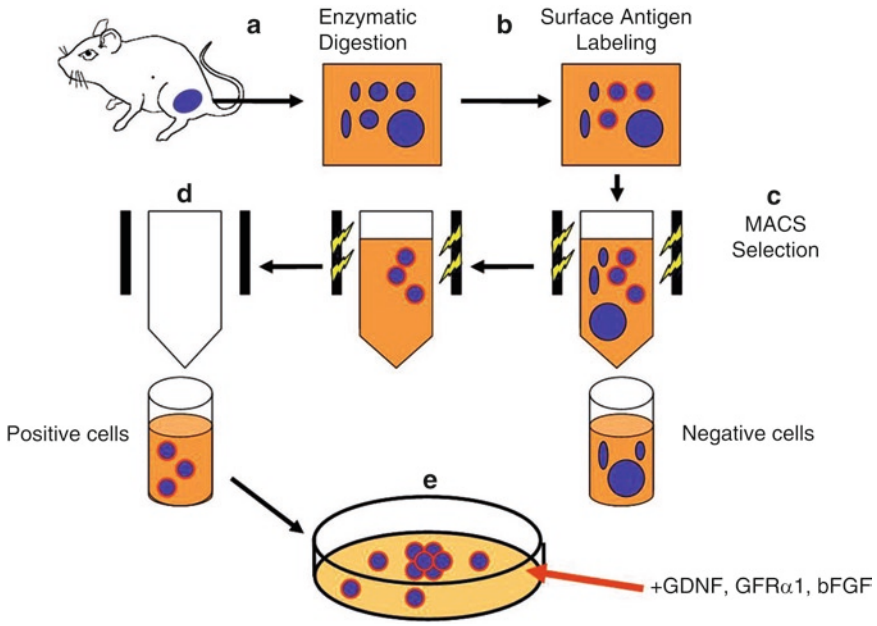

Fig. 5.5 Diagrammatic representation of the establishment of a typical rodent SSC culture using MACS selection. **(a)** Testes from transgenic mouse pups are enzymatically digested resulting in a heterogeneous cell population. **(b)** Cells are immunologically labeled with MACS conjugated antibodies for a specific germ cell surface antigen (Thy-1; mouse or EpCam; rat). **(c)** The labeled cells are then placed over a separation column within a magnetic field. Cells that are not bound to the specific MACS conjugated antibody flow through the column. **(d)** After removal of the magnetic field, the retained positively labeled cells flow through the column. **(e)** These enriched cells can then be used for SSC culture and maintained long-term in the presence of GDNF, GFRα1, and bFGF

signal transduction molecules, it was demonstrated that the mechanism of GDNF activation of these genes is through SRK family kinase signaling mechanisms and that GDNF activation of AKT is important for SSC survival (Oatley et al. 2007; Braydich-Stolle et al. 2007; Lee et al. 2007). For this type of experiment to study regulation of self-renewal, SSCs should be used as soon as contaminating somatic cells are absent from the culture, about 4–6 weeks after initiation. A scheme for initiation of SSC cultures is outlined in Fig. 5.5. Although culture will theoretically maintain SSCs indefinitely, cells can become modified, especially during long-term culture and lose nearly all testis colonizing and spermatogenic ability without an apparent change in culture phenotype. Therefore, it is critical to verify stem cell content of cultures during experimental procedures.

Culture of SSCs in a three-dimensional environment would more closely mimic the niche environment of the seminiferous epithelium, and the ability to induce germ cell differentiation and meiosis may be facilitated in a three-dimensional environment. A recent report describes the utilization of a three-dimensional soft agar culture system containing somatic testis feeder cells (Stukenborg et al. 2008).

The authors concluded that the technique supported the expansion of SSCs and the development of postmeiotic germ cells over a 15-day culture period. However, transplantation assays were not conducted to determine the level of SSC proliferation and no functional spermatozoa were produced.

5.3.4 Species

5.3.4.1 Rodent

The first long-term self-renewing SSC cultures that were validated with SSC transplantation were generated from mice in 2003 and 2004 (Kubota et al. 2004a; Nagano et al. 2003; Kanatsu-Shinohara et al. 2005a). These experiments demonstrated that GDNF was the essential molecule for SSC self renewal and that SSCs were maintained for extended periods of time. In some systems of rodent SSC culture, the starting cells come from newborn pups of day 0, 1, or 2 after birth (Kanatsu-Shinohara et al. 2003), which contain gonocytes that reside in the center of the seminiferous tubules. In other systems, the cells are recovered from pups at days 5–8 or from adults (Nagano et al. 2003); in both instances, the gonocytes have converted to spermatogonial stem cells and reside on the basement membrane of the seminiferous tubule. Shortly after birth the gonocytes migrate to the basement membrane of the seminiferous tubules and between day 0 and 6 in mice have begun to initiate the first wave of spermatogenesis and to differentiate into SSCs (Huckins and Clermont 1968; de Rooij and Russell 2000; Yoshida et al. 2007; McLean et al. 2003). For these reasons, is has been suggested that results from germ cells isolated at 6 days of age or older may differ from results obtained using cells isolated at day 0, 1, or 2 after birth. Identification of differences in these two populations could be informative regarding the transition from true gonocytes to stem cell. The defined culture system that was reported in 2004 (Kubota et al. 2004b) readily supports the maintenance of SSCs from both pup and adult testes from a variety of different mouse donor strains. In addition to the mouse, systems for the long-term culture of rat SSCs have been reported (Ryu et al. 2005; Hamra et al. 2005). Experiments using the rat SSC culture system, which is based on a defined medium very similar to that of the mouse system, have identified several important characteristics of rodent SSCs *in vitro* (Ryu et al. 2005). First, cultured mouse SSCs double approximately every 5 days; whereas, rat SSCs double every 11 days (Kubota et al. 2004a; Ryu et al. 2005), which may result from intrinsic differences in SSC proliferation between these two species. Second, different cell surface markers are utilized to enrich for mouse and rat SSCs. Mouse SSCs are readily enriched using MACS for the cell surface marker Thy1; whereas, isolation of EpCam is more effective for isolating SSCs from the rat testis. In the rat SSC culture system reported by Hamra et al. (2005) an undefined culture medium and different feeders are used, but long-term growth of rat SSCs is obtained. Additional work from this laboratory also describes detailed methodologies for rat SSC isolation and enrichment using

differential plating with plastic and collagen matrices (Hamra et al. 2008). Recently, it was reported that hamster SSCs can be cultured for long periods (Kanatsu-Shinohara et al. 2008a). The culture system for these cells is very similar to that for other rodents.

5.3.4.2 Non-rodent

Considerable effort has been devoted to the development of non-rodent SSC culture; however, confirmation of success is lacking. Recent reports exist describing SSC culture for bovine (Aponte et al. 2008) and porcine SSCs (Luo et al. 2006).

5.4 Summary

The development of techniques to transplant and culture the SSC have the potential to revolutionize reproductive management of various species, including humans, livestock, and endangered species. Furthermore, combined applications of these techniques are essential for amplification of SSCs and the efficient development of transgenic animals. Much progress has been made in the culture and transplantation of rodent SSCs; yet, progress in developing these techniques in other species of economical or societal value is minimal. However, translation of these techniques to species such as livestock and humans will open many doors for novel methods of reproductive management and are currently the next frontier in the study of SSC biology.

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Chapter 6
The Cluster-Forming Activity Assay: A Short-Term *In Vitro* Method to Analyze the Activity of Mouse Spermatogonial Stem Cells

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Abstract The previous two chapters discussed contrasting approaches to detect SSCs and investigate their biology. The approach described in Chap. 4 represents the attempt to identify SSCs prospectively by means of cell morphology, while the transplantation approach in Chap. 5 is based on the functional definition of stem cells (long-term self-renewal and differentiation) and represents retrospective SSC identification. This chapter will discuss another type of retrospective, functional SSC detection method, termed the "cluster-forming activity (CFA) assay." This technique was developed in the mouse model on the basis of the SSC culture system described in Chap. 5. Using this *in vitro* assay, SSC activity can be detected in a semi-quantitative manner within a short period of time, in marked contrast to the time-consuming and laborious transplantation assay. As with any technology, however, the CFA assay is not without limitations, and there are issues to be noted when one uses it and interprets the data obtained. The aim of this chapter is therefore twofold. First, we describe the conceptual framework of the CFA assay in order to justify its legitimacy as a reliable SSC detection method. Second, we discuss cautionary issues and relate them to the *in vitro* behavior of SSCs warranting further studies.

Keywords Quantitative stem cell assay • Cell culture • Cluster • Colony • Transplantation

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6.1 Parallelism Between the Results of *In Vitro* and *In Vivo* SSC Assays

As described in Chap. 5, when SSCs are cultured on a feeder layer in serum-free medium, supplemented with glial cell line-derived neurotrophic factor (GDNF), a secreted form of its receptor (GFRA1), and fibroblast growth factor 2 (FGF2), they form morphologically distinct, three-dimensional aggregates of spermatogonia (Fig. 6.1) (Kubota et al. 2004; Kanatsu-Shinohara et al. 2003; Yeh et al. 2007). Here, we call these aggregates "clusters" to distinguish them from "colonies" of spermatogenesis that emerge in recipient testes after transplantation (Chap. 5). These clusters can be serially passaged every 6–7 days and maintained for an extended period of time. Transplantation of clusters results in the regeneration and maintenance of complete spermatogenesis, indicating that clusters contain SSCs.

In the definitive SSC assay technique, spermatogonial transplantation, the establishment of colonies 2 months after transplantation qualitatively demonstrates SSC activity, while the number of colonies found in a recipient testis indicates the number of functional SSCs (Nagano et al. 1999; Zhang et al. 2003). Likewise, in the CFA assay (Yeh et al. 2007), the number of clusters indicates the relative SSC activity. Since clusters form within 6 days of culture, the CFA assay generates quantitative data in a far shorter period of time than the transplantation assay.

It is of note that, although the concept of this *in vitro* technique is simple and straightforward, it appears to contradict the functional definition of SSCs: i.e., the ability to regenerate and support the long-term maintenance of spermatogenesis. As spermatogenesis cannot be reproduced *in vitro* at present, the CFA assay is not based on cells' regenerative capacity. It is thus logical to raise two critical questions (1) Can SSCs indeed be detected on the basis of cluster formation without demonstrating completion of spermatogenesis and (2) Does an assay that is completed in only 6 days reliably detect long-term stem cell function? The following evidence provides answers to these questions (Yeh et al. 2007).

Figure 6.1b depicts the proliferation kinetics of clusters and SSCs *in vitro* over the period of 12 weeks. Clusters were derived initially from pup testis cells and, during the study period, counted visually after one cycle of culture (6–7 days) periodically. Proliferation kinetics of clusters were determined using the cluster number

Fig. 6.1 (continued) continue over the extended culture period, demonstrating that cluster numbers faithfully reflect colony numbers. (**c**) Numbers of colonies (*filled bars*, measured with spermatogonial transplantation) and clusters (*open bars*, measured with the CFA assay) derived from mouse pup testis cells that were exposed to a hypotonic solution for indicated times. Pup testis cells were first enriched for SSCs, and then incubated in the hypotonic solution for indicated times (Yeh et al. 2007). The numbers are expressed as a normalized value using the result of control (no exposure to the hypotonic solution) as a denominator. A significant decline in numbers of colonies and clusters is seen after a 20-min exposure. Note that both numbers are nearly identical under each experimental condition, indicating the faithfulness of the CFA assay to spermatogonial transplantation. (**b**, **c**) were reproduced and modified from (Yeh et al. 2007) with permission from the Society of Study for Reproduction

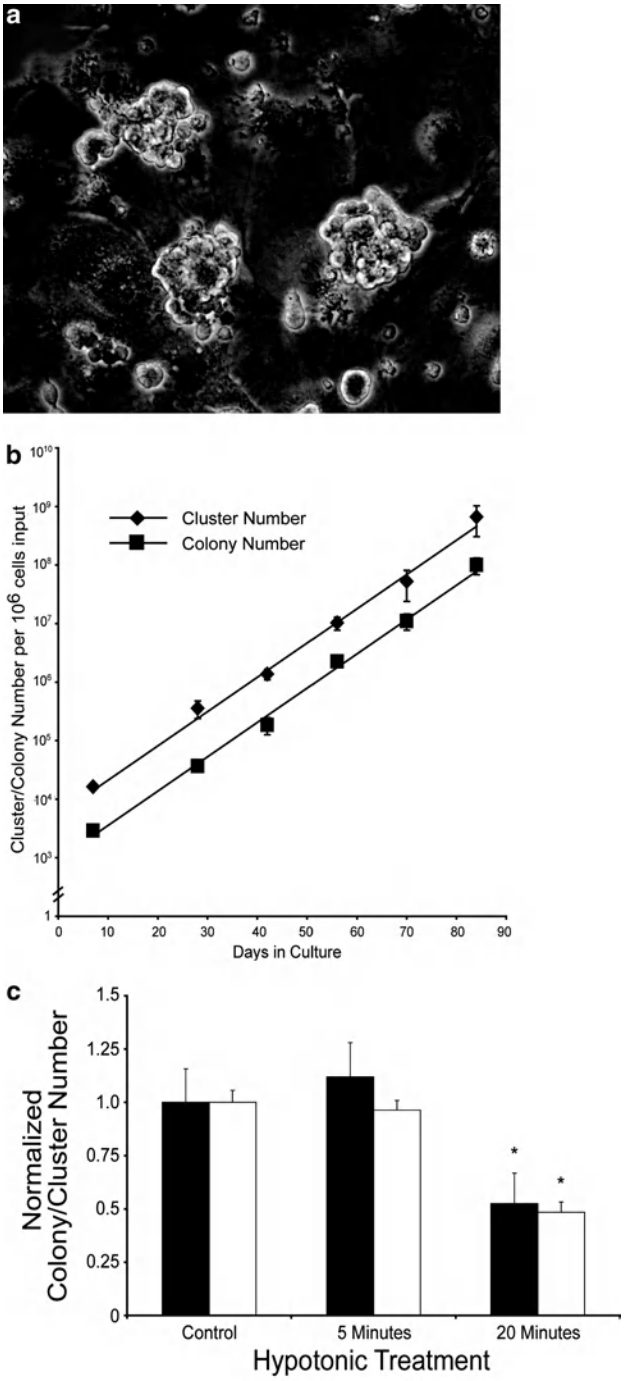

Fig. 6.1 (**a**) Appearance of germ cell clusters. (**b**) Proliferation kinetics of clusters and colonies. Clusters were cultured and serially passaged for 12 weeks and their numbers measured periodically. Clusters were also transplanted during the study period, and colonies in recipient testes were counted. Note the strong linearity and parallelism in proliferation kinetics of both parameters that

and a dilution factor at each passage (1:2–1:4). All cultured cells were also transplanted periodically, and SSC numbers were measured by counting the number of colonies in recipient testes 2 months later. As the figure shows, each parameter increases linearly over time, when cluster and colony numbers are expressed in $\log_{10}$ values. This observation indicates that cluster-forming cells are capable of long-term self-renewal and proliferate continuously *in vitro* while sustaining spermatogenic regeneration activity. Importantly, proliferation kinetics of clusters parallel with those of colonies determined by transplantation, demonstrating that cluster numbers directly correlate with SSC numbers. Using the data shown in Fig. 6.1b, therefore, it is calculated that both clusters and colonies increase in number with a near-identical population-doubling time: 5.5 days with clusters and 5.6 days with colonies (Yeh et al. 2007).

This strong correlation addresses the above two questions. First, the parallelism between the two proliferation kinetics assures that the cluster number faithfully reflects the number of functional SSCs, even though the regeneration of spermatogenesis is not demonstrated. Second, on this basis, the strong linearity of cluster proliferation kinetics that is consistent over an extended culture period demonstrates that relative SSC activity can be quantified by cluster counts at any single culture period, even though the period is only 6–7 days. Therefore, although the regeneration and long-term maintenance of complete spermatogenesis cannot be recapitulated *in vitro*, the CFA assay detects SSC activity in a semi-quantitative manner in a short time, based strictly on its correlation to the transplantation assay.

The faithfulness of the CFA assay to the transplantation assay can also be demonstrated under an experimental condition (Yeh et al. 2007). For example, we measured the number of SSCs surviving exposure to a cytotoxic hypotonic solution using both the CFA and transplantation assays, and obtained near identical results with both techniques (Fig. 6.1c). We have used the CFA assay in combination with the transplantation assay under various experimental situations, and data thus far obtained in our laboratory have been supportive of the correlation between the two methods.

6.2 Advantages of the CFA Assay

The CFA assay has several advantages over spermatogonial transplantation (Yeh et al. 2007). First, it generates results in 1 week. Although the transplantation assay is the unequivocal SSC detection method, its time-consuming nature makes it difficult to conduct a given experimental program in a stepwise manner; it often forces us to take a second step without the data of a first step in hand. Hence, the CFA assay facilitates research progress by markedly shortening the time until data acquisition, while allowing for semi-quantitative detection of SSC activity. Second, the culture technique is simple and enables an analysis of a wide range of experimental conditions at once. In contrast, the transplantation assay requires microinjection into individual testes, which limits the number of conditions to be examined at a time. Third, while the transplantation assay requires genetic compatibility between donor

cells and recipient animals to avoid immunological rejection, the CFA assay eliminates such a consideration all together. Finally, the variation of data obtained is less (approximately 50%) with the CFA assay than with the transplantation assay (Yeh et al. 2007), implying that the CFA assay can generate a consistent dataset with fewer samples than the transplantation assay.

6.3 Cautionary Issues

6.3.1 Does the CFA Assay Assess the Full Range of Stem Cell Characteristics?

As described above, the CFA assay relies solely on the self-renewing ability of SSCs and does not detect the regenerative capacity of SSCs. This *in vitro* technique therefore does not stand as an unequivocal SSC assay on its own. It is prudent to combine both the CFA and transplantation assays for efficiently generating convincing results. For example, when the effect of a given growth factor on SSCs needs to be investigated, one may need to examine a wide range of factor doses. Such a task is labor-intensive and time-consuming if the transplantation assay is the only means to derive data. Using the CFA assay, one can promptly evaluate the factor's effects across varied doses, taking advantage of the procedural simplicity of the assay; then, based on the outcome, the effect of the factor on SSCs can be confirmed with the transplantation assay only in a defined and limited number of factor doses. This is perhaps an ideal approach for the use of the CFA assay while circumventing its weakness, i.e., the inability to measure the regeneration activity of SSCs.

6.3.2 Do Clusters Arise Only from SSCs?

We do not yet have a definitive answer to this question. A possibility was raised recently that there is a subpopulation of spermatogonia that have initiated differentiation but still retain stem cell activity (Nakagawa et al. 2007). If such cells indeed exist, they may well form clusters in an *in vitro* environment. It is also possible that committed progenitors do have the ability to form clusters, at least for a short period of time. This is known to be the case in the assay of neural stem cells (NSCs) (Reynolds and Rietze 2005; Singec et al. 2006; Seaberg and van der Kooy 2002). NSCs can be maintained and propagated *in vitro* in the form of free-floating cell aggregates, called neurospheres. Importantly, NSCs are not the only cells that can form neurospheres, but progenitors committed to differentiation can also do so. Whether or not a similar situation applies to SSCs and clusters is unknown at present; however, the parallelism between the CFA and transplantation assays (Fig. 6.1) indicates that even if committed cells can produce clusters, it does not significantly interfere with the faithful measurement of relative SSC activity with the CFA assay.

6.3.3 Does the Number of Cells Placed in Culture Affect Assay Outcomes?

It is conceivable that the CFA assay can be affected by the number or density of cluster-forming cells placed in culture. If such cells are densely cultured, multiple cells located nearby may form one cluster, or multiple clusters may merge as they grow, causing an underestimation of cluster numbers. We addressed this issue in two ways (Yeh et al. 2007). The first was to examine the correlation between cluster numbers and testis cells that were seeded after SSC enrichment, ranging from 5 to 2.5×10^4 cells/cm^2 (i.e., limiting dilution of SSC-enriched testis cells). We found that these two parameters (both in a log scale) linearly correlated. Next, we cultured a 1:1 mixture of two populations of SSC-enriched testis cells, which can be distinguished from each other by the presence or absence of a transgene, and examined the proportion of clusters comprised of cells from both populations; clusters with both cell types were judged to have arisen from multiple cluster-forming cells placed nearby or merging of multiple clusters. The results showed that such clusters represented less than 10% of all clusters when the density of total SSC-enriched cells seeded was 0.5×10^4 cells/cm^2 or lower, while 14% of total clusters were made of the two cell types when the cell density was 2.5×10^4 cells/cm^2; 1.25×10^4 cells/cm^2 gave approximately 10% of such clusters. Total cluster numbers were ~160, ~343, and ~520 clusters/cm^2, at 0.5, 1.25, and 2.5 cells seeded/cm^2.

The strong linear correlation between the cell density and cluster number, as well as the observation that clusters composed of the cells with two different origins are a minority in general, indicate that an underestimation of SSC quantification by the CFA assay may not be a significant concern. Perhaps, this is because cluster-forming cells represent a rare cell population. Nevertheless, the cell-density- dependent increase in the proportion of multi-origin clusters implies that the possibility exists that a higher cell density affects the fidelity of assay outcomes. Based on the above results, the density of testis cells seeded at ~1×10^4 SSC-enriched cells/cm^2 or ~350 clusters/cm^2, with which multi-clonal clusters arise at a frequency of 10% or less, generate the most reliable assay condition.

6.3.4 Can the CFA Assay Distinguish SSC Proliferation and Maintenance?

To address this question, we want to introduce a hypothetical experimental situation. Here, the aim is to evaluate the effect of a growth factor (Factor A) on SSC activity *in vitro* in the presence of GDNF, GFRA1, and FGF2 (Fig. 6.2, "experimental culture"). Test cells were harvested from an established cluster culture, which had been maintained for experimental use. This culture was then split into to the hypothetical cultures with or without Factor A, and cluster numbers were measured after 6 days *in vitro*. The culture with Factor A produced 200 clusters, whereas the control group generated 100 clusters.

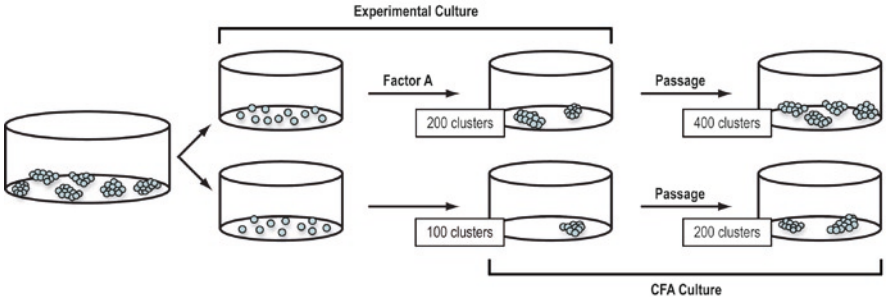

Fig. 6.2 A schematic representation of a hypothetical experiment to analyze the effect of Factor A on SSCs *in vitro*. Established clusters (the far left well), cultured under standard conditions, are digested and plated in two groups of culture. In one, target cells are initially incubated with Factor A for 6 days, producing 200 clusters (experimental culture). These clusters are digested into single cells, replated on a feeder layer, and cultured for an additional 6-day period under the standard cluster-inducing condition without Factor A (CFA culture), generating 400 clusters. In the other group (control), target cells are cultured similarly to the factor-treated group for two culture cycles, but Factor A is not used in the experimental culture. Under this condition, 100 and 200 clusters are derived in the experimental and CFA cultures, respectively. See text for discussion on the data interpretation

These results indicate that twofold more SSCs, which were placed in culture on day 0, survived for 6 days and formed clusters in the presence of Factor A, compared to the control culture. Therefore, an experimental culture generates information about maintenance or survival of SSCs and their cluster-forming capacity. However, it is of note that the twofold increase in cluster numbers cannot necessarily be attributed to SSC proliferation, because SSCs proliferate within a cluster.

In order to unmask SSC proliferation taking place inside clusters, they need to be digested so that SSCs are released from the cluster structure, and replated into a secondary culture: i.e., the CFA assay (Fig. 6.2). These secondary clusters are induced using the identical, standard condition in both groups (in this case, without Factor A). Thus, the degree of SSC proliferation *in each group* can be measured by the differential in cluster numbers between the experimental and CFA cultures (Fig. 6.2). As explained below, by comparing two values of the differential, derived in the factor-treated and control groups, the effect of Factor A on SSC proliferation can be revealed.

In this hypothetical experiment (Fig. 6.2), 400 clusters were found in the CFA culture in the factor-treated group and 200 clusters in the control group. This indicates that SSCs proliferated twofold in "both" groups: 200–400 in the factor-treated group and 100–200 in the control group. Together with the cluster counts in the experimental culture, therefore, the data indicate that Factor A did not stimulate SSC proliferation, but likely promoted SSC survival.

As such, SSC survival and proliferation can be distinguished more readily with the CFA assay than with the transplantation assay. To measure the degree of SSC proliferation with the transplantation assay, transplantation needs to be done at both the beginning and end of the experimental culture; thus, the CFA culture is equivalent to transplantation after the experimental culture.

6.3.5 Is Cluster Number the Only Readout of the CFA Assay?

The readout of the CFA assay discussed thus far was only cluster numbers, but the constituents of clusters (SSCs and non-stem spermatogonia) can be another assay outcome. It is conceivable that each cluster may represent a heterogeneous cell community, as 2% or less of cluster cells have been estimated to be SSCs (Yeh et al. 2007; Kanatsu-Shinohara et al. 2005; de Rooij 2006). This suggests that as SSCs divide and form clusters in culture, they not only self-renew but also generate cells that are committed to differentiation. Such committed cells may also proliferate in response to added growth factors, composing the vast majority of cluster cells. Based on the detection and quantification of committed cells in clusters, therefore, the CFA assay may potentially provide parameters to analyze the degree of spermatogonial differentiation. Since early spermatogenesis has been difficult to study experimentally *in vivo*, the CFA assay, which provides a defined environment *in vitro*, can be an ideal research tool to understand the regulatory mechanism of SSC/spermatogonial differentiation.

One approach to accomplish this may be to measure the size of clusters. Since the majority of cluster cells are apparently committed cells, the cluster size can be indicative of the differentiation activity and the production level of differentiating cells.

To evaluate differentiation events in a more definitive manner, we need to determine differentiation markers. The most common marker thus far used is KIT (c-kit proto-oncogene), since SSCs cannot be enriched in the KIT-positive cell population (Shinohara et al. 1999; Ebata et al. 2005); a recent study disputed this notion (Barroca et al. 2009), and further studies are required to clarify this issue. Thus, spermatogonial differentiation can be monitored by detection of differentiation marker expression. Alternatively, the loss of SSC markers can also allow for assessing differentiation. In both cases, immunohistochemical analyses will give endpoint readout, while the use of SSCs carrying fluorescent markers driven by a promoter of a marker molecule will support a real-time observation of differentiation events. Likewise, changes in cell cycle and apoptosis in clusters can be additional assay readouts. The CFA assay can thus be an important tool to analyze spermatogonial behavior and early spermatogenesis *in vitro* in a quantitative manner.

6.3.6 Other Considerations

The CFA assay is perhaps most effective when used in *ex vivo* studies, as shown in the above examples. When this assay is applied for *in vivo* studies (to measure SSC activity after *in vivo* manipulation), one issue that needs to be considered is the fact that cluster induction requires enrichment of testis cells for SSCs (see Chap. 5). The degree of SSC enrichment may vary, for example, if the expression of selection markers is affected by *in vivo* manipulation. Thus, the requirement of an additional procedure before the CFA assay represents a weakness of this technique when applied for *in vivo* studies.

Another issue is that the effectiveness of the CFA assay has not been evaluated using adult SSC-derived clusters. Since clusters can be more readily induced with pup than adult testis cells (Chap. 5), the CFA assay was established using pup SSCs. However, adult SSC-derived clusters can also be maintained for a long time, and SSCs amplified (Chap. 5). It is thus likely that the CFA assay is effective with adult SSCs, but this may need to be confirmed.

6.4 Future Prospects

While the CFA assay can be used immediately as a short-term functional assay for SSCs, the technique can be improved and extended. Here we discuss two possibilities.

In a published report (Yeh et al. 2007), we measured cluster numbers visually under a microscope, but this is a tedious process that reduces the value of the short-term assay. The use of SSCs or spermatogonia carrying a fluorescent marker (e.g., green fluorescent protein) should allow for a more facile detection of clusters. In addition, combined with a computer-assisted, automated fluorescence detection system, the assay can provide readouts more readily and in real-time.

Second, when the SSC culture and the CFA assay are used to analyze the effect of a growth factor, the presence of feeder cells becomes a confounding factor. It is possible that a factor affects SSC activity indirectly by changing the viability or actions of feeder cells. This problem can be resolved by using a feeder-free SSC culture. Since such a culture system has been reported (Kanatsu-Shinohara et al. 2005), its applicability for quantitative SSC analyses needs to be evaluated.

In this regard, it is important to establish a human SSC culture system. Then, a CFA assay for human SSCs will become possible and will be a powerful technique to analyze their biology in a short-term and semi-quantitative manner. Since regeneration of human spermatogenesis in experimental animals through spermatogonial transplantation has not been achieved, the CFA assay could become a critical method to study human SSCs. We realize that such an assay has great potential to evaluate the quality and quantity of human SSCs in the future, and expect that the clinical impact of such a system will be immense.

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Chapter 7
Molecular Mechanisms Regulating Spermatogonial Stem Cell Fate Decisions

Olga M. Ocón-Grove and Jon M. Oatley

Abstract Spermatogenesis in the mammalian testis is a classic stem cell dependent process relying on spermatogonial stem cells (SSCs) to undergo two distinct fate decisions referred to as self-renewal and differentiation. Deciphering the mechanisms that control these functions is crucial to understanding spermatogenesis and can provide insight into the biology of tissue-specific stem cells. In general SSC fate decisions are controlled extrinsically from influences of a niche microenvironment and internally by regulation of specific molecular pathways. In mice, the growth factor glial cell line-derived neurotrophic (GDNF) functions as an essential extrinsic stimulator of SSC self-renewal and survival by activating SRC family kinase, phosphoinositide-3-kinase/AKT, and RAS signaling pathways. Other known extrinsic stimulators of SSC self-renewal include fibroblast growth factor 2 and colony stimulating factor 1. Internally, expression of specific transcription regulators including *Bcl6b*, *Etv5*, and *Lhx1* are regulated by GDNF and these play roles in regulating SSC self-renewal and survival. Additionally, other non GDNF-regulated genes including *Mili, Ngn3, Nanos2, Oct3/4, and Taf4b* have also been implicated as regulators of SSC fate decisions.

Keywords Spermatogonial stem cell • Niche • Self-renewal • Signaling • Transcription factor

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7.1 Spermatogenesis and Spermatogonial Stem Cell Biology

In mammals, continual spermatogenesis is dependent on an adult tissue-specific stem cell population, termed spermatogonial stem cells (SSCs) whose biological activities provide the foundation for high output of spermatozoa throughout the life span of males. SSCs arise from gonocytes, a more undifferentiated precursor germ cell derived from primordial germ cells (PGCs) that are formed during fetal development (Clermont and Perey 1957; Wartenberg 1976; Curtis and Amann 1981; McLaren 2003). Transition from gonocytes to SSCs generally occurs during the first 6 days postpartum in male mice (Huckins and Clermont 1968; Bellve et al. 1977; de Rooij and Russell 2000), with the first biologically active SSCs appearing 3–4 days after birth (McLean et al. 2003). In the adult testis, SSCs are few in number, estimated to be present at a concentration of 1 in 3,000 cells in the mouse (Tegelenbosch and de Rooij 1993), and comprise a sub-fraction of the proliferating spermatogonial population that consists of A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) spermatogonia (Huckins 1971; Huckins and Oakberg 1978; Russell et al. 1990; de Rooij and Russell 2000).

