Stem Cell Biology and Regenerative Medicine

Kursad Turksen *Editor*

Adult Stem Cells

Second Edition

Humana Press

Stem Cell Biology and Regenerative Medicine

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Preface

Over the last decade, stem cells and their potential to be manipulated for regeneration of particular tissues and organs have become an intensely investigated topic. Notwithstanding the efforts, there are still many challenging issues in identifying the putative tissue-specific stem cell subpopulation(s) and coaxing them to adopt the fate or fates of interest. In this volume, the authors define in a variety of tissue types the specific stem cell populations, their locations, and approaches being developed and used to demonstrate their potential in physiologically relevant assays. I thank the contributors for their efforts in capturing in their chapters both the promise of the stem cells of interest and the obstacles in maximizing their potential utility.

Ottawa, ON, Canada — Kursad Turksen

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Signaling Pathways Maintaining Stemness in Adult Hematopoietic Stem Cells

Patricia Arreba-Tutusaus and Florian H. Heidel

Abstract Division of hematopoietic stem cells needs to generate daughter cells harboring identical developmental capacity as the mother cell. This step is necessary to maintain hematopoiesis over several decades and implicates the ability of stem cells to self-renew. Self-renewal is a key characteristic of hematopoietic stem cells (HSC). Signaling pathways, epigenetic modulators, cell cycle regulators, and transcription factors control the fine balance between self-renewal and differentiation. Self-renewal-associated signaling includes chemical modulators (e.g., prostaglandins, retinoic acid), developmental regulators (e.g., BMPs), and evolutionary conserved pathways such as canonical Wnt signaling, Notch signaling, or the Hedgehog pathway. These signaling nodes contribute to self-renewal in embryonic and stem cell development. However, in adult stem cells they seem to be dispensable to some extent. Members of the cellular polarity network may display a novel class of signaling molecules associated with self-renewal capacity in development and maintenance of hematopoietic stem cells.

Keywords Hematopoietic stem cell • Self-renewal • Signaling

Abbreviations

ACD	Asymmetric cell division
BMP	Bone morphogenetic proteins
CMML	Chronic myelomonocytic leukemia
Ctnnb1	β-Catenin
$dmPGE_2$	Di-methyl-prostaglandin E_2

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FLC	Fetal liver cells
GMP	Granulocyte–monocyte progenitor
Hh	Hedgehog
Hox genes	Homeobox genes
HSC	Hematopoietic stem cell
LT-HSC	Long-term hematopoietic stem cells
NICD	Notch intracellular domain
Ptch	"Patched" receptor
RA	Retinoic acid
Smo	"Smoothened"

1 Stemness and Self-Renewal

Maintenance of body tissue homeostasis is an important physiological process. It is regulated through programmed cell death with cells being eliminated from the organism and replaced by new ones. This replacement is guaranteed by the presence of self-renewing stem cells in different tissues that do not exhaust over a long period of time.

The hematopoietic system is a clear example of this cellular replacement, as most of the blood cells have a short life span. In hematopoiesis, new mature blood cells are formed in a hierarchical process [1]. At the top of this hierarchy are the hematopoietic stem cells (HSC), a limited pool of immature cells [2] that maintains hematopoiesis. The existence of stem cells was first proposed in the hematopoietic system by Ernest McCulloch and James Till in 1961 during their groundbreaking work on radiation sensitivity of normal mouse bone marrow cells [3]. Later, their and other studies described two key properties of these cells: self-renewal capacity [4] and the multipotential differentiation into more specialized and differentiated cells [5]. Thus, hematopoietic stem cells need to differentiate into blood and immune cells on one hand and to maintain hematopoiesis over decades on the other. This property—to maintain an immature state without exhaustion—is frequently referred to as "stemness" or "self-renewal capacity." "Self-renewal" is frequently discussed as a "single" phenomenon. However, it can probably be seen as a combination of three phenomena resulting in maintenance of a balanced number of stem cells: (1) proliferation, (2) inhibition of apoptosis, and (3) inhibition of differentiation [6]. To understand the genetic machinery implicated in regulation of self-renewal is crucial for stem cell biology, not only because of its physiologic role but also because of its implication in leukemia development. Focusing on hematopoietic stem cells, multiple studies have shown that their "stemness" is regulated by extrinsic and intrinsic stimuli [7]. Regarding cytokines or growth factors, extrinsic signals are implicated under environmental influence. On the other hand, transcription factors, chromatin modifiers, and cell-cycle regulators have also been described as intrinsic factors. Self-renewal of hematopoietic cells needs to be functionally assessed by transplantation assays. Transplantation of a single HSC into a

previously lethally irradiated host can reestablish all hematopoietic lineages. Transplantation of these HSC in secondary (or tertiary) recipients can assess for the degree of self-renewal capacity.

Several signaling nodes and pathways have been described as regulators of stemness or self-renewal in HSC. Many of these pathways (e.g., PI3K-AKT-mTOR signaling) fulfill various functions including proliferation, control of the apoptotic machinery, or differentiation of cells. On the other hand, they are also involved in maintenance of self-renewal and interact with evolutionary conserved pathways (e.g., Wnt, Hedgehog or Notch signaling). In this chapter, we will focus on murine evolutionary conserved signaling pathways implicated in regulation of hematopoietic stem cell self-renewal, their cross talk with other signaling pathways, and their downstream effectors.

2 Signaling Pathways Implicated in HSC Self-Renewal

2.1 Canonical Wnt Signaling

The Wnt signaling pathway plays a critical role in embryonic and hematopoietic development. Wnt proteins are secreted glycoproteins, which can be released or presented on cell surface, and induce different pathways, with the canonical pathway being the best described. Activation of the pathway occurs by binding of its physiological ligand Wnt to the cystine-rich domain of receptors of the Frizzled (Fz) and LRP (low-density lipoprotein receptor related) family (LRP 5 or LRP6). The canonical pathway is regulated through its central player β-catenin. In the absence of Wnt ligand, β-catenin is recruited by a multifactor complex formed by glycogen synthase kinase-3β (GSK-3), casein kinase 1 (CK1), adenomatous polyposis coli (APC) a tumor suppressor protein, and the scaffolding protein axin [8]. Axin promotes GSK-3β-dependent phosphorylation of β-catenin for the ubiquitin–proteasome pathway, maintaining low levels of the protein. The activation of canonical Wnt signaling blocks β-catenin phosphorylation and degradation, leading to an accumulation of unphosphorylated β-catenin in the cytoplasm and this unphosphorylated version is able to shuttle into the nucleus [9]. Nuclear β-catenin binds to T-cell factor (TCF)/lymphoid-enhancer factor (LEF) transcription factors and promotes gene expression. Bona fide downstream targets include Cyclin D1 (CCND1), c-jun, or c-myc. Functional analysis of β-catenin (Ctnnb1) loss could not be performed in a conventional knockout mouse model due to early embryonic lethality. Loss of Ctnnb1 leads to embryonic lethality due to lack of mesoderm formation and defects of the ectodermal cell layer.

Investigation of Wnt/β-catenin activity in early embryonic development showed that it is transiently required in the AGM to generate long-term HSCs and to produce hematopoietic cells derived from the AGM endothelial precursors in vitro. Genetic inactivation of β-catenin from the embryonic endothelium stage

Table 1 Requirement of evolutionary conserved signaling pathways during HSC development and in maintenance of adult HSC

Evolutionary conserved signaling pathways	HSC development	Adult HSC
Wnt signaling	+++ [10, 11]	– [14]
Notch signaling	+++ [16, 17]	(+) [18, 19]
Hedgehog signaling	+++ [20, 21]	– [22, 23]

(using VE-cadherin-Cre recombinase), but not from embryonic hematopoietic cells (using Vav1-Cre), precluded progression of mutant cells toward the hematopoietic lineage. These findings indicate that Wnt/β-catenin signaling is needed for the emergence but not the maintenance of HSCs in murine embryonic development [10].

Use of Vav1-Cre in conditional knockout mouse models leads to genetic inactivation of the respective gene in early hematopoietic development. Conditional genetic inactivation of β-catenin during HSC development generated hematopoietic cells impaired in long-term growth and maintenance following transplantation. These cells show a clear competitive disadvantage when transplanted against wild-type competitor cells [11]. Enforced Ctnnb1 activation leads to exhaustion of the LT-HSC pool [12] and is associated with loss of the GMP stage [13].

Analysis of canonical Wnt signaling in maintenance of adult HSC has been performed in very detail using conditional knockout mouse models. In contrast to its role in HSC development, genetic inactivation of β-catenin (and also γ-catenin to address potential compensatory effects) in adult HSC left steady state hematopoiesis unaffected. Maintenance of adult HSC was also not impaired as assessed by transplantation assays [14]. However, a recent publication could demonstrate convincingly that different levels of Wnt signaling are required to regulate steady-state hematopoiesis in a dosage-dependent fashion [15]. Here, depending on Wnt pathway activity, downstream signaling nodes are able to activate HSC self-renewal and to activate or block differentiation capacity.

Taken together, canonical Wnt signaling is crucial for HSC development but is not required for maintenance of self-renewal in fully developed adult HSC (Table 1). Precisely regulated levels of Wnt signaling seem to be crucial for maintenance of hematopoiesis. Canonical Wnt signaling connects with other HSC-relevant pathways and mediators such as retinoic acid signaling and prostaglandin signaling. Moreover, its activation leads to alteration in gene expression of both transcription factors (e.g., Cyclin D1, c-jun, or c-myc) as well as developmental gene sets (such as Hox genes).

2.1.1 Chemical Modulators Interacting with Canonical Wnt Signaling

Prostaglandins

Prostaglandins, a group of lipid compounds that are derived enzymatically from fatty acids have recently been shown to regulate HSC biology and to interact closely

with canonical Wnt signaling. Using zebrafish as a model organism, the Zon laboratory performed chemical screens to identify new regulators of hematopoietic stem cells. They found that stimulation with prostaglandin E2 (PGE_2) significantly increased HSC numbers [24]. Moreover, they were able to provide evidence that PGE_2 interacts with the canonical Wnt signaling pathway to regulate HSCs through cAMP–PKA-mediated β-catenin phosphorylation [25]. Stability of PGE_2 was a critical point that led to the use of the stable derivative $dmPGE_2$. Consistent with previous findings, $dmPGE_2$ increased the frequency of long-term repopulating HSCs in irradiated recipient mice. These experiments paved the way for a future therapeutic potential and $dmPGE_2$ is currently tested in preclinical [26] and clinical trials in the context of hematopoietic stem cell transplantation.

Retinoic Acid

Retinoic acid (RA) is another signaling molecule interacting with canonical Wnt signaling and inducing Hox genes during embryonic development [27, 28]. RA binds to its surface receptor and this leads to rapid internalization and translocation into the nucleus. Binding to the respective nuclear hormone receptors results in transcriptional modulation of homeobox genes (Hox genes, e.g., HoxA4, HoxB5, Meis1), zincfinger proteins (e.g., Egr-1, RARA), kinases (e.g., CSF1R, EGFR, LYN), and parts of the TGFβ pathway [29]. Retinoic acid has been shown to preserve self-renewal capacity during ex vivo culture and consecutively led to increased competitive advantage of HSC during serial transplantation [28]. In murine models, genetic inactivation of retinoic acid receptor gamma (RARg) resulted in pronounced impairment of HSC function [30].

2.2 Notch Signaling

The Notch signaling pathway is highly regulated and requires a specific cell–cell interaction between Notch ligand and its receptor. In the mammalian system four receptor isoforms (Notch1–4) exist, while five canonical notch ligands are classified into Jagged and Delta-like families (Jag1, Jad2, Dll1, Dll3, and Dll4). Upon ligand binding, the pathway is activated by a double proteolysis of the receptor. First, it becomes extracellularly modified (S2 site) by an "a-disintegrin-and-metalloprotease" (ADAM). Subsequently the notch intracellular domain (NICD) is cleaved by a gamma secretase/presenilin complex and released to the cytoplasm. The NICD is able to shuttle from the cytoplasm to the nucleus to contribute to an active transcription complex with the DNA-binding transcription factor CSL/RBP-Jk. This complex is stabilized by the coactivator Mastermind-like (MAML1-3), among others, switching on the transcription of target genes, such as Hes family genes, c-Myc, CCND1, or Notch1 and 3. In absence of Notch, CSL/RBP-Jk is found in a corepressor complex [31].

In the hematopoietic system, Notch is essential for the development of hematopoietic stem cells (HSCs) during embryonic hematopoiesis [17]. There is conflicting data on the role of Notch signaling in the function of adult stem cells (HSCs) and myeloid differentiation. These differences may be explained by the experimental approach and the extent of Notch modulation.

It has been shown that Notch signaling can suppress myeloid differentiation in vitro [32]. Moreover, it has been reported that Notch signaling can induce megakaryocyte differentiation [33]. Further studies have convincingly shown that Notch signaling can function as an antagonist of the granulocyte–monocyte progenitor (GMP) cell fate and that loss of Notch signaling biases commitment toward GMP differentiation [19].

In gain-of-function experiments, induction of canonical Notch signaling enhanced self-renewal of HSC and led to HSC expansion in vitro. Retroviral overexpression of members of the Notch signaling pathway (such as Notch1 or Hes1), has shown an induction of self-renewal capacity, an increase in stem cell numbers, and a preservation of long-term reconstitution capacity [34–36]. This accumulation of precursor cells has been also shown when HSC are co-cultered with an engineered Notch ligand Delta1 [37]. Therefore, upregulation of Notch signaling seems to promote self-renewal and to inhibit differentiation of HSC.

Genetic inhibition of Notch signaling revealed more inconsistent results. While an inducible knockout of murine Notch1 compromised T cell linage development, the other lineages of the bone marrow appeared not to be affected [38]. A conditional double knockout of Notch1 and its ligand Jagged1 also presented with normal steady-state hematopoiesis, hematopoietic stem cell self-renewal, and differentiation [18]. In both studies this lack of a phenotype could be caused through redundancy from other Notch receptors or ligands involved in the pathway. Moreover, modulation of Notch downstream effectors was also assessed in several models. Notch signaling was inhibited by the expression of a dominant-negative Master-mind-like1 construct (DNMAML) or by conditional deletion of Rbpj, both crucial components for the transcriptional complex of Notch target genes [39]. These models did not show any defect on self renewal capacity of HSC.

Inactivation of Notch signaling has also been investigated by conditional targeting of Nicastrin (Ncstn), a member of the gamma secretase complex reported to be one of the few nonredundant members of canonical Notch signaling. Genetic inactivation of Ncstn was achieved by the use of two different Cre recombinases (Mx1-Cre and Vav1-Cre) with consistent results: Genetic deletion of Ncstn in mouse hematopoietic stem cells (HSCs) resulted in an aberrant accumulation of granulocyte/monocyte progenitors (GMPs), extramedullary hematopoiesis, and the induction of a myeloproliferative/myelodysplastic phenotype (CMML-like) in vivo [19]. This is consistent with the discovery of Notch-inactivating mutations in patients suffering from CMML. Within the murine system, combination of Notch inactivation with other mutations (such as Tet2 mutations) resulted in overt leukemia [40].

Taken together, genetic deletion of Notch receptors or modulation of ligands and complex members results in no significant phenotype in adult HSC. Abrogation of Notch signaling by conditional deletion of Nicastrin seems to affect adult HSC, leading to aberrant self-renewal and transformation into a pre-leukemic state.

2.3 Hedgehog Signaling

Hedgehog (Hh) signaling pathway is highly conserved in vertebrates and takes place at the primary cilium, although this structure is expendable in hematopoietic cells. Hedgehog protein requires posttranslational modifications and three isoforms are described: Sonic (Shh), Desert (Dhh), and Indian (Ihh) hedgehog that are expressed in hematopoietic tissue. The receptor, Patched (Ptch), is a 12-transmembrane protein which acts as a negative modulator. In absence of Hedgehog, Ptch represses the signal transducer Smoothened (Smo), a 7-transmembrane G-like protein-couple receptor, through oxysterol trafficking. Once Hedgehog binds to Ptch, Smo inhibition is interrupted allowing its translocation to the membrane and activation by phosphorylation [41–43]. Downstream, Smo targets and stabilizes from ubiquitinylation zinc-finger transcription factors members of the Gli family, which translocate to the nucleus. Gli1 and Gli2, as activators, switch on the transcription of target genes important for proliferation and survival. On the other hand, Gli3 along with Gli2 serve as repressive transcription factors when Ptch is active and Smo is not able to inhibit their degradation.

Abrogation of the Hh pathway using conventional knockout mouse models was embryonic lethal: Smo knockout mouse embryos as well as Shh knockouts died during embryogenesis with severe neuronal malformations [44]. In order to investigate the impact of Smo on development of hematopoiesis and HSC, $Smo^{-/-}$ and $Ptch^{+/-}$ fetal liver cells (FLC) have been used and transplanted into recipient mice [21]. In these assays, $Smo^{-/-}$ FLC showed reduction of colony-forming potential in methylcellulose CFU-replating assays, while $Ptch^{+/-}$ FLC (in which Smo is activated) showed enhanced serial replating capacity [21]. Transplantation of these FLC into irradiated mice showed improved engraftment of $Ptch^{+/-}$ cells when compared with wild-type cells under homeostatic conditions. This was also detectable during acute regeneration indicating a functional role for the Hh pathway in HSC development and regeneration. The increase of Hh activity has been studied in a *Ptch*$^{+/-}$ mouse model, where HSC show activation of Hh signaling already during their development. Here, it was demonstrated that Hh signaling plays a role in expansion of hematopoietic stem cells, but constitutive activation of the pathway exhausted HSC by affecting cell cycle modulators [45]. The impact of Hh signaling was also investigated using conditional $Smo^{loxP/loxP}$ mice. These mice were crossed with Vav1-cre mice, in order to genetically inactivate Smo early in embryogenesis in the hematopoietic system. In this system no acute effect was observed on steady-state hematopoiesis or composition of the different cellular compartments. However, a defect in long-term HSC function could be detected through serial primary and secondary bone marrow transplants [20]. Conditional deletion of Smo has been performed in hematopoietic cells to study the impact of Hh signaling on adult HSC. Two recent papers used conditional $Smo^{loxP/loxP}$ mice that were intercrossed with Mx1-cre mice to conditionally inactivate Smo in hematopoietic cells after interferon stimulation. In contrast to the studies during HSC development, these reports demonstrated that the Hh pathway is dispensable for adult HSC function and

hematopoiesis [22, 23]. Consistently with these findings, pharmacological inhibition of Hh signaling—using a small molecule Smo inhibitor—had no effect on normal hematopoiesis in adult mice. Moreover, global gene expression profiling underlined that an HSC-specific gene expression signature was preserved in the Smo-deficient HSC and a Smo gain-of-function model (SmoM2) did not lead to expansion of HSC.

The impact of Hh signaling on HSC has been variable, depending on the experimental system, which reflects the importance of Hh signaling during specific periods of hematopoietic development. On the other hand, Hh signaling seemed to be dispensable for adult HSC in steady-state hematopoiesis.

2.3.1 Developmental Regulators Interacting with Hh Signaling

Bone morphogenetic proteins **(BMPs)** such as **Smad4** and the connected transformed growth factor beta **(TGFβ)** signaling have been described to cross talk with Hedgehog signaling in regulating HSC abundance [46]. Smad-signaling molecules are phosphorylated and regulated downstream of TGF receptor. Smad4 is a critical component of the downstream complex consisting of activating and inhibitory components. This complex can shuttle to the nucleus to activate transcriptional targets [47]. Here, TGFβ is thought to be involved in maintenance of HSC quiescence. The functional role of Smad4 in HSC had not been investigated because of the early embryonic lethality of the conventional knockout mouse model. Taken together, these pathways seem to have regulatory functions during embryonic development and organogenesis. Genetic inactivation of Smad4 in adult HSC was investigated using a conditional knockout mouse model. Conditional deletion of Smad4 was achieved by Mx1-Cre recombinase activation through interferon. Inactivation of Smad4 showed a severe defect especially in erythropoiesis. Serial transplantation experiments confirmed an important role for Smad4 in adult HSC self-renewal [48]. Downregulation of Notch1 and c-myc expression in $Smad4^{-/-}$ cells connects Smad4 to Notch and Wnt-signaling networks.

2.4 Hox Genes Regulate Self-Renewal Downstream of Evolutionary Conserved Signaling Pathways

Hox genes have been described as downstream effectors of self-renewal associated signaling pathways. Overexpression of **Hox genes** can increase self-renewal capacity of hematopoietic stem cells and has been shown to increase the immature cell compartment in several mouse models. Retroviral overexpression of HoxB4 or HoxA9 in murine bone marrow HSC revealed its relevance in proliferation and regeneration of primitive HSC without compromising their repopulation capacity [49, 50]. Recent studies with a similar approach confirmed that HoxB4 overexpression expands and increases cell growth of HSC without altering hematopoietic

homeostasis [51, 52]. However, while HoxB4 presents as a positive regulator of primitive hematopoietic cells and leaves the differentiation capacity largely unaffected, overexpression of HoxA9 led to expansion of the myeloid lineage and finally can initiate leukemic transformation. Conversely, a double knockout model for HoxB3 and HoxB4 led to a significant decrease in HSC proliferation without impairment of differentiation capacity [53]. This impact on proliferation was also observed in single HoxB4 knockout mice, although the effect was more moderate [54]. Modest overexpression of HoxA10 in murine HSC resulted in significant gain of self-renewal capacity; however, cell fate was affected in terms of a differentiation block toward megakaryopoiesis and erythropoiesis [55]. Cofactors that modulate Hox gene expression such as PBX- and CDX-genes as well as MEIS1 are known to drive leukemia development [56–59]. Overexpression of the respective genes in murine hematopoiesis leads to perturbation of myeloid differentiation and induction of self-renewal capacity in adult HSC [60, 61]. Taken together, there is clear evidence that self-renewal of HSC is linked to Hox gene expression.

2.5 Polarity Regulators and RNA-/DNA-Binding Proteins

Cell fate decisions are influenced by proteins, organelles, mRNAs, or microRNAs being segregated into one or the other daughter cell during cell division. These types of hematopoietic stem cell division have been described as “symmetric” or “asymmetric” [62]. Regulation of cell polarity may impact asymmetric cell division (ACD) of HSC. ACD has been described to be involved in differentiation of the progeny and maintenance of “stemness” and is an important part of cell polarity that may have an important impact on both hematopoiesis and leukemia development. ACD regulates the induction and maintenance of polarity during cell division, resulting in the generation of two daughter cells with different genetic properties. As a consequence, the asymmetrically localized proteins often include determinants of cell fate. In regular hematopoiesis, ACD is involved in maintenance of the HSC pool. When a stem cell divides, one daughter cell follows a genetic program inducing proliferation and differentiation, while the second daughter cell stays under a program, inducing quiescence and the capacity for longevity. ACD leads to asymmetric segregation of self-renewal to one daughter cell in HSC [62]. Therefore, regulation of balance between symmetric and asymmetric cell division is assumed to be crucial for hematopoietic stem cell (HSC) division and homeostasis.

Cell fate determinants, such as RNA-binding proteins or polarity regulators have been recently described as effectors in stem cell biology. The Sauvageau laboratory investigated the impact of 21 polarity regulators selected from the literature. A total of eight polarity-regulating genes evolved from a primary screen (Ctnna, Mapro3, Llgl1, Numb, Prox1, Pard6a, Prkcz, Msi2). Four of these genes have been validated and RNAi knockdown experiments revealed enhanced (Prox1) or decreased (Pard6a, Prkcz, Msi2) repopulation potential of purely sorted HSC in vivo [63]. Of these

candidates, several genes have been assessed for their functional impact on HSC self-renewal. The RNA-binding protein Msi2 was found to be highly expressed in adult HSC and its knockdown resulted in reduced engraftment and depletion of HSCs in vivo [64]. In contrast, overexpression of human *MSI2* in a mouse model increased HSC cell cycle progression and enforced leukemic transformation. Msi2 mutant mice (using a gene-trap model) showed a significant reduction in the frequency and absolute number of the HSC-enriched (LSK−) population [65]. Moreover, this reduction in Msi2 expression impaired leukemic transformation indicating a potential loss of self-renewal capacity.

Genetic inactivation of atypical PKCs such as aPKCzeta and aPKClambda in the Par complex did not have any impact on maintenance or self-renewal capacity of adult HSC [66]. Even double-knockout HSC did not reveal any impact of the respective aPKCs on competitive behavior. In contrast, genetic inactivation of the Scribble complex member Llgl1 led to enhanced fitness and self-renewal potential of adult hematopoietic stem cells in vivo. This advantage increased upon serial transplantation or when stress was applied to HSCs [67]. Llgl1 deletion was associated with transcriptional repression of transcription factors such as Krüppel-like factor 4 (Klf4) and early growth response 1 (Egr1), known inhibitors of HSC self-renewal [68, 69]. Moreover, self-renewal-associated genes, such as Hox gene cofactor Pbx3 were significantly upregulated. Both genes, aPKCs and Llgl1, are known to be involved in regulation of cell polarity, proliferation, and cell division. They belong to a group of three major polarity complexes: Par, Scribble and Crumbs complex [70]. In other cell types, this network has been reported to interact with several stem cell relevant pathways, such as canonical Wnt signaling, Notch signaling, and the Hippo pathway [70, 71]. However, none of these interactions have been functionally confirmed so far in hematopoietic stem cells. Moreover, these polarity nodes interact with Rac/Rho GTPases known effectors of stem cell mobility and viability. Cdc42 is an example of a small Rho-GTPase that has been reported to interact with the Par-polarity complex. This gene seems to play an essential role in embryonic development, as conventional genetic inactivation was embryonically lethal [72]. Recent publications were able to provide evidence that Cdc42 activity is significantly increased in aged hematopoietic stem cells [73]. Elevated level of Cdc42 in HSCs was causally linked to stem cell aging and correlated with a loss of polarity. These data provide evidence for a potential influence of the polarity network on HSC biology.

In summary, evolutionary conserved pathways such as canonical Wnt, Notch, or Hedgehog signaling contribute crucially to HSC self-renewal during embryonic development and stem cell development. However, in adult stem cells they seem to be dispensable to some extent. These signaling pathways interact with chemical modulators (e.g., PGE_2 signaling, RA signaling) and developmental regulators (such as TGFβ and BMP signaling). Members of the cellular polarity network interact with self-renewal-associated signaling molecules and therefore may display a novel class of signaling molecules associated with self-renewal capacity in development and maintenance of hematopoietic stem cells.

References

1. Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132(4):631–644
2. Ogawa M (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81(11):2844–2853
3. McCulloch EA, Till JE (1964) Proliferation of hemopoietic colony-forming cells transplanted into irradiated mice. Radiat Res 22:383–397
4. Siminovitch L, McCulloch EA, Till JE (1963) The distribution of colony-forming cells among spleen colonies. J Cell Physiol 62:327–336
5. Till JE, McCulloch EA, Siminovitch L (1964) A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc Natl Acad Sci U S A 51:29–36
6. Zhang J, Li L (2005) BMP signaling and stem cell regulation. Dev Biol 284(1):1–11
7. Zon LI (2008) Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. Nature 453(7193):306–313
8. Behrens J et al (1998) Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280(5363):596–599
9. Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. Cell 149(6):1192–1205
10. Ruiz-Herguido C et al (2012) Hematopoietic stem cell development requires transient Wnt/beta-catenin activity. J Exp Med 209(8):1457–1468
11. Zhao C et al (2007) Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12(6):528–541
12. Scheller M et al (2006) Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol 7(10):1037–1047
13. Kirstetter P et al (2006) Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol 7(10):1048–1056
14. Koch U et al (2008) Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. Blood 111(1):160–164
15. Luis TC et al (2011) Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell Stem Cell 9(4):345–356
16. Guiu J et al (2013) Hes repressors are essential regulators of hematopoietic stem cell development downstream of Notch signaling. J Exp Med 210(1):71–84
17. Robert-Moreno A et al (2008) Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. EMBO J 27(13):1886–1895
18. Mancini SJ et al (2005) Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood 105(6):2340–2342
19. Klinakis A et al (2011) A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature 473(7346):230–233
20. Zhao C et al (2009) Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 458(7239):776–779
21. Dierks C et al (2008) Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell 14(3):238–249
22. Gao J et al (2009) Hedgehog signaling is dispensable for adult hematopoietic stem cell function. Cell Stem Cell 4(6):548–558
23. Hofmann I et al (2009) Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. Cell Stem Cell 4(6):559–567
24. North TE et al (2007) Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 447(7147):1007–1011
25. Goessling W et al (2009) Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. Cell 136(6):1136–1147
26. Goessling W et al (2011) Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models. Cell Stem Cell 8(4):445–458