Similar to other tissue-specific stem cell populations, SSCs possess the capacity for both self-renewal and cellular differentiation. Self-renewal, a putatively infinite process, results in maintenance of a stem cell pool. The A_s spermatogonia have classically been considered SSCs, and during steady-state spermatogenesis their differentiation results in formation of A_{pr} and A_{al} spermatogonia, a process that marks the beginning of eventual spermatozoa production (Huckins 1971; Oakberg 1971; de Rooij and Russell 2000). Along this course of differentiation, A_{pr} spermatogonia undergo further mitotic divisions, becoming $A_{al(4)}$, $A_{al(8)}$, and $A_{al(16)}$ spermatogonia in the mouse testis. These $A_{al(16)}$ spermatogonia then give rise to the differentiating spermatogonia population, A_1, A_2, A_3, and A_4 spermatogonia. The A_4 spermatogonia transition into intermediate and type B spermatogonia, which enter meiosis, becoming primary and secondary spermatocytes, leading to the development of haploid spermatids and eventually transforming into spermatozoa (Russell et al. 1990). Mechanisms regulating the balance between SSC self-renewal and differentiation have been explored, though understanding of these processes is still limited. In general, SSC fate decisions are controlled extrinsically by a niche microenvironment that consists of a milieu of growth factors and internally by activation of specific molecular signaling and gene expression pathways. Currently, understanding of the characteristics of the SSC niche and mechanisms regulating SSC fate decisions is limited. Niches are formed by contributions of support cells (Spradling et al. 2001; Scadden 2006), and in the mammalian testis Sertoli cells have been regarded as the major contributor of this microenvironment. However, studies by Chiarini-Garcia et al. (2003) suggested that proliferating spermatogonia (A_s, A_{pr}, and A_{al}) in the rat testis are predominately present in areas of seminiferous tubules adjacent to interstitial tissue. Furthermore, results of Yoshida et al. (2007) indicate that proliferating spermatogonia in the mouse testes are focally located in seminiferous tubules bordering the vasculature. Together these observations indicate that the SSC niche in mammalian

testes is defined at least in part by contributions from cells of the interstitium. More recent evidence has strengthened this concept, showing that the cytokine colony stimulating factor 1 (CSF1) influences self-renewal of mouse SSCs *in vitro* and its production *in vivo* is localized to interstitial Leydig cells and peritubular myoid cells (Oatley et al. 2009).

Studying SSC fate decisions in mammals is challenging due to their scarcity, and lack of known specific morphological, phenotypical, or molecular markers to specifically identify SSCs (Oatley and Brinster 2008). Previous studies showed that nearly all SSCs (>90%) in both pre-pubertal and adult mouse testes are contained within the THY1+ (CD90) germ cell fraction (Kubota et al. 2004a, b). To date, the greatest enrichment of SSCs from mouse testes is achieved by isolation of THY1+ cells (Oatley and Brinster 2008). Using functional germ cell transplantation, Kubota et al. (2004a) showed that SSC concentration of the THY1+ cell fraction is 5- to 30-fold greater compared to the unselected total cell population of pre-pubertal and adult mouse testes, respectively (Kubota et al. 2004b). Other cell surface molecules reported to be expressed by rodent SSCs include α6-integrin (Shinohara et al. 1999) and GPR125 (Seandel et al. 2007). Examination of cell populations enriched for SSCs, such as the THY1+ germ cell population, compared to the total testis provides insights into characteristics of SSCs. Unfortunately, no isolated testis cell fraction based on any surface marker reported to date, including the THY1+, α6-integrin+, or GPR125+ cell population, is composed purely of SSCs and likely also contain A_{pr} and A_{al} spermatogonia that are produced upon SSC differentiation. For this reason, examination of SSCs should not rely on cell surface markers and must include functional transplantation analysis to draw unequivocal conclusions about the biology of these cells (Oatley and Brinster 2008).

Impairment of SSC function *in vivo* results in formation of seminiferous tubules devoid of germ cells, a phenotype referred to as Sertoli-cell-only (Fig. 7.1), which leads to sub-fertility or infertility. Progressive increase in the percentage of seminiferous tubules with this phenotype is regarded as a hallmark of impaired SSC self-renewal (Buaas et al. 2004; Costoya et al. 2004; Oatley et al. 2006). However, this interpretation can be misleading because disruption of SSC differentiation to A_{pr} spermatogonia, SSC death, or quiescence could cause an identical phenotype (Fig. 7.2). Because stem cell is a functional definition, the truest measure of an SSC is the ability to reestablish spermatogenesis following transplantation into a recipient testis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). However, this assay cannot distinguish between defects in SSC self-renewal, differentiation, or survival because disruption of any of these fates will result in impaired reestablishment of spermatogenesis. Thus, distinguishing between defective SSC self-renewal and differentiation by *in vivo* examination is challenging. Another means to evaluate SSC functions is use of *in vitro* culture systems that support their self-renewal and differentiation.

Methods to maintain mouse SSCs *in vitro* for extended periods of time have been devised, providing a tool to critically study their fate decisions (Kanatsu-Shinohara et al. 2003, 2005; Kubota et al. 2004a). Currently, techniques for isolation of SSC-enriched testis fractions and long-tem culture of SSCs are only available for

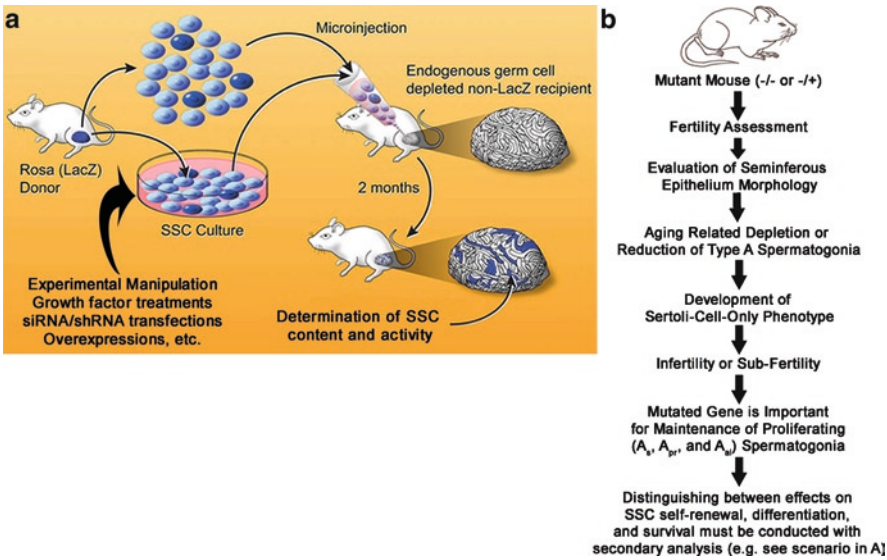

Fig. 7.1 Experimental approaches that have been used for studying SSC functions in mice. (**a**) Combining culture of mouse SSCs with functional transplantation assays to examine self-renewal and survival. With advent of long-term culture conditions the effects of exposure to specific growth factors and manipulating the expression of specific genes on the activity of SSCs can be examined. Unfortunately, all SSC culture methods reported to date support the growth and survival of stem cells and non-SSC germ cells. Thus, transplantation analyses should be conducted with cultured cell populations following experimental manipulation to determine treatment effects on SSCs specifically. Modified and reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 24 © 2008 by Annual Reviews, http//:www.annualreviews.org (Oatley and Brinster 2008). (**b**) Use of mutant mice for examination of gene-specific functions in SSCs. Knockout mice have been used to assess the importance of specific genes on spermatogenesis and SSC activity. Typically, null or heterozygous animals are examined for fertility defects followed by evaluating the morphology of the seminiferous epithelium. Mice with impaired expression of genes important for spermatogonial maintenance often show aging-related depletion or reduction of type A spermatogonia. This defect typically leads to a Sertoli-cell-only phenotype due to loss of all proliferating spermatogonia and could also be caused by a phagocytic response by Sertoli cells to impaired spermatogenesis, which eliminates the seminiferous epithelium of germ cells. Depending on the degree of spermatogonial elimination male mice will become infertile or sub-fertile, conditions that could manifest later in life. These phenotypes suggest that the mutated gene is important for maintenance of proliferating spermatogonia. However, distinguishing whether the importance is in regulating SSC self-renewal, differentiation, or survival should be conducted with secondary analyses such as culture and transplantation assay

rodents (Kanatsu-Shinohara et al. 2003, 2005, 2008; Kubota et al. 2004a; Ryu et al. 2005). Thus, the majority of what has been discovered about the biology of SSCs comes from studies using the mouse as a model. Over the last several years, major insights into the regulation of SSC fate decisions have been made using SSC culture and transplantation methods, in addition to evaluation of mutant mouse strains for Sertoli-cell-only phenotype (Fig. 7.1). Even with these recent advancements, the

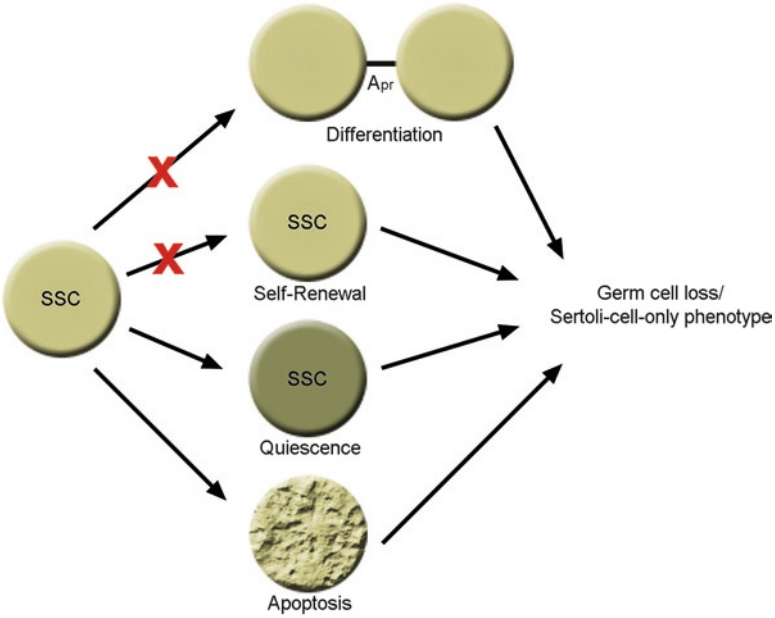

Fig. 7.2 Potential effects of impaired SSC fate decisions *in vivo*. Under normal conditions SSCs maintain spermatogenesis by undergoing self-renewal and differentiation. Disruption of either fate decision can result in germ cell loss leading to Sertoli-cell-only phenotype. Theoretically, impairment of either self-renewal or differentiation could cause SSCs to enter a quiescent state or undergo apoptosis, both of which fates would also result in germ cell loss and Sertoli-cell-only phenotype. Additionally, disruption of differentiation could shift the balance to greater self-renewal possibly resulting in germ cell tumorigenesis

current body of knowledge about molecular mechanisms controlling mammalian SSC fate decisions is limited.

7.2 Extrinsic Growth Factor Regulation of SSC Self-renewal

7.2.1 GDNF

Research over the past decade has established that glial cell line-derived neurotrophic factor (GDNF) is an essential extrinsic growth factor that stimulates the self-renewal of mouse, rat, hamster, and bull SSCs (Meng et al. 2000; Kanatsu-Shinohara et al. 2003, 2008; Nagano et al. 2003; Kubota et al. 2004a; Oatley et al. 2004; Ryu et al. 2005). A member of the transforming growth factor beta (TGFβ) super family, GDNF was originally shown to play an important role in neuronal progenitor cell function as well as kidney morphogenesis (Sariola and Saarma

2003; Dressler 2006). In 2000, Meng et al. reported that spermatogenesis is disrupted in mice carrying a single *Gdnf* null allele, while an accumulation of A_{pr} and A_{al} spermatogonia was observed in the testes of mice over-expressing GDNF. In serum-free culture conditions, supplementation with GDNF is essential for SSC self-renewal *in vitro* for extended periods of time (Kubota et al. 2004a). Additionally, studies by Kanatsu-Shinohara et al. (2003) showed a similar importance of GDNF in supporting survival and proliferation of mouse gonocytes *in vitro*. Moreover, the self-renewal of rat and hamster SSCs *in vitro* is also supported by exposure to GDNF (Hamra et al. 2005; Ryu et al. 2005; Kanatsu-Shinohara et al. 2008). Maintenance of proliferating spermatogonia in defined culture conditions with GDNF supplementation supports the formation of germ cell clumps. These clumps can be maintained and expanded for extended periods of time and upon transplantation, some of the cells can reestablish spermatogenesis in a recipient testis demonstrating the presence of SSCs within the germ cell clump (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004a; Ryu et al. 2005). However, the germ cell clumps are not composed purely of SSCs and also contain other non stem cell spermatogonia, which are likely A_{pr}-like and/or A_{al}-like spermatogonia produced upon SSC differentiation (Kubota et al. 2004a; Kanatsu-Shinohara et al. 2005; Dann et al. 2008). The concentration of SSCs within germ cell clumps can vary widely throughout a given culture period (Kubota et al. 2004a; Kanatsu-Shinohara et al. 2005), estimated to be as low as 0.02% of the cell population in one study (Kanatsu-Shinohara et al. 2005). Regardless, the truest measure of an SSC is the ability of a germ cell to reestablish spermatogenesis following transplantation and cells capable of fulfilling this criterion are present in cultured spermatogonial populations. These observations indicate that both self-renewal and differentiation of SSCs is supported in clump-forming spermatogonial cultures, which provides an excellent *in vitro* model system to study SSC fate decisions.

Self-renewal of stem cells can be defined by the ability of a cell to undergo mitosis producing two new daughter cells of which one is identical to the parent cell and does not enter a differentiation pathway, maintaining the capability to undergo future self-renewing divisions. Response to specific growth factors is one major regulatory mechanism of this fate decision and requires the binding and stimulation of specific receptor complexes expressed by stem cells. Although GDNF is an essential regulator of SSC proliferation, its receptor complex consisting of GDNF family receptor α 1 (GFRα1) and c-RET are expressed by A_s, A_{pr}, and A_{al} spermatogonia (Dettin et al. 2003; Naughton et al. 2006). The GFRα1+ cell fraction is lesser than twofold enriched for SSCs in the pre-pubertal mouse testis and depleted of SSCs in the adult mouse testis (Buageaw et al. 2005; Ebata et al. 2005; Grisanti et al. 2009). Additionally, fewer SSCs are found in the c-Ret+ cell population of both pre-pubertal and adult mouse testes compared to the total testis cell population (Ebata et al. 2005). Because GDNF action is not specific to SSCs, functional transplantation assays must be conducted when examining GDNF actions on spermatogonia to make unequivocal conclusions about the biology of SSCs.

7.2.2 FGF2 and CSF1

While GDNF is essential for SSC self-renewal *in vitro*, the rate of proliferation is slow, suggesting that other growth factors could also influence this fate decision. Additional exposure to both fibroblast growth factor 2 (FGF2) and CSF1 enhances mouse SSC self-renewal *in vitro*; however, exposure to either of these factors alone does not support SSC expansion (Kubota et al. 2004a; Oatley et al. 2009). Similarly, proliferation of mouse PGCs, the embryonic precursors of SSCs, *in vitro* also requires supplementation of culture media with FGF2 (Resnick et al. 1992). Also, self-renewal of rat and hamster SSCs *in vitro* requires the supplementation of both FGF2 and GDNF (Ryu et al. 2005; Kanatsu-Shinohara et al. 2008). Similar to GDNF, production of FGF2 has been localized to Sertoli cells in the mouse testis further supporting the belief that these cells are major contributors of the SSC niche (Mullaney and Skinner 1991).

Using microarray transcript profiling, specific genes with augmented expression in the SSC-enriched THY1+ germ cell fraction of pre-pubertal mouse testes were identified (Oatley et al. 2009). These analyses revealed that colony stimulating factor 1 receptor (*Csf1r*) gene expression is highly (>400-fold) enriched in THY1+ germ cells compared to other testis cell types (Oatley et al. 2009). Subsequent experiments showed that addition of recombinant CSF1, the specific ligand for CSF1R, to culture media significantly enhances the self-renewal of mouse SSCs (Oatley et al. 2009). In a similar study, microarray analysis of the Gfrα1+ cell fraction isolated from pre-pubertal mouse testes also revealed enriched expression (~2-fold) of *Csf1r* compared to other testis cells (Kokkinaki et al. 2009). *In vivo*, expression of CSF1 in both pre-pubertal and adult testes was localized to clusters of Leydig cells and selected peritubular myoid cells (Oatley et al. 2009). Collectively, these observations indicate that CSF1 is an extrinsic stimulator of SSC self-renewal and implicate Leydig and myoid cells as contributors of the testicular stem cell niche in mammals. A niche microenvironment that supports stem cell self-renewal is composed of a milieu of growth factors produced by multiple support cells. Currently, our understanding of these components for the SSC niche includes the growth factors GDNF, FGF2, and CSF1; with the supporting cell component consisting of Sertoli, Leydig, and myoid cells. Many more components of the SSC niche await discovery in the coming decades before complete understanding of the regulation of SSC fate decisions is made.

7.3 Internal Molecular Pathways Regulating SSC Self-Renewal

The effect of extrinsic stimuli from niche microenvironments, including growth factor stimulation on stem cell self-renewal, is mediated via activation of specific molecular pathways. Over the last 5 years these mechanisms in SSCs have begun to be examined, yet understanding is still very limited. A major area of study has

Table 7.1 Transcription factors implicated as important regulators in SSC function

Gene	GDNF regulated[a]	Fertility phenotype[b]	Germline defect	Citations
Bcl6b	Y	Sub-fertile	Loss of spermatogonia, Sertoli cell only phenotype	Oatley et al. (2006)
C-Fos	Y	Sub-fertile	Impaired development of meiotic germ cells	Johnson et al. (1992)
Etv5	Y	Infertile	Loss of spermatogonia Sertoli cell only phenotype	Chen et al. (2005)
Lhx1	Y	N/A[c]	Lack gonads	Shawlot and Behringer 1995
Ngn3	N	Undetermined	Undetermined	Gradwohl et al. (2000)
n-Myc	N	N/A[c]	Undetermined	Charron et al. (1992)
Plzf	N	Infertile	Loss of spermatogonia, Sertoli cell only phenotype	Buaas et al. (2004) Costoya et al. (2004)
Sohlh1	N	Infertile	Disrupted spermatogonial differentiation	Ballow et al. (2006)
Taf4b	N	Infertile	Loss of spermatogonia, Sertoli cell only phenotype	Falender et al. (2005)

[a]Based on microarray analysis of GDNF-regulated genes in cultured mouse SSCs (Oatley et al. 2006)
[b]As documented in knock-out mouse models
[c]Embryonic/neonatal lethality

been the importance of specific transcription factor encoding genes that regulate mouse SSC fate decisions (Table 7.1).

7.3.1 GDNF-Regulated Gene Expression in Mouse SSCs

Because GDNF is regarded as an essential extrinsic stimulator of SSC self-renewal, recent examination of internal molecular mechanisms regulating SSC fate decisions have focused on those activated or suppressed by GDNF stimulation. Using a DNA microarray approach, GDNF-regulated gene expression was explored in cultured mouse SSCs (Oatley et al. 2006). Expression levels of specific genes that dramatically decreased upon GDNF withdrawal and increased following GDNF replacement were identified. In total, expressions of 79 genes were up-regulated twofold or greater by GDNF stimulation (Oatley et al. 2006). Those showing the greatest level of GDNF-regulation included three transcription factors, B cell CLL/

lymphoma 6, member b (*Bcl6b*; also termed *Bazf*), Ets variant gene 5 (*Etv5*; also termed *Erm*), and Lim homeobox protein 1 (*Lhx1*, also termed *Lim1*) (Oatley et al. 2006). Transient reduction of *Bcl6b*, *Etv5*, or *Lhx1* transcript levels with siRNA treatment impaired SSC maintenance *in vitro* demonstrated by transplantation analysis showing loss of SSCs after one self-renewal cycle *in vitro* (Oatley et al. 2006). GDNF-regulated gene expression was also examined in cultured GFRα1+ germ cells isolated from testes of pre-pubertal mice (Hofmann et al. 2005; Braydich-Stolle et al. 2007; He et al. 2008). In those studies, up-regulation of several genes including *Numb*, a component of the notch signaling pathway, was identified in the cultured cells. Additionally, *c-Fos* and *n-Myc* gene expressions were up-regulated in cultured GFRα1+ testis cells after exposure to GDNF (Braydich-Stolle et al. 2007; He et al. 2008). In contrast, expression of either *Numb*, *c-Fos*, or *n-Myc* was not affected by GDNF stimulation in cultured THY1+ germ cell populations proven to contain SSCs by functional transplantation (Oatley et al. 2006). Unfortunately, the SSC composition of cultured GFRα1+ cell populations used in previous studies was not determined. Thus, while these factors are potentially regulators of SSC functions the true importance of GDNF-regulated *Numb, c-Fos, or n-Myc* expression is difficult to assess based on the analyses reported to date. The remainder of this chapter will focus on transcription factors with demonstrated regulation or function in SSCs.

7.3.2 GDNF-Regulated Transcription Factors

7.3.2.1 BCL6B

The transcriptional repressor BCL6B is a homolog of BCL6 in both the mouse (Okabe et al. 1998) and human (Sakashita et al. 2002). In the lymphocyte lineage, BCL6B is important for activating naive CD4+ T cells and mediating proliferation of CD8+ memory T cells (Manders et al. 2005). In cultured mouse SSCs, *Bcl6b* gene expression is down-regulated upon GDNF withdrawal and up-regulated by GDNF stimulation, suggesting a key role in SSC self-renewal (Oatley et al. 2006). To study a biological importance of BCL6B in SSC function, expression was experimentally reduced by siRNA treatment in cultured SSCs (Oatley et al. 2006). Using functional transplantation as an assay, reduction of BCL6B expression was shown to impair SSC maintenance *in vitro* over one self-renewal cycle of 7 days. Further examination showed that BCL6B siRNA treatment induced apoptosis in the cultured mouse SSCs (Oatley et al. 2006). Inactivation of BCL6B *in vivo* causes a sub-fertile phenotype in male mice (Oatley et al. 2006). At 3 months of age, testes of *Bcl6b*$^{-/-}$ mice are smaller compared to those of wild-type litter mates and contain varying percentages of seminiferous tubules with degenerating spermatogenesis including spermatozoa-only and Sertoli-cell-only phenotypes (Oatley et al. 2006). Collectively, these observations suggest that BCL6B plays an important role in promoting SSC survival.

7.3.2.2 ETV5

ETS transcription factors bind conserved N-box sequences within promoter regions to activate expression of specific genes important for a variety of developmental processes (Seth et al. 1992). The Pea3 subfamily of ETS transcription factors consists of three members including ER81, PEA3, and ETV5 (also termed ERM). Expression of ETV5 is localized to several tissues including brain, lung, and testis (Chotteau-Lelievre et al. 1997). In cultured mouse SSCs, *Etv5* gene expression is up-regulated upon exposure to GDNF and reducing expression by siRNA treatment impairs SSC maintenance *in vitro* over one self-renewal cycle (Oatley et al. 2006, 2007). In the mouse testis, ETV5 expression is localized to both germ cells (Oatley et al. 2007) and Sertoli cells (Chen et al. 2005). In the germ cell population expression was observed in most spermatogonial subtypes including both proliferating spermatogonia (i.e., A_s, A_{pr}, and A_{al}) and differentiating spermatogonia (Oatley et al. 2007). Targeted disruption of *Etv5* in mice causes male infertility, including progressive development of a Sertoli-cell-only phenotype (Chen et al. 2005). This condition is marked by complete loss of germ cells, presumably as an indirect effect from failure of SSCs to provide new cohorts of developing germ cells. Aging-related increase of seminiferous tubules with Sertoli-cell-only phenotype occurs in *Etv5*$^{-/-}$ mice, culminating in complete infertility by 10 weeks of age (Chen et al. 2005). However, several waves of spermatogenesis occur during early life, indicating that the progressive formation of seminiferous tubules with Sertoli-cell-only phenotype is due to impaired SSC self-renewal (Chen et al. 2005). Additionally, artificial insemination of wild-type females with sperm from young *Etv5*$^{-/-}$ males fail to produce offspring or fertilize wild-type oocytes *in vitro*, suggesting defects in sperm quality (Schlesser et al. 2008). Importantly, germ cells from *Etv5*$^{-/-}$ fail to generate colonies of spermatogenesis following transplantation into seminiferous tubules of recipient mice indicating impaired function of SSCs (Tyagi et al. 2009). The role of ETV5 in spermatogenesis appears to be complex as expression is important for function of both Sertoli cells and several germ cell types including SSCs. However, this function is yet to be fully defined.

7.3.2.3 LHX1

Lim homeobox protein 1 (LHX1, also referred to as LIM1) is a transcription factor essential for anatomical morphogenesis in the mouse (Shawlot and Behringer 1995). Disruption of *Lhx1* expression during embryogenesis impairs formation of the anterior head structure, though the remaining body axis develops normally (Shawlot and Behringer 1995). Most *Lhx1*$^{-/-}$ embryos die at 10 days of embryonic development; however, a few pups are born dead, appearing normal except head development is essentially absent. Upon necropsy, LHX1 deficient neonates also lack kidneys and gonads, suggesting a role in morphogenesis of the urogenital system (Shawlot and Behringer 1995). In cultures of wild-type mouse SSCs, *Lhx1* gene expression is up-regulated by GDNF stimulation and reduction of *Lhx1*

expression by siRNA treatment followed by functional transplantation analysis revealed impaired SSC maintenance *in vitro*, suggesting a role in SSC self-renewal and survival (Oatley et al. 2007). *In vivo*, LHX1 expression is localized to individual spermatogonia in the testes of both pre-pubertal and adult mice (Oatley et al. 2007). Unfortunately, further investigation of LHX1 importance in SSC function *in vivo* has been challenging due to severe defects in embryonic development of *Lhx1*$^{-/-}$ mice. Deletion of *Lhx1* expression in the germline specifically via methods such as Cre/Lox technology will be required to further examine the role of this molecule in SSC functions *in vivo*.

7.3.2.4 OCT6

Individual members of the octamer (OCT)-binding family of transcription factors, also referred to as POU domain transcription factors, display a spatiotemporal expression pattern and have diverse roles in cellular processes and stem cell functions. One POU domain protein expressed by SSCs and up-regulated by GDNF stimulation is POU3f1, also referred to as TST-1 or OCT6. Expression of this transcription factor was first identified in the testis (Meijer et al. 1990) and has been identified as a regulator of neural cell development (He et al. 1989; Suzuki et al. 1990). In the male mouse germline, OCT6 expression is localized to spermatogonia and regulated by GDNF through the phosphoinositide-3-kinase (PI3K)/AKT pathway (Wu et al. 2010). In addition, transient reduction of Oct6 expression by siRNA treatment impairs GDNF-induced maintenance of SSCs *in vitro* and increases apoptosis (Wu et al. 2010). Thus, OCT6 is another GDNF-regulated transcription factor playing a role in the regulation of SSC maintenance.

7.3.3 Non-GDNF Regulated Transcription/Translation Factors

7.3.3.1 MILI

The PIWI family of proteins regulate translation in a variety of cell types and are essential for maintenance of germline stem cells in the ovary and testis of *Drosophila melanogaster* (Lin and Spradling 1997; Cox et al. 1998; Lin 2007). The murine homologs of PIWI, termed MIWI and MILI (PIWIL1 and PIWIL2, respectively), are expressed in the germline of mouse testes and disruption of either molecule causes impaired spermatogenesis (Deng and Lin 2002; Unhavaithaya et al. 2009). MIWI expression is localized specifically to meiotic spermatocytes and spermatids rather than spermatogonia or SSCs (Deng and Lin 2002), and deletion in mice causes arrested germ cell development at the spermatid stage indicating a role in spermiogenesis, but not stem cell function (Deng and Lin 2002). MILI expression is localized to gonocytes in neonatal mice, and spermatogonia and early spermatocytes in the adult testis (Unhavaithaya et al. 2009). Spermatogenesis in

Mili$^{-/-}$ mice is arrested at early prophase of meiosis during the spermatocyte stage causing male infertility (Kuramochi-Miyagawa et al. 2004). This phenotype suggests that similar to MIWI, stem cell function in the mouse testis does not require MILI expression; however, recent studies suggest otherwise and imply a role in SSC function (Unhavaithaya et al. 2009). Examination of testes from both pre-pubertal and adult *Mili*$^{-/-}$ mice revealed reduced mitotic activity of the spermatogonial population. At 8 days of age, reduced spermatogonial proliferation was observed in *Mili*$^{-/-}$ mice, which was considered evidence of impaired SSC proliferation (Unhavaithaya et al. 2009). During the postnatal development period of 0–10 days of age in the mouse testis, gonocytes, which are the precursors to SSCs, give rise to both SSCs and differentiating spermatogonia (de Rooij 1998; Yoshida et al. 2006). Thus, reduction of the germ cell population beginning at 8 days of age and subsequent disruption of spermatogenesis in adulthood is likely a result of impaired proliferation of both SSCs and differentiating spermatogonia. Evaluation of spermatogenesis at 10 days of age and after puberty at 35 days of age in *Mili*$^{-/-}$ mice also revealed reduced proliferation of spermatogonia (Unhavaithaya et al. 2009). These analyses indicate a general role of MILI in spermatogonial proliferation. By 98 days of age, seminiferous tubules display a Sertoli-cell-only phenotype in *Mili*$^{-/-}$ mice, which is likely due to loss of all spermatogonia as a result of impaired proliferation or a secondary effect of changes in Sertoli cell function caused by degenerating germ cells. Specific examination of defects in SSC function due to impaired MILI expression using functional transplantation or other measures of SSC activity have not been reported. Thus, whether PIWI proteins, which are essential for self-renewal of germline stem cells in *D. melanogaster*, have conserved function in mammalian SSCs remains to be further defined.

7.3.3.2 NANOS2

Expression of the NANOS family of RNA binding proteins by germ cells is conserved for several vertebrate and invertebrate species. In the mouse testis, expression of NANOS2 is germ cell-specific beginning with PGCs after their migration to the genital ridge and formation of seminiferous cords (Tsuda et al. 2003). Deletion of NANOS2 causes PGC apoptosis and male mice contain only a few germ cells within seminiferous tubules after birth (Tsuda et al. 2003); thus, the undifferentiated spermatogonial population never develops. In wild-type mice, NANOS2 expression is restricted to the undifferentiated spermatogonial population including A_s, A_{pr}, and some A_{al} spermatogonia (Sada et al. 2009). Using a conditional deletion approach, Sada et al. (2009) showed that disruption of NANOS2 expression in germ cells of adult testes with active spermatogenesis results in rapid depletion of the undifferentiated spermatogonial population in accordance with increased apoptosis. Furthermore, overexpression of NANOS2 in the male germline resulted in accumulation of undifferentiated spermatogonia and reduction in the number of the differentiating spermatogonia (Sada et al. 2009). Collectively, these findings imply an important role for NANOS2 in maintenance of the undifferentiated spermatogonial population

including SSCs via regulation of cell survival and preventing differentiation. In cultured mouse SSCs NANOS2 expression is not effected by GDNF stimulation (Oatley et al. 2006) and other extrinsic factors that may influence its expression have not been described.

7.3.3.3 OCT3/4

OCT3/4, a homeobox transcription factor encoded by the *Pou5f1* gene, is critical for maintenance of embryonic stem cell pluripotency (Nichols et al. 1998; Niwa et al. 2000). Also, OCT3/4 is expressed by PGCs in the embryonic gonad and spermatogonia of pre-pubertal mice (Pesce et al. 1998). Because of these features, OCT3/4 has been considered a factor that is potentially essential for SSC function in mice. However, global disruption of *Oct3/4* expression causes embryonic lethality (Nichols et al. 1998) and tissue-specific *Oct3/4* disruption in PGCs induces apoptosis (Ohbo et al. 2003). Thus, these phenotypes present difficulty in studying the role of OCT3/4 function in SSCs of postnatal mice. However, transfection of cultured spermatogonia with an OCT3/4 shRNA vector reduced the colonizing ability of SSCs upon transplantation into recipient testes compared to cells treated with a control shRNA (Dann et al. 2008). These results suggest that OCT3/4 plays an important role in SSC function; however, whether that role is in regulating self-renewal, differentiation, or survival is undefined. Impairment of either these potential SSC fates caused by permanent reduction of OCT3/4 expression would inhibit colonization upon transplantation. Additionally, disrupted homing ability of SSCs to their cognate niche upon transplantation, an effect that is independent of self-renewal, would also cause impaired reestablishment of spermatogenesis. Permanent reduction of OCT3/4 expression by stable shRNA transduction compared to transient reduction by siRNA treatment could alter homing efficiency and this effect cannot be eliminated as a cause of reduced SSC colonization following transplantation; thus, the actual function of OCT3/4 in SSC biology remains to be further elucidated. Recently, studies by Wu et al. (2010) indicate that expression of OCT3/4 in cultured mouse SSCs is not needed for their maintenance or self-renewal *in vitro*. These conflicting reports regarding the importance of OCT3/4 in SSC functions await clarification with future experimentation.