27. Shiotsugu J et al (2004) Multiple points of interaction between retinoic acid and FGF signaling during embryonic axis formation. Development 131(11):2653–2667
28. Nordstrom U et al (2006) An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. PLoS Biol 4(8):e252
29. Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid Res 43(11):1773–1808
30. Purton LE et al (2006) RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. J Exp Med 203(5):1283–1293
31. Bigas A, Espinosa L (2012) Hematopoietic stem cells: to be or Notch to be. Blood 119(14): 3226–3235
32. de Pooter RF et al (2006) Notch signaling requires GATA-2 to inhibit myelopoiesis from embryonic stem cells and primary hemopoietic progenitors. J Immunol 176(9):5267–5275
33. Mercher T et al (2008) Notch signaling specifies megakaryocyte development from hematopoietic stem cells. Cell Stem Cell 3(3):314–326
34. Varnum-Finney B et al (2000) Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 6(11):1278–1281
35. Stier S et al (2002) Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 99(7):2369–2378
36. Kunisato A et al (2003) HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. Blood 101(5):1777–1783
37. Varnum-Finney B, Brashem-Stein C, Bernstein ID (2003) Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. Blood 101(5):1784–1789
38. Radtke F et al (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 10(5):547–558
39. Maillard I et al (2008) Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell Stem Cell 2(4):356–366
40. Lobry C et al (2013) Notch pathway activation targets AML-initiating cell homeostasis and differentiation. J Exp Med 210(2):301–319
41. Dahmane N et al (1997) Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. Nature 389(6653):876–881
42. Lee J et al (1997) Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 124(13):2537–2552
43. Ikram MS et al (2004) GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. J Invest Dermatol 122(6):1503–1509
44. Zhang XM, Ramalho-Santos M, McMahon AP (2001) Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. Cell 106(2):781–792
45. Trowbridge JJ, Scott MP, Bhatia M (2006) Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. Proc Natl Acad Sci U S A 103(38):14134–14139
46. Bhardwaj G et al (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat Immunol 2(2):172–180
47. Blank U, Karlsson G, Karlsson S (2008) Signaling pathways governing stem-cell fate. Blood 111(2):492–503
48. Karlsson G et al (2007) Smad4 is critical for self-renewal of hematopoietic stem cells. J Exp Med 204(3):467–474
49. Sauvageau G et al (1995) Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev 9(14): 1753–1765
50. Thorsteinsdottir U et al (2002) Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 99(1):121–129
51. Amsellem S et al (2003) Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med 9(11):1423–1427

52. Antonchuk J, Sauvageau G, Humphries RK (2002) HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109(1):39–45
53. Bjornsson JM et al (2003) Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4. Mol Cell Biol 23(11):3872–3883
54. Brun AC et al (2004) Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. Blood 103(11):4126–4133
55. Magnusson M et al (2007) HOXA10 is a critical regulator for hematopoietic stem cells and erythroid/megakaryocyte development. Blood 109(9):3687–3696
56. Faber K et al (2013) CDX2-driven leukemogenesis involves KLF4 repression and deregulated PPARgamma signaling. J Clin Invest 123(1):299–314
57. Li Z et al (2013) PBX3 is an important cofactor of HOXA9 in leukemogenesis. Blood 121(8):1422–1431
58. Rawat VP et al (2004) Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia. Proc Natl Acad Sci U S A 101(3):817–822
59. Scholl C et al (2007) The homeobox gene CDX2 is aberrantly expressed in most cases of acute myeloid leukemia and promotes leukemogenesis. J Clin Invest 117(4):1037–1048
60. Rawat VP et al (2008) Overexpression of CDX2 perturbs HOX gene expression in murine progenitors depending on its N-terminal domain and is closely correlated with deregulated HOX gene expression in human acute myeloid leukemia. Blood 111(1):309–319
61. Schnabel CA, Jacobs Y, Cleary ML (2000) HoxA9-mediated immortalization of myeloid progenitors requires functional interactions with TALE cofactors Pbx and Meis. Oncogene 19(5):608–616
62. Wu M et al (2007) Imaging hematopoietic precursor division in real time. Cell Stem Cell 1(5):541–554
63. Hope KJ et al (2010) An RNAi screen identifies Msi2 and Prox1 as having opposite roles in the regulation of hematopoietic stem cell activity. Cell Stem Cell 7(1):101–113
64. Kharas MG et al (2010) Musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia. Nat Med 16(8):903–908
65. Ito T et al (2010) Regulation of myeloid leukaemia by the cell-fate determinant Musashi. Nature 466(7307):765–768
66. Sengupta A et al (2011) Atypical protein kinase C (aPKCzeta and aPKClambda) is dispensable for mammalian hematopoietic stem cell activity and blood formation. Proc Natl Acad Sci U S A 108(24):9957–9962
67. Heidel FH et al (2013) The cell fate determinant Llgl1 influences HSC fitness and prognosis in AML. J Exp Med 210(1):15–22
68. Min IM et al (2008) The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. Cell Stem Cell 2(4):380–391
69. Park CS et al (2011) Loss of Krueppel-like factor 4 (KLF4) leads to increased self-renewal under stress conditions and improved survival of hematopoietic stem cell. Blood (ASH Annual Meeting Abstracts)
70. Humbert PO, Dow LE, Russell SM (2006) The Scribble and Par complexes in polarity and migration: friends or foes? Trends Cell Biol 16(12):622–630
71. Menendez J et al (2010) A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proc Natl Acad Sci U S A 107(33):14651–14656
72. Chen F et al (2000) Cdc42 is required for PIP(2)-induced actin polymerization and early development but not for cell viability. Curr Biol 10(13):758–765
73. Xing Z et al (2006) Increased hematopoietic stem cell mobilization in aged mice. Blood 108(7):2190–2197

The Adult Stem Cell Niche

Aravind Ramakrishnan, Manoj M. Pillai, and Beverly J. Torok-Storb

Abstract Tissue-specific adult stem cells generally exist in a quiescent state with only a small percentage actively dividing to meet the demand of homeostatic tissue replacement. However, a significant number of stem cells can be recruited into cycle in response to injury. The actively dividing stem cell pool will produce cells that differentiate to replace the mature cells that were damaged, but will also rigorously maintain a critical number of stem cells. Given that stem cells have tremendous potential for use in tissue repair and replacement, understanding of how these stem cell fates are controlled has become an area of intense research. However, our ability to expand stem cells ex vivo for therapeutic purposes is still poorly developed, probably due to a lack of understanding of critical factors that maintain pluripotency. Much of our understanding of stem cell regulation comes from studies of the hematopoietic system, including the concept of a stem cell niche, a specialized microenvironment that maintains stem cells in the pluripotent state. In this chapter, we will review the major concepts that have emerged regarding the identity of the cellular/secreted components that influence stem cell fate.

Keywords Stem cell • Niche • Microenvironment • Macrophage • Stromal cell • Osteoblast • Endothelium

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Abbreviations

BMPRIA	Bone morphogenic protein receptor type IA
CAR	CXCL12 abundant reticular cell
CFU-F	Colony-forming unit-fibroblast
CFU-S	Colony-forming unit-spleen
ColA1	Collagen A1
CXCL12	Chemokine, CXC motif, ligand 12
CXCR4	Chemokine, CXC motif, receptor 4
DNA	Deoxyribonucleic acid
DsRED	Red fluorescent protein from *Discosoma* species
GFP	Green fluorescent protein
HPV-E6/E7	Human papilloma virus
Hs27a	Human stromal cell line 27a
Hs5	Human stromal cell line 5
HSC	Hematopoietic stem cell
IRES	Internal ribosome entry site
LTC	Long-term culture
MCP-1	Monocyte chemotactic protein-1
MDS	Myelodysplastic syndrome
ME	Microenvironment
MMP-9	Matrix metalloproteinase 9
MPD	Myeloproliferative disorder
MSC	Marrow stromal cell
NGFR	Nerve growth factor receptor
PPR	Parathyroid hormone receptors
RARγ	Retinoic acid receptor gamma
RNA	Ribonucleic acid
SBDS	Shwachman–Bodian–Diamond syndrome protein
SCF	Stem cell factor
SDF1	Stromal derived factor 1
SL	Steel
SLAM	Signaling lymphocytic activation molecule
SNO	Spindle-shaped N-cadherin-positive osteoblasts
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late activation protein 4

1 Early Seminal Work

Much of our understanding of stem cell biology comes from the study of the hematopoietic system, and hematopoietic stem cell transplantation remains the only successful application of stem cell therapy in humans. Till and McCulloch were the first to identify an adult stem cell in the hematopoietic tissue by demonstrating that

colonies forming in the spleen (CFU-S) of irradiated mice after bone marrow transplantation were clonal [1, 2], and the contents of one colony could reconstitute a lethally irradiated mouse. Very soon after the discovery of the hematopoietic stem cell (HSC), it became abundantly clear that these stem cells do not exist in isolation and are not autonomous but, rather, require a specialized microenvironment.

Much of the early evidence for the requirement of a supporting environment comes from the study of naturally occurring mutant mice, particularly the SL/SL or "Steel" mutant, which die spontaneously of severe anemia, and the more viable SL/SLd mice which are severely anemic even though both have a normal stem cell compartment as evidenced by transplantation studies [3]. The defect in these mice lies in the microenvironment as the SL/SLd mouse cannot be rescued after irradiation by an infusion of bone marrow cells; however, transplantation of an intact spleen, which subsequently becomes the site of normal hematopoiesis, is able to rescue these animals [3, 4]. Subsequently, the mutated gene product that gives rise to the Steel phenotype was identified as Kit ligand, or stem cell factor (SCF), a critical cytokine expressed by bone marrow stromal cells [5, 6].

These studies in mutant mice, together with 40 years of experience with human marrow transplantation, established the critical concept that the stromal components of the microenvironment are not derived from HSCs and cannot be transplanted through intravenous infusion [7].

Studies by Wolf and Trentin also suggested that the microenvironment was critical in directing stem cell fate [8]. In their seminal experiments, small pieces of intact bone marrow were implanted within the spleen of a mouse prior to irradiation and stem cell transplantation. After recovery from transplantation, the spleen was harvested and microscopic evaluation showed that spleen colonies that bridged the two different microenvironments were mixed such that there was mostly erythroid differentiation on the splenic side while myeloid differentiation predominated on the bone marrow side [8].

While these studies highlighted the importance of the microenvironment in normal hematopoiesis, the identity of the cells and/or their secreted products that can support the expansion of stem cells without loss of their pluripotent potential has yet to be determined. This is most likely due to the requirement of multiple signals that must act in concert to generate the "stem cell niche." This concept was first hypothesized by Schofield in 1978, as he struggled to resolve inconsistent data regarding the interpretation of CFU-S as the ultimate HSC [9]. Schofield noted that bone marrow cells could be transplanted indefinitely, even from older mice, without loss of repopulating ability; however, the repopulation of ability of CFU-S was limited and eventually lost upon serial transplantation. To address this issue, Schofield postulated that the CFU-S, although it was pluripotent, was not the true HSC; rather, he proposed that the true HSC could only be found in the bone marrow in association within a specialized microenvironmental (ME) unit, or niche, where its stem cell potential could be maintained. He also postulated that once a stem cell occupies its niche, it becomes a "fixed" tissue; if a stem cell were to move out of its niche, it would start differentiating [9]. The identity of the cellular and secreted components of the niche, which traditionally has been thought to originate from non-hematopoietic-derived

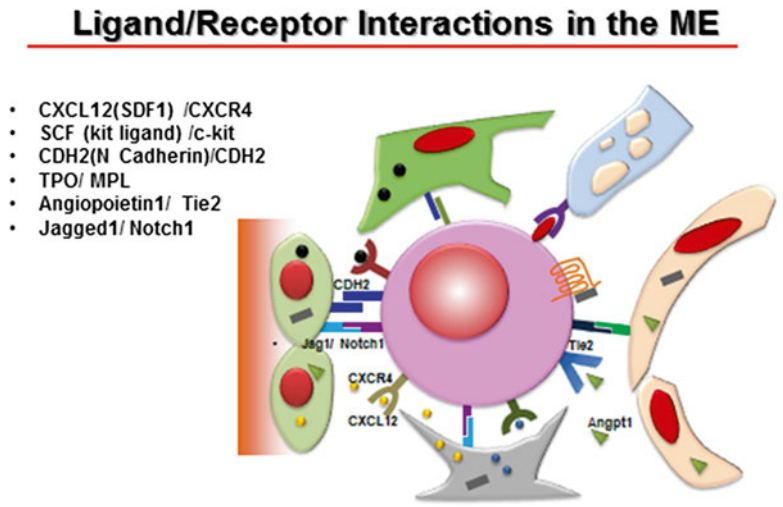

Fig. 1 Schematic representing the major ligand/receptor interactions in the stem cell niche

tissues, has been an area of active research using in vitro systems, genetic mouse models, and live imaging techniques and will be discussed below.

While there is general agreement that a number of signaling pathways including c-kit/SCF [10, 11], CXCR4/CXCL12 [12–14], VCAM1/VLA-4 [15, 16], Tie2/angiopoietin [17], c-mpl/thrombopoietin [18–20], notch/jagged-1 [21, 22], and osteopontin [23–25] contribute to the stem cell niche (see Fig. 1), they are not sufficient for this purpose. There is even less known about the identity of the cells that produce these activities and how they are regulated.

2 In Vitro Systems to Study the Hematopoietic Microenvironment

In vitro studies to approximate the ME have relied on the long-term culture (LTC) system, initially described by Dexter [26]. When aspirated bone marrow is plated in appropriate conditions, an adherent layer, thought to be composed of multiple cell types including fibroblasts, endothelial cells, macrophages, adipocytes, osteoclasts, and extracellular matrix, is established which can support in vitro hematopoiesis for several weeks (see Fig. 2). As mentioned above, the LTC only approximates the ME as myeloid cell production is generally favored over that of erythroid.

The LTC cultures are very complex, so it has been difficult to determine the contributions of individual cell types [27]. To dissect the stromal contribution, culture systems were developed to promote stromal growth over hematopoietic growth. When plated at low densities, a subset of stromal cells that are clonal can be identified (colony-forming unit-fibroblast/CFU-F assay) [28, 29]. These cultures are also commonly referred to as marrow stromal cell (MSC) cultures but are

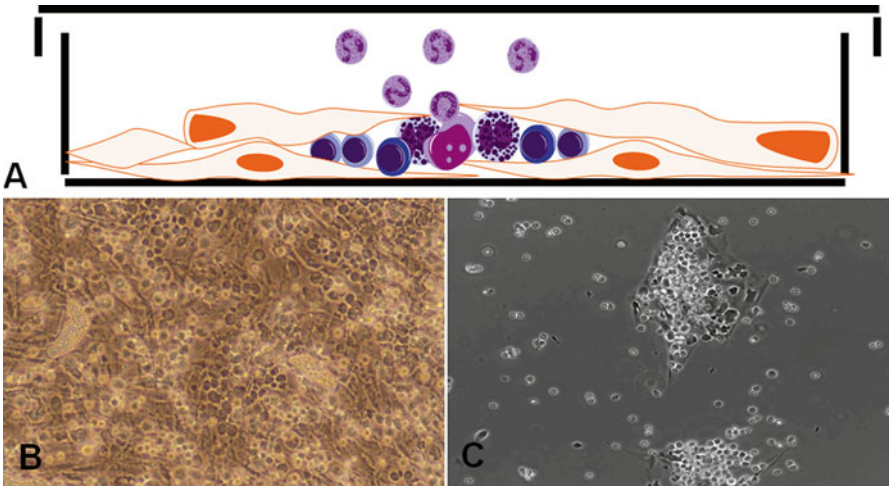

Fig. 2 Panel **a**: Schematic representation of Dexter long-term culture (LTC). A complex adherent layer of various stromal elements forms and supports the production of hematopoietic cells. As hematopoietic cells mature, they are released from the adherent layer into the media. Panel **b** depicts a phase-contrast photomicrograph of a typical human LTC showing its complexity. Panel **c** depicts a cobblestone forming area where progenitors crawl under the stromal layer and appear less refractile, and maturing forms are released from the layer and appear phase *bright*

still heterogeneous. To overcome the heterogeneity of stromal cell cultures, several groups have attempted to enrich for the CFU-F population using cell surface markers such as STRO-1, STRO-3, CD49a, CD63, CD90, NGFR, CD105, CD106, CD140b, and CD146 [30–38]. Of these markers, CD146 appears to be the most useful as recent studies demonstrate that cells expressing high levels of CD146 can transfer a functional hematopoietic microenvironment when transplanted into immunodeficient mice [38].

Another approach taken to overcome the heterogeneity of the stromal compartment is to generate stromal cell lines. Our group has contributed to this effort by generating cloned stromal cell lines by immortalizing cells from a normal LTC with HPV-E6/E7 [39]. Two such lines generated in our lab, designated Hs5 and Hs27a, are functionally distinct, have been extensively characterized, and have in vivo counterparts that can be identified in human marrow using immune histochemistry [39, 40]. Gene expression analysis and functional studies reveal that Hs27a expresses most of the activities associated with the stem cell niche such as CXCL12 and CD146 at high levels [40, 41]. It is also able to maintain primitive CD34+ cells in cobblestone forming areas. This is in contrast to Hs5 which secretes myelopoietic cytokines in large quantities and leads to differentiation of hematopoietic cells [39]. These lines continue to be useful in studying niche biology and recently were used to define a role for microRNAs in the niche [41]. Recent studies in our laboratory using DNase1 fingerprinting to determine the identity of these stromal cells suggest that Hs27a cells and their CD146-positive in vivo counterparts are actually fibroblasts and not osteoblasts (M Iwata, personal communication).

3 Mouse Models

Over the past several decades, knockout and transgenic mouse models have been used to identify the various signaling/adhesion molecules as well as cell types that are associated with stem cell niche function. However, the identity of the cells that make up the stem cell niche still remains an area of active debate (see Fig. 3). Two prevalent models have emerged, and there is compelling data to support both models. One model proposes that stem cell maintenance critically depends on osteoblasts that are found along the endosteal surface of bone. It was recognized as early as the late 1970s that hematopoietic progenitors were enriched in the endosteal region of bone marrow [42, 43]. Additional studies have demonstrated that cells expressing surface markers associated with HSC function home preferentially to the endosteal regions after transplantation [22, 44–46]. The "osteoblastic niche" model was strongly supported by two independent studies that documented that increases in osteoblast number also led to an increase in the size of the HSC pool. The first study showed that conditional inactivation of BMP receptor type IA (BMPRIA) led to an increased number of spindle-shaped N-cadherin-positive osteoblastic (SNO) cells [45]. In these mutant mice, HSCs expressing N-cadherin were found to interact with SNO cells in the bone marrow in a homotypic manner and also to be increased in number [45]. The second study showed that expression of constitutively active parathyroid hormone receptors (PPRs), using an osteoblast-specific promoter ColA1, led to increased expression of Notch ligand, Jagged1, in these cells and also increased numbers of HSC [22]. The other "endothelial niche" model proposes that HSCs localize close to endothelial cells that line marrow sinusoids. Evidence for the

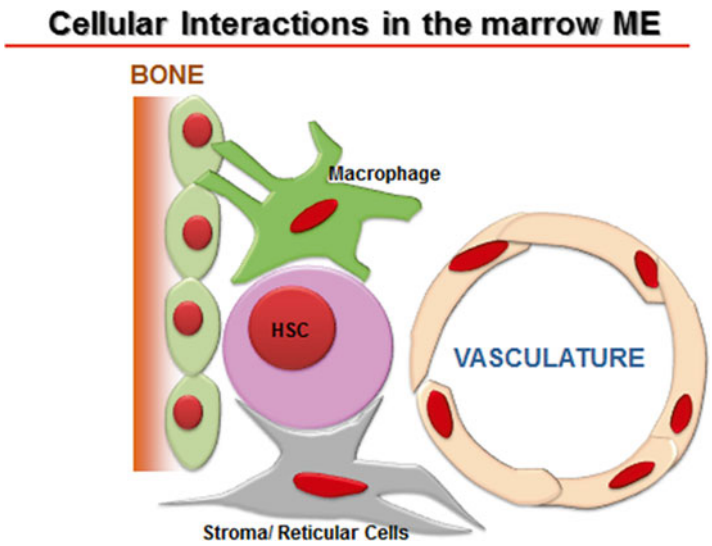

Fig. 3 Schematic representing the major cellular interactions in the stem cell niche

"endothelial niche" comes from experiments where cells expressing the SLAM family of surface receptors, which are highly expressed in HSCs, were detected in close association with vascular endothelium [46].

However, there are conflicting data that do not support either of the two proposed model systems. Studies in which osteoblasts are conditionally ablated have shown that there is no obvious effect on the HSC compartment [47]. Conditional deletion of N-cadherin also had no impact on hematopoiesis [48, 49]. Other murine studies using reporter mice suggest that reticular cells that express high levels of CXCL12/SDF1 (CAR cells) or Nestin+ mesenchymal progenitors essentially define the stem cell niche, and ablation of these cells results in reduced HSC numbers [13, 50].

The murine knockout models summarized above have helped to identify specific receptor–ligand combinations that are required for HSC niche regulation. However, important questions regarding the functional organization and regulation of the HSC niche components cannot be answered by simple knockout or gene-targeting models. Relevant factors are known to be produced by multiple cell types within the niche. For example, CXCL12 is expressed by osteoblasts, endothelial cells, while MSC and Angiopoietin1 are expressed by endothelial cells, MSC, and megakaryocytes [13, 38, 51–53]. Defining the source and regulated expression of these factors within the ME is as important as defining their function. Genetic models that rely on tissue specificity of promoters and site-specific recombinases, such as the cre-lox system, also have their limitations. Tissue specificity of promoters amongst cells of mesenchymal origin is clearly not absolute and likely exists in a spectrum of activity (e.g., from the primitive multipotent mesenchymal progenitors to fully differentiated osteoblasts or adipocytes). Relying on reported specificity of promoters to express fluorescent markers or to express tissue-specific recombinases is hence likely to lead to erroneous conclusions of how a biological function may be restricted to a particular cell type. Similarly, site-specific recombination techniques widely used to achieve deletion of genetic elements at specific stages of development are also often incomplete; even a small proportion of cells escaping recombination may result in erroneous interpretation of results.

Since some of the niche-defining factors, such as SCF, Angiopoieitn1, and CXCL12, are nonredundant in function, one refinement of the above murine models would be to define the cellular sources of the above factors within the marrow ME by appropriate genetic models in mice. Transgenic mice that utilize short stretches of endogenous promoter DNA elements that drive the expression of a fluorescent protein are clearly the easiest model to achieve the former, but this approach has serious limitations unless the promoter has been rigorously validated to recapitulate the tissue distribution of gene expression in vivo. Although this is often assumed, it is not always the case—as has been shown for transgenic mice using progressively longer constructs of SCF promoter without capturing the in vivo distribution of SCF in any of the constructs [54, 55]. This discrepancy is likely due to the effect of chromosomal elements much farther away than those included in the immediate upstream promoter or the importance of intronic regions, noncoding RNAs, RNA binding proteins, and other non-promoter elements which together result in the specific pattern of expression.

To faithfully reproduce the endogenous pattern of expression for genes such as SCF, one can express a fluorescent protein from the endogenous locus (either by replacing the gene or by adding an IRES-fluorescent protein construct; the former will result in fluorescent protein from one locus in hemizygotes, while homozygotes for fluorescent protein will be essentially gene knockouts. This is, however, a technically challenging endeavor needing several different knock-in mice that target endogenous loci of these factors. A report by Sean Morrison's group that focused on expression of SCF (SCF GFP) across multiple cell types, combined with a second report that focused on the expression of DsRed from the CXCL12 locus (CXCL12 DsRED) [56, 57], indicated that both factors seemed to be expressed at the highest level in a perivascular location within MSC and endothelial cells. These observations are consistent with one prevailing notion that the HSC niche is constituted by cells that are endothelial or at the very least perivascular [56, 57]. Tissue-specific deletion of both SCF and CXCL12 from specific populations of cells, including MSC, osteoblasts, osteoprogenitors, and endothelial cells, reported concurrently by Morrison's group and by Daniel Link's group, is in general agreement with this hypothesis [56–58]; however, they do suffer from the assumption of promoter specificity and have to be interpreted with caution for that reason alone.

4 Stem Cell-Derived Progeny Contribute to Niche Regulation

Traditionally, it has been thought that the cells that participate in the maintenance of the niche are derived from non-stem cell populations. However, data generated over the past few decades suggest that the progeny of stem cells play a significant role in the regulation of niche function in many different organs [59]. It has long been known that macrophages and T cells play an important role in stem cell function. T cells are also the only known source of interleukin 3, a critical factor for HSC maintenance and differentiation [60]. Hematopoietic stem cell transplants are usually unsuccessful if recipients fail to obtain high donor T-cell chimerism [61]. Recent murine in vivo imaging studies suggest that T regulatory cells are found in close association with and are necessary for maintaining allogeneic HSC after transplantation [62].

The monocyte/macrophage also plays a significant role in the regulation of hematopoiesis and has long been known to be an important component of the Dexter Culture [27]. A specialized macrophage, also known as the "nurse cell," is an important component of the erythroblast island [63–65]. Osteoclasts, a specialized type of macrophage, are critical in Ca+ homeostasis in the bone, which has also been implicated in stem cell maintenance [66]. As illustrated in Fig. 4, CD68-positive macrophages have a significant presence in the marrow and have numerous cell processes that interact with many cell types, suggesting a crucial role in the regulation of hematopoiesis. Furthermore, as shown in the Fig. 5, many

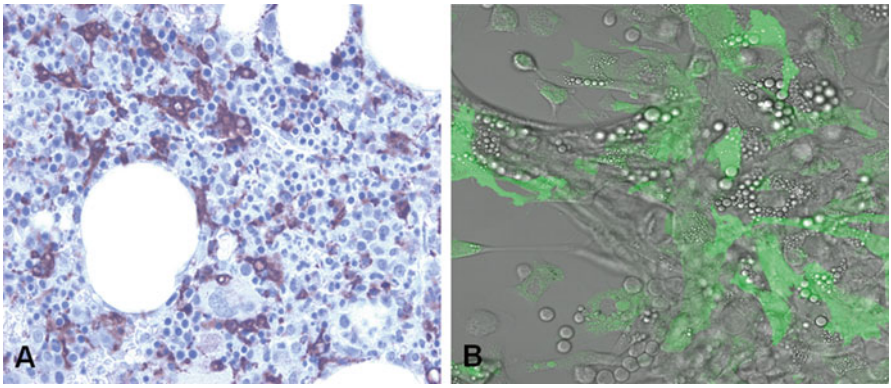

Fig. 4 Panel **a** shows immune histochemistry for CD68 from a normal marrow biopsy. As shown in the figure, CD68-positive macrophages (*brown cells*) had a prominent presence in the bone marrow and interact with numerous other cells types, suggesting a critical role in hematopoiesis. Panel **b** shows a long-term culture from a transgenic mouse where GFP is under the control of the monocyte/macrophage-specific human CD68 promoter. GFP-positive macrophages are prominent in the culture, often resembling fibroblasts. They also have numerous cellular processes which are not appreciated well under standard phase microscopy and appear to interact with many different hematopoietic cell types

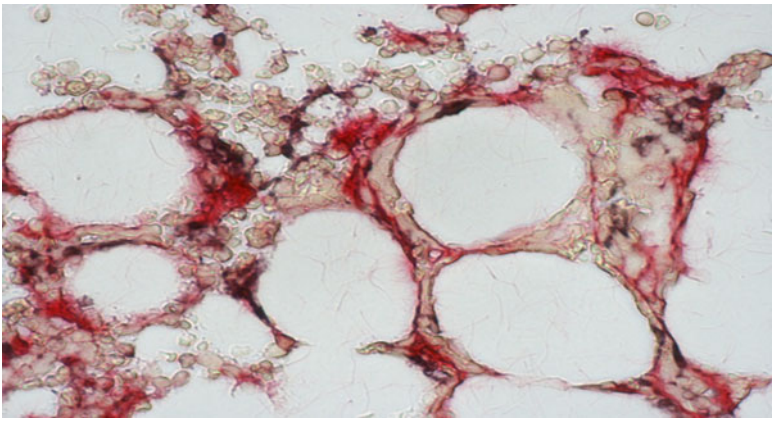

Fig. 5 Depicts immune histochemistry from a normal marrow biopsy for CD146 (shown in *red* and marking stromal cells) and CD68 (shown in *brown* and marking macrophages). As shown in the figure, CD146-positive stromal cells and CD68-positive macrophages appear to have significant interactions, suggesting an important regulatory role in hematopoiesis

CD68-positive macrophages are shown in close association with CD146-positive mesenchymal stromal cells often around vessels, suggesting that they may play a significant role in the regulation of the stem cell niche. Since studies to understand interactions between stromal cells and macrophages in Dexter cultures were hampered by the heterogeneous nature of this culture system, our lab has used stromal

cell lines described previously, Hs5 and Hs27a, to study stromal–monocyte interactions [23, 39, 40]. Data from our lab indicate that these stromal cells secrete factors that affect monocyte function and that the converse is also true: monocytes modulate stromal function. For example, we have demonstrated that monocytes stimulated by HS27a, the stromal cell line associated with niche function, secrete osteopontin, which downregulates Notch-1 expression on CD34+ cells [23]. This leads to the logical speculation that downregulation of Notch on progenitors can limit Jagged-Notch signaling, thereby making CD34+ cells more responsive to differentiation signals. Two recent studies from mouse models have also implicated macrophages in niche function. In one study, the loss of endosteal lining macrophages or "osteomacs" led to the reduction of osteoblasts and HSC trophic cytokines and thus HSC mobilization [67]. In the other study, CD169+ macrophages were found to interact with Nestin+ mesenchymal progenitor cells and promote retention of HSC within the marrow. Depletion of macrophages with clodronate liposomes led to mobilization of HSC, but in this study, there was no effect on osteoblasts [68].