7.3.3.4 PLZF

In mice, expression of the transcriptional repressor promyelocytic leukemia zinc finger protein (*Plzf*, also termed *Zbtb16*) is important for several development pathways. PLZF belongs to the BTB/POZ-domain family of transcription factors and functions as a repressor at the level of chromatin remodeling (Hong et al. 1997; Payne and Braun 2006). Initial studies of *Plzf* null mice revealed disrupted limb and axial skeleton development via regulation of HOX and bone morphogenic protein gene expression (Barna et al. 2000). In the germline of male mice PLZF expression

is restricted to proliferating spermatogonia including A_s, A_{pr}, and A_{al} spermatogonia (Buaas et al. 2004; Costoya et al. 2004). Disruption of PLZF expression in male mice causes impaired spermatogenesis leading to infertility (Buaas et al. 2004; Costoya et al. 2004). Testes of adult *Plzf*$^{-/-}$ mice are reduced in size; yet, testis morphogenesis and germline development during embryogenesis are normal (Costoya et al. 2004). Postnatally, development of the seminiferous epithelium is grossly normal in *Plzf*$^{-/-}$ mice and several rounds of spermatogenesis occur without disruption (Buaas et al. 2004; Costoya et al. 2004). However, the appearance of seminiferous tubules with a Sertoli-cell-only phenotype gradually increases with age in *Plzf*$^{-/-}$ mice, suggesting a defect in SSC functions to supply the next generation of germ cell cohorts. Additionally, germ cells from *Plzf*$^{-/-}$ donor male mice showed reduced ability to restore spermatogenesis in testes of wild-type recipient mice upon transplantation, further indicating SSC malfunction due to lack of PLZF expression (Costoya et al. 2004).

In vivo, studies with mutant mice suggest that PLZF has a role in SSC function; however, whether this regulation is at the level of self-renewal, differentiation, or survival has not been elucidated. As discussed previously, distinguishing between these SSC fate decisions by *in vivo* experimentation is challenging. Formation of Sertoli-cell-only phenotype and inability to reestablish spermatogenesis after transplantation could occur if SSC self-renewal, differentiation, or survival is disrupted. Also, impaired transition of A_{al} spermatogonia into differentiating A_1 spermatogonia would cause a phenotype similar to Sertoli-cell-only. This disruption could also lead to seminiferous tubules with Sertoli-cell-only phenotype as a secondary response of Sertoli cells to cease function or phagocytize germ cells, a response that would become more pronounced with age.

An additional means of evaluating SSC function is to explore activity in culture systems that support their self-renewing proliferation for extended periods of time. Currently, the effects of experimentally manipulating *Plzf* expression on SSC self-renewal *in vitro* have not been reported. Additionally, expression of *Plzf* is not regulated by GDNF in cultured mouse SSCs (Oatley et al. 2006, 2007). Recently, studies suggest that PLZF acts as repressor of c-KIT receptor expression in male germ cells (Filipponi et al. 2007). KIT ligand (also termed stem cell factor) binds the c-KIT receptor and is important for PGC development during embryonic gonad formation and spermatogenesis in the adult mouse. Expression of c-KIT is essential for development of differentiating spermatogonia and becomes apparent in A_{al} spermatogonia as they transition into A_1 spermatogonia (Tajima et al. 1994; Ohta et al. 2000, 2003). In the mouse, SSCs do not express c-KIT (Shinohara et al. 1999; Kubota et al. 2003) and exposure to KIT ligand does not influence SSC self-renewal (Kubota et al. 2004a, b). Additionally, self-renewal and differentiation of mouse SSCs in to A_{pr}/A_{al} spermatogonia occurs independent of KIT ligand stimulation (Ohta et al. 2003). Thus, acquiring c-KIT expression is a function of A_{al} spermatogonia that is necessary for transition into A_1 spermatogonia. The observation that PLZF represses c-KIT expression suggests a role in preventing the differentiation of A_s, A_{pr}, and A_{al} spermatogonia, not just SSCs.

7.3.3.5 TAF4B

Transcription activity of RNA polymerase II requires the general transcription factor complex TFIID, which is composed of TBP and TAFII subunits. TBP associated factor 4b (TAF4B) is a component of this complex and expressed in select tissues including the testis (Verrijzer and Tjian 1996). Disruption of *Taf4b* expression impairs fertility in both male (Falender et al. 2005) and female mice (Freiman et al. 2001). In males, inactivation of *Taf4b* expression causes formation of seminiferous tubules with a Sertoli-cell-only phenotype (Falender et al. 2005). Similar to *Plzf*$^{-/-}$ mice, *Taf4b*$^{-/-}$ mice are fertile initially with normal spermatogenesis but seminiferous tubules deficient of germ cells gradually appear in accordance with advancing age indicating a role in maintenance of normal SSC functions (Falender et al. 2005). Under natural mating conditions, *Taf4b*$^{-/-}$ mice sire a similar number of litters compared to TAF4B expressing mice when exposed to wild-type females at 1 month of age; however, at 2–6 months of age *Taf4b*$^{-/-}$ mice fail to sire offspring (Falender et al. 2005). Functional transplantation experiments showed that wild-type germ cells are capable of undergoing normal spermatogenesis in testes of *Taf4b*$^{-/-}$ males, indicating that fertility defects due to TAF4B deficiency are inherent to the germ cells (Falender et al. 2005).

In the mouse germline, TAF4B expression is localized to multiple spermatogonial sub-types including proliferating spermatogonia (A_s, A_{pr}, A_{al}) and differentiating spermatogonia, in addition to round and elongating spermatids (Falender et al. 2005). Because of this diverse expression in a multitude of germ cells it is unlikely that TAF4B has an SSC-specific function. Formation of a Sertoli-cell-only phenotype could be a secondary response by Sertoli cells to rid the seminiferous epithelium of degenerating germ cells and not due specifically to failed SSC self-renewal. Similar to *Plzf*, *Taf4b* expression is not regulated by GDNF in cultured mouse SSCs (Oatley et al. 2006) and whether its expression is important for SSC self-renewal *in vitro* has not been defined. Alternatively, TAF4B could play distinct roles in germ cells at different stages of development.

7.3.4 Transcription Factors with Putative Roles in SSC Differentiation

While several genes have been examined for their role in SSC self-renewal, little advancement in the knowledge of those involved in SSC differentiation has been made. Because GDNF has an essential role in promoting SSC self-renewal one could reason that genes suppressed by GDNF stimulation would be those that encode for molecules essential for SSC differentiation. In cultured SSC-containing germ cell populations, exposure to GDNF suppressed the expression of 79 transcripts (Oatley et al. 2006). Currently, the roles of these genes have not been studied in detail. However, a few genes including *Neurogenin 3* (*Ngn3*) and *spermatogenesis*

and oogenesis helix-loop-helix 1 (*Sohlh1*) have been investigated for roles in spermatogenesis (Yoshida et al. 2004; Ballow et al. 2006).

7.3.4.1 NGN3

One of the genes suppressed by GDNF exposure in cultured mouse SSCs is *Ngn3*, a class B bHLH transcription factor, also expressed by multiple types of germ cells in the mouse germline including A_s, A_{pr}, and A_{al} spermatogonia as well as differentiating spermatogonia (Yoshida et al. 2004, 2006). While NGN3 expression has been characterized in detail and utilized for tracing SSC and A_{pr}/A_{al} spermatogonial activity *in vivo*, its role in regulating SSC fate decision has not been examined. *Ngn3* gene expression dramatically increases in cultured mouse SSCs upon removal of GDNF stimulation (Oatley et al. 2006) suggesting a role in SSC differentiation.

7.3.4.2 SOHLH1

In the mouse, expression of SOHLH1 is localized to the proliferating spermatogonial population, similar to NGN3 (Ballow et al. 2006). SOHLH1 is also a class B bHLH transcription factor believed to have a role in cellular differentiation (Ballow et al. 2006). In the male germline, expression of SOHLH1 is first observed by PGCs at embryonic day 15.5 (Ballow et al. 2006), and similar to PLZF, is not detected at birth with expression being exclusively localized to proliferating spermatogonia in both pre-pubertal and adult mouse testes (Ballow et al. 2006). Disruption of *Sohlh1* expression in mice causes infertility due to impaired formation of spermatocytes, indicating a role in spermatogonial differentiation (Ballow et al. 2006). Additionally, NGN3 expression is reduced in SOHLH1 deficient germ cells, suggesting a connection between these two transcription factors in regulating spermatogonial differentiation (Ballow et al. 2006). Because both NGN3 and SOHLH1 are expressed by A_{pr} and A_{al} spermatogonia it is possible they have roles in SSC differentiation but this has yet to be explored in detail.

7.4 GDNF-Activated Signaling Pathways in Cultured Mouse SSCs

GDNF binds to a heterodimeric receptor complex composed of a GFRα1 and the tyrosine kinase transmembrane molecule c-RET. Binding of GDNF causes transphosphorylation of specific tyrosine residues in the kinase domain of c-RET, which subsequently activates down-stream intercellular signaling cascades (Airaksinen and Saarma 2002). In neurons, GDNF stimulation activates the mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways (Kaplan and Miller 2000; Airaksinen and Saarma 2002). Exposure of cultured SSCs to GDNF results in

activation of the SRC family kinase (SFK) and PI3K/AKT signaling pathways (Braydich-Stolle et al. 2007; Lee et al. 2007; Oatley et al. 2007). Pharmacological impairment of SFK signaling blocks GDNF up-regulation of *Bcl6b*, *Etv5*, and *Lhx1* gene expression, without effecting cell survival or expression of non-GDNF-regulated genes, indicating a specific role in SSC self-renewal (Oatley et al. 2007). In contrast, pharmacological impairment of PI3K/AKT signaling induces apoptosis in cultured SSCs and impairs expression of both GDNF and non-GDNF-regulated gene expression, indicating a general role of PI3K/AKT signaling in cell survival rather than SSC self-renewal (Oatley et al. 2007).

Recent studies by Lee et al. (2009) have identified RAS proto-oncogene as a downstream signaling target of GDNF and FGF2 induced SFK stimulation in cultured mouse gonocytes. Supplementation of culture medium with either GDNF or FGF2 was equally effective at activating RAS. Additionally, gonocytes harboring overexpression of an activated form of RAS are able to proliferate in the absence of both GDNF and FGF2 stimulation and treatment with a pharmacological inhibitor of MAPK signaling prevented RAS-induced cell growth. These findings are surprising given that FGF2 effectively stimulates RAS activity but is unable to support stem cell self-renewal in cultures of wild-type spermatogonia (Kubota et al. 2004b; Kanatsu-Shinohara et al. 2005). Also, pharmacological inhibition of MAPK signaling in cultures of wild-type gonocytes did not affect their growth, suggesting *in vitro* adaption of the RAS overexpressing cells that may not reflect self-renewal mechanisms of stem cells *in vivo* (Lee et al. 2009). Transplantation analyses of cultured gonocyte populations overexpressing activated RAS revealed that stem cell content was reduced by greater than 50% after 2 months of *in vitro* maintenance (Lee et al. 2009). This finding suggests that RAS signaling alone is unable to completely replace growth factor requirements for prolonged SSC self-renewal.

Stem cell proliferation in most tissue is an infrequent occurrence and self-renewal cues may act by regulating progression of the cell cycle. Lee et al. (2009) found that gonocytes overexpressing activated RAS had elevated levels of cyclin D2 expression. Similar to RAS overexpression, cyclin D2 overexpression in cultured gonocytes promoted growth factor independent proliferation, and transplantation analysis revealed no decline in stem cell content after 2 months *in vitro* (Lee et al. 2009). These observations indicate that GDNF and FGF2 induction of SSC self-renewal occurs, at least in part, through regulation of entry into G1 phase of the cell cycle. Because RAS lacks a DNA binding domain, direct regulation of cyclin D2 transcription must occur through other intermediaries that have yet to be defined.

7.5 Summary

In the mammalian germline, SSCs undergo both self-renewal and differentiation to support continual spermatogenesis from puberty until old age in males. Self-renewal of SSCs is dependent on extrinsic stimulation by the growth factor GDNF. Our current understanding of internal molecular mechanisms regulating SSC self-renewal

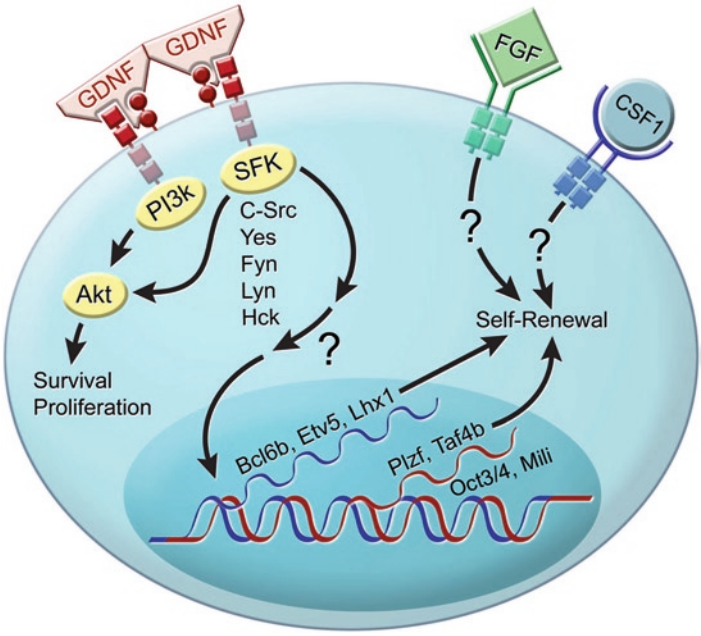

Fig. 7.3 Current understanding of molecular mechanisms regulating mouse SSC self-renewal and survival. Extrinsic stimulation by GDNF is essential for SSC self-renewal and this fate decision is enhanced by further stimulation from FGF2 and CSF1. GDNF binding to the receptor complex of c-Ret tyrosine kinase and GFRα1 activates PI3K and SFK intercellular signaling pathways causing down-stream activation of AKT signaling, which influences SSC survival. SFK signaling also activates a second pathway, yet to be defined, that regulates expression of specific genes important for SSC self-renewal. The transcription factor encoding genes *Bcl6b*, *Etv5*, and *Lhx1* are regulated through this pathway and are important for maintenance of SSCs *in vitro*. Additionally, the transcription/translation factors MILI, NANOS2, OCT3/4, PLZF, and TAF4B have been implicated as regulators of SSC self-renewal in mice. The expression of these molecules is not influenced by GDNF stimulation in cultured SSCs, and their importance in SSC self-renewal *in vitro* has not been determined. SFK stimulation by either GDNF or FGF2 also leads to down-stream activation of RAS proto-oncogene, which up-regulates cyclin D2 expression to influence SSC entry into G1 phase of the cell cycle. Intermediaries of RAS activation of cyclin D2 expression have not been elucidated. To date, mechanisms by which CSF1 influence self-renewal and survival of SSCs have not been reported. Image is modified and reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 24 © 2008 by Annual Reviews, http//:www.annualreviews.org (Oatley and Brinster 2008)

and survival is summarized in Fig. 7.3. GDNF stimulation activates SFK and PI3K/AKT signaling pathways followed by down-stream regulation of *Bcl6b*, *Etv5*, and *Lhx1* gene expression to control SSC self-renewal and survival. Additionally, downstream of SFK signaling is activation of the proto-oncogene RAS, which influences cyclin D2 expression to regulate cell cycle progression of cultured SSCs. Furthermore, the non-GDNF-regulated genes *Mili*, *Ngn3*, *Oct3/4*, *Plzf*, and *Taf4b* have also been implicated as regulators of SSC function through currently undefined mechanisms.

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Chapter 8
Stem Cell Niche System in Mouse Spermatogenesis

Shosei Yoshida

Abstract Mammalian spermatogenesis endures on the persistent activity of stem cells, i.e., their self-renewal and production of differentiating progeny. The normal functioning of stem cells explicitly requires a particular microenvironment within the tissue – *the stem cell niche* – as an indispensable element. While the mammalian spermatogenic stem cell niche system remains to be fully elucidated, recent knowledge has improved our understanding of the nature of stem cells and their niche and the underlying molecular mechanisms. Our improved knowledge encompasses the functional identification of stem cells, anatomical implications of the stem cell niche in relation to blood vessels, and identification of several molecular mechanisms involved in stem cell regulation. In this chapter, these findings are summarized along with the historical background, with an emphasis on the stem cell niche. For describing the characteristic features of the mammalian system, comparison with those of the *Drosophila* germline stem cell system will be helpful.

Keywords Niche • Stem cells • Spermatogonia • Seminiferous tubules • Blood vessels

8.1 Introduction

Mammalian spermatogenesis is supported by the persistence of stem cells that achieve self-renewal and production of progeny differentiating into sperm. In general, stem cells ensure the homeostasis and regeneration of a tissue during adulthood.

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It is generally established that the normal functioning of stem cells explicitly requires a particular microenvironment within the tissue – *the stem cell niche* – as an indispensable element (Spradling et al. 2001; Morrison and Spradling 2008). The integrated view for the stem cell niche system was established substantially in the *Drosophila* germline stem cells (GSCs) in both the testis and ovary (Spradling et al. 2001; Fuller and Spradling 2007), as well as a number of other systems, including mammalian hematopoiesis (Morrison and Spradling 2008). Although it is clear that the mammalian spermatogenic stem cell system also involves the niche microenvironment in the testis, its cellular and molecular nature has not been characterized to the extent that has been performed in the abovementioned systems. However, in recent years, mammalian spermatogenic stem cell research has experienced several breakthroughs, leading to a greater understanding of the nature of stem cells and their niche. The concept of niche may not be limited solely for the self-renewing stem cells, but can be extended to broader populations such as so-called progenitors. This idea is acknowledged because it is becoming apparent that progenitors are also involved in the stem cell system as an indispensable component.

This chapter summarizes the anatomical and historical backgrounds as well as ongoing research regarding the mammalian (mostly mouse) spermatogenic stem cell system, with an emphasis on the stem cell niche. In addition, a comparison with the characteristics of the *Drosophila* GSC system will be helpful.

8.2 *Drosophila* Germline Stem Cell Niche System

The germline of the fruit fly, *Drosophila melanogaster*, represents a typical stem cell niche system (Spradling et al. 2001; Fuller and Spradling 2007). In this organism, gamete production in both sexes is based on typical stem cell systems. The ovary and testis demonstrate a clear polarity, with one end opening to outside of the body while the other is a blind end, which provides the niche region that not only tethers the stem cells but also controls their growth and differentiation (Fig. 8.1a, b) (Morrison and Spradling 2008; Fuller and Spradling 2007). Figure 8.1c is a schematic representation of the testis tip, where highly specialized somatic cells, namely the hub cells, create the niche for GSCs. Hub cells form a tight contact with GSCs and control the orientation of GSC division to occur perpendicularly relative to hub cells. This results in one daughter that remains in contact with the hub and the other that loses the contact: The former persists as GSC, while the latter enters the process of differentiation, thus representing a typical asymmetric "stem cell division." Along with differentiation, the differentiating cells gradually leave behind the niche and finally exit as the mature sperm. As a result, the stem cells and the differentiating cells are arranged so that they recapitulate the chronological order of differentiation from the distal niche region toward the proximal opening. In the ovary, similarly, highly specialized somatic cells (cap and terminal filament cells) comprise the niche that supports the female GSC system in an essentially identical, but somewhat more complex, manner (Fuller and Spradling 2007). The *Drosophila*

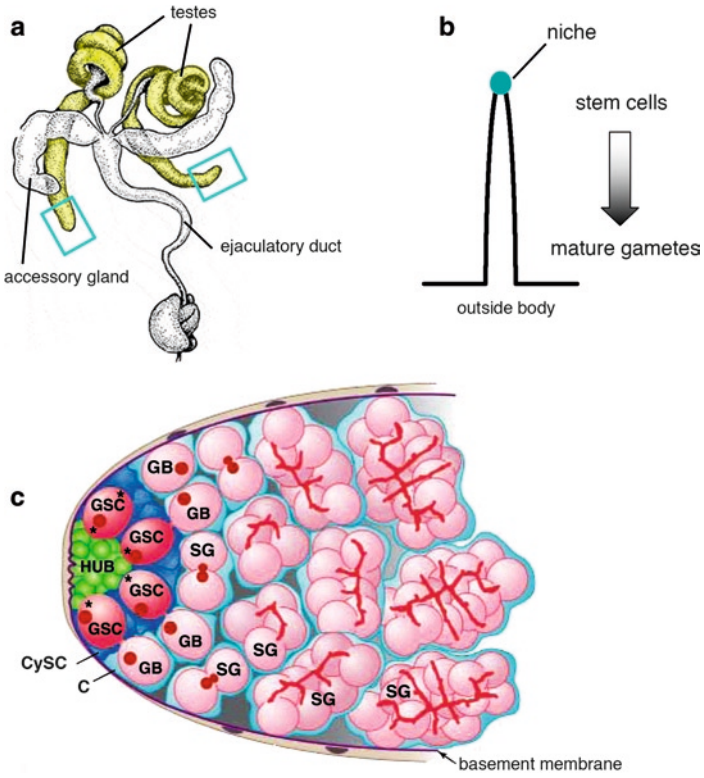

Fig. 8.1 Germline stem cell niche system in the *Drosophila* testis. (**a**) Structure of the male reproductive system of the *Drosophila melanogaster*, in which the pair of testes are colored *yellow*. The distal blind tips (*rectangles*) include the niche structure that harbors the stem cells (schematically shown in (**c**)). (**b**) A simplified model of a testis. Stem cells reside in the distal, niche region (*green circle*), while differentiating cells are arranged toward the proximal opening to outside the body. Thus, one can observe a polarity over the entire gonad (schematically shown by the *arrow*). (**c**) Schematic of the stem cell niche system at the testis tip. A group of highly specialized gonadal somatic cells, termed hub cells (*green*), comprise a niche to which germline stem cells (*GSCs*) are attached. As a result of asymmetric division of a GSC, the cell that remains in contact with the hub persists as a GSC, while the other that loses the contact becomes a gonialblast (*GB*), the first step in cell differentiation. As a result of the subsequent four synchronized incomplete cell divisions of GB, two-, four-, eight-, and 16-cell cysts are formed; these interconnected mitotic cells are termed spermatogonia or SG. The 16-cell cysts enter meiosis to form haploid sperms. *Drosophila* testis also contains cystocytes ((**c**), shown in *light blue*), somatic cells that support the differentiating germ cells. Cystocytes also have stem cells (cyst stem cells, *CySC*), which are also in touch with and under the control of hub cells. (**a**, **c**), modified from Patterson (1943) and Fuller and Spradling (2007), respectively, with permission

niche cells are established as a result of a programmed process of organogenesis during the developmental stages (Kitadate et al. 2007). In *Drosophila* germline, mechanisms important for the maintenance of the stem cell niche system have been revealed to a greater extent than any other systems. These include the contact between a stem cell and the niche mediated by adhesion molecules such as cadherin,

the stem cell niche intercellular signaling mediated mainly by Upd (male) and Dpp (female) ligands, and the intracellular molecular mechanisms in stem and niche cells [for detail, see (Fuller and Spradling 2007)].

The findings obtained from the *Drosophila* GSC research will provide much information for the future investigation of the mammalian spermatogenic stem cell system. However, it is not believed that the mammalian system will be a copy of that in *Drosophila* because the biological contexts of these organisms look different. What is important will be to understand the common basis among species and to clarify the characteristic features for the mouse spermatogenic stem cell niche system.

8.3 Architecture of the Mouse Testis

The anatomy of the testis of the mouse and other mammals makes a good contrast to that of the fruit fly. Spermatogenesis proceeds inside the seminiferous tubules – long, convoluted tubules that have a diameter of up to 200 μm (Russell et al. 1990). This structure is common in amniotes (i.e., mammalians, birds, and so-called reptiles). Seminiferous tubules form loops that open into the rete testes at both the ends, with a total length of up to 2 m per testis in the case of mice (Fig. 8.2a). Blood vessels, which nourish the tubules, never penetrate them but run in the interstitial spaces between the tubules to form a network (Fig. 8.2c, d). The interstitial space also includes Leydig cells (the major producers of androgens), macrophages, lymphoid epithelial cells, and connective tissue cells (Russell et al. 1990; Hinton and Turner 1993).

Spermatogenesis progresses uniformly all over the inner surface of the tubules or the seminiferous epithelium (Russell et al. 1990). Therefore, one can hardly recognize the overall polarity that governs the entire gonads or particular segments within the tubules where stem cells are accumulated (Fig. 8.2b, compare with Fig. 8.1b). In addition to a far bigger size than the *Drosophila* gonads, such a uniform architecture of the seminiferous tubules makes it difficult to demonstrate stem cell localization based on the presumptive niche structure. Indeed, based on transplantation and regeneration experiments, it has been suggested that stem cells are scattered all through the tubules (de Rooij and Russell 2000).

As shown in Fig. 8.2e, the anatomical framework of seminiferous epithelium is composed of the basement membrane and two types of somatic cells, Sertoli and peritubular myoid cells, which line the inside and outside of the basement membrane, respectively. Sertoli cells form a beautiful epithelium with tight junctions between them, which is the anatomical basis of the blood–testis-barrier. Sertoli-cell tight junctions separate the tubules into basal and adluminal compartments (Fig. 8.2e). The basal compartment between the tight junction and the basement membrane is occupied with all the stages of spermatogonia, defined as mitotic germ cells in the mature testis (Fig. 8.2f). Note that there is a discrepancy in terminology between mice and fruit fly. In *Drosophila*, the single, differentiation-oriented cells (a differentiating daughter of GSC) and the subsequent interconnected cysts are

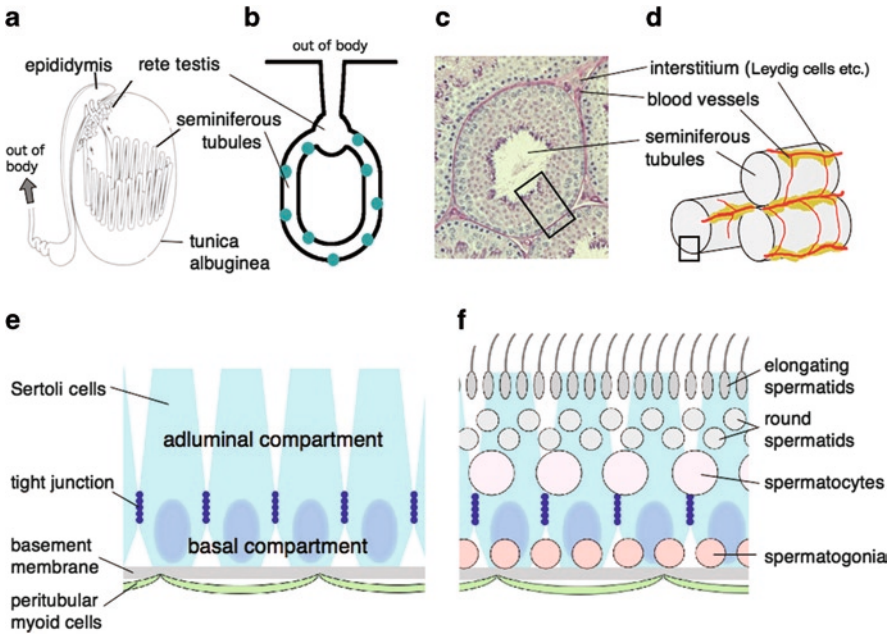

Fig. 8.2 Anatomical aspect of spermatogenesis in the mouse testis. (**a**) Schematic overview of the mouse testis. A single seminiferous tubule out of approximately 20, each of which forms loops with both ends that open into the rete testis, is shown. (**b**) A highly simplified diagram of mouse seminiferous tubule topology. As shown by *green dots*, stem cells are scattered throughout the tubule loops, which do not show apparent overall polarity. (**c**, **d**) Seminiferous tubules and the surrounding network of vasculature along with the interstitium. The blood vessels (*red*), which are surrounded by Leydig cells and other types of interstitial cells (*yellow*), form a network between the seminiferous tubules. (**e**, **f**) Scheme of the seminiferous epithelium architecture, corresponding to the area shown by *rectangles* in (**c**, **d**). (**e**) Representation of the anatomical framework composed of somatic components, while spermatogenic cells are included in (**f**). See text for details. Panels, modified from Yoshida (2008a)

termed "gonialblasts" (GB) and "spermatogonia" (SG), respectively, as a distinct entity from GSC. In the mouse, "spermatogonia" is a wider entity defined as mitotic stages of spermatogenic cells in the mature testis, including chained cells, single cells and the stem cells.

Upon entering into meiosis, the spermatogenic cells, now designated as spermatocytes translocate to the adluminal compartment across the tight junction, in a manner not fully understood yet. This is followed by movement toward the lumen along with the process of meiotic division and the formation of round and elongating haploid spermatids. As a result, a multilayered organization of differentiating germ cells appears among the Sertoli cell epithelium [Fig. 8.2f, see (Russell et al. 1990) for detail]. This basic architecture of seminiferous epithelium is found in the entire circumference and throughout the length of the tubules.

Thus, the microenvironment for the stem cells, which is a yet-to-be-identified small subpopulation of spermatogonia, is common to that of the basal compartment,

showing the closest relationship with Sertoli cells, myoid cells, and the basement membrane (Fig. 8.2f). However, given that most of the spermatogonia are not stem cells, some mechanism should provide uneven feature within the basal compartment to specify the niche microenvironment for stem cells (Ogawa et al. 2005; Hess et al. 2006).

8.4 Mouse Spermatogenic Stem Cells

The following is a review of the current knowledge regarding spermatogenic stem cells in the mouse. Figure 8.3 is a schematic representation of the mouse spermatogenic differentiation process. As mentioned above, there is no doubt that stem cells comprise a small subset of spermatogonia among those that occupy the basal compartment. However, we are currently unable to identify them in the architecture of seminiferous tubules. In the classical view known as the "A_s model," it has been proposed and widely accepted that the A_s or A_{single} spermatogonia, the singly isolated spermatogonia with an undifferentiated morphology, act as the stem cells (de Rooij and Russell 2000; Meistrich and Van Beek 1993; Huckins 1971; Oakberg 1971; de Rooij 1973). An A_s is expected to give rise to two A_s cells after a regular cell division, or a pair of interconnected daughters (A_{pair} or A_{pr}) due to incomplete cytokinesis. While the first division is considered to be self-renewing, the latter is of a differentiating type (de Rooij and Russell 2000; Meistrich and Van Beek 1993). A_{pr} subsequently give rise to chains of 2^n cells interconnected via intercellular bridges, as a result of incomplete division that occurs synchronously (Russell et al. 1990; de Rooij and Russell 2000). According to the A_s model, A_{pr} and other interconnected cells are believed to be committed for differentiation and their stem cell potential is lost.

In addition to such "morphological recognition," function-based identifications of stem cells have also been achieved. The first is based on posttransplantation colony formation. Brinster and coworkers developed a transplantation technique in which dissociated stem cells in the donor cell suspension gave rise to persisting spermatogenic colonies after transplantation into germ-cell-depleted host testis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). This was a great breakthrough that enabled the quantitative analyses of mammalian spermatogenic stem cells, and it is due to this system that the concentration and purification of stem cells could be performed (Shinohara et al. 2000). It is generally expected to be true that the stem cells detected by transplantation are equal to the population of A_s. However, it is by definition impossible to evaluate this idea, unless one can purify the A_s and non-A_s fractions and test their colony-formation activities.

The current consensus is that a vast majority of the stem cell activity (posttransplantation colony forming activity) resides within the population of "undifferentiated spermatogonia" or "A_{undiff}." A_{undiff} is a collective entity originally emerged from the morphological features that lack apparent heterochromatin condensation in their nuclei, and includes A_s, A_{pr}, and short chains of four, eight, 16, or up to

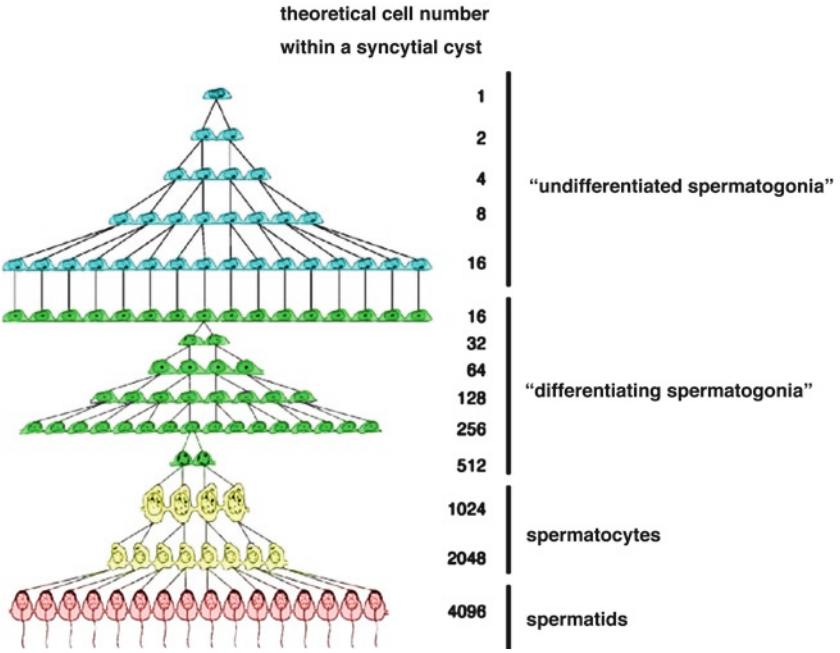

Fig. 8.3 Steps of the spermatogenic differentiation in mice. The mouse spermatogenic process is divided into the stage of spermatogonia (mitotic cells), spermatocytes (meiotic cells), and spermatids (haploid cells). The singly isolated spermatogonia are considered to be the most primitive cells. The subsequent mitotic and meiotic divisions accompany incomplete cytokinesis to form syncytia of 2^n cells as indicated. See text for details

32 cells ($A_{aligned}$ or A_{al} spermatogonia) (Russell et al. 1990; de Rooij and Russell 2000) (see Fig. 8.3). Upon differentiation of A_{undiff} to more advanced "differentiating spermatogonia," the expression of c-Kit is highly up-regulated from essentially no expression (Schrans-Stassen et al. 1999). Transplantation assay demonstrated that the c-Kit negative (~A_{undiff}) population exhibited most of the colony-forming activity (Shinohara et al. 2000; Ohbo et al. 2003), while investigations for the narrower fractions have not been achieved.

The use of the posttransplantation colony formation as an assay for the stem cell detection was essential for the establishment of long-term culture of the spermatogonia with stem cell activity (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004). This success is also owed to the discovery of the important roles of GDNF (glial cell line-derived neurotrophic factor) signaling, which was obtained from the loss-of-stem-cell-maintenance phenotype observed in the mutants (Meng et al. 2000). It can be said that the *in vitro* culture system mimics some, although not the entire, essential aspects of the *in vivo* stem cell niche system. Further, the stem cell behavior in response to these signals can be assessed using the *in vitro* system (Yeh et al. 2007).

A different approach for the functional identification of the stem cells was the *in vivo* pulse-chase strategy (Nakagawa et al. 2007; Yoshida et al. 2007a). This

system is advantageous in that it can detect the behaviors of the cells of interest in the process of homeostasis, without disturbing the normal architecture and functioning of the seminiferous tubules. Pulse-labeling of A_{undiff} marked by the expression of NGN3 (Yoshida et al. 2004) revealed that A_{undiff} include both stem and transit amplifying progenitor cells, the latter of which are defined by their proliferation and differentiation without self-renewal. It is also shown that some A_{undiff} that are destined for differentiation in homeostasis do contribute to post-transplantation colony-formation and postinsult regeneration (i.e., "potential stem cells") (Nakagawa et al. 2007; Yoshida et al. 2007a). Therefore, it may be difficult to provide a universal definition of "spermatogenic stem cells." Although the central role of A_s can be acknowledged intuitively, it is also strongly suggested that, within the A_{undiff} population, varying sets of cells play active roles in different aspects of stem cell functions (such as homeostasis versus regeneration/colony formation) (Nakagawa et al. 2007; Yoshida et al. 2007a). Recently, it has also been suggested that some of the so-called differentiating spermatogonia may retain the stem cell potential to form colonies after transplantation or *in vitro* cultivation (Barroca et al. 2009).

The stem cell niche system in the mouse spermatogenesis may not be as simple as that in *Drosophila* gonads. This issue needs to be further investigated carefully.

8.5 Niche Microenvironment in the Mouse Testis

Sertoli cells, peritubular myoid cells, and the basement membrane (components of the basal compartment of seminiferous tubules, see Fig. 8.2f) are often described to comprise the mammalian spermatogenic stem cell niche (Ogawa et al. 2005; Hess et al. 2006). This is correct but not a fully adequate description. This cannot explain why only a limited number of spermatogonia are stem cells, prompting the notion of the lack of uniformity over the basal compartment.

Given the seemingly uniform morphology of the seminiferous tubules, a straightforward strategy for investigating the stem cell niche is to localize the candidate stem cell populations. Along this line of thought, a series of experiments have been performed to address the localization of A_{undiff}. Chiarini-Garcia and colleagues examined the localization of A_{undiff} based on the observation of plastic-embedded thin sections, and found that A_{undiff}, within the basal compartment, preferentially localized to the area adjacent to the interstitium, compared to attaching to the neighboring tubules (Chiarini-Garcia et al. 2001, 2003). More recently, the author's group has extended this finding by three-dimensional reconstitution and an originally developed *in vivo* live imaging system that allows one to trace the live behaviors of A_{undiff} without disturbing the normal architecture (Yoshida et al. 2007b). As shown in Fig. 8.4, A_{undiff} preferentially localized to the area adjacent to interstitial spaces, namely to the blood vessels with medium thickness (i.e., arterioles and venules). It is noteworthy that these testicular vessels accompany the Leydig cells and other interstitial cells (Hinton and Turner 1993). Interestingly, A_{undiff}

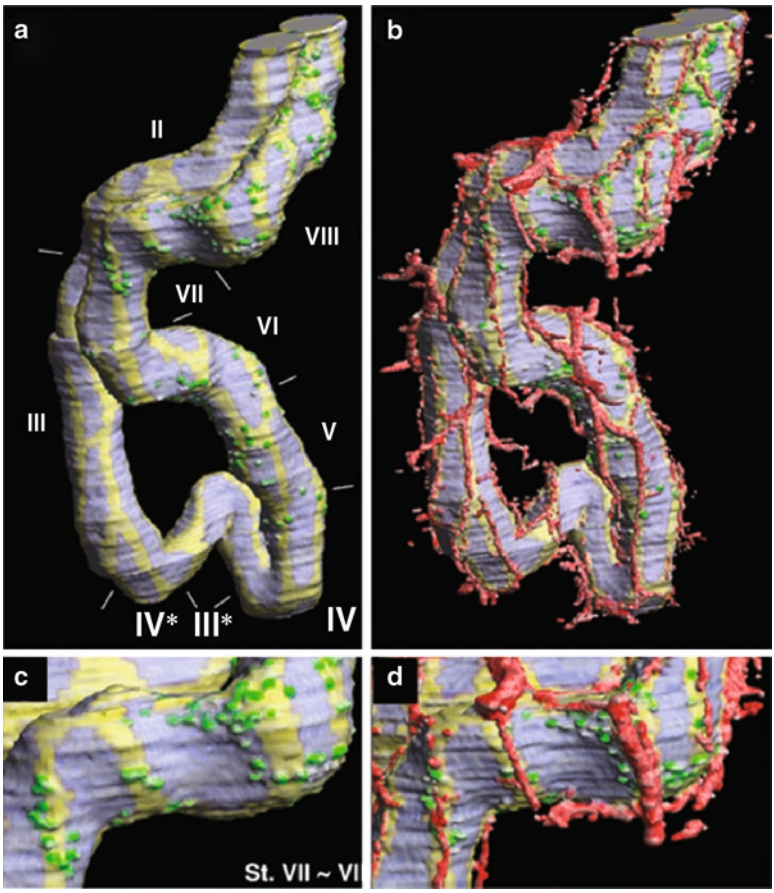

Fig. 8.4 Localization of A_{undiff} in a three-dimensional reconstruction. Computationally reconstituted three-dimensional image of the seminiferous tubules based on 280 serial sections. A_{undiff} (*green*) show biased localization to the blood vessel network (*red*) and the area adjacent to the interstitium (*yellow*). (**a**, **c**) and (**b**, **d**), without or with blood vessels, respectively. *Roman numerals* indicate the stage of the seminiferous epithelium. Reprinted with permission from Yoshida et al. (2007b)

showed a further preference to the branching point of these vessels. The live imaging clearly revealed that, upon differentiation, the spermatogonia migrate out of these specified areas and spread to all over the basal compartment (Fig. 8.5) (Yoshida et al. 2007b).

Based on these observations, we concluded that the area of the basal compartment that faces the surrounding vasculature serves as the niche for A_{undiff} (Fig. 8.6a) (Yoshida et al. 2007b; Yoshida 2008b). Moreover, because the actual stem cells consist a part of A_{undiff}, these sites might also act as a niche for the stem cells. However, we could not deny the possibility that actual stem cells are localized independently of the vasculature, and that the A_{undiff} spermatogonia that are destined

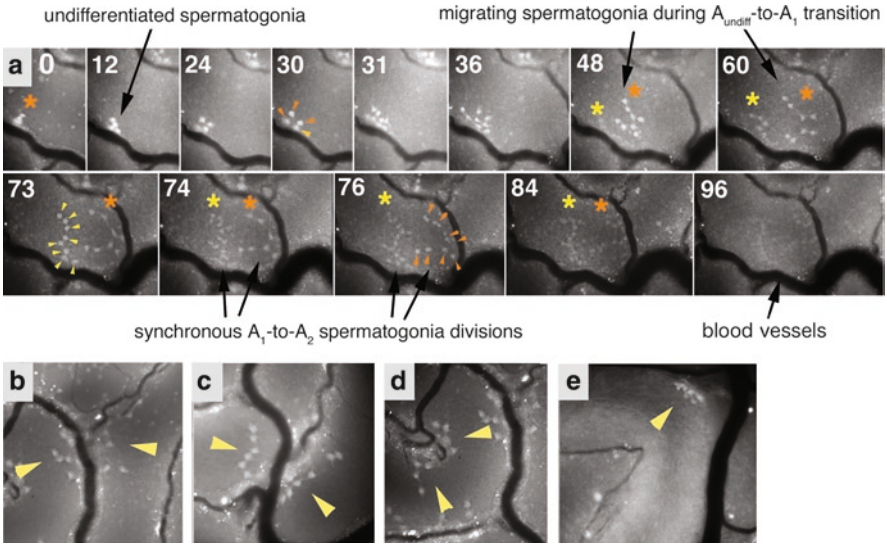

Fig. 8.5 A live imaging study of GFP-labeled Ngn3^{+} A_{undiff} upon transition into differentiating spermatogonia. (**a**) Selected frames of a live imaging study of spermatogonia upon A_{undiff}-to-A_1 transition. The elapsed time is indicated in each panel in hours. Before transition (0–12 h), A_{undiff} preferentially localized to the area adjacent to the blood vessels (seen as a *black line*). Upon transition into A_1, two chains of eight-cell cysts (A_{al-8}; indexed in *yellow* and *orange*) migrated from this position to spread all over the basal compartment of the tubule (by ~36–60 h), followed by synchronous divisions. See (Yoshida et al. 2007b) for details. (**b**–**e**) Examples of the vasculature-proximal localization of A_{undiff} (*arrowheads*), more characteristically to their branch points. Modified from Yoshida et al. (2007b)

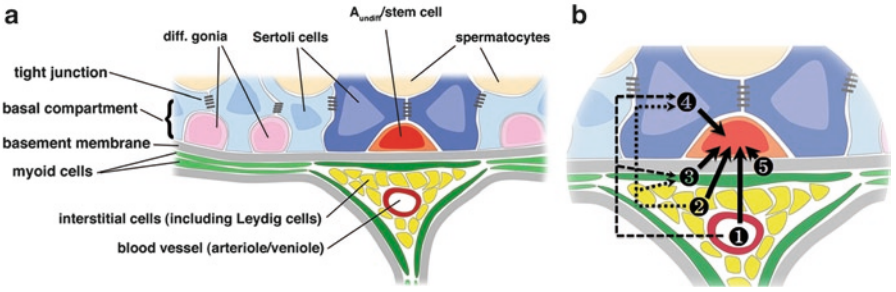

Fig. 8.6 Schematic of the vasculature-associated niche for A_{undiff}. (**a**) A schematic of the proposed vasculature-associated microenvironmental niche for A_{undiff} (and possibly the stem cells), shown in *red*. Important components include blood vessels, interstitial cells, myoid cells, Sertoli cells, and the basement membrane. Sertoli and myoid cells in this region might be "specialized," as schematically shown in *deeper blue* and *green*, respectively. (**b**) The possible controlling pathways of A_{undiff}/stem cells, shown in *arrows 1–5*. *Dotted arrows* indicate a possible indirect effect from the blood vessels/interstitium. Cells are not shown proportionally in size. See text for details

for differentiation could show preferred localization to the vasculature region before.

Thus, in addition to Sertoli cells, peritubular myoid cells, and basement membrane, the components common to the entire basal compartment, blood vessels, and/or interstitial cells possibly specify the niche microenvironment (Fig. 8.6b). This may be a direct, distant effect mediated by soluble factors (Fig. 8.6b, *arrows 1* and *2*), or may be an indirect effect relayed via myoid or Sertoli cells (Fig. 8.6b, *arrows 3* and *4*). In the latter case, myoid and/or Sertoli cells that are located in the proximity of the vasculature would be endowed with special characters under the influence of the blood vessels/interstitial cells (Fig. 8.6b, *dotted arrows*). Additionally, the specific characteristics of the basement membrane could be important (Fig. 8.6b, *arrow 5*). Intriguingly, transplantation of seminiferous tubule has suggested that the remodeling of the vasculature may accompany the relocation of the A_{undiff} to the newly formed vessels (Yoshida et al. 2007b), suggesting that the niche region is not fixed but is plastic and reversible. In accordance with this, testis of golden hamster is suggested to remodel the vasculature and accompanying niche region in response to short photoperiod (do Nascimento et al. 2009). This is in a contrast to that in *Drosophila* GSC niches that are specified as a result of a strict organogenesis program and do not regenerate once damaged.

Currently, identification of the components that specify the niche microenvironment and regulate the A_{undiff}/stem cell behaviors has not been achieved to an extent comparable to those for *Drosophila*. However, a number of molecules are known to functionally contribute to stem cell control. In the next section, considering these factors together, readers will gain an insight into the functional aspects of the stem cell niche.

8.6 Molecular Mechanisms That Could Be Involved in the Niche Functions

8.6.1 Soluble Factors

8.6.1.1 GDNF (Glial Cell Line-Derived Neurotrophic Factor) Signaling

GDNF is the most important currently known controlling factor of mouse spermatogenic stem cells. GDNF is a soluble ligand and a diverged member of TGF-beta superfamily, which transmits signals through a receptor complex consisting of c-Ret tyrosin kinase and GFRα1 co-receptor (Paratcha and Ledda 2008). The importance of GDNF signaling in the control of spermatogenic stem cells was first recognized from the mutant phenotype (Meng et al. 2000). Mutant mice heterozygous for the *Gdnf*- or *c-Ret*-targeted allele exhibited a gradual degeneration in spermatogenesis following essentially normal establishment of spermatogenesis. This phenotype

suggested the essential role of GDNF signaling in the maintenance of the stem cell compartment *in vivo* rather than its establishment (Meng et al. 2000).

This work was a milestone and, following this study, mutations in several genes have been shown to exhibit similar phenotypes. Some such genes function in the somatic cells [such as GDNF, Etv5/ERM (see below)], while others function in the germ cells [such as c-Ret, GFRα1, mUtp14b [mutated in *jsd* (*juvenile spermatogonial depletion*) (Beamer et al. 1988; Rohozinski and Bishop 2004; Bradley et al. 2004)], Plzf [mutated in *luxoid* (Buaas et al. 2004; Costoya et al. 2004)], Taf4b (Falender et al. 2005)]. The former group of genes may be involved in the control of the *niche* microenvironment. This chapter, which focuses on the *stem cell niche*, describes the genes that act in somatic cells in the following sections.

The GDNF ligand is expressed in Sertoli cells, while c-Ret and GFRα1 are expressed in spermatogonia, namely, in the least matured subsets of A_{undiff} (Hofmann et al. 2005; Tokuda et al. 2007). So far, the precise localization of GDNF expression has not been achieved due to its low expression levels. However, the forced expression of GDNF in Sertoli cells causes the abnormal accumulation of stem cell-like spermatogonia with immature phenotypes (Meng et al. 2000; Yomogida et al. 2003). These loss-of-function and gain-of-function experiments indicate that GDNF signaling mediates stem cell regulation by Sertoli cells. Importantly, the identification of GDNF signaling as an essential factor *in vivo* has led to the successful establishment of a spermatogonial culture that retains the stem cell potential by addition of GDNF ligand and sometimes a soluble form of GFRα1 (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004). Altogether, GDNF is crucial for the expansion, survival, and/or maintenance of spermatogenic stem cells. The downstream cascade of GDNF signaling would affect the intracellular machinery of the stem cells per se, and this is one of the current foci of interest in this field (Hofmann 2008; Jijiwa et al. 2008; Oatley et al. 2007).

8.6.1.2 CSF1 (Colony Stimulating Factor 1/Macrophage Colony-Stimulating Factor or M-CSF) Signaling

Recently, gene expression profiling has revealed that the receptor for CSF1 (Csf1r) is highly expressed in candidate stem cell populations (Oatley et al. 2009; Kokkinaki et al. 2008). In contrast, the CSF1 (Colony Stimulating Factor 1) ligand is expressed in Leydig cells and a subset of myoid cells (Oatley et al. 2009). The addition of CSF1 to the GDNF-containing spermatogonial stem cell culture increased the content of the posttransplantation colony-forming cells (Oatley et al. 2009). Although the *in vivo* function of this signaling pathway remains to be elucidated, it is suggested that CSF1 may cooperate with GDNF in supporting the self-maintenance of stem cells. CSF1 expression in the interstitium suggests that it could be involved in the function of the vasculature-associated niche, which possibly regulates the stem cells. Further investigations would be warranted regarding this pathway and its relationship with GDNF and other signals.

8.6.2 FGF (Fibroblast Growth Factor) and Other Factors

In vitro culture of the spermatogonia revealed the important role of Fibroblast Growth Factor (FGF) signaling in the maintenance/expansion of the stem cells; however, it is only observed in the presence of GDNF (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004). While bFGF (FGF2) demonstrates a clear function in these cultures, it is obscure whether FGF signaling actually plays an active role *in vivo*, and, if it does, which member of the FGF family is involved. The expression profiles of the FGF family ligands and their receptors are somewhat complicated. In addition, the *in vitro* culture has revealed the effect of other factors, such as leukemia inhibitory factor (LIF) or epidermal growth factor (EGF) (Kanatsu-Shinohara et al. 2003, 2007); how these factors are involved in the *in vivo* niche also needs to be elucidated.

8.7 Cell–Cell Contact and Basement Membrane Binding

In the case of *Drosophila* germline and other stem cell systems in mammals, cell–cell contact plays an essential role in their niches, which is mediated by adhesion molecules such as cadherins (Song et al. 2002). In these systems, cadherins tether the stem cell to the niche cells via homophilic binding. In the mouse spermatogenesis, E-cadherin is expressed in A_{undiff} (Tokuda et al. 2007), raising the possibility of a similar tethering mechanism via cadherin molecules being involved in the mouse spermatogenic stem cells. However, recently, Shinohara and colleagues clearly demonstrated that E-cadherin is dispensable for the normal functioning of the stem cells, i.e., the transplantation of cultured stem cells lacking E-cadherin successfully colonized the host seminiferous tubules and supported persisting spermatogenesis (Kanatsu-Shinohara et al. 2008). This finding agrees with the observation that E-cadherin gene expression is not detected in Sertoli or myoid cells (Yoshida, unpublished data). Similarly, our live imaging studies have suggested that E-cadherin-expressing A_{undiff}, move around in the testis, suggesting that they are not tethered to a fixed position [(Yoshida et al. 2007b) and data not shown].

In contrast, Shinohara's transplantation assay has also demonstrated the essential role of β1-integrin in posttransplantation colony formation (Kanatsu-Shinohara et al. 2008). β1-integrin is expressed in a spermatogonial population, including stem cells, and mediates the attachment to the basement membrane via binding to laminins, probably as a heterocomplex with α6-integrin (Shinohara et al. 2000). GS cells lacking β1-integrin fail to develop spermatogenic colonies after transplantation. This suggests the important role of interaction with the basement membrane in stem cell functioning. The posttransplantation homing of stem cells and subsequent colony formation includes multiple steps (attachment to the Sertoli cell surface, retrograde translocation to the basal compartment across the tight junction, migration to the presumptive stem cell niche, survival,

proliferation, and self-renewal within the niche, expansion of the transient amplifying spermatogonia and differentiation into sperm); it would be interesting to determine which step actually requires β1-integrin.

8.8 Transcriptional Regulator in Somatic Cells

8.8.1 Etv5 (ets Variant Gene 5/ERM or ets-Related Molecule)

(*Etv5*)-homozygous testes have apparently normal spermatogenesis in the initial cycles after puberty; however, it does not persist in the following cycles, resulting in the loss of germ cells in older animals (Chen et al. 2005). The *Etv5* gene encodes a transcriptional regulator that is detected, according to this initial report, only in the Sertoli cell nuclei in the adult testis. Therefore, it can be postulated that Etv5 can regulate Sertoli cell functions that mediate the regulation of germ cells. Etv5 expression in Sertoli cells appears to be uniform and does not show any apparent position-specificity (Chen et al. 2005). However, it has been recently reported that Etv5 is also expressed in some germ cells in younger stages, and that Etv5 mutant germ cells either do not form colonies after transplantation or exhibit an impaired response to GDNF (Tyagi et al. 2009), raising the possibility that the stem cell maintenance phenotype could be germ cell-autonomous. Clarifying the site of action of this gene in terms of cell type and developmental stages would be essential in order to explain such an intriguing phenotype and to achieve a greater understanding of the stem cell niche system.

8.9 Conclusion

This chapter has the current knowledge regarding the mammalian spermatogenic stem cell niche system. Readers should recognize that we have a number of pieces of knowledge in our hands. Several further investigations will be required before a comprehensive view of this system can be obtained, where anatomical, functional, and molecular aspects of stem cell niche somatic cell interactions will be integrated into a single scheme. This is a challenge and includes a number of essential issues such as the precise identification of stem cells and localization in the seminiferous tubules. Once this is achieved, however, the mammalian spermatogenic stem cell niche system will definitely provide invaluable insights for stem cell research in general.

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Part III
Translation and Regenerative Potential of Spermatogonial Stem Cells

Chapter 9
Regenerative Potential of Spermatogenesis after Gonadotoxic Therapies

Gunapala Shetty, Gensheng Wang, and Marvin L. Meistrich

Abstract Gonadotoxic therapies, particularly radiation and chemotherapy used in the treatment of cancer, are extremely damaging to spermatogenesis. They may result in prolonged or permanent oligospermia or azoospermia and consequent infertility. Transient oligo- or azoospermia is generally observed due to killing of the rapidly proliferating differentiating spermatogonia. High doses of some of these agents can also kill spermatogonial stem cells, but their sensitivity to killing by different agents varies with the species. Any surviving stem cells may repopulate the tubules and produce differentiating spermatogenic cells and restore fertility. However the kinetics of this process, which has been studied in mice, rats, monkeys, and humans, is generally gradual, again showing wide variation between species. There are no universal methods to enhance the regeneration of spermatogenesis from the surviving stem cells, but suppression of testosterone and possibly gonadotropins enhances the recovery process in rodents. An understanding of the mechanisms controlling this regenerative process and how the results might be applied to humans is needed.

Keywords Spermatogonia • Radiation • Chemotherapy • Stem cells • Regeneration

9.1 Introduction

Chemotherapy and radiotherapy used in the treatment of cancer, in some autoimmune and kidney diseases, and in other nonmalignant disorders can cause long-term or permanent gonadal toxicity in male patients. For children and young adults

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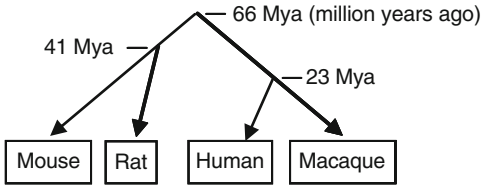

Fig. 9.1 Evolutionary distances between mice, rats, macaques, and humans

who have been cured of these diseases, the quality of life, which includes the ability to have a normal child, then becomes a major issue. The major concern is the prolonged reduction in sperm count to the point of azoospermia, as endocrine dysfunction (e.g., testosterone reduction) only occurs in limited instances (Shalet et al. 1989) and damage to other aspects of sperm function, such as loss of motility or morphological abnormalities are less pronounced than the loss of sperm production (Gandini et al. 2006; Meistrich et al. 1992). When sperm count recovers following cytotoxic therapy, fertility is generally restored. However, when the damage is severe, sperm count sometimes plateaus in an oligospermic range and the sperm may have morphological abnormalities (Anserini et al. 2002), both of which may be incompatible with fertility.

The adverse effects of chemotherapeutics and radiation on sperm production have been studied in mice, rats, macaque monkeys, and human patients undergoing treatment. Although many aspects of spermatogenesis and its regulation are conserved across mammalian species, interspecies differences can result in variations in response to and recovery from gonadotoxic therapies. Quantitative interspecies comparisons are best done using radiation, because similar doses to the target organ can be compared (Meistrich 1989, 1992). In this chapter, when the species is not designated, this will represent general principles across these four species; we will clarify which aspects apply to a more limited subset of them. Although it is expected that the primates will be more similar to each other and the same for rodents, the evolutionary distance between humans and macaques is 35% of that between rodents and primates (Fig. 9.1) (Kumar and Hedges 1998). Thus, some limited aspects in the response of the human testis to a gonadotoxic insult might possibly be more similar to one of the rodents than to macaques. Overall, there must be unifying biological processes that dictate the regenerative capacity of spermatogonial stem cells after injury, but there are likely to be details that differ between species (Meistrich 1992) causing the different outcomes.

9.2 Immediate and Short-Term Effects of Gonadotoxic Therapies

The sequence of spermatogenic cells consists of the stem spermatogonia, differentiating spermatogonia, spermatocytes, spermatids, and spermatozoa. Among these cells, the differentiating spermatogonia proliferate most actively and are extremely

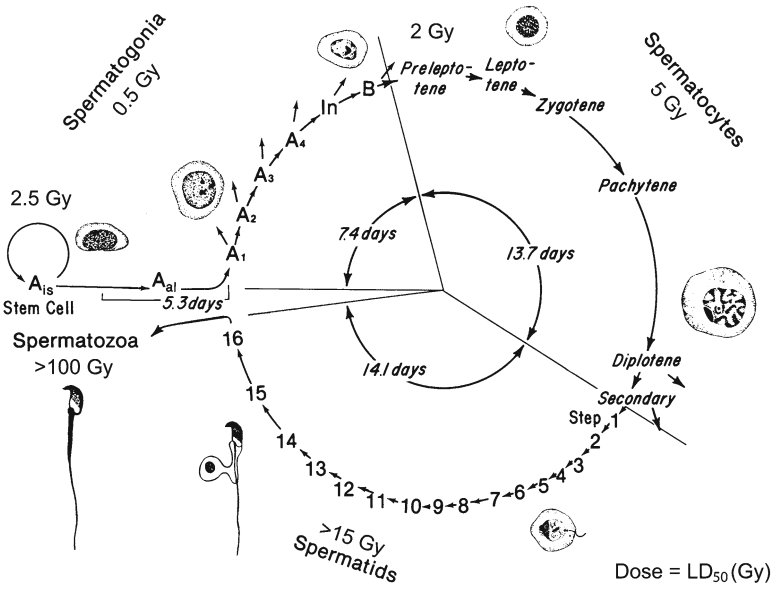

Fig. 9.2 Sequence, kinetics, and radiation sensitivities of spermatogenic cells in the mouse. The LD_{50} is the radiation dose necessary to kill 50% of the cells [modified with permission from (Meistrich et al. 1978)]

susceptible to cytotoxic agents (Fig. 9.2) (Oakberg 1957). The later stage germ cells (spermatocytes and especially spermatids) are less sensitive to killing by most of these cytotoxic agents (Meistrich et al. 1982; Oakberg and Diminno 1960). The somatic cells of the testis also survive most cytotoxic therapies; however, these cells may suffer functional damage (Zhang et al. 2006).

After cytotoxic treatment, the time course of changes in sperm count depends on the sensitivities of the different spermatogenic cells, and their kinetics and efficiency of maturation to sperm in the testicular environment. Once the progeny of the stem spermatogonia differentiate to the point at which cells are related to specific stages of the cycle of the seminiferous epithelium (A_1 spermatogonial stage in rodents, B spermatogonial stage in primates), they progress with the same kinetics as in the normal testis.

Because of the relative resistance of the later stage germ cells, the immediate effect of cytotoxic exposure on sperm count is minor (at low doses) or gradual (at higher doses) (Fig. 9.3). However, at the times that the differentiating spermatogonia would have become sperm, ranging from 35 days in mice to 60 days in humans, sperm counts often decline dramatically. This occurs with the doses of a highly gonadotoxic agent like radiation shown in Fig. 9.3. Even mildly gonadotoxic forms of chemotherapy, which do not affect stem cells or the recovery of spermatogenesis from the stem cells, can cause transient reductions in sperm count lasting until 2–3 months from the end of treatment because they kill differentiating spermatogonia (Lu and Meistrich 1979; Meistrich et al. 1997a).

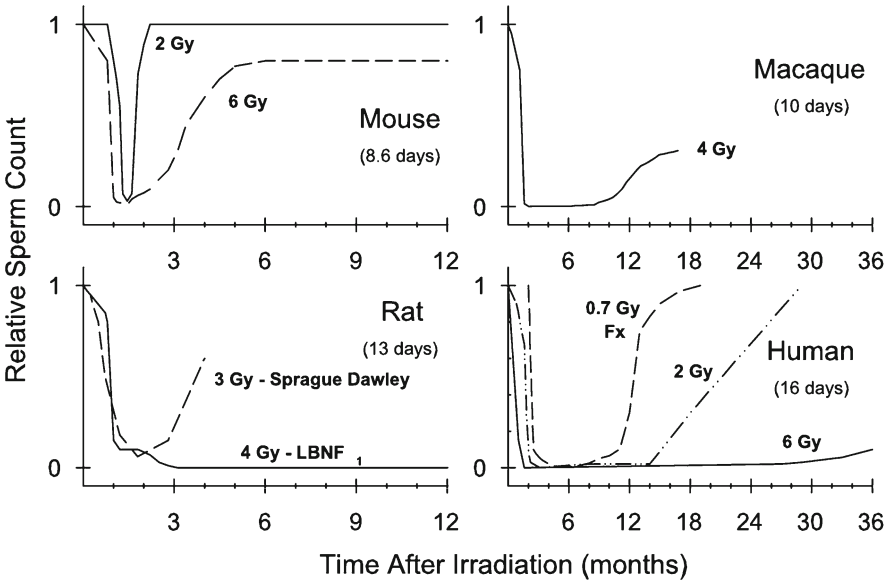

Fig. 9.3 Diagrammatic representation of time courses of changes in relative sperm counts in different species. Rodent data are based on testicular and epididymal counts; the primate data on ejaculated sperm counts. Note the different time scales in rodents and primates. The durations of a single cycle of the seminiferous epithelium in each species are given in parentheses. The data were obtained in mice (Meistrich et al. 1978; Meistrich and Samuels 1985; Searle and Beechey 1974), Sprague–Dawley rats (Dym and Clermont 1970; Velez de la Calle JF et al. 1989), $LBNF_1$ rats (Kangasniemi et al. 1996b; Meistrich et al. 2001), macaques (Kamischke et al. 2002) and human single dose (Rowley et al. 1974) and fractionated (Fx) (Dubey et al. 2000; May et al. 2000) irradiations

The effects of gonadotoxic therapies on the somatic cells of the testis and on the hypothalamic-pituitary-gonadal axis are not immediate and generally appear to be secondary to the loss of germ cells. Germ cell loss causes reduced inhibin secretion by the Sertoli cells, resulting in increased follicle-stimulating hormone (FSH) secretion by the pituitary (Boekelheide and Hall 1991). Testosterone production is usually unaffected, but germinal aplasia reduces testis size and consequently testicular blood flow, resulting in less testosterone being distributed into the circulation (Wang et al. 1983) and hence increased luteinizing hormone (LH) secretion by the pituitary.

9.3 Stem Spermatogonial Survival–Methods

Regeneration of spermatogenesis requires the survival of spermatogonial stem cells. Several different approaches have been taken to assess their survival.

Morphological criteria have been used to quantify numbers of putative spermatogonial stem cells. In rodents, it is generally accepted that isolated undifferentiated type A spermatogonia, designated A_s, are the stem cells (Huckins 1971a). When

they divide and rearrange their topology to become A-paired (A_{pr}) and A-aligned (A_{al}) cells, they show molecular changes and have initiated their differentiation process (Yoshida et al. 2007). They further divide to become A_1, A_2, A_3, and A_4 spermatogonia, which most certainly lack stem cell potential. In contrast, in primates, there are two categories of A spermatogonia, A-dark (A_{dark}) and A-pale (A_{pale}) (Clermont 1969). The A_{pale} are believed to be self-renewing stem cells, which normally give rise to the differentiating cells, the B spermatogonia (Clermont 1966, 1969). The A_{dark} are believed to be reserve stem cells, which are only active after the A_{pale} are depleted, but this model has not been definitively proven (Ehmcke et al. 2005b).