5 The Niche and Disease

Recent murine studies have also suggested that abnormalities in the stromal niche can cause disease in the hematopoietic compartment. In these studies, alterations of RARγ, Dicer, or SBDS in osteoprogenitors led to the development of myelodysplastic/myeloproliferative neoplasms (MDS/MPD) [69, 70]. Transplantation of normal hematopoietic cells into the abnormal microenvironment also led to the development of disease in transplanted cells [70]. However, whether this situation is clinically relevant in humans is questionable as patients with these hematologic malignancies can be cured by hematopoietic stem cell transplantation. Furthermore, if an abnormal niche induced abnormalities in donor cells, one would expect a much higher incidence of donor-derived MDS/MPD, which is exceedingly rare [71]. However, since monocytes are derived from the abnormal clone and have significant interactions with stromal cells, it is reasonable to conclude that abnormal monocytes contribute to altered niche function. For example, we have previously reported that monocytes from patients with MDS fail to respond appropriately to stromal signals and upregulate MMP-9 gene expression [72]. Given the role of MMP-9 in facilitating the egress of cells from marrow, it is reasonable to conclude that, as the proportion of nonresponsive monocytes increases, levels of MMP-9 decrease, resulting in hypercellularity. We also determined that the stromal signal that induced MMP-9 is most likely MCP-1; however, we have not as yet identified the compromised monocyte signaling pathway that fails to respond. Clearly, a better delineation of signaling pathways that are responsible for normal responses between stromal cells and monocytes, as well as the activities that trigger these pathways, are needed.

6 HSC Niche as a Functional Concept

The ongoing controversy about the precise anatomic location of the HSC niche, the cellular components of the niche, and the multiple functional compartments within the niche confound some fundamental observations of how the niche might be organized in higher vertebrates. Importantly, defining a specific cell type, the osteoblast, endothelium, or the MSC, to be "the" niche-defining cell would ignore the fact that the total number of true stem cells in higher vertebrates, as extrapolated from stochastic models, would be far fewer than the total number of any of these cell types [73–76]. Since there is no experimental evidence to suggest that the total number of niches within an organism is far in excess of the total number of HSCs, one explanation would be that, within these cell types, subpopulations of specialized niche-defining osteoblasts, MSC, or endothelium exist. An alternative explanation would be that the HSC is not merely a passive player in its interaction with its niche, but also actively instructs the microenvironment to be supportive by elaborating the niche-defining factors in appropriate quantities. Such a two-way communication between the niche and HSC would accommodate a model in which the niche is not defined by just one specialized cell type but a combination of cell types that elaborates different factors, a combination that could vary with variations of homeostasis, stress, or disease. Such a model could explain how the HSC niche moves out of the adult bone marrow in disease conditions, giving rise to extramedullary hematopoiesis.

Finally, although several aspects of human hematopoiesis at the tissue, cellular and molecular levels are closely mirrored in the murine system, important differences exist between the two organisms that would prompt caution while extrapolating murine in vivo data to human physiology, particularly concerning the issue of scale. Mouse marrow has little reserve for stress hematopoiesis, relying instead on splenic production, particularly for erythropoiesis. Whether or how this compartmentalization impacts the location and composition of the HSC niche is purely speculative. Since regulation of hematopoiesis requires both stage- and lineage-specific controls that include both membrane bound and secreted activities, the latter often acting through a gradient, it is easy to imagine an ME unit that provides cell–cell contact via several receptor–ligand interactions that function to retain "stem cell-ness", with subsequent daughter cells losing that contact and becoming subject to increasing concentrations of signals for commitment and maturation. Examples of such stem cell control are abundant in model organisms, including nematodes and fruit flies [77]. The cells of mice, man, worms, and flies are about the same size, and these organisms share the challenge of retaining a stem cell population while satisfying developmental or daily demand; yet the magnitude of cell production among these animals is vastly different. It is reasonable to speculate that ME units are probably packaged and distributed differently to accommodate differences in scale. To date, the composition of signals that retain stem cell-ness while permitting stem cell division has not been defined.

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References

1. McCulloch EA, Till JE (1960) The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 13:115–125
2. Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222
3. McCulloch EA, Siminovitch L, Till JE, Russell ES, Bernstein SE (1965) The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype sl-sld. Blood 26(4):399–410
4. Russell ES (1979) Hereditary anemias of the mouse: a review for geneticists. Adv Genet 20:357–459
5. Copeland NG, Gilbert DJ, Cho BC, Donovan PJ, Jenkins NA, Cosman D, Anderson D, Lyman SD, Williams DE (1990) Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell 63(1):175–183
6. Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, Wellner D, Leder P, Besmer P (1990) The hematopoietic growth factor KL is encoded by the sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell 63(1):225–233
7. Awaya N, Rupert K, Bryant E, Torok-Storb B (2002) Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. Exp Hematol 30(8):937–942
8. Wolf NS, Trentin JJ (1968) Hemopoietic colony studies. V. effect of hemopoietic organ stroma on differentiation of pluripotent stem cells. J Exp Med 127(1):205–214
9. Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4(1–2):7–25
10. Barker JE (1994) Sl/sld hematopoietic progenitors are deficient in situ. Exp Hematol 22(2):174–177
11. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109(5):625–637
12. Nagasawa T (2000) A chemokine, SDF-1/PBSF, and its receptor, CXC chemokine receptor 4, as mediators of hematopoiesis. Int J Hematol 72(4):408–411
13. Sugiyama T, Kohara H, Noda M, Nagasawa T (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25(6):977–988
14. Ara T, Tokoyoda K, Sugiyama T, Egawa T, Kawabata K, Nagasawa T (2003) Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity 19(2):257–267
15. Papayannopoulou T, Craddock C, Nakamoto B, Priestley GV, Wolf NS (1995) The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. Proc Natl Acad Sci U S A 92(21):9647–9651
16. Jiang Y, Bonig H, Ulyanova T, Chang K, Papayannopoulou T (2009) On the adaptation of endosteal stem cell niche function in response to stress. Blood 114(18):3773–3782
17. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118(2):149–161
18. Kimura S, Roberts AW, Metcalf D, Alexander WS (1998) Hematopoietic stem cell deficiencies in mice lacking c-mpl, the receptor for thrombopoietin. Proc Natl Acad Sci U S A 95(3): 1195–1200
19. Qian H, Buza-Vidas N, Hyland CD, Jensen CT, Antonchuk J, Månsson R, Thoren LA, Ekblom M, Alexander WS, Jacobsen SE (2007) Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1(6):671–684

20. Yoshihara H, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, Gomei Y, Iwasaki H, Matsuoka S, Miyamoto K, Miyazaki H, Takahashi T, Suda T (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1(6):685–697
21. Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L, Marcovina S, Friedman C, Trask BJ, Hood L, Torok-Storb B (1998) The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. Immunity 8(1): 43–55
22. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT et al (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425(6960):841–846
23. Iwata M, Awaya N, Graf L, Kahl C, Torok-Storb B (2004) Human marrow stromal cells activate monocytes to secrete osteopontin, which down-regulates Notch1 gene expression in CD34+ cells. Blood 103(12):4496–4502
24. Stier S, Ko Y, Forkert R, Lutz C, Neuhaus T, Grünewald E, Cheng T, Dombkowski D, Calvi LM, Rittling SR, Scadden DT (2005) Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 201(11):1781–1791
25. Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, Bertoncello I, Bendall LJ, Simmons PJ, Haylock DN et al (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106(4):1232–1239
26. Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91(3):335–344
27. Chabannon C, Torok-Storb B (1992) Stem cell-stromal cell interactions. Curr Top Microbiol Immunol 177:123–136
28. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2(2):83–92
29. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. cloning in vitro and retransplantation in vivo. Transplantation 17(4):331–340
30. Simmons PJ, Torok-Storb B (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78(1):55–62
31. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC (2007) A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. Stem Cells Dev 16(6):953–963
32. Deschaseaux F, Charbord P (2000) Human marrow stromal precursors are alpha 1 integrin subunit-positive. J Cell Physiol 184(3):319–325
33. Stewart K, Monk P, Walsh S, Jefferiss CM, Letchford J, Beresford JN (2003) STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) as markers of primitive human marrow stromal cells and their more differentiated progeny: a comparative investigation in vitro. Cell Tissue Res 313(3):281–290
34. Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19(3):180–192
35. Majumdar MK, Banks V, Peluso DP, Morris EA (2000) Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. J Cell Physiol 185(1):98–106
36. Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 116(Pt 9):1827–1835
37. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2(4):313–319
38. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131(2):324–336

39. Roecklein BA, Torok-Storb B (1995) Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. Blood 85(4): 997–1005
40. Graf L, Iwata M, Torok-Storb B (2002) Gene expression profiling of the functionally distinct human bone marrow stromal cell lines HS-5 and HS-27a. Blood 100(4):1509–1511
41. Pillai MM, Yang X, Balakrishnan I, Bemis L, Torok-Storb B (2010) MiR-886-3p down regulates CXCL12 (SDF1) expression in human marrow stromal cells. PLoS One 5(12):e14304
42. Lord BI, Testa NG, Hendry JH (1975) The relative spatial distributions of CFUs and CFUc in the normal mouse femur. Blood 46(1):65–72
43. Gong JK (1978) Endosteal marrow: a rich source of hematopoietic stem cells. Science 199(4336):1443–1445
44. Nilsson SK, Johnston HM, Coverdale JA (2001) Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood 97(8):2293–2299
45. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L (2003) Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425(6960):836–841
46. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121(7):1109–1121
47. Zhu J, Garrett R, Jung Y, Zhang Y, Kim N, Wang J, Joe GJ, Hexner E, Choi Y, Taichman RS, Emerson SG (2007) Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. Blood 109(9):3706–3712
48. Kiel MJ, Radice GL, Morrison SJ (2007) Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell Stem Cell 1(2):204–217
49. Kiel MJ, Acar M, Radice GL, Morrison SJ (2009) Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance. Cell Stem Cell 4(2):170–179
50. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466(7308):829–834
51. Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, Arenzana-Seisdedos F, Magerus A, Caruz A, Fujii N, Nagler A, Lahav M, Szyper-Kravitz M, Zipori D, Lapidot T (2000) Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J Clin Invest 106(11):1331–1339
52. Li JJ, Huang YQ, Basch R, Karpatkin S (2001) Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost 85(2):204–206
53. Saulle E, Guerriero R, Petronelli A, Coppotelli E, Gabbianelli M, Morsilli O, Spinello I, Pelosi E, Castelli G, Testa U, Coppola S (2012) Autocrine role of angiopoietins during megakaryocytic differentiation. PLoS One 7(7):e39796
54. Yoshida H, Hayashi S, Shultz LD, Yamamura K, Nishikawa S, Nishikawa S, Kunisada T (1996) Neural and skin cell-specific expression pattern conferred by steel factor regulatory sequence in transgenic mice. Dev Dyn 207(2):222–232
55. Cairns LA, Moroni E, Levantini E, Giorgetti A, Klinger FG, Ronzoni S, Tatangelo L, Tiveron C, De Felici M, Dolci S, Magli MC, Giglioni B, Ottolenghi S (2003) Kit regulatory elements required for expression in developing hematopoietic and germ cell lineages. Blood 102(12): 3954–3962
56. Ding L, Morrison SJ (2013) Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495(7440):231–235
57. Ding L, Saunders TL, Enikolopov G, Morrison SJ (2012) Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 481(7382):457–462
58. Greenbaum A, Hsu YM, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, Nagasawa T, Link DC et al (2013) CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature 495(7440):227–230

59. Hsu YC, Fuchs E (2012) A family business: stem cell progeny join the niche to regulate homeostasis. Nat Rev Mol Cell Biol 13(2):103–114
60. Niemeyer CM, Sieff CA, Mathey-Prevot B, Wimperis JZ, Bierer BE, Clark SC, Nathan DG (1989) Expression of human interleukin-3 (multi-CSF) is restricted to human lymphocytes and T-cell tumor lines. Blood 73(4):945–951
61. McSweeney PA, Niederwieser D, Shizuru JA, Sandmaier BM, Molina AJ, Maloney DG, Chauncey TR, Gooley TA, Hegenbart U, Nash RA, Radich J, Wagner JL, Minor S, Appelbaum FR, Bensinger WI, Bryant E, Flowers ME, Georges GE, Grumet FC, Kiem HP, Torok-Storb B, Yu C, Blume KG, Storb RF (2001) Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. Blood 97(11):3390–3400
62. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, Gao W, Saito TI, Lo Celso C, Tsuyuzaki H, Sato T, Côté D, Sykes M, Strom TB, Scadden DT, Lin CP et al (2011) In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. Nature 474(7350):216–219
63. Bessis M (1958) Erythroblastic island, functional unity of bone marrow. Rev Hematol 13(1): 8–11
64. Chasis JA, Mohandas N (2008) Erythroblastic islands: niches for erythropoiesis. Blood 112(3): 470–478
65. Chow A, Huggins M, Ahmed J, Hashimoto D, Lucas D, Kunisaki Y, Pinho S, Leboeuf M, Noizat C, van Rooijen N, Tanaka M, Zhao ZJ, Bergman A, Merad M, Frenette PS (2013) CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat Med 19(4):429–436
66. Adams GB, Chabner KT, Alley IR, Olson DP, Szczepiorkowski ZM, Poznansky MC, Kos CH, Pollak MR, Brown EM, Scadden DT (2006) Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 439(7076):599–603
67. Winkler IG, Sims NA, Pettit AR, Barbier V, Nowlan B, Helwani F, Poulton IJ, van Rooijen N, Alexander KA, Raggatt LJ, Lévesque JP (2010) Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. Blood 116(23): 4815–4828
68. Chow A, Lucas D, Hidalgo A, Méndez-Ferrer S, Hashimoto D, Scheiermann C, Battista M, Leboeuf M, Prophete C, van Rooijen N, Tanaka M, Merad M, Frenette PS (2011) Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J Exp Med 208(2):261–271
69. Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, Westmoreland SV, Chambon P, Scadden DT, Purton LE (2007) A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell 129(6):1097–1110
70. Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, Ebert BL, Al-Shahrour F, Hasserjian RP, Scadden EO, Aung Z, Matza M, Merkenschlager M, Lin C, Rommens JM, Scadden DT (2010) Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature 464(7290):852–857
71. Sala Torra O, Loeb KR (2011) Donor cell-derived leukemia and myelodysplastic neoplasm: unique forms of leukemia. Am J Clin Pathol 135(4):501–504
72. Iwata M, Pillai M, Ramakrishnan A, Hackman RC, Joachim Deeg H, Opdenakker G, Torok-Storb B (2007) Reduced expression of inducible gelatinase B/matrix metalloproteinase-9 in monocytes from patients with myelodysplastic syndrome: correlation of inducible levels with the percentage of cytogenetically marked cells and with marrow cellularity. Blood 109(1): 85–92
73. Abkowitz JL, Persik MT, Shelton GH, Ott RL, Kiklevich JV, Catlin SN, Guttorp P (1995) Behavior of hematopoietic stem cells in a large animal. Proc Natl Acad Sci U S A 92(6): 2031–2035
74. Abkowitz JL, Catlin SN, McCallie MT, Guttorp P (2002) Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. Blood 100(7):2665–2667