Stem cell survival can also be inferred from the numbers of colonies containing differentiated germ cells, which must have been derived from surviving stem cells after treatment with cytotoxic therapies. Since germ cells are absent from many tubule cross-sections, which contain only Sertoli cells, spermatogonial stem cell survival in the mouse and rat has been assessed by the percentages of cross-sections of seminiferous tubules showing differentiating spermatogenic cells 5–11 weeks after cytotoxic therapies (Delic et al. 1987; Kramer et al. 1974; Meistrich and van Beek 1993; Withers et al. 1974). This inference has been rigorously validated in the mouse testis (de Ruiter-Bootsma et al. 1976), but there are instances where it is not valid in the rat. Surviving spermatogonial stem cells are unable to produce differentiating cells or repopulate the tubules after irradiation of certain inbred strains (Kangasniemi et al. 1996b) or even after other cytotoxic treatments of outbred Sprague–Dawley rats (Boekelheide and Hall 1991).

In addition, the prolonged depression of sperm count when, following gonadotoxic exposure, surviving stem cells should be producing sperm can be used as a surrogate measure for stem cell killing in mice (Meistrich 1982, 1986a). This approach has application to studies in the human where sperm counts are much more readily obtained than is testicular material.

Recently it has become possible to assess spermatogonial stem cell survival directly by transplanting the cells (Brinster and Zimmermann 1994; Nagano et al. 2001) in a suspension taken from the testis of an animal treated with the gonadotoxic agent into a recipient testis that lacks endogenous germ cells and counting colonies of germ cells. Mutant mice lacking endogenous germ cells (Kanatsu-Shinohara et al. 2003) or busulfan-treated mice (Zhang et al. 2003) have been used as recipients to assess stem cell survival in donor mice treated with gonadotoxic agents. Busulfan-treated nude mice have been used as recipients to quantify the spermatogonial stem cell survival in the donor testes from other species (Hermann et al. 2007).

9.4 Stem Spermatogonial Survival-Results

9.4.1 Counts of Type A Spermatogonia

In rats and mice, A_s spermatogonial numbers were not affected 7–8 days after 2 Gy irradiation, but by that time the numbers fell to about 40% of control after 6 Gy

(Erickson 1976, 1981). However, these numbers are an underestimate of killing of A_s spermatogonia because in the mice the surviving stem cells undergo about three self-renewing divisions within this time (Van Beek et al. 1986; Van Beek and Meistrich 1990). After 6 Gy, there is also a subsequent progressive loss of A_s spermatogonia in both rodent species to roughly 10% of control at 8 weeks after irradiation (Erickson and Hall 1983). The functional significance of this decline is not known because, at least in the mouse, sperm production shows progressive recovery during this time period (Meistrich et al. 1978).

In monkeys, after radiation doses in the 1–4 Gy range, there is a rapid decline in the numbers of A_{pale} spermatogonia to about 20% within 10 days and then to about 2% of control at 6 weeks, whereas the numbers of A_{dark} spermatogonia are unaffected for 10 days but then decline to about 3% of control at 6 weeks (van Alphen et al. 1988a). In human, the decline in A_{dark} spermatogonia after irradiation is also gradual, reaching about 7 or 1% of control at about 26 weeks after 1 or 6 Gy irradiation, respectively (Clifton and Bremner 1983; Meistrich and van Beek 1990; Rowley et al. 1974). The numbers of A_{pale} spermatogonia also decline but there is disagreement as to whether their numbers parallel the gradual A_{dark} decline or rapidly decline (Meistrich and van Beek 1990; Paulsen 1973; Rowley et al. 1974).

9.4.2 Assessing Stem Cell Survival by Recovery Potential

The reduction in the percentage of tubules containing differentiated germ cells derived from stem cells (repopulation index) has been used to quantify the killing of stem spermatogonia by irradiation in mice (de Ruiter-Bootsma et al. 1976; Lu and Meistrich 1979). This assay demonstrated the sensitivity of mouse spermatogonial stem cells to some chemotherapeutic drugs, such as certain alkylating agents (busulfan and thio-TEPA), and doxorubicin, but not other drugs (Bucci et al. 1985; Lu et al. 1980; Meistrich 1986a; Meistrich et al. 1982) (Table 9.1). Killing of stem spermatogonia by single doses of procarbazine and chlorambucil could not be demonstrated by this assay; however, there was an indication of stem cell killing as assessed by reduced sperm counts, and multiple doses of these drugs clearly resulted in stem cell killing as demonstrated by reduced repopulation indices (ML Meistrich, unpublished data). Other chemotherapeutic agents, such as some alkylating agents (e.g., cyclophosphamide), cisplatin, antimetabolites, microtubule inhibitors, and topoisomerase inhibitors (e.g., amsacrine, Table 9.1) (Da Cunha et al. 1985), were not toxic to mouse stem cells.

The effects of these agents on stem cell survival have also been studied in the rat by assessment of the ability of spermatogenesis to recover at 8–11 weeks after treatment. Complete recovery indicates that few, if any stem cells were killed, but the absence of recovery may indicate stem cell killing but may, in part, result from damage to the somatic environment preventing recovery of spermatogenesis from the surviving stem cells (Zhang et al. 2006). Similar to the mouse, radiation, procarbazine, doxorubicin, and chlorambucil (Delic et al. 1986a, b, 1987; Lui et al. 1986)

Table 9.1 Effectiveness of anticancer gonadotoxic agents in different species at killing stem spermatogonia

Agent	Mouse	Rat	Macaque	Human
Radiation	++ (Withers et al. 1974)	++ (Delic et al. 1987)	+++ (van Alphen et al. 1988a)	+++ (Rowley et al. 1969)
Busulfan	++ (Bucci and Meistrich 1987)	+ (Ogawa et al. 1999)	+ (Hermann et al. 2007)	++ (Sanders 2004)
Cyclophosphamide	− (Lu and Meistrich 1979)	± (Meistrich et al. 1995)	n.d.	++ (Meistrich et al. 1992)
Chlorambucil	+ (Meistrich et al. 1982)	+ (Delic et al. 1986b)	n.d.	++ (Clark et al. 1995)
Cisplatin	− (Meistrich et al. 1982)	− (Huang et al. 1990)	n.d.	++ (Hansen et al. 1989; Meistrich et al. 1989)
Melphalan	n.d.	n.d.	n.d.	+ (Jacob et al. 1998)
Procarbazine	+ (Meistrich et al. 1982)	+ (Delic et al. 1986a; Parchuri et al. 1993)	(+) ([a]Sieber et al. 1978)	+ (Da Cunha et al. 1984; Sherins and DeVita 1973)
Thio-TEPA	++ (Meistrich et al. 1982)	n.d.	n.d.	+ (Anserini et al. 2002)
Doxorubicin	++ (Lu and Meistrich 1979)	++ (Lui et al. 1986)	n.d.	± (Da Cunha et al. 1983)
Amsacrine	− (Da Cunha et al. 1985)	−[b]	n.d.	− (Da Cunha et al. 1982)

+, ++, +++: Relative effectiveness at stem cell killing

±: Stem cell killing cannot be demonstrated with the agent given alone but has additive effect on stem cell killing with other agents,

−: No stem cell killing

n.d: Testing of that agent was not done in that species

[a] Germinal aplasia observed while receiving drug treatment but no analysis was performed after cessation of drug administration

[b] M.F. da Cunha and M.L. Meistrich, unpublished results

all resulted in non-repopulating tubules in rat testes, suggesting stem spermatogonial killing. Busulfan also appears to kill stem spermatogonia, as assessed by a prolonged reduction in fertility (Jackson et al. 1961), but it appears less able to produce extensive tubular atrophy than it did in the mouse (Ogawa et al. 1999). Although cisplatin and cyclophosphamide did not on their own produce stem cell killing in rats (Huang et al. 1990; Meistrich et al. 1995), some additional stem cell killing could be observed when cyclophosphamide was given along with irradiation. Amsacrine produced no detectable stem cell killing (Pegg et al. 1996).

Stem cell killing due to radiation and busulfan on monkey testes has been assessed by histological methods. Data have been taken from rhesus (*Macaca mulatta*), cynomolgus (*Macaca fascicularis*), and stump-tailed (*Macaca arctoides*) macaques, which seem to show similar responses but this has not yet been proved. Tubules totally devoid of germ cells have been observed 7 months after radiation doses of 2 Gy (Schlatt et al. 2002; van Alphen et al. 1988a) and even more extensive depletion was observed after 4 or 7 Gy (Boekelheide et al. 2005; Kamischke et al. 2003). Busulfan produced complete depletion of germ cells in nearly all tubules and prolonged reduction in sperm counts, but only at doses that produced some animal mortality (Hermann et al. 2007). Procarbazine likely produces stem spermatogonial killing, as long-term treatment produced complete atrophy of the germinal epithelium, but those studies did not include a post-treatment recovery period (Sieber et al. 1978). The killing of stem cells in monkey testes was also indirectly shown by the prolonged reduction in sperm counts after radiation (Fig. 9.3) and busulfan (Hermann et al. 2007).

In human, there are limited studies in which testicular biopsies of treated patients have been examined. Biopsies from azoospermic patients several years after the end of treatment with regimens containing cyclophosphamide or procarbazine often showed the complete absence of germinal cells, indicating complete stem cell killing in those tubules, although occasionally tubules with various levels of spermatogenesis are observed, indicating that some stem cells survived (Kreuser et al. 1989; Van Thiel et al. 1972).

However, most of the information on stem spermatogonial killing in human is indirect, based on reduction of sperm counts after treatment of patients with radiation and chemotherapy (Meistrich et al. 2005). With some types of chemotherapy, sperm count returns to normal levels within 3 months after the completion of the therapy (Da Cunha et al. 1982; Meistrich et al. 1997a). Since 2–3 months is sufficient for stem spermatogonia to mature to sperm that will appear in the ejaculate, we conclude that these regimens did not kill stem spermatogonia (Meistrich et al. 2005). In contrast, other chemotherapy agents and regimens induce prolonged azoospermia, for periods longer than 1 year. Based on the above histological findings, we assume that these likely killed stem spermatogonia.

Radiation is one of the agents that produce prolonged azoospermia. When given as a single dose, greater than 6 Gy to the testis is required to produce permanent azoospermia (Fig. 9.3), but with multifraction radiotherapy, only 2.5 Gy can also achieve this effect (Dubey et al. 2000; Meistrich and van Beek 1990; Sandeman 1966; Speiser et al. 1973). Many alkylating chemotherapeutic agents (procarbazine,

busulfan, cyclophosphamide, chlorambucil, and melphalan), and cisplatin, also produce prolonged azoospermia (Meistrich et al. 2005). Others, particularly topoisomerase inhibitors (e.g., amsacrine), antimetabolites (e.g., methotrexate), and microtubule inhibitors do not produce prolonged azoospermia in most combinations, but doxorubicin, microtubule inhibitors (e.g., vincristine), and select antimetabolites (e.g., cytosine arabinoside) can have additive effects on azoospermia induction when given with the highly gonadotoxic agents listed above (Meistrich et al. 1989).

9.4.3 Functional Assay of Stem Cells by Transplantation

The survival of stem cells after gonadotoxic insult has also been assessed by spermatogonial transplantation. In mice, busulfan at 15 mg/kg reduced functional stem cell numbers to a minimum of about 4% of control at 3 days after treatment (Kanatsu-Shinohara et al. 2003). This agrees with the functional assessment of the stem cells left in situ, based on subsequent sperm head counts, which were reduced to 8% of control (Bucci and Meistrich 1987).

This assay was also used to demonstrate that the spermatogonia remaining in 6-Gy-irradiated testes of rats, which did not produce differentiated cells in the irradiated rat testis, were functional when transplanted to a nude mouse testes depleted of endogenous germ cells (Zhang et al. 2006).

The killing of functional spermatogonial stem cells in busulfan-treated macaques was assessed by the reduction of their ability to form colonies after transplantation into depleted nude mouse testes (Hermann et al. 2007). However, the sensitivity of this assay may be limited since one of the donor monkeys showed endogenous recovery of spermatogenesis in about half of the tubules, but the spermatogonia did not form colonies after transplantation.

9.5 Recovery of Spermatogenesis from Surviving Stem Spermatogonia

The eventual recovery of sperm production depends on the survival of the spermatogonial stem cells, the regeneration of their numbers, and their ability to differentiate (Table 9.2).

In the mouse, after irradiation stem spermatogonia (A_s) initially divide with about a 2-day cell cycle time and a high probability of self-renewal, thereby increasing their numbers, but they also begin to produce differentiating A_{pr} and A_{al} spermatogonia within the first week after irradiation (Van Beek et al. 1986, 1990). Differentiation continues and progressive recovery of sperm production begins at 5 weeks (Meistrich and Samuels 1985). It is puzzling that the numbers of spermatogonial stem cells, assayed either by endogenous colonization ability (Meistrich et al. 1978) or counts of A_s spermatogonia (Erickson and Hall 1983), does not begin continuous progressive recovery until about 8 weeks after 6 Gy irradiation.

Table 9.2 Comparison of effects of comparable doses of radiation on the spermatogenic cells of mouse, rat, monkey, and human

	Stem spermatogonia		Differentiating spermatogonia		Later germ cell stages	
Cell stage: species (strain)	Survival	Recovery of numbers	Immediate effects	Development during recovery	Immediate effects	Development during recovery
Mouse	Some survive doses up to 16 Gy, Killed by some alkylating agents, Adriamycin, and radiation	Progressive recovery (immediate or after 8 weeks)	Very sensitive to direct killing by radiation and chemotherapy	Develop normally from surviving stem cells	Spermatocytes moderately resistant and spermatids highly resistant to radiation	Develop normally although efficiency may be reduced
Rat (several inbred strains)	Some survive doses up to 10 Gy	(No data on recovery of stem cell numbers)	Very sensitive to direct killing by radiation Sensitive to chemotherapy too	Strong block of differentiation after radiation and some chemothera-peutic drugs	Relatively resistant to irradiation	Failure of cells to progress through differentiation
Rat (Sprague–Dawley)	Some survive doses up to 10 Gy	Progressive recovery (may be delayed until 8 weeks)	Very sensitive to direct killing by radiation Sensitive to chemotherapy too	Variable, intermediate between mice and inbred rats	Resistant (no cytotoxic effects of 3 Gy)	Develop normally after 3 Gy
Monkey (macaque)	Most killed by doses of 1–4 Gy. Killing of A_{dark} is delayed	Recovery begins about 6 weeks after 0.5–2 Gy	Very sensitive to direct killing by radiation	Develop from surviving stem cells after transient block (~3 months)	Resistant (negligible cytotoxic effects of 4 Gy)	Develop normally after 4 Gy

Human	More sensitive to radiation than rodents Killing of A_{dark} is delayed Killed by some alkylating agents	Recovery begins about 5–6 months; slower than in rodents or monkeys	Very sensitive to direct killing by radiation Most sensitive stage to chemotherapy	Differentiation is transiently and partially inhibited after radiation	Relatively resistant to radiation	Efficiency may be reduced

Chemotherapy effects are included but it was not possible to compare doses

Recovery of stem cells and sperm production continues for about 25 weeks and then reaches a plateau. Complete recovery is observed after doses of 6 Gy or less, but the plateau becomes progressively lower after higher doses. Recovery kinetics of sperm production, based on equivalent levels of stem cell survival, are similar after chemotherapy as was described above for radiation (Meistrich 1982, 1986b). The recovery of the numbers of functional stem cells measured by the transplantation assay, showed a fourfold increase between 3 and 15 days after busulfan treatment (Kanatsu-Shinohara et al. 2003).

In rats, complete regeneration of the numbers of A_s spermatogonia occurs between 8 and 16 weeks after 6 Gy irradiation (Erickson and Hall 1983). However, the extent of recovery of spermatogenesis from these surviving stem cells depends on the strain (Fig. 9.3). After radiation or procarbazine exposure, recovery is more vigorous in outbred, such as Sprague–Dawley, than in inbred rats such as Wistar, Lewis, PVG, and $LBNF_1$ (Delic et al. 1987; Dym and Clermont 1970; Kangasniemi et al. 1996b; Parchuri et al. 1993; Ward et al. 1989, 1990). Morphological examination of the atrophic testes in the sensitive $LBNF_1$ rats after these gonadotoxic therapies revealed that many type A spermatogonia, including the A_s spermatogonia, were still present and actively proliferating, but their progeny underwent apoptosis when they attempted to differentiate (Meistrich et al. 1999; Shuttlesworth et al. 2000). This block continued for at least 60 weeks after irradiation (Kangasniemi et al. 1996b). Spermatogonial transplantation revealed that these stem spermatogonia were functional and that their failure to undergo differentiation was due to damage to the somatic environment of the testis (Zhang et al. 2006). We presume that radiation produces this spermatogonial block in other strains, but to a lesser degree, as Sprague–Dawley rats show a lower percentage of tubules with differentiating cells (Delic et al. 1987) than do mice (Withers et al. 1974) given the same dose of radiation, despite similar levels of stem cell survival (Erickson and Hall 1983). When Sprague–Dawley or Fischer 344 rats are treated with hexanedione (Allard and Boekelheide 1996) or testicular heating (Setchell et al. 2001), a high percentage of seminiferous tubules do lack differentiating germ cells even though they contain type A spermatogonia.

In monkeys, regeneration and recovery are dose dependent. At doses below 2 Gy, the A spermatogonia have already begun to increase their numbers and produce type B spermatogonia or later cell types at 3 months after irradiation. In contrast, at 2 Gy or more, there is less regeneration of type A spermatogonia and 80% of the colonies of these cells have not yet begun differentiation at this time (van Alphen et al. 1988b). However, by 8 months they all have produced differentiating cells. The gradual progressive recovery of sperm count, which lasts for 1 year after 4 Gy irradiation, may also indicate a very gradual regeneration of stem cell numbers and/or a gradual reinitiation of spermatogenesis from those surviving stem cells (Kamischke et al. 2003). Although a few stem cells survived a dose of 7 Gy, no progressive recovery was observed (Boekelheide et al. 2005).

In human, the regeneration of the numbers of type A spermatogonia begins at about 160 days (5–6 months) after irradiation, for single doses up to 6 Gy (Clifton and Bremner 1983; Paulsen 1973; Rowley et al. 1974). They may take about

2 years to reach pre-irradiation levels after a single dose of 1 Gy (Meistrich and van Beek 1990) and longer after higher doses of irradiation (Paulsen 1973).

The recovery of sperm counts reflects the survival and regeneration of the stem spermatogonia to some extent after low doses of irradiation (≤1 Gy). The nadir of sperm count occurs about 6 months after irradiation and there is progressive recovery of sperm counts after that. But at this time, the ratio of spermatocytes to spermatogonia is tenfold lower than control levels (Meistrich and van Beek 1990), indicating that spermatogonial differentiation is not blocked but there is a reduced efficiency of production of differentiated cells.

There seems to be a dissociation between the presence of stem spermatogonia and recovery of sperm count after higher radiation doses. There are also occasional examples where histological analysis of testes following treatment with chemotherapy reveals tubule cross-sections containing only spermatogonia (Kreuser et al. 1989). In addition, testicular sperm are present in nearly half of the azoospermic post-chemotherapy patients undergoing testicular sperm extraction for fertility treatments (Chan et al. 2001). Spontaneous recovery of sperm production can occur at about 1 or 2 years after 2 Gy or 4–6 Gy of radiation given as single fractions (Fig. 9.3) (Clifton and Bremner 1983; Rowley et al. 1974). Individuals can also completely recover sperm production after being azoospermic for 2–5 years after toxicant exposure (Marmor et al. 1992; Meistrich et al. 1992; Potashnik and Porath 1995; Pryzant et al. 1993). It has been suggested that the numbers of stem spermatogonia must first reach a critical number for differentiation to take place; however, this cannot be the complete explanation (Meistrich and van Beek 1990; Paulsen 1973; Rowley et al. 1974). The observation in rats that radiation damages the somatic environment of the testis, which blocks the differentiation of spermatogonia (Zhang et al. 2006), may also apply to this situation in humans. The somatic damage may cause the failure of differentiation of spermatozoa from spermatogonia but may spontaneously resolve itself in the human testis after several years.

9.6 Modulation of the Regenerative Process

The use of hormone suppression treatments to reduce gonadotropins (FSH and LH) and intratesticular testosterone levels were originally based on the hypothesis that these treatments would protect the survival of stem spermatogonia from killing by cytotoxic treatments and thereby enhance the subsequent recovery of spermatogenesis (Glode et al. 1981). However, studies in rats disproved this theory; on the contrary, hormone suppression does not alter spermatogonial kinetics (Meistrich et al. 1997b) or stem spermatogonial survival, but protects or enhances the subsequent ability of the somatic cells of the testis to support the recovery of spermatogenesis from surviving stem spermatogonia (Meistrich et al. 2000; Zhang et al. 2006).

Although protection of spermatogenesis in mice from cyclophosphamide by treatment with a GnRH (gonadotropin releasing hormone) agonist before and during chemotherapy was claimed (Glode et al. 1981), later studies failed to reproduce those

results (Da Cunha et al. 1987) or show that other gonadotropin suppression protocols protected spermatogenesis in mice (Crawford et al. 1998; Kangasniemi et al. 1996a; Nonomura et al. 1991) (Table 9.3). However, recently, we have been able to demonstrate that treatment with a GnRH antagonist and an antiandrogen starting immediately after irradiation significantly enhanced the ability of surviving stem spermatogonia to produce differentiated germ cells (Wang et al. 2010). Hormonal suppressive treatments for up to 11 weeks resulted in about a twofold increase in the percentage of seminiferous tubules containing differentiated germ cells. Hormonal suppressive treatment for 10 weeks after irradiation also resulted in a twofold increase in epididymal sperm numbers about 20–30 weeks later and an increase in the percentage of fertile males from 7 to 80%.

In contrast to the scant positive results and modest effect in the mouse, numerous reports demonstrated that hormone suppression prior to and during radiation, procarbazine, doxorubicin, indenopyridine, or heat treatments, markedly enhanced the subsequent recovery of spermatogenesis and fertility (Delic et al. 1986a; Hild et al. 2001; Jegou et al. 1991; Kangasniemi et al. 1995; Manabe et al. 1997; Morris and Shalet 1990; Parchuri et al. 1993; Setchell et al. 2002; Weissenberg et al. 1995). Note that in none of these studies was the protection of the survival of stem spermatogonia directly assessed. We proposed that the mechanism by which hormone suppression enhances the subsequent recovery of spermatogenesis is by prevention of the pronounced block in differentiation of surviving stem spermatogonia in rat testes after exposure to cytotoxic agents (Meistrich et al. 2000).

Accordingly, when the hormone suppression was administered to the rats only after irradiation, the differentiation of stem spermatogonia, which would have been otherwise blocked, was restored (Meistrich and Kangasniemi 1997; Shuttlesworth et al. 2000). Similar stimulation of recovery following procarbazine (Meistrich et al. 1999), busulfan (Udagawa et al. 2001), heat (Setchell et al. 2001) or hexanedione (Blanchard et al. 1998) treatment has been observed. This hormone suppression-stimulated spermatogonial differentiation led, after subsequent restoration of hormone levels, to increased sperm counts and fertility (Meistrich et al. 2001).

Studies using hormone suppression in monkeys have not convincingly demonstrated enhanced recovery of spermatogenesis following gonadotoxic injury. Although one preliminary report based on a total of only three baboons suggested that hormone suppression might decrease the gonadal damage from cyclophosphamide (Lewis et al. 1985), two larger studies using an adequate number of macaques showed neither protection (Kamischke et al. 2003) nor stimulation (Boekelheide et al. 2005) of recovery of spermatogenesis from radiation damage by hormone treatment.

Seven clinical trials have been performed in attempts to demonstrate improvement in the recovery of spermatogenesis in human males by hormone suppression treatment before and during cytotoxic therapy (Brennemann et al. 1994; Fossa et al. 1988; Johnson et al. 1985; Kreuser et al. 1990; Masala et al. 1997; Redman and Bajorunas 1987; Waxman et al. 1987). Only one of these studies showed that hormone-suppression preserved subsequent sperm production of men, who received cyclophosphamide in this case (Masala et al. 1997), but this study has not been

Table 9.3 Summary of effects of hormone suppression on protection and/or stimulation of recovery of spermatogenesis after cytotoxic treatment in different species

Species	Treatment	Effect of timing of hormone suppression relative to cytotoxic treatment			References
		Before	Immediately after	After a delay	
Mouse	Procarbazine, doxorubicin, cyclophosphamide	–		n.d.	(Crawford et al. 1998; Da Cunha et al. 1987)
	Cisplatin	–	n.d.	n.d.	(Nonomura et al. 1991)
	Radiation	–	+	n.d.	(Crawford et al. 1998; Kangasniemi et al. 1996a; Wang et al. 2010)
Rat	Radiation[a]	++	++	+	(Kurdoglu et al. 1994; Meistrich and Kangasniemi 1997)
	Procarbazine[a]	++	++	n.d.	(Delic et al. 1986a; Jegou et al. 1991; Meistrich et al. 1999; Morris and Shalet 1990; Parchuri et al. 1993)
	Busulfan	–	+	n.d.	(Udagawa et al. 2001, 2006)
Monkey	Radiation	–	–	n.d.	(Boekelheide et al. 2005; Kamischke et al. 2003)
Human	Chemotherapeutic drug combinations (± radiation)	–/?		–	(Fossa et al. 1988; Johnson et al. 1985; Kreuser et al. 1990; Redman and Bajorunas 1987; Thomson et al. 2002; Waxman et al. 1987)
	Radiation	–		n.d.	(Brennemann et al. 1994)
	Cyclophosphamide	++		n.d.	(Masala et al. 1997)

+, ++: Relative effectiveness at protection or stimulation

–: No protection/stimulation

?: No protection observed but could not be demonstrated or ruled out with experimental design, n.d.: The effect not tested

[a] Instances in which the type A spermatogonia showed a block in differentiation

subsequently replicated. The one attempt to restore spermatogenesis by hormone suppression after cytotoxic therapy was also unsuccessful (Thomson et al. 2002). However, it should be noted that there were shortcomings in the clinical studies that may have masked subtle effects. The use of testosterone or medroxyprogesterone either alone (Fossa et al. 1988; Redman and Bajorunas 1987; Thomson et al. 2002) or combined with a GnRH analogue (Waxman et al. 1987) is suboptimal given that, in animal studies, both of these steroids reduce the stimulatory effects of GnRH analogs on the recovery of spermatogenesis after cytotoxic damage (Shetty et al. 2002, 2004). Some treatment regimens were not sufficiently gonadotoxic to cause prolonged sterility (Brennemann et al. 1994; Kreuser et al. 1990); conversely some regimens may have delivered doses well above those that would ablate all spermatogonial stem cells, since no evidence of spermatogenesis was observed in almost all patients even after many years (Johnson et al. 1985; Thomson et al. 2002; Waxman et al. 1987).

9.7 Analysis of Interspecies Differences

The above data indicated the many similarities in the effects of gonadotoxic effects in the four different species considered. In all cases, the somatic cells are highly resistant to killing by the cytotoxic agents; the later stage germ cells are also relatively resistant to killing; the differentiating spermatogonia are most sensitive; and the stem cells have intermediate sensitivity. The time courses of depletion of sperm production are proportional because the surviving post-spermatogonial cells differentiate with kinetics determined by the cycle of the seminiferous epithelium, and then sperm count is reduced at the time when the sensitive differentiated spermatogonia would have produced sperm. Also the stem cells are highly sensitive to radiation and the alkylating agents procarbazine, busulfan, and chlorambucil, and relatively resistant to topoisomerase inhibitors, antimetabolites, and microtubule inhibitors. In all species, there is some evidence for progressive loss of putative stem type A spermatogonia for several months after the toxic insult, and this affects sperm production in primates but not in mice.

The differences between species occur with respect to the survival of the stem cells, regeneration of their numbers, and recovery of spermatogenesis from the surviving stem cells.

Killing of putative stem spermatogonia by radiation, as assessed by histological counts of cells, was similar in mice and rats, but the stem cells in monkeys and human were more sensitive than those in rodents as indicated by reductions in spermatogonial counts occurring at lower doses of radiation (Table 9.1). The reasons for these differences in sensitivity are not known. Whereas the spermatogonial stem cells of the rodents are almost exclusively in the A_s and to a limited extent in the A_{pr} populations, less is known about the distribution of stem cells among the spermatogonial subtypes in primates. The stem cells in primates appear to be limited to the A_{dark} and A_{pale} spermatogonia, which have a molecular phenotype corresponding

the A_s and A_{pr} of rodents (Hermann et al. 2009). However, there are important kinetic differences. The A_{dark} spermatogonia are largely nonproliferative (Clermont and Antar 1973; Ehmcke et al. 2005b), whereas in rodents all of the A_s spermatogonia are proliferative (Huckins 1971b). Furthermore, whereas in rodents the majority of the stem cells are single A spermatogonia, in primates the self-renewing A_{pale} spermatogonia usually have a minimum clonal size of two or four cells (Ehmcke et al. 2005a).

Although killing of stem spermatogonia may also be determined indirectly by the prolonged reduction of recovery of sperm production, this assay can be differentially affected by the efficiency of recovery of spermatogenesis from surviving stem cells in different species. Whereas in mice, there is an excellent correlation between stem cell survival and sperm counts, in some cases in rats, there may be a complete block in the differentiation of surviving stem cells, resulting in no sperm being produced despite surviving stem cells. In the limited studies done with monkeys, sperm count recovery appears to reflect the stem cell killing. Although in human, prolonged azoospermia is observed despite the presence of surviving stem cells, the duration of azoospermia may be an indicator of the degree of stem cell killing. Nevertheless these assays do appear to show interspecies differences in the sensitivity of stem spermatogonia to some chemotherapeutic drugs. In particular, rodent stem cells are more resistant than those in human to killing by cyclophosphamide and cisplatin, but are more sensitive to killing by doxorubicin. The reasons for these differences are not known.

There appear to be some interspecies differences in regeneration of stem cell number. In mice, there are data indicating immediate progressive recovery of stem cell number after radiation and busulfan and other data indicating that, in the case of radiation, there may then be some decline until 2 months later, followed by progressive recovery. In irradiated monkeys and humans there are clear declines in the numbers of type A spermatogonia lasting about 3 and 6 months, respectively, after which recovery begins.

The recovery of spermatogenesis from surviving stem cells shows major inter- and intra-species differences. The initiation of differentiation appears to occur quite rapidly (within 1 week) from surviving stem cells in the mouse. In rats, rapid initiation of differentiation occurs in some cases, but in other situations depending on the strain, dose, and toxicant there is a prolonged (at least 60-week) block in the ability of the somatic environment to support spermatogonial differentiation. In monkeys there is an initial period of about 2–3 months during which type A spermatogonia show little differentiation, but subsequently spermatogonia differentiate without a block. In human, the prolonged absence of sperm in the ejaculate followed by excellent recovery of sperm counts indicates that there is a block at some point in the differentiation or sperm transport process.

The dramatic stimulation of recovery of spermatogenesis by hormone suppression in toxicant-treated rats is in contrast to the less marked effects observed in mice, the absence of stimulation in macaques, and generally negative but variable results in human. One contribution to this difference may be the interspecies differences in the block in differentiation of spermatogonia. This block is most dramatically

observed in rats. There is some evidence that there is a block in spermatogenic cell differentiation in human males treated with gonadotoxic agents (Clifton and Bremner 1983; Kreuser et al. 1989; Pryzant et al. 1993). Although this can sometimes be reversed spontaneously, gradually over time, or perhaps by some unknown perturbation, there is no consistent evidence that it can be reversed by hormone suppression.

A second contribution to the interspecies difference in stimulation by hormone suppression may be a result of differences in the effects of intratesticular testosterone and FSH on the normal progression of spermatogenesis. In normal rodents, suppression of these hormones has very little effect on the development of spermatogonia to the spermatocyte stage. The first step in the stimulation of spermatogenesis by hormone suppression in toxicant-treated rats is progression of spermatogonial differentiation and spermatocyte formation; this may be necessary to effectively "jump-start" the process. In contrast, in both normal monkeys and humans, suppression of testosterone and FSH markedly inhibits the differentiation of type A to type B spermatogonia (McLachlan et al. 2002), but this effect appears to be primarily a result of the FSH suppression (Simorangkir et al. 2009). This inability of spermatogonial differentiation to occur in hormone-suppressed primates may therefore explain the failure of hormone suppression to stimulate spermatogenic recovery in those species.

9.8 Conclusion

The observations of interspecies differences in sensitivity and recovery of spermatogenesis can lead to focusing future research to answer specific questions that can improve our understanding of, and ability to enhance, spermatogenic recovery in humans.

The failure of recovery in primates is in part due to the greater sensitivity of human (and monkey) stem spermatogonia, compared to rodent stem spermatogonia, to radiation and certain chemotherapeutic agents. Future research should focus on elucidating the reasons for greater sensitivity of primate stem spermatogonia to radiation and certain chemotherapeutic agents in order to develop methods to protect them.

The failure of recovery in primates also appears to be due to the slow repopulation of the stem cell compartments and their inability to differentiate to produce spermatozoa. This may be in part due to somatic damage to the stem cell niche and the support of spermatogenic cell differentiation. Such damage to the somatic support of differentiation is also most apparent in certain strains of rat. The results of future research on the mechanisms involved in the spermatogonial block in rats may therefore be applicable to stimulating recovery of spermatogenesis in humans exposed to gonadotoxic agents.

Finally, the mechanism by which hormone suppression protects or reverses the damage to the somatic elements of the testis to stimulate spermatogenic recovery

in rodents is not yet known. Knowledge of the mechanism should allow the identification of treatment targets downstream from the initial action of the hormones. It may be possible to modulate those targets to release the blockade in spermatogenic differentiation, while maintaining hormone levels, which should allow spermatogonial differentiation in primates. This approach may be useful to enhance the recovery of spermatogenesis and sperm count in toxicant-exposed human males.

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Chapter 10
Testicular Xenografting

Stefan Schlatt, Jose R. Rodriguez-Sosa, and Ina Dobrinski

Abstract Mammalian spermatogenesis is sustained by spermatogonial stem cells. Although our knowledge of spermatogenesis and the biology of spermatogonial stem cells has increased in the last decades, many aspects of spermatogonial function still remain unclear, especially in the primate testis. The lack of suitable systems to study testis development and spermatogenesis *ex vivo* has limited our ability to elucidate these processes. Ectopic grafting of testis tissue from immature animals into immunodeficient mice provides a system to recapitulate spermatogenesis and to harvest fertilization-competent sperm from many mammalian species, including primates. More recently, it was shown that isolated testis cells from immature males are able to organize and rearrange into seminiferous cords that subsequently undergo complete development, including production of viable sperm. While few studies have been performed with xenografting human testis, xenografting of non-human primate testis tissue has provided a novel approach to study prepubertal maturation of the testis, optimize cryopreservation of testis tissue, evaluate gonadotoxic effects, and produce fertilization competent primate sperm. These novel strategies to generate sperm from spermatogonial stem cells or immature testicular tissue could provide an option for fertility preservation by cryopreservation of testicular tissue fragments from young patients whose future fertility is at risk due to oncological treatment or other gonadotoxic exposures. Here we review comparative aspects of testis tissue xenografting and clinical as well as experimental applications to explore spermatogenesis and novel strategies for preservation of male fertility in primates.

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10.1 Introduction

Spermatogonial stem cells sustain the male germ lineage. They represent a pool of diploid reserve germ cells for reconstitution of spermatogenesis [for review see Ehmcke et al. (2006)] and contain the genetic material that is transmitted to the next generation. In most systems studied so far, stem cells reside in a specialized environment, called the stem cell niche, which governs their proliferation and differentiation. The existence of a testicular stem cell niche is well documented but its cellular and molecular components are poorly understood and might vary between species. However, settlement and colonization of testicular stem cells from a large variety of species in the mouse testis reveals that stem cell recognition and colonization are highly conserved among different species (Dobrinski et al. 2000). The importance of the niche and its plasticity to accept various germline cells has been demonstrated. In addition to spermatogonia, primordial germ cells or teratocarcinoma cells have the potential to enter the niche and initiate spermatogenesis (Nayernia et al. 2004; Chuma et al. 2005).

In primate testes, three types of spermatogonia are distinguished by morphological criteria: A_{dark}, A_{pale}, and B spermatogonia (Clermont and Leblond 1959; Clermont 1966, 1969). The number of subsequent divisions of B-spermatogonia differ between primate species. For example, one division is described in men and four divisions (B1–B4) in macaques. Various models for spermatogonial kinetics have been described and are currently under debate, signifying that the exact details of spermatogonial turnover in the primate testis are still largely unresolved (Ehmcke and Schlatt 2006; Amann 2008). However, it is generally agreed that A_{dark} spermatogonia are mitotically quiescent and act as reserve stem cells since they become proliferatively active during pubertal expansion (Simorangkir et al. 2005) and following depletion of spermatogonia due to irradiation or toxic exposure (van Alphen et al. 1988, 1989). On the other hand, A_{pale} spermatogonia proliferate regularly and are considered self renewing progenitors (Ehmcke et al. 2005a, b).

Rapidly dividing premeiotic germ cells are highly sensitive to irradiation and toxins in adult and immature monkeys (van Alphen et al. 1989; Jahnukainen et al. 2006a). Low doses of cytotoxic drugs or irradiation deplete the differentiating spermatogonia while less sensitive spermatogonial stem cells as well as spermatocytes and spermatids survive. Recovery of spermatogenesis occurs from the remaining stem cells and relies on the type, dose, and fractionation of cytotoxic drugs and irradiation (van Alphen et al. 1988). During recovery, testicular histology reveals an all-or-nothing pattern with areas of full spermatogenesis and areas with a Sertoli-cell-only pattern. This histological pattern during spermatogenic recovery indicates a critical role of spermatogonial stem cells for re-initiation of spermatogenesis and shows that under doses used in these studies in primates the somatic environment is not heavily affected by chemotherapy or radiation exposure (Schlatt et al. 2009).

Several strategies for protection of spermatogonial cells could be developed for application in a clinical setting [for review see Jahnukainen et al. (2006b) and Schlatt et al. (2009)]. While cryopreservation of sperm offers a standardized and routine option for fertility preservation in adult patients, prepubertal children cannot donate sperm for cryostorage. Moreover, cryopreservation of sperm represents a finite source of gametes. Several alternatives have been discussed based on the high regenerative potential of the seminiferous epithelium that is supported by spermatogonial stem cells. Although the somatic environment can also be affected by irradiation and toxic exposures, this appears to be more resistant than germ cells (Bar-Shira Maymon et al. 2004; Zhang et al. 2008). Autologous transplantation of spermatogonial stem cells, presented in Chap. 11 of this book, represents an option for fertility preservation in young patients. Other alternatives are the xenografting of testicular tissue and cells into immunodeficient mice. Especially prior to puberty, when the testis consists of cords and the only germ cells are spermatogonia, the developing testis appears to be tolerant to hypoxia and temporary insufficient blood supply rendering it liable to manipulation of cells and tissue that maintain their full developmental potential. This creates a very promising scenario for ectopic or orthotopic testicular (auto or xeno)-grafting of immature testis tissue fragments and cells. Besides preservation of fertility, xenotransplantation of testicular tissue and cells open new avenues for experimental investigation in a way that was not possible before or logistically very difficult; a small number of donors is needed to perform valid comparative studies. Instead of exposing groups of males to different gonadotoxic strategies, groups of mice carrying xenografts from few juvenile donors are exposed to such treatments. Grafts can be collected then at different points for subsequent analysis. This chapter focuses on testicular grafting and summarizes primarily the advances achieved in non-human primates and humans with respect to the application of testicular grafting for research and fertility protection.

10.2 Testicular Tissue Grafting: A Comparative Overview

Grafting of any tissue can be considered a specialized form of organ "culture." The host serves as a living bioincubator. Not only is the environment well controlled but also the blood supply to the grafted tissue is restored. At first glance, the testis does not appear to be a suitable tissue for grafting because of the complexity of the seminiferous epithelium and the architecture of its vascular and duct systems. However, several reports on testicular transplantation of the organ as a whole or of testicular tissue were published in the 1920s and have been reviewed by Gosden and Aubard (1996a, b). These studies were performed in a variety of species but most of these experiments were performed in sheep, rats, and guinea pigs. They have provided important insights into testicular function, especially on Leydig cells and effects of temperature on spermatogenesis. An important breakthrough for grafting came through the availability of immunodeficient recipient mice. They allow xenotransplantation of testicular and other tissues (Paris et al. 2004) without

strong immunorejection (Dobrinski et al. 2000). Xenotransplantation of testicular tissue (human fetal testis into the abdominal wall of adult nude mice) was first performed in 1974 (Skakkebaek et al. 1974) and revealed survival of the tissue but no progression of germ cell development beyond the gonocyte stage. Subsequently Hochereau-de-Reviers and Perreau (1997) transplanted ovine fetal testis into the scrotum of intact nude mice and reported differentiation of gonocytes into spermatogonia and primary spermatocytes (Hochereau-de-Reviers and Perreau 1997). However, complete cross-species spermatogenesis was first reported in 2002 (Honaramooz et al. 2002). In that report, fragments of testis tissue from newborn pigs and goats were able to survive and displayed complete development with production of sperm. Testicular xenografting has since been tested in numerous species (Table 10.1), and testes from almost all of them appear to be responsive to mouse gonadotropins as demonstrated by initiation of spermatogenesis after xenografting. A notable exception is the marmoset, which appears insensitive to mouse LH due to a deletion in exon 10 of its luteinizing hormone-receptor gene (Michel et al. 2007). This blocks androgen production and results in poor spermatogenesis after grafting (Schlatt et al. 2002; Wistuba et al. 2004). The limited development of marmoset xenografts indicates a response to the stimulation by mouse FSH in the absence of androgen-dependent differentiation processes. Co-grafting experiments combining marmoset and hamster tissue, however, revealed no beneficial impact of well developing hamster xenografts on marmoset testis tissue (Wistuba et al. 2004). Nonetheless, such studies promote interesting experimental approaches to explore hormonal regulation of testicular development.

In most studies, the testicular grafts consist of small fragments of ~0.5–1 mm^3 weighing 3–10 mg (Honaramooz et al. 2002; Schlatt et al. 2002; Schmidt et al. 2006a, b), but the use of bigger fragments (9×5×1 mm) is also feasible (Rodriguez-Sosa et al. 2010). Two to eight fragments of tissue are commonly transplanted into multiple sites under the dorsal skin on either side of the spinal column. As immunodeficient recipients, Nude (T-cell deficient) mice (Honaramooz et al. 2002; Schlatt et al. 2002; Oatley et al. 2004, 2005; Rathi et al. 2005, 2006; Zeng et al. 2006), SCID (T- and B-cell-deficient) mice (Honaramooz et al. 2004; Snedaker et al. 2004; Rathi et al. 2005, 2006; Schlatt et al. 2006) and RAG-1 (T- and B-cell-deficient) mice (Rodriguez-Sosa et al. 2010) have been used. No difference has been found between xenografts transplanted into Nude and SCID mice (Rathi et al. 2005, 2006; Geens et al. 2006).

The recipients are usually adult males that are castrated prior to or during the transplantation surgery. Turner (1938) found that survival of homologous testicular grafts in rats was better (less degeneration, more sperm) when the recipient was castrated (Turner 1938). Rathi et al. (2006) observed that horse xenografts under the dorsal skin of mice did not develop in intact males (Rathi et al. 2006). In contrast, Shinohara et al. (2002) obtained functional sperm from rabbit testis orthotopic xenografts in intact mice (Shinohara et al. 2002). These data indicate that xenografting works in principle irrespective of the sex and gonadal status of the recipient. In addition to improved graft survival, castration of recipients at the time of grafting has several more advantages. It avoids interference of the host testis and the grafted donor testis tissue towards the hormonal stimulation of the recipient and

Table 10.1 Xenografting of testis tissue and spermatogenesis in non-rodent donor species except primates

Species	Donor age	Collection time[a]	%Tubules with complete spermatogenesis[b]	Onset of spermatogenesis[c]	References
Pig	1–2 weeks	7–8 months	52 (7–98)	Advanced	Honaramooz et al. (2002) and Zeng et al. (2006, 2007)
Cattle	1–8 weeks	24–36 weeks	<15	Similar or slightly advanced	Oatley et al. (2004, 2005), Rathi et al. (2005), and Schmidt et al. (2006a, b)
Goat	4 weeks	Not mentioned	Not mentioned	Not determined	Honaramooz et al. (2002)
Sheep	1–2 weeks	12–28 weeks	64 (2–92)	Similar or advanced	Zeng et al. (2006), Arregui et al. (2008a), and Rodriguez-Sosa et al. (2010)
Horse	2 weeks to 4 years	8 months	~5[d]	Not determined	Rathi et al. (2006)
Cat	1 week to 15 months	35–50 weeks	6–25[e]	Delayed	Snedaker et al. (2004) and Kim et al. (2007)

[a] Collection time at which full spermatogenesis was observed
[b] Presence of elongated spermatids and/or sperm
[c] In comparison with testis in situ
[d] Only in a 10-month-old donor
[e] In donors younger than 7 months of age

allows estimation of androgen production by the grafted tissue. Androgenic activity of the grafted testicular tissue can be monitored by serum androgen levels or the size of the host seminal vesicles that are highly sensitive to androgens (Gosden and Aubard 1996a). In addition, removal of the host gonads releases the negative feedback of the recipient's gonad on the pituitary resulting in increased levels of FSH after grafting (Schlatt et al. 2003). Increased gonadotropin stimulation may support Sertoli cell proliferation and graft development until a feedback axis is reestablished between the grafted tissue and the host hypothalamus and pituitary. Xenografting of hamster tissue into young and old nude mice was performed to explore a potential impact of the recipient's age. This study revealed a surprisingly better outcome of graft survival and function in older mice (Ehmcke et al. 2008). Less efficient immunological interference or a change in the endocrine milieu in aged recipients might be reasons for this unexpected finding.

Other factors that affect testis tissue survival and function after transplantation are the donor species, and the developmental or functional stage of the donor testis. The time to achieve full maturation of immature testicular tissue and the number of tubules displaying full spermatogenesis after grafting depends on the donor species. While in grafts from rodent tissue (mouse, hamster, rat) it takes only several weeks until sperm are generated, the period until active spermatogenesis occurs and first elongated spermatids can be observed is several months in grafts of larger species. However, time to the first appearance of spermatids is generally advanced when compared with normal tissue in situ. Such an acceleration of testicular development is especially notable in species with long periods until onset of puberty and is attributed to the immediate response of the xenografts to the host gonadotropins. Two examples of this are xenografts of immature pigs and monkeys (Honaramooz et al. 2002, 2004), while two notable exceptions are grafts from cattle and cats. In cattle, onset of spermatogenesis is slightly advanced or similar to testes in situ (Oatley et al. 2004, 2005; Rathi et al. 2005). In cats, onset of spermatogenesis in xenografts is delayed (Snedaker et al. 2004; Kim et al. 2007). It therefore appears that xenografting can accelerate testicular maturation by premature initiation of pubertal development in 1- to 2-year-old macaques but not by acceleration of pubertal maturation (Table 10.2). Interestingly, even bovine and feline xenografts that show a delay in full development initiate pubertal differentiation prematurely until the onset of meiosis when compared to age-matched in situ controls. However, bovine germ cells in xenografts frequently arrest at meiosis with only a small percentage of tubules producing elongated spermatids (Rathi et al. 2005). In cats, delay of testicular maturation appears to be controlled by intrinsic factors of the grafted tissue and may indicate a delayed development of specific components (Snedaker et al. 2004; Kim et al. 2007).

The efficiency of spermatogenesis in xenografts is also species dependent. While the number of spermatozoa produced by pig and goat testicular xenografts was similar to that produced in normal testes on a "per gram of tissue" basis (Honaramooz et al. 2002), complete spermatogenesis does not occur in all seminiferous tubules in xenografts of cattle (Oatley et al. 2004, 2005; Rathi et al. 2005; Schmidt et al. 2006a, b), horses (Rathi et al. 2006), cats (Snedaker et al. 2004; Kim et al. 2007) sheep (Zeng et al. 2006; Arregui et al. 2008a; Rodriguez-Sosa et al. 2010), and

Table 10.2 Outcome of testicular grafting using primate testicular tissue

Primate species and type of grafting	Age of donors and status of spermatogenesis	Cryopreservation before grafting	Treatment of recipient	Status of grafted testicular tissue and spermatogenic activity in grafted tissue	References
Rhesus monkey (xenologous)	Infantile/juvenile (3/6 months)	No	Hormone injections	– Testicular grafts increased in size and showed normal tubular organization 6 months post-grafting – Differentiation of seminiferous tubules and initiation of spermatogenic activity relied on hormone treatment – PMSG and hCG treatment had similar effects – Grafts from untreated mice contained spermatogonia as most advanced germ cells – Following hormone stimulation, tissue from 6-month-old donors showed mature sperm as most advanced germ cells; 3-months-old donor tissue developed up to spermatocytes	Rathi et al. (2008)
Human (xenologous)	Juvenile (prepubertal, 10–11 years)	No	None	– Normal somatic tubular morphology – No active spermatogenesis – Few A-spermatogonia – No difference between 4 and 9 months post-grafting	Goossens et al. (2008)
Human (xenologous)	Immature/pubertal	Yes	None	– 6 months post-grafting, 55% of seminiferous tubules showed normal morphology – Spermatogonial numbers were reduced to 4% of fresh donor tissue – High rate of spermatogonial proliferation (35%) in grafts – Spermatocytes and spermatids were detected as most advanced germ cells in grafted tissue – Spermatids showed disturbed marker expression	Wyns et al. (2008)

(continued)

Table 10.2 (continued)

Primate species and type of grafting	Age of donors and status of spermatogenesis	Cryopreservation before grafting	Treatment of recipient	Status of grafted testicular tissue and spermatogenic activity in grafted tissue	References
Rhesus monkey (xenologous)	Pubertal/adult (3/6/11/12 years)	No	None	– Mostly atrophic tissue with some SCO tubules in grafts from donors older than 6 years – Few tubules (0.3%) with spermatocytes in grafts from 6-year-old donor – Some tubules with spermatids (0.1/1%) and spermatocytes (20/60%) at 12 and 24 weeks post-grafting in grafts from 3-year-old donor	Arregui et al. (2008b)
Marmoset (autologous)	Immature (4 weeks) and adult (21 months)	Yes	No	– Good recovery and survival of grafts from immature testes, poor survival and atrophy of adult testis grafts – Most advanced germ cells were spermatocytes in ectopic grafting sites and mature spermatids in scrotal grafting sites – Cryopreservation induced complete atrophy of testis grafts	Luetjens et al. (2008)
Rhesus monkey (xenologous)	Juvenile (16/19 months)	No	None, but exposure of testicular tissue fragments to irradiation	– 67–78% of testicular grafts were recovered after 4 months showing good structural preservation – B-spermatogonia were encountered as most advanced germ cell types – Spermatogonial numbers in grafts were reduced after exposure to 0.5 Gy of irradiation – Spermatogonia were almost depleted after exposure to 1 and 4 Gy of irradiation – Graft growth was diminished after exposure to 4 Gy of irradiation	Jahnukainen et al. (2007b)

Human (xenologous)	Immature (2–12 years) crypt-orchid	Yes	None	– Three weeks after thawing and grafting, testicular tissue showed only minor degenerative changes – Spermatogonial numbers are reduced to 15% compared to fresh tissue after cryopreservation and grafting – Spermatogonia and Sertoli cells were more frequently proliferating in grafts compared to fresh tissue	Wyns et al. (2007)
Rhesus monkey (xenologous)	Juvenile (18–21 months)	Yes	None	– 50–70% of testicular grafts were recovered from fresh tissue showing good structural preservation 3 and 5 months post-grafting – Depending on the type of cryoprotectant and details of the protocol, less grafts were recovered following cryopreservation, thawing, and grafting – Structural preservation of thawed grafts was good – Most advanced germ cells were B-spermatogonia after 3 months and spermatocytes after 5 months – Spermatogonial numbers were better maintained in tissue cooled for 24 h when compared to cryopreservation with DMSO	Jahnukainen et al. (2007a)

(continued)

Table 10.2 (continued)

Primate species and type of grafting	Age of donors and status of spermatogenesis	Cryopreservation before grafting	Treatment of recipient	Status of grafted testicular tissue and spermatogenic activity in grafted tissue	References
Human (xenologous)	Fetal (20/26 weeks of gestation)	No	None	– Integrity of the testicular tissue in grafts was normal and changed from fetal to pre-pubertal developmental status 116 and 135 days post-grafting – Germ cells were mostly located at the basement membrane – Seminiferous tubules increased in diameter and presented a small lumen	Yu et al. (2006)
Rhesus monkey (xenologous)	Juvenile (16/19 months)	No	Busulfan injections	– Integrity of the testicular tissue in grafts was normal 28 and 32 weeks post-grafting – Most advanced germ cell types were B-spermatogonia, spermatocytes and at 32 weeks also spermatids – Busulfan treatment of mice reduced germ cell numbers in xenografts	Jahnukainen et al. (2006a)
Marmoset (autologous)	Infantile (4 weeks)	No	None	– 5 of 16 grafts were recovered 17 months post-grafting – 35% of seminiferous tubules were SCO – 30/25% contained spermatogonia/ spermatocytes	Wistuba et al. (2006)
Human (xenologous)	Adult (intact spermatogenesis)	No	None	– Complete atrophy and sclerosis in 60% of the grafts- Complete SCO in 10–20% of grafts – Some A-spermatogonia in 20% of grafts – No difference between mouse recipients (SCID-NOD versus Swiss Nude) – More atrophy with time (40% at 30–119 days versus 70% at 120–195 days post-grafting)	Geens et al. (2006)

Human (xenologous)	Adult (intact and disturbed spermatogenesis)	No	None	– Donors with obstructive azoospermia, hypospermatogenesis, or SCO: complete atrophy and hyalinization, no germ cells- Donor with testicular cancer: mostly SCO tubules, some A-spermatogonia – Transsexual donors post estradiol treatment: some A- and few B-spermatogonia	Schlatt et al. (2006)
Rhesus monkey (autologous)	Juvenile (prepubertal, 13 months)	Yes	Hormone injections	Spermatocytes after several weeks of hCG stimulation	Orwig and Schlatt (2005)
Rhesus monkey (xenologous)	Juvenile (prepubertal, 13 months)	No	No	– After 2 months: sp ermatogonia only- After 4 months: spermatocytes in 30% and spermatids in 3% of seminiferous tubules – After 7–12 months: complete spermatogenesis and sperm in 4% of seminiferous tubules – ICSI with sperm from xenografts and *in vitro* matured eggs led to blastocyst development	Honaramooz et al. (2004)
Marmoset (xenologous)	Neonate, infantile and juvenile (0, 1, 3, 7 months)	No	Co-grafting of hamster tissue	– 30% complete atrophy of tissue – 60% SCO tubules in surviving grafts – A-spermatogonia in 40% of surviving grafts – No spermatocytes or spermatids – No benefit through co-grafting of hamster testes	Wistuba et al. (2004)

(continued)

Table 10.2 (continued)

Primate species and type of grafting	Age of donors and status of spermatogenesis	Cryopreservation before grafting	Treatment of recipient	Status of grafted testicular tissue and spermatogenic activity in grafted tissue	References
Marmoset (xenologous)	Neonatal	No	Hormone injections	– Spermatogonia at 100 days post-grafting without hormonal stimulation – Spermatocytes as most advanced germ cells at day 135 post-grafting after hormonal stimulation (Pergonal, 1.5 IU/week for 5 weeks)	Schlatt et al. (2002)
Human (xenologous)	Fetal (14–20 weeks of gestation)	No	None	– After 4–8 weeks survival of the grafted tissue consisting of primitive Sertoli cells and gonocyte – No germ cell differentiation beyond the gonocyte stage – Gonocytes were less frequent in seminiferous cords from grafts (73–90%) compared to pre-grafting (99%)	Skakkebaek et al. (1974)

rhesus monkeys (Honaramooz et al. 2004; Rathi et al. 2008). One potential reason for failure to achieve complete spermatogenic differentiation appears to be incomplete Sertoli cell maturation in the grafted tissue (Rathi et al. 2008).

Several other factors that depend on the status of the donor are also of importance for graft survival and development. One of the most significant parameters is the developmental or functional stage of the donor testis. Survival of xenografts declines with increasing maturity of the donor tissue. Tissue from adult donors shows poor survival and a marked tendency to degenerate, making it unsuitable for transplantation (Schlatt et al. 2002; Geens et al. 2006; Kim et al. 2007; Arregui et al. 2008b). Some spermatozoa were produced from transplanted adult mouse and human testicular tissue. However, these spermatozoa were believed to be from differentiating germ cells that completed spermatogenesis after grafting, rather than arising de novo from spermatogonial stem cells (Schlatt et al. 2002, 2006; Geens et al. 2006). Degeneration of adult testis xenografts occurs faster compared to immature donor tissue (Arregui et al. 2008b). Several reasons have been proposed to explain poor survival of adult testis tissue, including lack of proliferation of Sertoli cells, increased sensitivity to ischemia and a decreased angiogenic ability of the adult tissue (Schlatt et al. 2002; Arregui et al. 2008a, b). Once meiosis is initiated the developmental capacity of the tissue declines dramatically (Rathi et al. 2006; Kim et al. 2007). Ischemic damage is problematic for any type of transplantation. Initial depletion of germ cells and necrotic damage following xenografting have been noted (Rathi et al. 2006; Rodriguez-Sosa et al. 2010). Post-grafting establishment of spermatogenesis in testis xenografts requires intense and coordinated proliferation of somatic components and recolonization of spermatogonial stem cells (Rathi et al. 2006; Huang et al. 2008; Rodriguez-Sosa et al. 2010). It can be speculated that differences in stem cell cohorts between prepubertal and pubertal donors may contribute to the variable ability of graft survival and development. Furthermore, the developmental capacity may be influenced by post-grafting damage of somatic components (Kim et al. 2007; Rodriguez-Sosa et al. 2010). At initiation of meiosis, Sertoli cells have reached a more mature degree of physiological and morphological differentiation and their proliferative activity decreases (Meachem et al. 2005). This may render Sertoli cells more sensitive to grafting-related injuries and certainly limit their ability to repair tissue damage through extensive proliferation. A negative impact of degenerating maturing germ cells is also likely in pubertal and postpubertal donor tissue. Meiotic and postmeiotic germ cells are killed by exposure to hypoxia since they are highly depending on oxidative metabolism (Rathi et al. 2006; Kim et al. 2007; Rodriguez-Sosa et al. 2010). It is interesting to note that the grafting success was markedly different between testicular tissue from pubertal and photoinhibited Djungarian hamsters. While xenografts from adult hamsters showed the expected poor graft survival, grafts from immature hamster testes showed excellent capacity to grow, differentiate, and initiate full spermatogenesis (Schlatt et al. 2002). Surprisingly, photoinhibited hamster testes in which spermatogenesis is blocked at the level of premeiotic germ cells, like in immature hamsters, showed poor survival and no initiation of spermatogenesis. The fact that the postpubertal hamster testis reveals poor developmental capacity

irrespective of its spermatogenic activity indicates that the mechanisms responsible for survival of hamster testis grafts are primarily dependent on developmentally controlled differentiation events and are not related to the spermatogenic activity of the seminiferous epithelium. In contrast, suppression of spermatogenesis in adult mouse testes by treatment with gonadotropin antagonists or experimentally induced cryptorchidism prior to grafting resulted in improved graft survival and resurgence of spermatogenesis compared to allografting of adult testis tissue with full spermatogenesis, indicating that poor graft survival is largely due to the increased metabolic activity and therefore heightened sensitivity to hypoxic damage in testis tissue undergoing full spermatogenesis, and is not intrinsic to the adult testis (Arregui et al. 2008b). Therefore, species-specific differences as well as different mechanisms of suppression of spermatogenesis prior to grafting may influence graft survival and germ cell differentiation after grafting of adult testis tissue.

10.3 Testicular Xenografting Using Testicular Cell Suspensions

Xenografting of testicular tissue fragments maintains the integrity of the seminiferous epithelium but does not open routes for manipulation of selected cell types. The strong morphogenetic ability of isolated testis cells to reconstitute cord-like structures after grafting creates new opportunities to target specific cell types and their role during testis development and initiation of spermatogenesis. Grafting of isolated testis cells has only been described recently. This approach has thus far been evaluated using cells from newborn donors in pigs (Honaramooz et al. 2007), rodents (Dufour et al. 2002; Gassei et al. 2006, 2008; Kita et al. 2007) and sheep (Arregui et al. 2008a). For this strategy, cells obtained after enzymatic digestion of testicular tissue are injected subcutaneously in recipient mice as pellets or cultured for several days embedded in extracellular matrix gel and then injected with the matrix.

Formation of seminiferous cords and tubules from transplanted testicular cell suspensions was first reported in experiments testing immunoprotection of Sertoli cells for co-transplanted pancreatic islets cells (Kin et al. 2002). Using this approach, spermatogenesis up to and beyond the level of round spermatids was obtained in testicular tissue that was reconstituted from crude cell suspensions of immature pigs, sheep, and embryonic or neonatal rats and mice (Honaramooz et al. 2007; Kita et al. 2007; Arregui et al. 2008a). The rodent studies revealed an opportunity to manipulate the system by adding mouse germ line stem cells carrying a GFP marker to the cell suspension prior to grafting into mouse hosts. The GFP-positive spermatids obtained in this system were successfully used for assisted fertilization (Kita et al. 2007). Recently, the ability to form seminiferous tubules from isolated Sertoli cells after grafting into mouse hosts has also been demonstrated for bovine Sertoli cells (Zhang et al. 2008). A combination of *in vitro* culture of Sertoli cells and grafting has demonstrated the importance of neurotrophic tyrosine receptor kinases controlled aggregation of Sertoli cells for formation of cord-like

structures (Gassei et al. 2008). Isolated testis cells from neonatal males transplanted under the dorsal skin of mice rearrange to generate testis-like structures that exhibit not only spermatogenic but also endocrine function (Gassei et al. 2006; Honaramooz et al. 2007; Kita et al. 2007). These studies demonstrate an amazing capability of isolated postnatal testis cells to recapitulate testis development, rearrange into seminiferous cords, and initiate steroidogenesis and spermatogenesis. These approaches offer fascinating novel scenarios to explore morphogenetic events during testicular development (Dobrinski 2005). Furthermore, since it has been shown that sperm obtained from testis tissue xenografts could be used for intracytoplasmic sperm injection to produce embryos and offspring (Honaramooz et al. 2002, 2004, 2007; Schlatt et al. 2003) these *in vivo* strategies represent new approaches for preserving the germline of valuable males or endangered species (Dobrinski 2005).

10.4 Xenografting of Primate Testicular Tissue: Clinical and Experimental Perspectives

Survival of fetal human testicular tissue as xenograft was described shortly after nude mice became available as immunodeficient recipients of tissue grafts (Skakkebaek et al. 1974). The grafts survived but no developmental progression of spermatogenesis was observed in the grafted tissue. Improved grafting methods and a better understanding of the critical steps came from studies using immature testis tissue from domestic animals and rodents. These studies revealed a promising developmental capacity of the grafted tissue. Therefore, xenografting of non-human primate and human tissue was revisited. A schematic representation of primate testis grafting with recovery of functional spermatozoa from primate testis grafts is presented in Fig. 10.1. The potential to generate sperm from prepubertal testes (Honaramooz et al. 2004) created novel opportunities to explore primate testis development and offered a clinically relevant strategy for fertility preservation in boys undergoing oncological therapies. Table 10.2 summarizes the strategies and outcome of studies that were performed using testicular xenografting with postnatal monkey or human as tissue donors. From 2002 to 2005 the studies revealed that xenografted monkey tissue survives as xenografts and has a promising ability to differentiate in the mouse recipient (Schlatt et al. 2002; Honaramooz et al. 2004; Orwig and Schlatt 2005). As discussed above, a prominent exception was the marmoset (Wistuba et al. 2004). Xenografts of immature marmosets show limited capacity to differentiate. As for other species it was shown that xenografting of immature testes from monkeys and humans is much superior compared to adult tissue that undergoes almost complete degeneration after grafting (Arregui et al. 2008b; Schlatt et al. 2006; Geens et al. 2006). Xenografting of monkey tissue then became a strategy for preservation of fertility and to study the effects of hormonal manipulation or exposure to radiation or gonadotoxins on testicular development.