75. Shepherd BE, Kiem HP, Lansdorp PM, Dunbar CE, Aubert G, LaRochelle A, Seggewiss R, Guttorp P, Abkowitz JL (2007) Hematopoietic stem-cell behavior in nonhuman primates. Blood 110(6):1806–1813
76. Catlin SN, Busque L, Gale RE, Guttorp P, Abkowitz JL (2011) The replication rate of human hematopoietic stem cells in vivo. Blood 117(17):4460–4466
77. Torok-Storb B (1988) Cellular interactions. Blood 72(2):373–385

Adult Stem Cells: Adult Skeletal Muscle Stem Cells

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Abstract Maintenance, repair and regeneration of adult skeletal muscle are mediated by stem or precursor cells within the muscle. In addition to the satellite cell, which is the archetypal muscle stem cell, there are other stem cells within skeletal muscle that can contribute to muscle regeneration under experimental conditions. We describe these different cells within skeletal muscle and evaluate the experimental evidence for them being skeletal muscle stem cells. Further studies will be needed to determine the roles of different skeletal muscle resident cells to repair, maintain and regenerate skeletal muscle.

Keywords Adult skeletal muscle • Satellite cells • Blood vessel-associated stem cells • Muscular dystrophy

Abbreviations

MAPCs	Multipotent adult progenitor cells
MDSCs	Muscle-derived stem cells
MECs	Myoendothelial cells
PICs	PW1(+)/Pax7(−) interstitial cells
SP cells	Side population cells
VSELs	Very small embryonic-like stem cells

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1 Introduction

Skeletal muscle is the largest organ within the human body, comprising 30–40 % of the body mass [1] and is essential for movement and posture. Muscle fibres that contain the contractile elements are formed during development by the fusion of myoblasts to form multinucleated muscle fibres, in which the nuclei (myonuclei) are postmitotic. Postnatal growth, repair and maintenance of skeletal muscle are mediated by satellite cells; however, there are other stem cells within skeletal muscle that are also capable of differentiation into skeletal muscle. In this chapter, we review the different stem cells present within adult skeletal muscle and their contribution to skeletal muscle regeneration.

2 Satellite Cells

The classical stem cells within adult skeletal muscle are satellite cells, which were first identified by Mauro [2] and defined by their position between the basal lamina and sarcolemma of the muscle fibre. Early work provided evidence that satellite cells are the source of new myofibre nuclei during muscle growth [3, 4] and regeneration [5]. Only recently, with the availability of reliable antibodies [6–14] and genetically modified mice [15–17], have satellite cells been established as muscle stem cells, able to both contribute to muscle growth [18, 19], regeneration [20, 21] and to functionally reconstitute the satellite cell niche [22, 23].

Studies of satellite cells in mice have been facilitated by the relative ease by which they may be separated from other cells present within skeletal muscle. Isolated muscle fibres, bearing their complement of satellite cells under the basal lamina [24] enable studies of satellite cells in their niche [25–27]; satellite cells may also be physically [22, 23, 28] or enzymatically [29] removed from their niche on the fibre for in vitro or in vivo studies. There are also protocols for satellite cell purification from enzymatically disaggregated skeletal muscle either on the basis of size and granularity [30] or using cell-surface satellite cell-specific antibodies combined with antibodies against other cell types to enrich for satellite cells [31–33]. However, there are caveats in using cell-sorting techniques—the sub-population isolated may not be 100 % pure and satellite cells may be activated during the procedure and thus not express particular markers. In addition, some antibodies used for cell sorting are not ideal, e.g. the monoclonal antibody SM/C-2.6 [9], which is frequently used for satellite cell purification [10, 34, 35], is not commercially available, nor is the antigen that it recognises known.

The term ‘satellite cell’ is often used incorrectly in the literature. By definition, a satellite cell is a quiescent cell underneath the basal lamina of muscle fibres. When it is no longer under the basal lamina, it is therefore no longer a satellite cell, but some studies refer to cells in tissue culture as satellite cells [36, 37]. If the cell is

under the basal lamina of the fibre but has divided and is expressing myogenic regulatory factors such as MyoD, this cell is the progeny of a satellite cell (a myoblast), not a satellite cell [27].

An important caveat is that not all satellite cells are capable of contributing to muscle regeneration [23, 38], suggesting the existence of a 'stem' satellite cell sub-population [33] that survives into old age [39, 40]. Whether the 'stem' satellite cell sub-population really exists, or if satellite cell functional characteristics are stochastic, is still not clear. Despite age-related decrease in satellite cell number [40–42] and changes in signalling factors, hormones, cytokines and growth factors that modulate their function [43], efficient muscle regeneration in old age can occur, provided the local or systemic environment is modulated appropriately [39, 40, 44]. The extent to which the aged or dystrophic environment [45] and the satellite cell niche itself [39, 46] affect satellite cell function is the focus of much current research (reviewed [21, 47–49]).

Although there is good evidence that satellite cells are required for postnatal skeletal muscle regeneration [20], they lose their regenerative capacity following culture [30], only have a very local effect after intra-muscular injection and do not seem to be systemically deliverable, so much work has focussed on other skeletal muscle stem cells that might be more appropriate for treating conditions such as muscular dystrophies (reviewed [50, 51]). However, other stem cells within skeletal muscle have been less intensively studied, largely due to challenges in identifying and purifying them. To complicate matters, the same, or similar, stem cells are often given different names or acronyms, e.g. pericytes [52] and muscle-derived cells (mdcs) [53], satellite cells and their putative stem cell sub-population, muscle stem cells (MuSCs) [33]. An additional problem in studying different cell types is that, if cells have to be expanded in culture, they may change their phenotype, so it is always best to study them either in vivo or immediately following their direct isolation.

3 Other Stem Cells Within Skeletal Muscle

Stem cells other than satellite cells that have been shown to contribute to skeletal muscle regeneration include blood vessel-associated stem cells, such as muscle side population (SP) cells, myoendothelial cells (MECs) and pericytes/mesoangioblasts; stem cells of unknown origin, such as muscle-derived stem cells (MDSCs), multipotent adult progenitor cells (MAPCs), CD133+ cells, PW1(+)/Pax7(−) interstitial cells (PICs) and very small embryonic-like stem cells (VSELs). But the extent to which these cells contribute to muscle regeneration is often slight [54–56] and whether these cells participate in muscle growth, maintenance, repair and regeneration in non-experimental conditions often remains unclear (Table 1).

Table 1 Stem cells within skeletal muscle—location, identification and potential

Cell type	Location	Markers	Differentiation potential (in vitro and in vivo)	Participation in muscle growth and maintenance	Muscle formation after transplantation	Satellite cell formation after transplantation	Systemically deliverable
Satellite cell	Underneath basal lamina of myofibre	Pax7+, CD34+, Myf5+, c-Met+, M-Cad+, α7 integrin+, syndecan3 and 4+, Caveolin1+	Skeletal muscle	Yes	Robust	Yes	No
Pericyte	Outside endothelium of the blood vessel	ALP+, NG2+, PDGFRβ+, CD146+	Skeletal and smooth muscle, osteoblasts, adipocytes	Yes	Yes	Yes	Yes
PIC	Interstitial	Pax7−/PW1+	Skeletal and smooth muscle	Not determined	Equivalent to satellite cells	Yes	Not determined
MEC	Blood vessel associated	CD56+/CD34+ /CD144+	Skeletal and cardiac muscle	Not determined	Robust, better than endothelial cells and myoblasts	Not determined	Not determined
MDSC	Not known	Desmin+, MyoD+, CD34+, Sca-1+ Bcl-1+, CD45− and c-kit−	Skeletal and cardiac muscle, haematopoietic, osteogenic, endothelial and neuronal	Not determined	Robust	Not determined	Not determined
MAPC	Not known	Cd13+, CD44−, CD45−, MHC I− and II−, c-kit−	Endothelium, neurons, glia, hepatocytes	Not determined	Limited	Not determined	Not determined
SP cell	Not known	Hoechst low	Muscle and haematopoietic	Not determined	Limited	Not determined	Yes
CD133+ cell	Not known	CD133+	Muscle	Not determined	Robust, better than myoblasts	Yes	Yes
VSEL	Not known	Oct-4+, SSEA-4+, Nanog+, Sox-2+, Rex-1+, Tert+, CD133+, CXCR4+	Haematopoietic, cardiomyocytes	Not determined	Not determined	Not determined	Not determined

3.1 Blood Vessel-Associated Stem Cells

3.1.1 Side Population Cells

Side population (SP) cells were identified by a low Hoechst staining 'tail' in their FACS profile [57]. The 'tail' disappears in the presence of a calcium channel blocker, verapamil. SP cells have been found in a wide variety of mammalian tissues and in many cases this cell population has been shown to contain multipotent stem cells [58]. Skeletal muscle SP cells express the stem cell marker Sca-1, but no myogenic markers and are located outside the basal lamina of muscle fibres, apparently associated with the vasculature [59]. The fact that they are present in mice in which Pax7, expressed in satellite cells, is knocked out [14] is compelling evidence that they are not derived from satellite cells.

Murine muscle SP cells do not differentiate into skeletal muscle in vitro, but after co-culture with myogenic cells or on intra-muscular transplantation, they do give rise to skeletal muscle [54, 59]. They are also capable of differentiating into haematopoietic cells in vitro [59] and can reconstitute the haematopoietic system of lethally irradiated mice [60]. Skeletal muscle SP cells are systemically deliverable to skeletal muscle [61–63], but not to any therapeutically significant levels [63, 64].

3.1.2 Endothelial/Myoendothelial Cells

Myoendothelial cells (MECs), co-expressing both myogenic and endothelial markers (CD56, CD34 and CD144) have been derived from human (but not mouse) skeletal muscle by flow cytometry [65]. However, it has been suggested that mouse MDSCs and human MECs are in fact the same, as they have a similar phenotype and ability to contribute to muscle regeneration [66]. Human MECs gave rise to significantly more skeletal muscle regeneration following intra-muscular grafting in mice than either endothelial cells (CD56−CD34+CD144+) or myoblasts (CD56+). When MECs cells were transplanted into infarcted myocardium, they stimulated angiogenesis, attenuated scar tissue, and promoted proliferation and survival of endogenous cardiomyocytes more effectively than either myoblasts or endothelial cells [67]. However, although blood vessel associated, there is no evidence that myoendothelial cells can transmigrate to skeletal muscle if transplanted systemically.

3.1.3 Pericytes/Mesoangioblasts

Myogenic cells derived from the mouse embryonic dorsal aorta, which co-expressed endothelial and myogenic markers, were shown to contribute to skeletal muscle growth and regeneration [68]. These cells, termed mesoangioblasts, are multipotent stem cells [69], able to differentiate into several mesodermal tissues and might be the origin of postnatal mesodermal stem cells. Mesoangioblasts have been shown to contribute to muscle regeneration and improve the muscle function after intra-arterial transplantation into either dystrophic mice [70] or dogs [71].

Pericytes are ALP+ cells located along the blood vessels and may be the adult counterpart of embryonic mesoangioblasts. However, unlike mesoangioblasts that express endothelial markers, they express pericyte markers such as alkaline phosphatase (ALP), NG2 and PDGFR-β [52]. Pericytes may also be isolated from skeletal muscle and other tissues by direct sorting of CD146+ CD34− CD45− CD56− cells [72, 73]. Like mesoangioblasts, skeletal muscle-derived pericytes are myogenic and can contribute extensively to skeletal muscle regeneration after intra-arterial [52] and intra-muscular [53] transplantation into dystrophin-deficient immunodeficient mice. In addition to myogenic differentiation, pericytes can also give rise to many other mesenchymal lineages, suggesting a close relationship with mesenchymal stem cells (MSCs) [52, 73].

Recent work using genetically modified mice has provided evidence that pericytes, but not endothelial cells, contribute to muscle fibres and to satellite cells during normal postnatal development [74].

3.2 Other Skeletal Muscle Stem Cells

3.2.1 Multipotent Adult Progenitor Cells

MAPCs were first isolated from human and mouse adult bone marrow (BM) [75], then from other postnatal tissues such as brain and muscle [55] and have the potential to differentiate into cells of all the three germ layers, including skeletal muscle. Skeletal muscle-derived MAPCs can be expanded up to 75 population doublings in vitro and similar to mouse- and human BM-derived MAPC, muscle MAPCs are CD13+, Flk1dim, c-kit−, CD44−, CD45−, MHC class I− and MHC class II−. Human and mouse MAPCs were reported to improve ischemic limb function after transplantation intramuscularly to C57BL/6 mice or BALB/c-nu/nu mice after artery ligation [76]. Although they did give rise to donor-derived muscle fibres, the percentage was low, suggesting the positive effects of these cells were most likely via their immunomodulatory or trophic effects, e.g. by increasing angiogenesis and endogenous stem cell proliferation, than by making a direct contribution to skeletal muscle fibres.