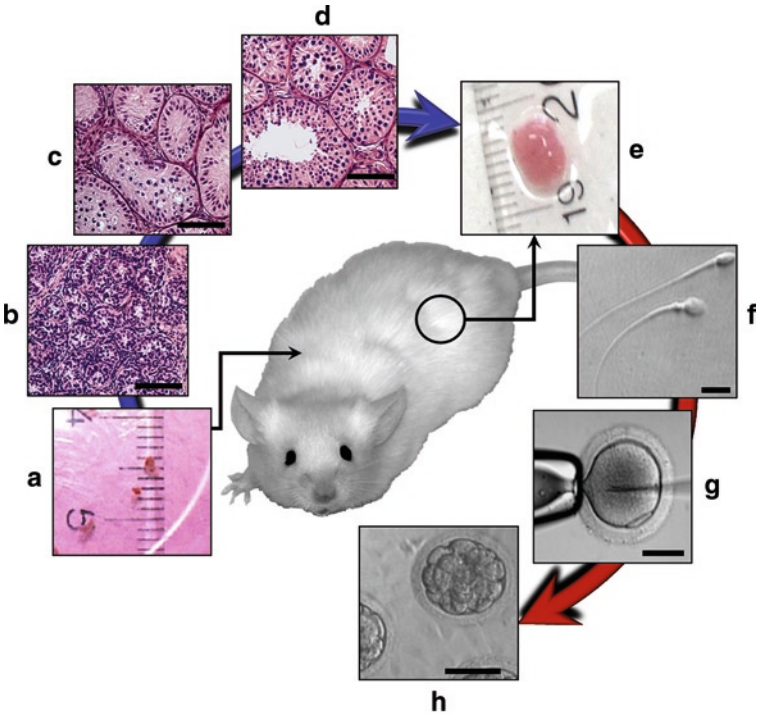

Fig. 10.1 Ectopic xenografting of immature primate testis tissue into immunodeficient mice. Fragments of immature donor testis (~1 mm^3) transplanted under the dorsal skin of immunodeficient mice (**a, b**) are able to survive and respond to gonadotropins. As a result, testis tissue undergoes complete development, including formation of fertilization competent sperm (**c–f**). Once testis xenografts are collected (**e**) they can be used for analysis or to obtain sperm for ICSI (**g**) and embryo production (**h**). *bars* equal 50 μm (**b–d, g, h**) or 10 μm (**f**)

Since the time of sperm retrieval is often many years earlier than their estimated use, excellent protocols for cryopreservation and cryostorage are important prerequisites when testicular grafting will become a clinical tool. To maintain several options for future use of the cryopreserved material, testicular tissue is best cryopreserved as small fragments as well as enzymatically dispersed single cell suspensions. This will maintain options to use the intact tissue as grafts or to create a single cell suspension for reaggregation of testicular tissue or isolation of spermatogonial stem cells for *in vitro* approaches or germ cell transplantation. Protocols for cryopreservation of cell suspensions and testicular fragments from adult and cryptorchid testes using propanediol, glycerol, ethylene glycol, or DMSO were described (Brook et al. 2001; Keros et al. 2005, 2007; Kvist et al. 2006). However, none of these studies had assessed the *in vivo* stem cell capacity of the cryopreserved and thawed primate spermatogonia. Xenografting was applied to optimize procedures for cryopreservation of testicular tissue (Jahnukainen et al. 2007a). In addition to showing that immature primate testis tissue can best be cryopreserved

using 1.4 M DMSO as cryoprotectant, a comparison of various strategies revealed that juvenile testicular tissue can be maintained on ice for 24 h prior to xenografting without diminishing the developmental potential of the tissue fragment. Simplifying the preservation procedures and creating options for safe shipping of testicular tissue provides clinically relevant strategies to centralize these procedures and to generate testis tissue banks from patient tissue.

The use of testis tissue xenografting as an experimental strategy was illustrated when the gonadotoxic effects of busulfan were demonstrated in subcutaneous monkey xenografts (Jahnukainen et al. 2006a). Combining grafting for exploration of long-term effects and organ culture to describe the immediate cellular injury, a dose-dependent gonadotoxic effect incurred by radiation was shown (Jahnukainen et al. 2007b). Stimulation of grafted infant primate testis tissue with exogenous gonadotropins supported Sertoli cell maturation, thereby terminating the unresponsive phase of the germinal epithelium, and allowed complete spermatogenesis in testis tissue from infant rhesus monkeys (Rathi et al. 2008). The use of testis tissue xenografting requires a smaller number of monkeys to perform valid comparative studies. While differences in pharmacokinetics between primates and rodents have to be considered, groups of mice carrying xenografts from few juvenile donors can be exposed to different regimens rather than exposing large groups of monkey to different gonadotoxic treatments. Analysis of the grafted tissue then reveals valid parameters to determine developmental failures of the testis and spermatogenic damage.

Further studies are needed to optimize the many variables that affect the success of grafting in non-human primates and humans. Two reports in marmosets have described the outcome of autologous grafting (Wistuba et al. 2006; Luetjens et al. 2008). While it appeared that ectopic placement of grafts was associated with a developmental block at the meiotic stage of spermatogenesis (Wistuba et al. 2006), a more promising development of grafts up to the level of spermatids was observed when the scrotum was used as an orthotopic grafting site (Luetjens et al. 2008). Other studies revealed that additional supplementation of human gonadotropins to mouse recipients promoted graft development and function (Rathi et al. 2008). Other studies have focused on xenografting of fetal and immature human testicular tissue (Goossens et al. 2008; Wyns et al. 2007, 2008; Yu et al. 2006). These studies showed good survival of testicular tissue and maintenance of integrity of the tubular architecture. As yet only very limited initiation of spermatogenesis in human tissue has been achieved but survival of human spermatogonia has been determined. Grafting of adult human tissue, in contrast, revealed a very poor survival and development of xenografts (see Table 10.2). So far the human xenografts have always been placed ectopically, which may have a negative influence on grafting outcome. Further improvement of xenografting strategies will prepare the ground for using the amazing regenerative potential of immature primate testicular tissue. This will create exciting clinically applicable strategies for fertility preservation. The future development of xenografting as a clinical tool will focus on safety aspects associated with any xenotransplantation approach and improving the efficiency to generate sperm. The perspective of novel strategies to generate sperm from spermatogonial stem cells or immature testicular tissue should stimulate more widely applied

cryopreservation of testicular tissue fragments from young patients whose future fertility is at risk due to oncological treatment or other gonadotoxic exposures.

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Chapter 11
Translating Spermatogonial Stem Cell Transplantation to the Clinic

Brian P. Hermann and Kyle E. Orwig

Abstract The spermatogenic lineage is maintained by a pool of spermatogonial stem cells (SSCs) that balance self-renewing and differentiating divisions to meet the biological demand of the testis. In animal models (mice, rats, goats, pigs, and dogs), SSCs transplanted into infertile recipient testes exhibit the remarkable potential to regenerate spermatogenesis. Tremendous progress has been made towards applying SSC transplantation techniques to primates (human and nonhuman), which may have applicability in the human fertility clinic. This chapter will review the approaches used to isolate and preserve donor SSCs from primate testes, generate infertile recipient nonhuman primates for transplantation studies, transplant donor SSCs into primate testes, and evaluate transplant outcomes. In addition, we discuss some of the clinical considerations for future application of these techniques in patients.

Keywords Spermatogonial stem cells • Primates • Male infertility • Spermatogenesis • Transplantation

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11.1 Introduction—Clinical Possibilities for Male Fertility Preservation

Since the 1970s, oncology patient survival rates have improved as a result of cooperative protocol-driven clinical research, particularly in young patient categories. This has propelled the overall event-free survival rate for childhood cancer patients to 79% in 2004 (Ries et al. 2007), and as a result, research efforts are beginning to shift towards improving patient quality-of-life after cure. In particular, patients receiving chemotherapy and radiotherapy for cancer are often at risk for infertility, catapulting fertility preservation to the forefront of quality-of-life concerns (see Chap. 9). Progress to minimize the unwanted side effects of current treatment regimens without decreasing their effectiveness against the oncologic disease has allowed many cancer survivors to have children following spontaneous recovery of fertility (van den Berg et al. 2004). However, some oncological diseases require rigorous treatment regimens, which will almost always lead to permanent infertility. In addition, ablative conditioning prior to hematopoietic stem cell transplantation for malignant and non-malignant disorders is highly gonadotoxic. In particular, treatment regimens that include high-dose alkylating chemotherapy (e.g., busulfan, melphalan, cyclophosphamide, nitrosoureas, cisplatin, chlorambucil, carmustine, lomustine, cytarabine, ifosfamide, and procarbazine) result in the highest risk of long-term infertility [(Wallace et al. 2005; Lee et al. 2006; Mitchell et al. 2009); see Chap. 9]. Recently reported longitudinal male infertility data from the Childhood Cancer Survivor Study indicates that boys who receive radiation doses greater than 7.5 Gy, high cumulative alkylating agent doses, and those receiving cyclophosphamide or procarbazine are at highest risk for future infertility as adults (Green et al. 2010).

In contrast to the efficient treatment regimens for a patient's primary disease, very few and limited options are available to prevent the loss of fertility. However, promising therapies are currently in the research pipeline that may one day offer these cancer survivors the hope of future fertility. For men, cryobanking of semen before the initiation of treatment is possible and allows for future *in vitro* fertilization (IVF), including intracytoplasmic sperm injection (ICSI), but this is a finite resource and does not allow for natural conception. Furthermore, some men are not able to provide an adequate semen sample at the time of diagnosis. For these men, it is possible to isolate epididymal sperm directly via percutaneous epididymal sperm aspiration (PESA) or microsurgical epididymal sperm aspiration (MESA) (Patrizio et al. 1988; Silber et al. 1990; Craft et al. 1995). PESA involves sperm aspiration through a needle inserted into the cauda epididymis, while MESA is performed as open surgical procedure under the operating microscope to aspirate sperm from a single caput epididymal tubule. Alternatively, testicular sperm can be isolated by fine-needle aspiration via testicular sperm aspiration (TESA) or through a surgical biopsy for testicular sperm extraction (TESE) (Devroey et al. 1994). In the TESE procedure multiple biopsies are obtained surgically from the testicular parenchyma, biopsies are minced in buffered solutions, and any sperm are identified by microscopic examination (Tournaye 1999). Sperm identified using these techniques can be prospectively isolated by micromanipulation and used for ICSI

to generate embryos for uterine implantation or cryopreservation. Alternatively, aliquots containing sperm can be frozen for future ICSI in the embryology laboratory. While these approaches (sperm banking, isolation, and ICSI) are standard of care, they are not options for prepubertal boys who are not yet producing sperm.

For prepubescent boys (and perhaps some men), cryopreservation of spermatogonial stem cells (SSCs) prior to treatment and reintroduction of these cells into the testis upon its completion is an approach that may permanently restore natural fertility following successful treatment of their underlying disease (Fig. 11.1). SSCs are the adult tissue stem cells of the testis that give rise to sperm through the process of spermatogenesis. In animal models (rodents, pigs, goats, and dogs), transplantation of SSCs into the testes of infertile males can lead to restoration of spermatogenesis (Brinster and Avarbock 1994; Ogawa et al. 2000; Shinohara et al. 2001; Nagano et al. 2001a; Brinster et al. 2003; Honaramooz et al. 2003b; Orwig and Schlatt 2005; Mikkola et al. 2006; Kim et al. 2008). Stem cells from all ages, newborn to adult, are competent to produce complete spermatogenesis following transplantation into recipient testes (Shinohara et al. 2001; Ryu et al. 2003). In addition SSCs from a variety of species can be cryopreserved and retain spermatogenic function upon thawing and transplantation (Brinster 2002). Thus, through cryopreservation of SSCs prior to cancer therapy and autologous transplantation of SSCs after cancer cure, it may be feasible for male cancer patients to preserve and recover natural fertility and father their own genetic offspring (see Fig. 11.1). While recovery of natural fertility is the ideal result of SSC transplantation, a more realistic outcome may be production of low-level donor spermatogenesis that could be recovered by TESE and used for ICSI to give childhood cancer survivors the opportunity to have their own genetic children. While successes in lower animal species (e.g., mice, rats, goats, pigs, dogs) support application of this approach in humans, safety and efficacy studies are needed before this cell-based therapy can be translated to the bedside. For this purpose, studies in nonhuman primate models that more closely resemble the anatomy, endocrinology, and spermatogonial complement of the human testis (Plant and Marshall 2001; Hermann et al. 2010) are ideal.

Grafting of testicular tissue is a promising technique that may provide an alternative therapeutic option for prepubertal cancer patients (see Chap. 10). Undifferentiated spermatogonia in testicular tissue grafts from immature mice, rats, hamsters, pigs, goats, and nonhuman primates are competent to produce complete spermatogenesis following ectopic transplantation under the skin of mouse hosts [reviewed by (Rodriguez-Sosa and Dobrinski 2009)]. Sperm retrieved from grafts are capable of supporting normal development and producing normal male and female offspring following ICSI (Schlatt et al. 2003). Thus, ectopic grafting sites can provide a suitable environment to orchestrate complete spermatogenesis from testicular tissue isolated from immature donors. Moreover, cryopreserved tissue fragments from immature testes are capable of producing spermatogenesis in host mice after thawing (Schlatt et al. 2002b). For a prepubertal cancer patient, testicular tissue could be retrieved by TESE, processed into small fragments (≤ 1 mm^3), which could be xenografted immediately or cryopreserved for future grafting. While testicular xenografting does not restore natural spermatogenesis and requires state-of-the-art assisted reproductive techniques (i.e., ICSI), it may be

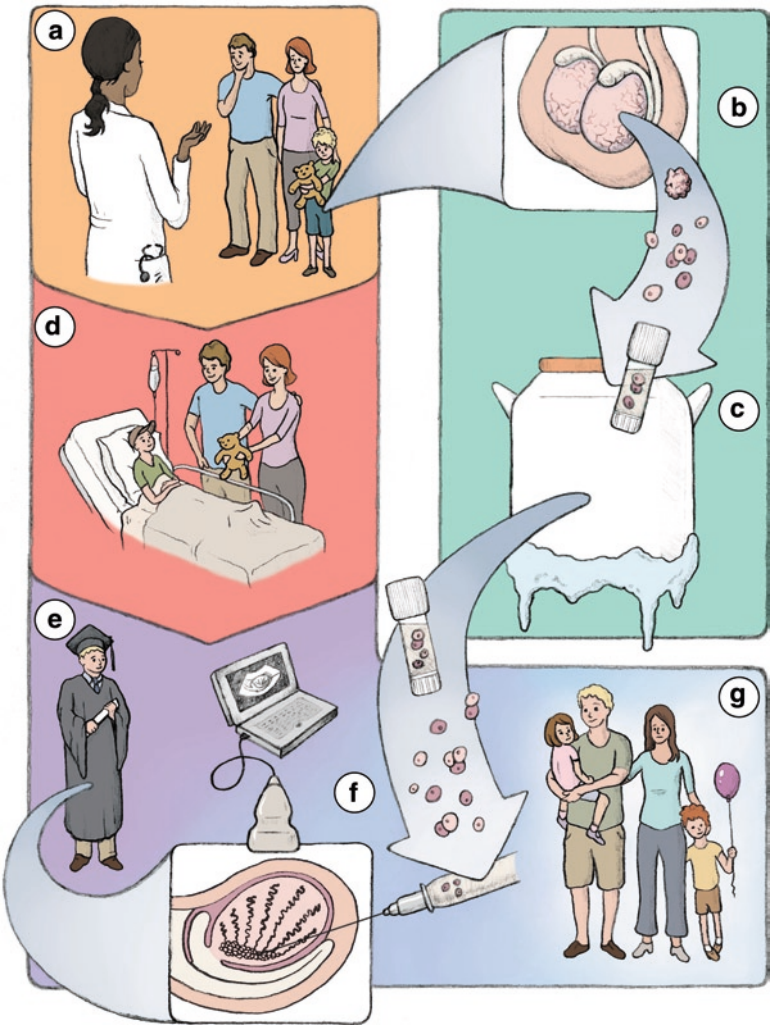

Fig. 11.1 Fertility preservation and restoration in prepubertal boys. This flow diagram presents a hypothetical case of preserving and restoring fertility in a prepubertal boy who will receive gonadotoxic therapy (e.g., chemotherapy or radiation) to treat his primary disease or condition. (**a**) Patients in this category are identified by their physician prior to gonadotoxic therapy. In this setting, the patient and/or their parent(s)/guardian(s) are counseled of the reproductive risks of the treatment and are referred for fertility preservation. (**b**) Surgical biopsy of the testis would occur in an expedited manner prior to therapy to recover a small amount of testicular parenchyma that contains potentially therapeutic SSCs. Enzymes would then be used to disrupt the seminiferous tubules and produce a suspension of testis cells. (**c**) The testis cell suspension would be cryopreserved and stored in liquid nitrogen for future use to restore fertility. (**d**) Gonadotoxic treatment for the patient's primary diagnosis (e.g., chemotherapy or radiation) would commence immediately after the patient recovers from testis biopsy surgery (typically within one week and perhaps as early as the next day). (**e**) After the patient has survived his primary disease or condition (likely five or more years after treatment), the patient's fertility status would be assessed to determine if intervention is necessary. (**f**) For infertile

an avenue to future fertility for young cancer survivors. Additional studies are needed to determine the optimal conditions to achieve complete spermatogenesis in xenografts of immature human testicular tissue (see Chap. 10).

In order to solidify the foundation for clinical translation of fertility-preserving therapies such as SSC transplantation and xenografting, we must gain additional fundamental insights into the biology of SSCs in the primate testis, including human. Thus, this chapter begins with an introduction of the current understanding of primate SSC biology. The majority of this chapter is devoted to describing approaches for isolating primate SSCs, nonhuman primate models for SSC transplantation, methods for introducing donor SSCs into primate testes, and evaluation of transplant outcomes. We will also discuss some of the clinical implications and considerations for responsible translation of SSC transplantation to the fertility clinic, including safety, efficacy, and ethics.

11.2 Primate Spermatogonial Stem Cells

SSCs are undifferentiated germ cells that occupy a niche in testicular seminiferous tubules (see Chap. 8) and balance self-renewing and differentiating divisions to maintain spermatogenesis throughout adult life. Several experimental approaches are used to distinguish rodent SSCs from other germ cells and testicular somatic cells, including their functional capacity to establish and maintain spermatogenesis in a transplantation assay (see Chap. 5), expression of a variety of specific molecular markers (see Chap. 7), and their clonal arrangement on the basement membrane of seminiferous tubules (A_{single} and some A_{paired}; see Chap. 4).

In contrast, relatively little is known about primate SSCs, including humans, but recent advances are expanding our understanding of the primate germline stem cell system [reviewed by (Hermann et al. 2010)]. Primate testes contain two distinct types of undifferentiated (Type-A) spermatogonia classified morphologically as A_{dark} and A_{pale}, based on differences in nuclear architecture and staining intensity with hematoxylin (Clermont and Leblond 1959; Clermont 1972; Clermont and Antar 1973; Cavicchia and Dym 1978). A_{dark} and A_{pale} are both found on the basement membrane of primate seminiferous tubules, but there is limited data describing their molecular characteristics or clonal arrangement (Clermont and Leblond 1959;

Fig. 11.1 (continued) patients, a SSC transplant could potentially restore fertility. For this purpose, vials of cryopreserved testis cell suspension would be thawed and introduced into the seminiferous tubules of the patient's testis by ultrasound-guided rete testis injection. (**g**) Any transplanted SSCs that find a niche in the patient's seminiferous tubules could engraft and regenerate spermatogenesis. In the ideal scenario, this could lead to the production of sperm from the transplanted testis, allowing the patient to father his own genetic children, possibly through normal coitus. Alternatively, transplanted SSCs could produce small foci of donor spermatogenesis from which functional sperm could be retrieved by TESE for use in assisted reproduction by ICSI. Artwork produced by Molly Feuer (http://www.feuerillustration.com)

Ehmcke et al. 2005) and conflicting views about whether these "undifferentiated" spermatogonia function as SSCs.

The prevailing model of spermatogenesis in primates suggests that A_{dark} and A_{pale} are reserve and renewing stem cells, respectively (Clermont 1969; Clermont and Antar 1973; Fouquet and Dadoune 1986; van Alphen and de Rooij 1986; Plant and Marshall 2001; Ehmcke et al. 2005; Simorangkir et al. 2005, 2009). This "reserve stem cell" model holds that A_{pale} function as "renewing stem cells" that divide regularly to maintain spermatogenesis under normal conditions. A_{dark} are considered "reserve stem cells" that rarely divide in the adult and only function to rescue spermatogenesis in cases where spermatogenesis is destroyed by cytotoxic insult [e.g., radiation; (van Alphen and de Rooij 1986)]. A recent comparative study examined molecular markers of rodent spermatogonia in the rhesus macaque testis and found a substantial proportion of A_{dark} and A_{pale} spermatogonia in the adult rhesus testis that exhibited a molecular phenotype similar to rodent SSCs (Hermann et al. 2009). This study raised the possibility that A_{dark} and A_{pale} are part of the same functional cell population rather than distinct populations of reserve and renewing stem cells. Numerous other recent studies have sought to elucidate the fundamental characteristics of undifferentiated spermatogonia in the nonhuman primate testis [(Hermann et al. 2007, 2009, 2010; Ehmcke et al. 2006; Ehmcke and Schlatt 2006; Muller et al. 2008; Maki et al. 2009; Simorangkir et al. 2009)]. This momentum has laid a foundation of fundamental biological information about primate SSCs upon which to build future studies investigating their regenerative capacity at a preclinical/translational level. Certainly, identification of the human SSC and clear understanding of its role in normal human spermatogenesis are key goals of future studies.

11.3 Nonhuman Primate Model for Developing SSC Transplantation

11.3.1 SSC Transplantation in Rodents

In rodents, the definitive endpoint demonstrating that a given spermatogonium exhibits the functional property of a SSC is the ability to produce and maintain spermatogenesis in a transplantation paradigm (see Chap. 5). As originally described by Brinster and colleagues for mice, suspensions of testis cells containing SSCs are isolated from the testes of donor animals and transplanted into the testes of infertile recipients where stem cells produce colonies of normal spermatogenesis and functional sperm (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). By definition, only a stem cell can produce and maintain a colony of spermatogenesis and each colony arises from the clonogenic proliferation and differentiation of a single SSC (Dobrinski et al. 1999; Zhang et al. 2003; Kanatsu-Shinohara et al. 2006). Therefore, the SSC transplantation technique is a functional assay to

characterize stem cell activity in any donor testis cell population. In order to translate SSC transplantation to assess the fundamental biology and therapeutic application of primate SSCs, it is necessary to optimize methods for primate testis cell isolation, cryopreservation, and transplantation.

11.3.2 Primate Testis Cell Isolation

Studies of the fundamental biology and regenerative capacity of primate SSCs require isolated suspensions of cells from primate testes. Several protocols have been developed using enzymes to digest primate testicular parenchyma to a single cell suspension (Nagano et al. 2001b, 2002; Schlatt et al. 2002a; Hermann et al. 2007, 2009; Muller et al. 2008; Maki et al. 2009). In general, a two-step enzymatic digestion procedure is employed to liberate cells, although an alternative protocol employs mechanical disruption and a single, extended incubation with collagenase IV to generate a suspension of marmoset testis cells (Muller et al. 2008).

Nagano and colleagues were first to report a method for generating a single cell suspension of nonhuman primate testis cells. In their protocol, fragments of baboon seminiferous tubules were generated by digestion with collagenase II (1 mg/ml, Worthington) at 33°C for 15–20 min, followed by digestion with trypsin (0.5 mg/ml, Sigma) at 33°C for 15–20 min and addition of DNase I (1 mg/ml, Sigma) with vigorous trituration to disrupt cell clumps and straining through a nylon mesh to produce a single-cell suspension (Nagano et al. 2001b). The resulting testis cell suspension was resuspended in DMEM and provided cells suitable for xenotransplantation. This protocol was later modified to produce a single-cell suspension of rhesus macaque testicular cells (Hermann et al. 2007, 2009), including a first step digestion with collagenase type IV (1 mg/ml, Sigma), a second-step digestion with trypsin (2 mg/ml) containing EDTA (1.04 mM) (Invitrogen) plus DNase I (1.4 mg/ml, Sigma) and resuspension in minimum essential medium alpha containing 10% FBS. Yields of rhesus testis cells using this protocol are highly reproducible and result in suspensions of cells with high viability [Table 11.1; (Hermann et al. 2007, 2009)]. Differences in cell yield between developmental stages are primarily attributed to the progressive increase in difficulty of enzymatic digestion during postnatal testis development accompanying formation of a basement membrane, increase in seminiferous cord-tubule diameter, and increasing tissue complexity.

Different iterations of a two-step enzymatic digestion protocol have also been reported for generation of cell suspensions from testes of rhesus macaques (Schlatt et al. 2002a; Maki et al. 2009). Schlatt and colleagues generated rhesus testis cells by digesting cultured testicular tissue fragments with collagenase I (Sigma), followed digestion with hyaluronidase (Sigma) and DNase I (Sigma). In the protocol established by Maki and colleagues, seminiferous tubules were finely minced and digested with collagenase A (1 mg/ml, Roche) plus DNase (10 U/ml, Invitrogen) at 37°C for 15 min plus agitation (Maki et al. 2009). The undigested seminiferous tubules are

Table 11.1 Yields and viability of fresh and cryopreserved/thawed rhesus macaque testis cells

	Fresh cells[a]		Cryopreserved cells[b]		
Developmental Stage	Cells/g testis ($\times 10^6$)	Viability (%)	Recovery (%)	Viability (%)	Duration (mean days)
Neonate rhesus	754 ± 175	92.1 ± 0.9	Nd.	Nd.	Nd.
Juvenile rhesus	468 ± 42	96.8 ± 0.3	75.8 ± 3.7	80.8 ± 0.9	176 ± 33
Adult rhesus	189 ± 16	94.8 ± 0.6	71.7 ± 5.0	66.2 ± 2.8	228 ± 29

[a]Adult and juvenile testis cells were prepared from 26 and 25 individual animals, respectively. Adult testis cell data are means of the four cell isolations reported in (Hermann et al. 2007), together with 22 isolations performed subsequently. Juvenile testis cell yields and recovery are as reported (Hermann et al. 2009). Neonatal rhesus testis cells were prepared from eight males (average 2.75 days postnatal)

[b]Recovery and viability of cryopreserved juvenile testis cells was performed in 37 individual thaws from a total of 20 animals. From adults, data are from 29 thaws from 13 individual animals

then digested with four enzymes to generate a single-cell suspension: collagenase A (1.5 mg/ml, Roche), hyaluronidase type V (1.5 mg/ml, Sigma), trypsin (0.5 mg/ml, Worthington) and DNase (10 U/ml. Invitrogen) at 37°C for 20 min with mild agitation. Undigested tissue and cell clumps are removed by straining and isolated cells are suspended in DMEM + 10% FBS. Cell yields using these approaches were not reported, but a lower concentration of trypsin [fourfold; (Maki et al. 2009)] or its absence altogether (Schlatt et al. 2002a) may be beneficial for characterizing cell surface markers that are sensitive to trypsin cleavage (e.g., cKIT).

11.3.3 Cryopreservation

Autologous transplantation of SSCs in primates to study the regenerative capacity of SSCs in infertile recipients (and eventual clinical application) requires storage of cells between the times of collection and transplant. Several protocols have been reported to cryopreserve primate testicular cells (Nagano et al. 2001b, 2002; Schlatt et al. 2002a; Hermann et al. 2007, 2009). Initial reports using baboon and human testis cell suspensions employed methods developed for cryopreservation of rodent SSCs (Avarbock et al. 1996). With this approach, 10% DMSO is used as the cryoprotectant and freezing is performed in a controlled-rate freezing device. DMSO penetrates cell membranes and acts as a cryoprotectant by preventing cellular dehydration and formation of intracellular ice crystals (Lovelock and Bishop 1959). Cells frozen with this approach and subsequently thawed were competent to colonize mouse testes in xenotransplantation studies, however, cell recovery, viability, and comparative phenotypic and functional attributes were not reported (Nagano et al. 2001b, 2002).

The first protocol for rhesus monkey testis cell cryopreservation utilized 1.5 M glycerol as a cell-permeant cryoprotectant in the presence of 4% autologous monkey serum and freezing using a controlled-rate freezing machine (Schlatt et al. 2002a).

We subsequently adapted the baboon, human and rodent protocol for cryopreservation of rhesus testis cells at high concentrations (20×10^6/ml) in medium containing 10% DMSO by freezing 1–2 ml aliquots in isopropanol controlled-rate freezing devices [−1°C/min, Nalgene-Nunc International; (Hermann et al. 2009, 2007)]. Larger cryovials (5 ml) can also be used when frozen using electronic controlled-rate freezing devices (e.g., CryoMed, ThermoFisher). Importantly, these methods result in high recovery and viability of cells that retain normal phenotypic and functional characteristics [Table 11.1; (Hermann et al. 2007, 2009)].

After storage in liquid nitrogen, cryopreserved cells are thawed rapidly at 37°C and the cell suspension is diluted ~5- to 10-fold by the drop-wise addition of excess medium (MEMα + 10% FBS). Dilution with medium is performed in a drop-wise fashion to reduce osmotic damage due to addition of the relatively hypotonic medium. Cells are then washed several times in medium to eliminate DMSO prior to experimentation. Viability of cryopreserved testis cells after thawing varies with the developmental stage from which they were isolated (Table 11.1), but is generally good. In our hands, testis cells from prepubertal animals typically survive a freeze-thaw cycle better than those from adult testes (Table 11.1), likely due to the absence of relatively fragile differentiating germ cells. Rhesus-to-nude mouse xenotransplantation was used to assess the colonization potential of cryopreserved/thawed rhesus testis cells compared to freshly isolated cells (Hermann et al. 2007) and no statistically significant difference was observed following cryopreserved of rhesus testis cells. Furthermore, cryopreserved rhesus testis cells exhibited similar phenotypic profiles for the cell surface marker THY-1 (CD90) before and after freezing based on FACS analysis (Hermann et al. 2009). Thus, rhesus SSCs appear to retain normal phenotypic and functional attributes after cryogenic storage. A similar approach was used to ship and store baboon testis cells prior to xenotransplantation, but recovery, viability, phenotype, and function of frozen-thawed testis cells were not reported (Nagano et al. 2001b).

While the data obtained to date suggest that surviving nonhuman primate SSCs retain normal functional attributes following cryopreservation (using standard 10% DMSO as a cryoprotectant and controlled-rate cooling), not all cells survive the freeze-thaw process, and thus, some of the starting regenerative pool is lost (see recovery and viability results, Table 11.1). To address this potential drawback of freezing cells, additional experiments are ongoing in rodents, monkeys, and humans to evaluate alternative cryoprotectants and additives. For example, inclusion of long-chain oligosaccharides or trehalose (a disaccharide of glucose), in addition to DMSO, results in improved cell recovery after cryopreservation of various cell types (Buchanan et al. 2004; Miyamoto et al. 2006; Katenz et al. 2007). Trehalose is a non-permeant cryoprotectant that is thought to safeguard lipid membranes from freeze-thaw damage and protect labile cell-surface proteins, although the most pronounced effects of trehalose are obtained with intracellular loading (Katenz et al. 2007). Thus, there is potential for increased recovery and viability of frozen-thawed primate testis cells with optimization of the cryopreservation medium (e.g., addition of Trehalose).

11.3.4 Recipient Models for SSC Transplantation

The best way to study the regenerative capacity of SSCs is using a transplantation paradigm. To facilitate SSC engraftment, donor cells are typically transplanted into recipient seminiferous tubules that lack endogenous spermatogenesis and, therefore, have niches available for occupancy by donor cells. Several approaches for depleting endogenous spermatogenesis in recipients have been described, including cytotoxic treatment (de Rooij and Kramer 1970; Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Ogawa et al. 1997, 2000; Nagano and Brinster 1998; Zhang et al. 2006a) and genetic mutation (Ohta et al. 2001; Shinohara et al. 2001). In rodents, the cytotoxic treatments used to deplete endogenous spermatogenesis in recipients involve anti-mitotic treatment with chemotherapy or local irradiation (de Rooij and Kramer 1970; Brinster et al. 2003; Zhang et al. 2006a). The gonadotoxicity of various chemotherapy and radiation treatment regimens is reviewed extensively in Chap. 9.

11.3.5 Busulfan Treatment

To evaluate the full regenerative potential of rhesus SSCs and study stem cell/niche interactions, we developed a similar germ cell depleted (infertile) rhesus testis model. Experiments were conducted to identify a busulfan treatment dose that is compatible with long-term survival and which results in germ cell and SSC ablation (Hermann et al. 2007). Adult rhesus macaques were treated with high doses of the alkylating chemotherapeutic agent busulfan (Busulfex IV, ISP Pharma), which led to long-term loss of sperm in the ejaculate beginning ~10 weeks after treatment and lasting for more than 1 year [Fig. 11.2a, (Hermann et al. 2007)]. Loss of sperm production after high-dose busulfan treatment also correlated with a complete depletion of spermatogenesis in histological sections of the testis (Fig. 11.2b). VASA (DDX4) and DAZL (Ruggiu et al. 1997; Castrillon et al. 2000; Reijo et al. 2000; Toyooka et al. 2000) are germ cell markers, and both were absent following busulfan treatment (Hermann et al. 2007). Furthermore, GFRα1 (GDNF receptor) and PLZF, consensus markers of stem and progenitor spermatogonia (Meng et al. 2000; Buaas et al. 2004; Ryu et al. 2004; Costoya et al. 2004; Buageaw et al. 2005; Ryu et al. 2005; Naughton et al. 2006), were also lost coincident with spermatogenic depletion (Hermann et al. 2007).