3.2.2 Muscle-Derived Stem Cells

Cells with stem cell capabilities have been isolated from mouse skeletal muscle on the basis of their adhesion and proliferative capabilities. MDSCs or long-term proliferating cells [77] were purified as a multipotent stem cell from neonatal mouse muscle by serial pre-plating, the less adherent cells being MDSCs. These cells derived from both mouse [77, 78] and human [56] contribute to muscle regeneration after transplantation into dystrophin-deficient mdx [the mouse homologue of Duchenne muscular dystrophy (DMD)] muscles. However, MDSCs derived from human muscle were phenotypically different from mouse MDSCs and gave rise to fewer donor-derived dystrophin+ fibres than did mouse MDSCs.

Mouse MDSCs are non-tumorigenic and can be expanded in vitro up to 300 population doublings without entering senescence [79]. Clones of MDSCs express myogenic markers (desmin and MyoD) and some stem cell markers such as CD34, Sca-1 and Bcl-1 and lack the haematopoietic stem cell marker CD45, c-kit and blood lineage markers [80]. These cells can reconstitute the haematopoietic system [78, 81] and elicited significant improvement in cardiac function in comparison to myoblasts following transplantation in a mouse cardiac injury model [82, 83]. MDSCs exist only in vitro and the cell within skeletal muscle from which they are derived is not known.

3.2.3 PW1(+)/Pax7(–) Interstitial Cells

PW1, also known as paternally expressed gene 3 (Peg3), a zinc finger protein which regulates two key cell-stress pathways, TNF and p53 signalling [84], is a key regulator of muscle atrophy. PW1 expression initiates in the early embryonic mesoderm and is down-regulated in tissues as they differentiate. It was recently suggested that PW1 might represent a pan-marker for multiple adult stem cells within mammalian tissue [85]. In mouse skeletal muscle immediately after birth, PW1 expression was detected not only on satellite cells but also on some Pax7– interstitial cells, termed PICs [86]. PICs are bipotent in vitro, generating both smooth and skeletal muscle and were able contribute to muscle regeneration in vivo within injured host mouse muscle [86]. However, PICs do not seem to be present within adult mouse muscle and their human counterparts have not yet been identified.

3.2.4 CD133+ Cells

CD133 is a pentaspan transmembrane glycoprotein (5-transmembrane, 5-TM), which specifically localises to cellular protrusions. The function of CD133 (also known as prominin-1 and AC133) is currently unknown. However, there is great interest in this marker, as it is expressed on many different types of stem cell, including haematopoietic stem cells [87], neural stem cells [88], endothelial progenitor cells [89, 90] and very small embryonic-like stem cells (VSELs) [91, 92]. CD133+ cells isolated from human skeletal muscle are able to contribute to muscle regeneration after both intra-muscular delivery to injured immunodeficient mouse muscle [93] and systemic administration to dystrophic immunodeficient mice [94]. But, as human muscle CD133+ cells were isolated by enzymatic disaggregation, the origin of these cells is unclear. As skeletal muscle is heavily vascularised, it is possible that the skeletal muscle-derived AC133+ cells isolated by Benchaouir et al. were blood borne and the same, or similar to, blood-derived AC133+ cells that can contribute to muscle regeneration [95]. A limitation to the study of these cells is that, although there are antibodies that can be used for FACS or MACS isolation [94], the anatomical location of these cells within skeletal muscle remains unknown. Skeletal muscle-derived CD133+ cells share some cell surface markers, such as CXCR4 and CD34, with satellite cells [93, 94] and cultured CD133+ cells express not only myoblast

markers but also the smooth muscle marker α-SMA and pericyte markers NG2 and PDGFRβ (Meng, unpublished data), suggesting the heterogeneity of this cell population. The majority of freshly isolated mouse CD133+ cells are very small in size—2–6 μm (Meng and Asfahani, unpublished observations)—a size very similar to those reported for VSELs [91, 92, 96]. In addition, their robust myogenic potential and ability to form Pax7+ cells in the satellite cell position [93] suggests a very close relationship of CD133+ with satellite cells, but whether they are a satellite cell sub-population, or a precursor of satellite cells, remains to be elucidated. Whether CD133+ cells derived from mouse skeletal muscle are equivalent to human CD133+ cells remains to be seen.

3.2.5 VSELs

Recently, a population of stem cells termed 'very small, embryonic-like stem cells' (VSELs) was discovered within many tissues, including skeletal muscle [91]. These cells are approximately 6.5 μm in diameter in the human [96] (i.e. smaller than red blood cells) and can be purified by flow cytometry [97] (reviewed [98]). VSELs express several markers of pluripotent stem cells, including Oct-4, cell surface protein SSEA-4, Nanog, Sox-2, Rex-1 and Tert [99] and form embryoid body-like spheres in vitro [97]. VSELs derived from the mouse bone marrow are radiation resistant and may be long-term repopulating haematopoietic stem cells [100] as well as differentiating to cardiomyocytes in vitro [101]. VSELs therefore show intriguing similarities to 'stem' satellite cells, which are also of small size [23, 30] and radiation resistant [39, 102, 103]. But their function and relationship to other cells within skeletal muscle is at present unknown.

4 The Relationships Between Stem Cells Resident in Skeletal Muscle

Some muscle stem cells clearly have close relationships (e.g. pericytes and satellite cells, PICs and satellite cells), whereas the hierarchy, if any, between other skeletal muscle resident stem cells is not clear. Even if one cell type does not directly give rise to another, they may affect each other's function. The close proximity of satellite cells to blood vessels [104] will facilitate satellite cell interactions with endothelial cells, pericytes and other blood vessel-associated cells [105, 106].

5 Why Are We Interested in Skeletal Muscle Stem Cells?

Interest in skeletal muscle stem cells was initiated because of the possibility of using them, or their progeny, to treat muscular dystrophies such as DMD [50, 107]. For this purpose, cells derived either from a normal donor or from the patient,

genetically modified to express the defective gene (e.g. dystrophin), could be used. Ideally, cells would be able to be systemically delivered to skeletal muscle and repair or replace dystrophic muscle fibres, thus restoring dystrophin expression within fibres that have donor-derived myonuclei. If the donor stem cells also reconstituted the skeletal muscle stem cell pool, they could contribute to muscle repair and regeneration and restoration of dystrophin protein throughout the lifetime of the individual.

Donor muscle stem cells might also be a therapeutic option for sarcopenia (the age-related loss of skeletal muscle mass and strength). However, the systemic or local environment as a result of age or dystrophy-related changes may prevent efficient stem cell function. There is therefore a pressing need to understand the effect of age or different muscular dystrophies on the satellite cells themselves and on their environment. Modification of pathways that promote muscle stem cell function could be an alternative means to alleviate the loss of muscle that occurs as a result of ageing or muscular dystrophies.

6 Conclusions

In this review, we have summarised the stem cell types within the skeletal muscle and the evidence for them being skeletal muscle stem cells. Skeletal muscle contains many stem or precursor cells that can contribute to muscle regeneration under experimental conditions, but, apart from satellite cells and pericytes, their contribution (if any) to 'normal' muscle growth, maintenance and repair is not known. Further studies will be needed to determine the roles of different skeletal muscle-resident cells within both normal and dystrophic muscles and how to augment their function to prevent or delay the loss of skeletal muscle fibres that occurs as a consequence of both age and muscular dystrophies.

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References

1. Janssen I, Heymsfield SB, Wang ZM, Ross R (2000) Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. J Appl Physiol 89:81–88
2. Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493–495
3. Moss FP, Leblond CP (1970) Nature of dividing nuclei in skeletal muscle of growing rats. J Cell Biol 44:459–462
4. Cardasis CA, Cooper GW (1975) An analysis of nuclear numbers in individual muscle fibers during differentiation and growth: a satellite cell-muscle fiber growth unit. J Exp Zool 191: 347–358
5. Snow MH (1978) An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. Cell Tissue Res 186:535–540

6. Capkovic KL, Stevenson S, Johnson MC, Thelen JJ, Cornelison DD (2008) Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation. Exp Cell Res 314:1553–1565
7. Cornelison DD, Filla MS, Stanley HM, Rapraeger AC, Olwin BB (2001) Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. Dev Biol 239:79–94
8. Cornelison DD, Wilcox-Adelman SA, Goetinck PF, Rauvala H, Rapraeger AC, Olwin BB (2004) Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. Genes Dev 18:2231–2236
9. Fukada S, Higuchi S, Segawa M, Koda K, Yamamoto Y, Tsujikawa K, Kohama Y, Uezumi A, Imamura M, Miyagoe-Suzuki Y, Takeda S, Yamamoto H (2004) Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. Exp Cell Res 296:245–255
10. Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S (2007) Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells 25:2448–2459
11. Gnocchi VF, White RB, Ono Y, Ellis JA, Zammit PS (2009) Further characterisation of the molecular signature of quiescent and activated mouse muscle satellite cells. PLoS One 4:e5205
12. Irintchev A, Zeschnigk M, Starzinski-Powitz A, Wernig A (1994) Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn 199:326–337
13. Ozeki N, Lim M, Yao CC, Tolar M, Kramer RH (2006) Alpha7 integrin expressing human fetal myogenic progenitors have stem cell-like properties and are capable of osteogenic differentiation. Exp Cell Res 312:4162–4180
14. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102:777–786
15. Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151:1221–1234
16. Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112(Pt 17):2895–2901
17. Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G (2011) Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development 138:3625–3637
18. Lipton BH, Schultz E (1979) Developmental fate of skeletal muscle satellite cells. Science 205:1292–1294
19. Seale P, Rudnicki MA (2000) A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev Biol 218:115–124
20. Lepper C, Partridge TA, Fan CM (2011) An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138:3639–3646
21. Relaix F, Zammit PS (2012) Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139:2845–2856
22. Boldrin L, Zammit PS, Muntoni F, Morgan JE (2009) Mature adult dystrophic mouse muscle environment does not impede efficient engrafted satellite cell regeneration and self-renewal. Stem Cells 27:2478–2487
23. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122:289–301
24. Collins CA, Zammit PS (2009) Isolation and grafting of single muscle fibres. Methods Mol Biol 482:319–330
25. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA (1995) Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim 31:773–779

26. Yablonka-Reuveni Z, Anderson JE (2006) Satellite cells from dystrophic (mdx) mice display accelerated differentiation in primary cultures and in isolated myofibers. Dev Dyn 235: 203–212
27. Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166:347–357
28. Shefer G, Yablonka-Reuveni Z (2005) Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. Methods Mol Biol 290:281–304
29. Ono Y, Boldrin L, Knopp P, Morgan JE, Zammit PS (2010) Muscle satellite cells are a functionally heterogeneous population in both somite-derived and branchiomeric muscles. Dev Biol 337:29–41
30. Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M (2005) Direct isolation of satellite cells for skeletal muscle regeneration. Science 309:2064–2067
31. Yi L, Rossi F (2011) Purification of progenitors from skeletal muscle. J Vis Exp (49):pii: 2476
32. Danoviz ME, Yablonka-Reuveni Z (2012) Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. Methods Mol Biol 798: 21–52
33. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM (2008) Self-renewal and expansion of single transplanted muscle stem cells. Nature 456:502–506
34. Mizuno Y, Chang H, Umeda K, Niwa A, Iwasa T, Awaya T, Fukada S, Yamamoto H, Yamanaka S, Nakahata T, Heike T (2010) Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. FASEB J 24:2245–2253
35. Ikemoto M, Fukada S, Uezumi A, Masuda S, Miyoshi H, Yamamoto H, Wada MR, Masubuchi N, Miyagoe-Suzuki Y, Takeda S (2007) Autologous transplantation of SM/C-2.6(+) satellite cells transduced with micro-dystrophin CS1 cDNA by lentiviral vector into mdx mice. Mol Ther 15:2178–2185
36. Perruchot MH, Ecolan P, Sorensen IL, Oksbjerg N, Lefaucheur L (2012) In vitro characterization of proliferation and differentiation of pig satellite cells. Differentiation 84:322–329
37. Huang S, Wang Z (2012) Platelet-rich plasma-derived growth factors promotes osteogenic differentiation of rat muscle satellite cells: in vitro and in vivo studies. Cell Biol Int 36(12): 1195–1205
38. Neal A, Boldrin L, Morgan JE (2012) The satellite cell in male and female, developing and adult mouse muscle: distinct stem cells for growth and regeneration. PLoS One 7:e37950
39. Boldrin L, Neal A, Zammit PS, Muntoni F, Morgan JE (2012) Donor satellite cell engraftment is significantly augmented when the host niche is preserved and endogenous satellite cells are incapacitated. Stem Cells 30:1971–1984
40. Collins CA, Zammit PS, Ruiz AP, Morgan JE, Partridge TA (2007) A population of myogenic stem cells that survives skeletal muscle aging. Stem Cells 25:885–894
41. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z (2006) Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294:50–66
42. Shefer G, Rauner G, Yablonka-Reuveni Z, Benayahu D (2010) Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. PLoS One 5:e13307
43. Brack AS, Rando TA (2007) Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. Stem Cell Rev 3:226–237
44. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433:760–764
45. Morgan JE, Zammit PS (2010) Direct effects of the pathogenic mutation on satellite cell function in muscular dystrophy. Exp Cell Res 316:3100–3108
46. Chakkalakal JV, Jones KM, Basson MA, Brack AS (2012) The aged niche disrupts muscle stem cell quiescence. Nature 490:355–360
47. Yablonka-Reuveni Z (2011) The skeletal muscle satellite cell: still young and fascinating at 50. J Histochem Cytochem 59:1041–1059