To augment the immunohistochemical evidence that busulfan depletes the primate SSC pool, the rhesus-to-nude mouse xenotransplantation assay was used to measure the effects of busulfan on the putative stem cell pool (Hermann et al. 2007). As previously demonstrated, germ cells from primate species including [rhesus macaques (Hermann et al. 2007, 2009), baboons (Nagano et al. 2001b) and humans (Nagano et al. 2002)] produce chains and patches of spermatogonia that resemble early rodent transplant colonies [reviewed in (Hermann et al. 2010)]. While primate xenotransplant colonies do not produce complete spermatogenesis,

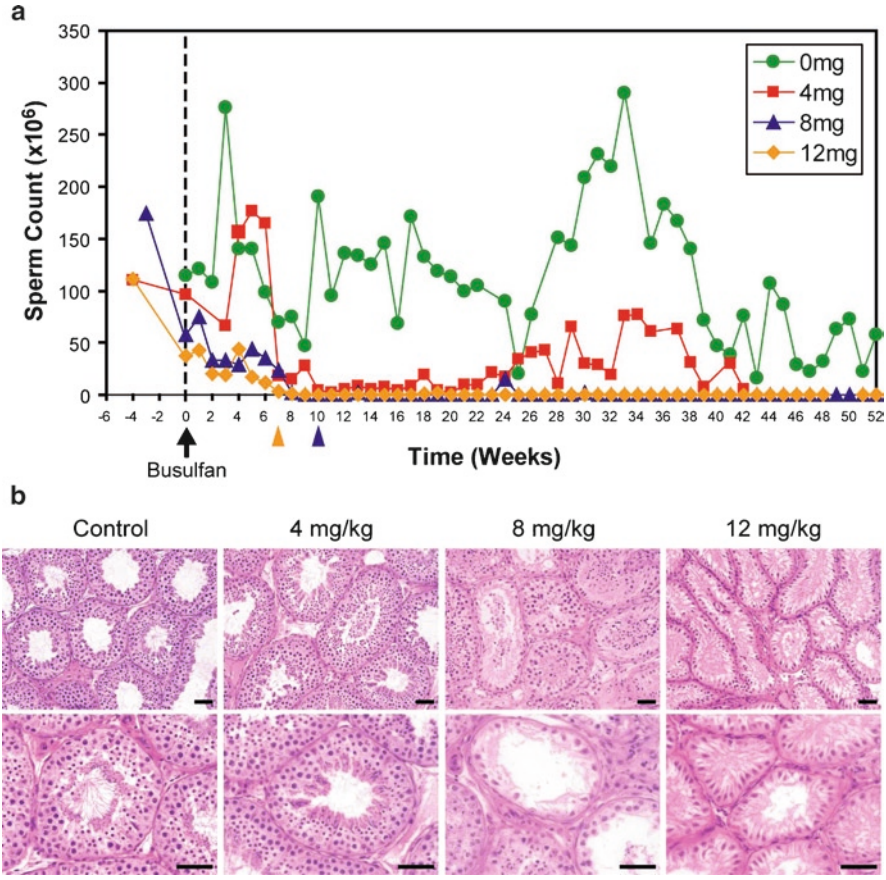

Fig. 11.2 Busulfan conditioning to generate an infertile recipient model. (**a**) To assess the effects of busulfan chemotherapy on spermatogenesis, sperm counts were measured weekly after treatment at doses of 0–12 mg/kg. Mortality was observed with animals treated at the higher doses of busulfan (8 and 12 mg/kg, *yellow* and *blue arrowheads*). (**b**) Hematoxylin & eosin staining was used to evaluate the effects of busulfan treatment on the extent of spermatogenesis in experimental animals before or after treatment. Scale bar = 50 μm. As noted (*black arrow* below x-axis), busulfan was administered at week 0. Note: samples for week 0 were collected prior to busulfan administration. Modified from (Hermann et al. 2007)

the resulting patches of spermatogonia are maintained long-term, and thus, may constitute a bioassay for primate SSCs that is more experimentally tractable than primate-to-primate transplantation. While normal adult rhesus testis cells produced 4.64 colonies/10^6 viable cells, testis cells isolated from males more than one year following high-dose busulfan treatment failed to produce any colonies of spermatogonia in the xenotransplantation assay (Hermann et al. 2007). The conclusion from these data was that high-dose busulfan treatment depleted SSCs in rhesus testes, resulting in a loss of endogenous spermatogenesis.

Busulfan doses that cause long-term infertility (e.g., 8–12 mg/kg) also cause severe hematopoietic deficits in some animals, sometimes with life-threatening and mortal consequences (Hermann et al. 2007). While the data also indicate that these negative side-effects are variable, prophylactic treatments (e.g., antibiotic regimen, blood transfusions) concurrent with and following busulfan treatment, along with peripheral blood mononuclear cell (PBMC) transplantation to restore the hematopoietic system, help to maintain animal health (Hermann and Orwig, unpublished).

11.3.6 Testicular Irradiation

Testicular irradiation is another approach that depletes the seminiferous epithelium of primate testes and may generate a suitable environment for SSC transplantation. In rhesus macaques, numbers of A_{dark}, A_{pale}, and B spermatogonia decline acutely to less than 5% of starting numbers in the first several weeks after low doses of radiation (1–4 Gy) (van Alphen et al. 1988). However, spermatogonial numbers return to 70% of pretreatment levels over the course of several months to a year after treatment with low-dose radiation (1–2 Gy) (van Alphen and de Rooij 1986). Thus, similar to results of 4 mg/kg busulfan treatment (see Fig. 11.2), low doses of testicular irradiation do not permanently ablate primate SSCs, and may not be suitable for testing the regenerative capacity of transplanted stem cells due to competition with remaining SSCs. In the prepubertal rhesus testis, radiation doses greater than 6 Gy are required for permanent depletion of germ cells, although the effect is heterogeneous between animals (de Rooij et al. 2002). High-dose irradiation (10 Gy) of immature rhesus monkeys led to a failure to achieve a pubertal increase in testis volume, although spermatogenesis was observed in one animal (Schlatt et al. 2009). While local irradiation to the testis avoids the potentially lethal side-effects observed with whole-animal chemotherapy treatments, evidence from rats suggests that radiation may damage the testicular somatic environment, leading to reduced ability of transplanted SSCs to engraft and produce spermatogenesis [(Zhang et al. 2006b); see Chap. 9]. Additional studies are needed to determine whether this complication is unique to the rat or extends to other species. In the end, radiation and chemotherapy may prove to be complimentary approaches to generate recipient animals with testes devoid of endogenous spermatogenesis suitable for SSC transplantation studies.

11.3.7 Ultrasound-Guided Rete Testis Injection

Ultimately, autologous SSC transplantation in primates is the only way to test the full regenerative potential of primate SSCs and evaluate the interaction between SSCs and their true niches located in seminiferous tubules. In rodents, the rete testis is readily visible on the surface of the testis and the efferent duct bundle can be cannulated to allow retrograde injection into recipient seminiferous tubules (Ogawa et al. 1997).

In contrast, primate caput epididymides are tightly adhered to the testis, the efferent ducts are not easily accessible, and rete testes are more centrally located in the testis. Thus, to visualize and access the rete testis, Schlatt and colleagues (Schlatt et al. 1999) initially described a method employing ultrasound to guide a needle into the rete testis space [Fig. 11.3a; (Hermann, Rodriguez and Orwig, unpublished)].

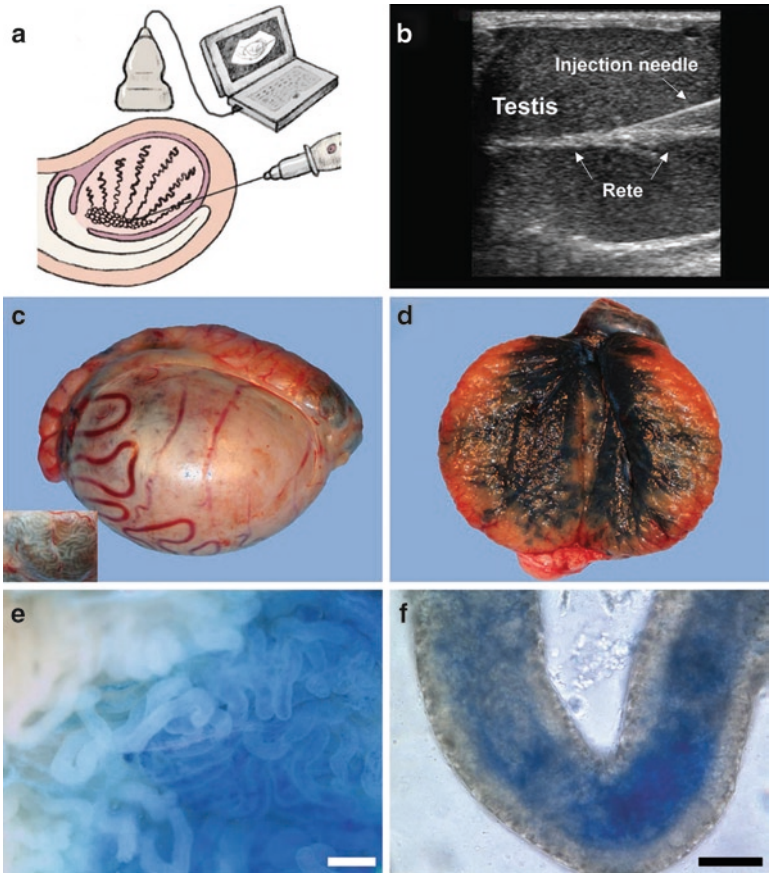

Fig. 11.3 Ultrasound-guided rete testis injection for SSC transplantation in primates. (**a**) To test the regenerative potential of nonhuman primate SSCs to produce complete spermatogenesis in the ablated testes of infertile recipient animals, it is necessary to transplant recipient testes using an ultrasound-guided rete testis injection procedure. (**b**) A needle is introduced transcrotally into the testicular parenchyma and the tip of the needle is placed into the rete testis space for retrograde injection of testis cell suspensions that contain SSCs. For demonstration purposes, trypan blue dye (10%) was injected into a rhesus testis, allowing visualization of injection success. (**c**) After recovering the injected testis, the testis is grossly normal and trypan blue dye was observed in the ductules of the caput epididymis (*inset*), which is contiguous via the efferent ducts with the rete testis, confirmed the success of these injections. (**d**) Bisection of the transplanted testis revealed that blue dye radiated from the rete testis into approximately 60–80% of seminiferous tubules. (**e**, **f**) Microscopic evaluation of intact seminiferous tubules from this recipient testis confirmed the presence of trypan blue dye in the lumen of the seminiferous tubules. Hermann, Rodriguez and Orwig, unpublished

An example of an ultrasound image of an adult rhesus testis is shown in Fig. 11.3b, where the rete testis space is visualized as an echodense line. An injection needle (22 Ga) is inserted into the testis trans-scrotally and the tip of the needle is placed within the rete testis space (Fig. 11.3b). Ultrasound-guided rete testis injection is percutaneous, and, thus, is considered a noninvasive procedure because the injection needle is simply passed through the scrotum into the testis. To illustrate this technique, the vital dye trypan blue was injected into rhesus macaque testes using this approach and testes were recovered immediately after injection by castration. After recovery, trypan blue dye could be visualized in the ductules of the caput epididymus, which is contiguous via the efferent ducts with the rete testis, confirming the success of these injections (Fig. 11.3c). Bisection of the transplanted testis revealed that blue dye radiated from the rete testis into approximately 60–80% of seminiferous tubules (Fig. 11.3d). Subsequent evaluation of intact seminiferous tubules confirmed the presence of blue dye in the lumen of seminiferous tubules (Fig. 11.3e, f). Thus, ultrasound-guided rete testis injection can be used to introduce donor SSCs into infertile recipient testes to test the regenerative potential of primate SSCs.

Dobrinski and co-workers have demonstrated the feasibility of performing SSC transplants in various other large animal models using ultrasound-guided rete testis injection (Honaramooz et al. 2002, 2003a, b; Kim et al. 2008). In goats, transplanted donor cells ultimately produced complete spermatogenesis, established fertility and transmitted the donor haplotype to progeny (Honaramooz et al. 2003b). Furthermore, transplants by ultrasound-guided rete testis injection were successfully performed between out-bred, immune competent goats and dogs, suggesting that the testis is an immune privileged site (D'Alessio et al. 2001, 2004; Kim et al. 2008).

In primates, injection of dye into seminiferous tubules using ultrasound-guided rete testis injection was initially performed in cynomolgus monkey and human testes, *ex vivo* (Schlatt et al. 1999). Furthermore, when donor germ cells preloaded with BrdU were transplanted into recipient monkey testes, labeled B-spermatogonia could be identified in recipient seminiferous tubules four weeks after transplant, demonstrating successful engraftment of donor cells into recipient monkey testes (Schlatt et al. 1999). Similar injections to assess SSC engraftment and spermatogenic regeneration have also been described in adult cynomolgous monkeys (*Macaca fascicularis*) that received 2 Gy of X-radiation (Schlatt et al. 2002a). In this study, transplanted testes exhibited modest increase in testis volume compared to contralateral testis (sham transplanted) during the 9 months after transplant (Schlatt et al. 2002a). Remarkably, the testes of two animals exhibited complete spermatogenic recovery. Despite promising results in these autologous SSC transplants, it was impossible to definitively identify donor spermatogenesis because donor cells were not marked. Thus, spermatogenesis arising from transplanted SSCs and recovering endogenous spermatogenesis could not be discriminated because the cells were identical. Similar autologous SSC transplants were performed in juvenile rhesus macaques that received 10 Gy radiation (Schlatt et al. 2009), but again, results of these transplant were equivocal since donor cell engraftment could not be definitively identified.

11.3.8 Definitive Autologous SSC Transplants in Primates

As discussed above, in an autologous monkey-to-monkey SSC transplant paradigm, detecting successful engraftment of transplanted stem cells is impossible if the cells are not marked to allow their discrimination from endogenous cells. In our experience, significant biological variability between out-bred monkeys causes some amount inconsistency in the degree of spermatogenic depletion following high-dose chemotherapy [(Hermann et al. 2007) and (Hermann and Orwig, unpublished)]. Thus, it is not adequate to assume that recovering spermatogenesis in treated animals following SSC transplants necessarily arises from transplanted cells. To address this problem, donor SSCs can be genetically marked with lentiviral vectors. The value of using lentiviral vectors to mark SSCs is their ability to genetically modify non-dividing cells (SSCs are thought to rarely divide). Vectors carrying marker genes (e.g., eGFP, dsRED) under the control of ubiquitous transcriptional regulatory sequences (e.g., the *UBC* promoter) are particularly useful for future evaluation of donor cell engraftment. Viral, marker, and promoter sequences can be detected in DNA prepared from ejaculated sperm of transplant recipients to provide confirmation of successful engraftment. Further, fluorescent reporter proteins can be used to identify sites and extent of donor-derived spermatogenesis in recipient testes. The efficiency of marking primate SSCs with lentivirus vectors, however, is unknown. Lentivirus transduction of SSCs can be as high as 40% in mouse and rat testis cell suspensions (Ryu et al. 2007), and so it is likely that a majority of primate SSCs would not be genetically marked for identification of donor cell engraftment. Alternatively, donor primate testis cells could be preloaded with a vital fluorescent dye (e.g., PKH26 or CFDA) prior to transplantation in recipient testes (Honaramooz et al. 2002; Maki et al. 2009). The fluorescent loading approach would mark most donor cells, but analysis is limited to 2–3 weeks after transplantation to minimize the dilution of the fluorescent dye through cell divisions.

11.3.9 Allogeneic SSC Transplants

To circumvent the limitation of efficient donor cell marking, it may be possible to utilize an allogeneic transplant paradigm where donor testis cells from an unrelated individual animal are transplanted into the recipient's testis. A potential drawback to the allogeneic transplant approach is that some or all donor cells could be immunologically rejected in allogeneic recipient primates. However, there are reports that transplantation of allogeneic testis cells are tolerated in large animal models allowing engraftment of unrelated donor SSCs (Honaramooz et al. 2002, 2003b; Kim et al. 2008). In primates, if the donor's cells are tolerated by the recipient's immune system, engraft, and produce spermatogenesis that results in ejaculated sperm, it is possible to discriminate sperm originating from donor and recipient germ cells using genetic techniques such as microsatellite DNA fingerprinting, SNP genotyping, or HLA allotyping. Microsatellite fingerprinting or SNP genotyping of ejaculated sperm DNA

can reliably detect donor chimerism in recipient ejaculates at levels of 1–5% and was used recently to detect donor sperm production in SSC-transplanted dogs (Kim et al. 2008). Pilot studies using rhesus macaque sperm have demonstrated that we can detect at least 1% donor chimerism in mixed samples of ejaculated sperm using microsatellite fingerprinting (Fig. 11.4). Alternatively, HLA allotyping PCRs are able

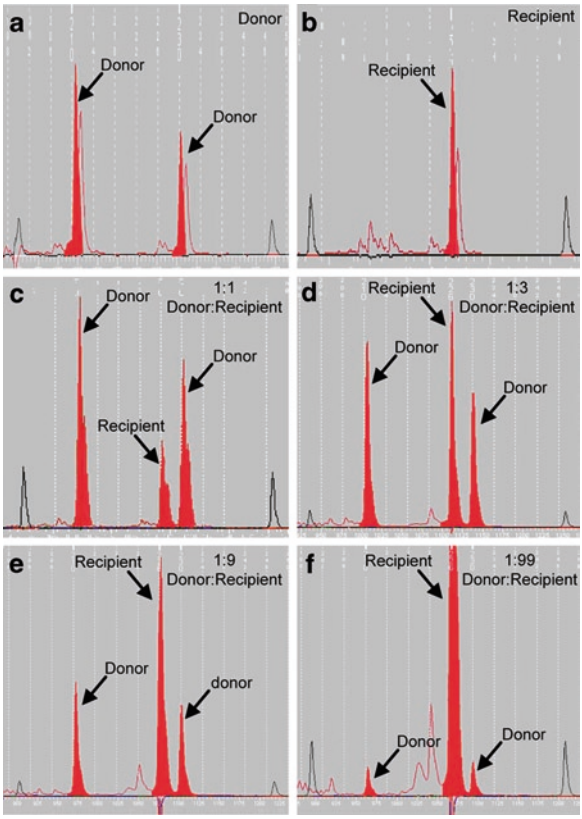

Fig. 11.4 Assessing transplanted SSC engraftment in allogeneic recipients by DNA microsatellite fingerprinting. Allogeneic rhesus-to-rhesus SSC transplants performed between unrelated individuals may offer the benefit of maximizing the detection of donor-derived spermatogenesis in recipient testes. If donor SSCs are tolerated by the recipient immune system, engraft, and produce sperm that are present in the ejaculate, it is possible to take advantage of the genetic differences between donor and recipient to definitively determine the presence of donor spermatogenesis. To demonstrate this phenomenon, sperm from two unrelated animals (putative donor and recipient) were genotyped at Veterinary Genetics Laboratory at the University of California at Davis. (**a**) The "donor" animal possesses the 212bp and 232bp alleles of microsatellite locus D13S765, while (**b**) the "recipient" animal possesses the 228bp allele of D13S765. Mixtures of sperm from the donor and recipient animals at defined ratios of (**c**) 1:1 donor:recipient (50% donor), (**d**) 1:3 donor:recipient (25% donor), (**e**) 1:9 donor:recipient (10% donor), (**f**) 1:99 donor:recipient (1% donor) were used to extract DNA for microsatellite fingerprinting to determine the threshold to detect donor chimerism. We found that we could detect as low as 1% donor chimerism. Therefore, microsatellite fingerprinting of ejaculated sperm could provide a sensitive, noninvasive method for detecting donor spermatogenesis in allogeneic transplant recipients

to detect different alleles of the MHC Class I antigens that vary between unrelated individuals, a strategy developed to identify peripheral blood chimerism (≥1%) following hematopoietic stem cell transplantation (Kean et al. 2007). To date there have been no reports of primate-to-primate SSC transplantation that provided definitive identification of donor-derived spermatogenesis.

11.4 Clinical Implications and Considerations for SSC Transplantation

As discussed at the beginning of this chapter, there is potential for utilizing SSCs to preserve the fertility of patients who will undergo sterilizing therapies for the treatment of a disease or condition. For this purpose, patient testicular tissue containing SSCs would need to be obtained prior to therapy, processed appropriately, and cryopreserved for future use. Considerations for clinical application of SSC transplant technologies include: (1) when and how to obtain patient testicular tissue, (2) how much tissue is required for future use, (3) appropriate techniques for tissue processing and cryopreservation, and (4) risks of malignant cell contamination in cancer patient testicular tissue.

11.4.1 Patient Testis Accrual

Obtaining testicular tissue from patients for preserving testicular cells must occur prior to potentially gonadotoxic therapies. In most cases, testicular tissue would be recovered from patients using a subcapsular, "open" biopsy approach with care taken to access the parenchyma through an avascular region of the tunica albuginea (e.g., towards the middle of the medial, lateral, or anterior surface of the testis) to minimize bleeding and potential scarring/fibrosis to the remaining tissue. The amount of testicular tissue recovered by biopsy should meet the expected need for producing a cell suspension useful for eventual SSC transplantation without any additional manipulations. The biopsy surgery, though, should balance the amount of tissue removed with the goal of leaving the remaining tissue potentially functional.

For patients who will receive chemotherapy or radiation for treatment of their primary disease, the patients' surgeon(s) would need to determine hemostasis after surgery and provide clearance for initiation of therapy, thus introducing the possibility of delaying their primary therapy. In most cases, treatments would begin in a timeframe dictated by clinical management of their primary disease or condition, typically within one week. In some cases, chemotherapy or radiation treatments have been initiated as soon as one day following testicular biopsy surgery (Bahadur et al. 2000). Besides the inherent risks of surgery, the potential costs to patients is another concern

since SSC preservation and transplantation are not likely to be covered by patients' healthcare insurance while the techniques are in the experimental stage. Efforts should be made to minimize or eliminate the potential financial burdens to patients.

11.4.2 Testis Cell Isolation in the Clinic

Isolation of testicular cells from human patients intended for eventual retransplantation back into the patient falls under the purview of the U.S. Food and Drug Administration's Center for Biologics Evaluation and Research (CBER), by the authority of the Public Health Service (PHS) Act §351(a). However, autologous cryopreserved testicular tissues and cells isolated, prepared, and cryopreserved in particular ways could be considered exempt from PHS Act §351 regulation and would fall under PHS Act §361. In order to qualify for §361 exemption, testicular cell "products" would have to meet all of the criteria in FDA regulations 21 CFR 1271.10(a), including: (1) the product is minimally manipulated, (2) the product is intended for homologous use (i.e., spermatogenesis), (3) product "manufacture" does not involve combination with another article, except for water, crystalloids, or a sterilizing, preserving, or storage agent (not raising new clinical safety concerns for the HCT/P), and (4) the product does not have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function or, if it has such an effect, it is intended for autologous use or allogeneic use in close relatives or for reproductive use. Thus, human testicular cells intended for ultimate retransplantation into a patient must be processed using reagents suitable for human use and in Clinical Laboratory Improvement Amendment (CLIA)-certified facilities by certified personnel.

Patient testicular cells for eventual autologous transplant must also be cryopreserved for long-term storage. There is limited information about cryopreservation of human testicular cells. Human testis cells cryopreserved for 3 months have been shown to exhibit similar xenotransplant colonization potential to freshly isolated cells, although viability and recovery of frozen-thawed cells is not known (Nagano et al. 2002). Similar outcomes were observed for cryopreserved adult and juvenile rhesus macaque testis cells, which exhibited similar phenotypic and functional attributes to fresh testis cells (Hermann et al. 2007, 2009). Additional studies are necessary to determine the best conditions for freezing human testicular cells to maximize recovery of healthy SSCs for future therapeutic application.

11.4.3 Human SSC Culture

In vitro SSC amplification using culture is one potential method for maximizing the therapeutic potential of SSCs recovered from a testicular biopsy. Robust protocols for culturing mouse and rat SSCs have been reported over the past several years

(Kanatsu-Shinohara et al. 2003; Kubota et al. 2004a, b; Ryu et al. 2005; Hamra et al. 2005). These methods allow for rodent SSCs to be maintained for a very long time (perhaps indefinitely) with a significant amplification in numbers [doubling time: mouse, 5.6 days (Kubota et al. 2004a); rat, 3–4 days (Hamra et al. 2005) or 11 days (Ryu et al. 2005)]. Progress was also reported recently in two studies that established conditions for culturing human spermatogonia, potentially including SSCs (Sadri-Ardekani et al. 2009; He et al. 2010). In the first study, testicular tissue from six adult men who underwent orchiectomy for prostate cancer treatment was used for cell isolation and culture (Sadri-Ardekani et al. 2009). Testicular cell suspensions from these donors were initially depleted of somatic cells by differential plating on plastic overnight and subsequent maintenance on uncoated plastic in StemPro-34 medium containing EGF, LIF, GDNF, and soluble GFRα1. Cultures of testicular cells established in this way contained cells expressing spermatogonial markers (e.g., PLZF) and could be maintained in short-term culture in the absence of feeder cells for up to 15 weeks with up to 53-fold amplification in colonization activity as determined by xenotransplantation (Sadri-Ardekani et al. 2009). Subsequent subculture of these cells onto laminin-coated dishes allowed for longer maintenance of up to 28 weeks and significant amplification of xenotransplant colonization activity (up to 18,450-fold) (Sadri-Ardekani et al. 2009). The latter study by He et al. used testicular tissue from five organ donors to isolate cells for culture (He et al. 2010). Testicular cell suspensions from organ donors were depleted of somatic cells by differential plating on plastic for 3 h (He et al. 2010). Subsequently, GPR125-positive spermatogonia were enriched by MACS and positive cells were maintained on gelatin-coated plates in StemPro-34 medium supplemented with bFGF (NUDT6), EGF, LIF, GDNF, soluble GFRα1, TGFbeta, and Nodal. Cultures containing cells expressing spermatogonial markers (e.g., GPR125, ITGA6, THY1, and GFRA1) could be maintained for 2 weeks with a fivefold increase in numbers (He et al. 2010). It is important to note that the testicular tissue for both studies was initially cryopreserved and thawed prior to cell isolation in a way that might be suitable for future translation to the clinic. If these methods are broadly applicable to human donor testis tissue and reproducible, it could be possible to isolate a small testicular biopsy from a patient and produce enough therapeutic SSCs in culture for future transplantation.

11.4.4 Risk of Reintroducing Malignant Cells During SSCs Transplants

Transplantation of cryopreserved testis cells isolated from patients with malignant diseases carries an inherent risk of reintroducing contaminating malignant cells back into patients. Caution is certainly warranted as Jahnukainen and co-workers have demonstrated that as few as 20 leukemic cells transplanted to a rat testis can result in the development of terminal leukemia within 21 days (Jahnukainen et al. 2001). A recent report indicated that MACS sorting for CD4 was ineffective for

removing cancer cells from the testis cells isolated from leukemic rats prior to transplantation (Hou et al. 2009). Similarly, *in vitro* "contaminated" mouse and human testis cell suspensions were not completely depleted of malignant cells using combinations of FACS or MACS strategies, although the degree of sorting purity in this study was poor (Geens et al. 2007). In contrast, when MHC class I (H-2K^{b}/H-2D^{b})$^{-}$, CD45^{-} germ cells were isolated from leukemic mouse whole testis cell suspensions using a fluorescence-activated cell sorter (FACS), all recipient mice survived (Fujita et al. 2005). In addition, those recipient mice displayed functional donor-derived spermatogenesis as demonstrated with the production of live progeny by ICSI. Similarly, germ cells from human testis cell suspensions could be separated from five leukemia and three lymphoma cell lines based on FACS sorting for CD45 and MHC Class I when the sorting strategy was appropriately tailored to the phenotype of the cancer cells (Fujita et al. 2006). Thus, it appears feasible to separate germ cells from cancer cells prior to autologous SSC transplantation when optimal sorting strategies are employed. Effective sorting strategies to enrich SSCs and remove malignant cells require detailed examination of the SSC cell surface phenotype and the corresponding phenotype of the particular malignancy for each patient.

11.4.5 Ethical Considerations

Development and implementation of testicular tissue/cell cryopreservation for fertility preservation must involve a discussion of the risks and benefits to potential patients. First and foremost, harvesting and freezing testicular tissue from patients who will undergo potentially gonadotoxic therapies is considered experimental and may involve risks. The potential risks include general anesthesia and surgical biopsy of the testis, the potential for vascular damage or fibrosis to the remaining testicular tissue, or the possibility of loss of the biopsied testis entirely due to infection or necrosis. Although the risk of infertility is high in patients undergoing certain chemotherapy and radiation regimens, there is always a chance that patients can retain or spontaneously recover spermatogenesis in spite of the potentially sterilizing treatments. In this case, surgery to remove testicular tissue would have been unnecessary and patients would be exposed to undue risk without any benefit. Moreover, since potential complications of testicular biopsy include infection and reduced testosterone (Bruun et al. 1987; Dieckmann et al. 2005; Manning et al. 1998), it is theoretically possible for patients to experience reduced testicular function, and potential infertility due to testicular tissue harvesting, and, thus, the fertility preservation procedure could potentially be detrimental. It should be noted that a recent study reported no negative outcomes during or after surgery in 14 boys who received testicular biopsies for fertility preservation prior to gonadotoxic therapy (Ginsberg et al. 2010).

On the flipside, cryopreserving testicular tissue or cells provides several potential benefits for patients. Currently there are no therapies to preserve the future

fertility of preadolescent boys and some adult men who cannot utilize standard of care approaches (e.g., sperm banking or TESE). However, as discussed extensively in this volume and chapter, there are new reproductive therapies under development and may one day offer "fertile hope" to those patients that do not currently have access to fertility preserving therapies. When no established fertility sparing or preserving options are available, it may be reasonable to offer harvesting and cryopreservation of gonadal tissue as a possible means of fertility preservation. In this way, patients who bank their testicular tissue prior to gonadotoxic therapy have a potential resource for future fertility. For instance, patients may have the opportunity to utilize their stored gonadal tissue or cells for fertility restoration procedures in the future. Since autologous SSC transplantation in humans is experimental and not proven effective for restoring spermatogenesis or fertility, though, the potential benefit is not clearly defined. One clinic in the United Kingdom preserved testicular tissue from 12 patients prior to treatment for Hodgkin's lymphoma and subsequently 7 patients received autologous transplants of cryopreserved cells into their rete testes (Brook et al. 2001; Radford 2003). There have been no follow-up reports to indicate whether these transplants restored spermatogenesis. Regardless of the uncertainty about whether SSC preservation and subsequent transplantation will safeguard fertility in human cancer survivors, there is a *potential* benefit to patients by cryopreserving their tissue that seems to outweigh the risks.

A growing body of literature suggests that parents of boys at risk for future infertility are interested in fertility-preservation options and place a strong value on the potential future benefits. A retrospective survey indicated nearly two thirds of parents of boys with childhood cancer would have allowed collection and cryopreservation of their sons' testicular tissue for fertility preservation, had it been available even as an experimental intervention (van den Berg et al. 2007). Thus, there is perceived acceptability and desire to undergo experimental therapy to preserve fertility, as long as treatment for the primary disease is not compromised (Oosterhuis et al. 2008). More recently, a study performed the University of Pennsylvania reported that 76% of parents of eligible boys (3 months to 14 years) consented to testicular biopsy prior to gonadotoxic therapy (Ginsberg et al. 2010).

In addition to the potential fertility-preserving direct benefits to subjects, there may also be psychological benefits [reviewed by (Schover 2009a, b)]. As discussed previously, there is clear parental desire to preserve fertility in childhood cancer patients, even in cases where only experimental interventions are available (van den Berg et al. 2007; Oosterhuis et al. 2008). Furthermore, around 75% of cancer patients who are of reproductive age also express a desire for future children and many are interested in exploring options for fertility preservation (Zebrack et al. 2004). Many patients that were not informed of the infertility risks before treatment are angry to learn of their infertility years later (Lee et al. 2006). Therefore, patients or parents of children who elect to participate in experimental fertility-preserving options will have the peace-of-mind that they have prospectively done *something* to give themselves or their children *a chance* for future fertility where there was a high risk of infertility in the absence of intervention.

11.5 Conclusions

Progress in animal models has generated tremendous excitement about the potential of SSC transplantation to treat some cases of male infertility (e.g., secondary to cancer therapy), if responsibly developed. Specifically, progress in nonhuman primate models is providing valuable preclinical data that will facilitate translation to the clinic, including: development of primate testis cell isolation techniques, refinement of recipient conditioning protocols to create animals devoid of spermatogenesis, optimization of the SSC transplant technique in large animal models, and development of methods for evaluating transplant. Application of these approaches in the clinical setting will require special consideration of procedure cost, patient safety, and regulatory oversight, which have implications for the methods of tissue accrual, processing, and preservation. These challenges are the subject of vigorous investigation in several laboratories around the world and will help to make the goal of fertility preservation a reality for patients facing the prospects of life-long infertility resulting from lifesaving gonadotoxic therapies.

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