48. Brack AS, Rando TA (2012) Tissue-specific stem cells: lessons from the skeletal muscle satellite cell. Cell Stem Cell 10:504–514
49. Pannerec A, Marazzi G, Sassoon D (2012) Stem cells in the hood: the skeletal muscle niche. Trends Mol Med 18:599–606
50. Meng J, Muntoni F, Morgan JE (2011) Stem cells to treat muscular dystrophies – where are we? Neuromuscul Disord 21:4–12
51. Patel K, Morgan J (2012) 185th ENMC International Workshop: stem/precursor cells as a therapeutic strategy for muscular dystrophies 3-5 June 2011, Naarden, The Netherlands. Neuromuscul Disord 22:447–452
52. Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R, Li S, Belicchi M, Peretti G, Chamberlain JS, Wright WE, Torrente Y, Ferrari S, Bianco P, Cossu G (2007) Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 9:255–267
53. Meng J, Adkin CF, Xu SW, Muntoni F, Morgan JE (2011) Contribution of human muscle-derived cells to skeletal muscle regeneration in dystrophic host mice. PLoS One 6:e17454
54. Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394
55. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904
56. Chirieleison SM, Feduska JM, Schugar RC, Askew Y, Deasy BM (2012) Human muscle-derived cell populations isolated by differential adhesion rates: phenotype and contribution to skeletal muscle regeneration in Mdx/SCID mice. Tissue Eng Part A 18:232–241
57. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806
58. Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. Stem Cells 24: 3–12
59. Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA (2002) Myogenic specification of side population cells in skeletal muscle. J Cell Biol 159:123–134
60. Jackson KA, Mi T, Goodell MA (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci U S A 96:14482–14486
61. Bachrach E, Li S, Perez AL, Schienda J, Liadaki K, Volinski J, Flint A, Chamberlain J, Kunkel LM (2004) Systemic delivery of human microdystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells. Proc Natl Acad Sci U S A 101:3581–3586
62. Bachrach E, Perez AL, Choi YH, Illigens BM, Jun SJ, Del NP, McGowan FX, Li S, Flint A, Chamberlain J, Kunkel LM (2006) Muscle engraftment of myogenic progenitor cells following intraarterial transplantation. Muscle Nerve 34:44–52
63. Perez AL, Bachrach E, Illigens BM, Jun SJ, Bagden E, Steffen L, Flint A, McGowan FX, Del NP, Montecino-Rodriguez E, Tidball JG, Kunkel LM (2009) CXCR4 enhances engraftment of muscle progenitor cells. Muscle Nerve 40:562–572
64. Muskiewicz KR, Frank NY, Flint AF, Gussoni E (2005) Myogenic potential of muscle side and main population cells after intravenous injection into sub-lethally irradiated mdx mice. J Histochem Cytochem 53:861–873
65. Zheng B, Cao B, Crisan M, Sun B, Li G, Logar A, Yap S, Pollett JB, Drowley L, Cassino T, Gharaibeh B, Deasy BM, Huard J, Peault B (2007) Prospective identification of myogenic endothelial cells in human skeletal muscle. Nat Biotechnol 25:1025–1034
66. Chen CW, Corselli M, Peault B, Huard J (2012) Human blood-vessel-derived stem cells for tissue repair and regeneration. J Biomed Biotechnol 2012:597439
67. Okada M, Payne TR, Zheng B, Oshima H, Momoi N, Tobita K, Keller BB, Phillippi JA, Peault B, Huard J (2008) Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium. J Am Coll Cardiol 52:1869–1880

68. De AL, Berghella L, Coletta M, Lattanzi L, Zanchi M, Cusella-De Angelis MG, Ponzetto C, Cossu G (1999) Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. J Cell Biol 147:869–878
69. Minasi MG, Riminucci M, De AL, Borello U, Berarducci B, Innocenzi A, Caprioli A, Sirabella D, Baiocchi M, De MR, Boratto R, Jaffredo T, Broccoli V, Bianco P, Cossu G (2002) The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development 129:2773–2783
70. Sampaolesi M, Torrente Y, Innocenzi A, Tonlorenzi R, D'Antona G, Pellegrino MA, Barresi R, Bresolin N, De Angelis MG, Campbell KP, Bottinelli R, Cossu G (2003) Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. Science 301:487–492
71. Sampaolesi M, Blot S, D'Antona G, Granger N, Tonlorenzi R, Innocenzi A, Mognol P, Thibaud JL, Galvez BG, Barthelemy I, Perani L, Mantero S, Guttinger M, Pansarasa O, Rinaldi C, Cusella De Angelis MG, Torrente Y, Bordignon C, Bottinelli R, Cossu G (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature 444: 574–579
72. Crisan M, Huard J, Zheng B, Sun B, Yap S, Logar A, Giacobino JP, Casteilla L, Peault B (2008) Purification and culture of human blood vessel-associated progenitor cells. Curr Protoc Stem Cell Biol Chapter 2:Unit-2B
73. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313
74. Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, Antonini S, Sambasivan R, Brunelli S, Tajbakhsh S, Cossu G (2011) Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat Commun 2:499
75. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49
76. Aranguren XL, McCue JD, Hendrickx B, Zhu XH, Du F, Chen E, Pelacho B, Penuelas I, Abizanda G, Uriz M, Frommer SA, Ross JJ, Schroeder BA, Seaborn MS, Adney JR, Hagenbrock J, Harris NH, Zhang Y, Zhang X, Nelson-Holte MH, Jiang Y, Billiau AD, Chen W, Prosper F, Verfaillie CM, Luttun A (2008) Multipotent adult progenitor cells sustain function of ischemic limbs in mice. J Clin Invest 118:505–514
77. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, Mytinger J, Cao B, Gates C, Wernig A, Huard J (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157:851–864
78. Cao B, Zheng B, Jankowski RJ, Kimura S, Ikezawa M, Deasy B, Cummins J, Epperly M, Qu-Petersen Z, Huard J (2003) Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. Nat Cell Biol 5:640–646
79. Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, Huard J (2005) Long-term self-renewal of postnatal muscle-derived stem cells. Mol Biol Cell 16: 3323–3333
80. Cao B, Huard J (2004) Muscle-derived stem cells. Cell Cycle 3:104–107
81. Huard J, Cao B, Qu-Petersen Z (2003) Muscle-derived stem cells: potential for muscle regeneration. Birth Defects Res C Embryo Today 69:230–237
82. Payne TR, Oshima H, Sakai T, Ling Y, Gharaibeh B, Cummins J, Huard J (2005) Regeneration of dystrophin-expressing myocytes in the mdx heart by skeletal muscle stem cells. Gene Ther 12:1264–1274
83. Oshima H, Payne TR, Urish KL, Sakai T, Ling Y, Gharaibeh B, Tobita K, Keller BB, Cummins JH, Huard J (2005) Differential myocardial infarct repair with muscle stem cells compared to myoblasts. Mol Ther 12:1130–1141

84. Relaix F, Wei X, Li W, Pan J, Lin Y, Bowtell DD, Sassoon DA, Wu X (2000) Pw1/Peg3 is a potential cell death mediator and cooperates with Siah1a in p53-mediated apoptosis. Proc Natl Acad Sci U S A 97:2105–2110
85. Besson V, Smeriglio P, Wegener A, Relaix F, Nait OB, Sassoon DA, Marazzi G (2011) PW1 gene/paternally expressed gene 3 (PW1/Peg3) identifies multiple adult stem and progenitor cell populations. Proc Natl Acad Sci U S A 108:11470–11475
86. Mitchell KJ, Pannerec A, Cadot B, Parlakian A, Besson V, Gomes ER, Marazzi G, Sassoon DA (2010) Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol 12:257–266
87. Kobari L, Giarratana MC, Pflumio F, Izac B, Coulombel L, Douay L (2001) CD133+ cell selection is an alternative to CD34+ cell selection for ex vivo expansion of hematopoietic stem cells. J Hematother Stem Cell Res 10:273–281
88. Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H (2003) Isolation and characterization of neural progenitor cells from post-mortem human cortex. J Neurosci Res 74: 838–851
89. Eggermann J, Kliche S, Jarmy G, Hoffmann K, Mayr-Beyrle U, Debatin KM, Waltenberger J, Beltinger C (2003) Endothelial progenitor cell culture and differentiation in vitro: a methodological comparison using human umbilical cord blood. Cardiovasc Res 58:478–486
90. Salven P, Mustjoki S, Alitalo R, Alitalo K, Rafii S (2003) VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. Blood 101:168–172
91. Ratajczak MZ, Zuba-Surma EK, Machalinski B, Ratajczak J, Kucia M (2008) Very small embryonic-like (VSEL) stem cells: purification from adult organs, characterization, and biological significance. Stem Cell Rev 4:89–99
92. Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Ratajczak J, Kucia M (2008) Very small embryonic-like stem cells: characterization, developmental origin, and biological significance. Exp Hematol 36:742–751
93. Negroni E, Riederer I, Chaouch S, Belicchi M, Razini P, Di SJ, Torrente Y, Butler-Browne GS, Mouly V (2009) In vivo myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. Mol Ther 17:1771–1778
94. Benchaouir R, Meregalli M, Farini A, D'Antona G, Belicchi M, Goyenvalle A, Battistelli M, Bresolin N, Bottinelli R, Garcia L, Torrente Y (2007) Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell 1:646–657
95. Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, Tonlorenzi R, Porretti L, Gavina M, Mamchaoui K, Pellegrino MA, Furling D, Mouly V, Butler-Browne GS, Bottinelli R, Cossu G, Bresolin N (2004) Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 114:182–195
96. Zuba-Surma EK, Ratajczak MZ (2010) Overview of very small embryonic-like stem cells (VSELs) and methodology of their identification and isolation by flow cytometric methods. Curr Protoc Cytom Chapter 9:Unit 9
97. Rodgerson DO, Harris AG (2011) A comparison of stem cells for therapeutic use. Stem Cell Rev 7:782–796
98. Zuba-Surma EK, Kucia M, Ratajczak J, Ratajczak MZ (2009) "Small stem cells" in adult tissues: very small embryonic-like stem cells stand up! Cytometry A 75:4–13
99. Bhartiya D, Shaikh A, Nagvenkar P, Kasiviswanathan S, Pethe P, Pawani H, Mohanty S, Rao SG, Zaveri K, Hinduja I (2012) Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. Stem Cells Dev 21:1–6
100. Ratajczak J, Wysoczynski M, Zuba-Surma E, Wan W, Kucia M, Yoder MC, Ratajczak MZ (2011) Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after coculture over OP9 stromal cells. Exp Hematol 39: 225–237

101. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Milewski K, Wallace-Bradley D, Kazmierski M, Buszman P, Hrycek E, Cybulski W, Kaluza G, Wieczorek P, Ratajczak J, Ratajczak MZ (2010) Cardiomyocyte differentiation of bone marrow-derived Oct-4+CXCR4+SSEA-1+ very small embryonic-like stem cells. Int J Oncol 37:237–247
102. Gross JG, Morgan JE (1999) Muscle precursor cells injected into irradiated mdx mouse muscle persist after serial injury. Muscle Nerve 22:174–185
103. Heslop L, Morgan JE, Partridge TA (2000) Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J Cell Sci 113(Pt 12):2299–2308
104. Mounier R, Chretien F, Chazaud B (2011) Blood vessels and the satellite cell niche. Curr Top Dev Biol 96:121–138
105. Christov C, Chretien F, Abou-Khalil R, Bassez G, Vallet G, Authier FJ, Bassaglia Y, Shinin V, Tajbakhsh S, Chazaud B, Gherardi RK (2007) Muscle satellite cells and endothelial cells: close neighbors and privileged partners. Mol Biol Cell 18:1397–1409
106. Rhoads RP, Johnson RM, Rathbone CR, Liu X, Temm-Grove C, Sheehan SM, Hoying JB, Allen RE (2009) Satellite cell-mediated angiogenesis in vitro coincides with a functional hypoxia-inducible factor pathway. Am J Physiol Cell Physiol 296:C1321–C1328
107. Tedesco FS, Cossu G (2012) Stem cell therapies for muscle disorders. Curr Opin Neurol 25:597–603

Adult Cardiac Stem Cells: Identity, Location and Potential

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Abstract The adult myocardium harbours a population of resident (endogenous) multipotent cardiac stem–progenitor cells. Manipulation of these cells in situ and ex vivo has opened new therapeutic avenues for anatomical and functional myocardial regeneration. In this chapter we will summarise the identity, potency and location of the different cardiac stem–progenitor cells documented thus far in the developing through to adult heart. We discuss the origin of cardiac stem–progenitor cells, determined through genetic lineage-tracing experiments, and methods for deriving them from both rodents and human subjects. Ageing and senescence of the cardiac stem–progenitor cells determine their function and regenerative capacity. Regulation of this parameter will impact the efficacy of myocardial regenerative therapies. Therefore, we discuss the alterations to cardiac stem–progenitor cell activity and

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potency with physiological remodelling, ageing and disease. Finally, we elucidate the clinical potential of these unique cells and the translation of their use, which will lead to better approaches to treat or prevent heart failure.

Keywords Cardiac • Stem cells • Adult stem cells • Ageing • Regeneration

Abbreviations

ABCG2	Adenosine triphosphate-binding cassette transporter G2
AdvSca1	Adventitial Sca1
ALCADIA	AutoLogous human CArdiac-Derived stem cell to treat Ischaemic cArdiomyopathy
α-MHC	Alpha-myosin heavy chain
AMI	Acute myocardial infarction
ATP	Adenosine triphosphate
5-AZA	5-Azacytidine
BAC	Bacterial artificial chromosome
β-gal	Beta galactosidase
bFGF	Basic fibroblast growth factor
BMDCs	Bone marrow-derived cells
BMP2	Bone morphogenic protein 2
CADUCEUS	CArdiosphere-Derived aUtologous Stem CElls to reverse ventricUlar dySfunction
CD	Cluster of differentiation
CDCs	Cardiosphere-derived cells
CDKIs	Cyclin-dependant kinase inhibitors
cMRI	Cardiac magnetic resonance imaging
CPCs	Cardiac progenitor cells
Cre	Cre recombinase
CS	Cardiospheres
CSP	Cardiac side population
CXCR4	Chemokine (C-X-C motif) receptor 4
DKK-1	Dickkopf WNT signalling pathway inhibitor 1
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eCSCs	Endogenous cardiac stem cells
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-to-mesenchymal transition
EPDCs	Epicardial-derived cells
ES	Embryonic stem
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FGF4	Fibroblast growth factor 4

FGF8	Fibroblast growth factor 8
Flk-1	Fetal liver kinase 1
GATA-4	GATA-binding protein 4
GFP	Green fluorescent protein
GG2	Glial growth factor 2
GMT	Gata4, Mef2c and Tbx5
H_2O_2	Hydrogen peroxide
HGF	Hepatocyte growth factor
iCMs	iPSC-derived cardiomyocytes
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iPSC	Induced pluripotent stem cell
iPSC-CMs	iPSC-derived cardiomyocytes
Isl-1	Islet-1
ISO	Isoproterenol
JNK1	c-Jun N-terminal kinase 1
Klf4	Kruppel-like factor 4
LA	Left atrium
Lin	Lineage
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MCP-1	Mast cell proteinase-1
MDR-1	Multidrug resistance 1
MEF2C	Myocyte-specific enhancer-binding factor 2C
Mer	Modified oestrogen receptor
MI	Myocardial infarction
miRNAs	Micro-ribonucleic acids
neg	Negative
Nkx2.5	NK2 homeobox 5
NRG-1	Neuregulin
O_2	Oxygen
Oct-4	Octamer-binding transcription factor-4
$p16^{INK4a}$	p16 kinase inhibitor 4a
$p21^{Cip1}$	p21 cyclin-dependent kinase inhibitor 1
PDGFrα	Platelet-derived growth factor receptor alpha
Pecam-1	Platelet/endothelial cell adhesion molecule 1
Pim-1	Proviral integration site 1
pos	Positive
QT	Q-wave to T-wave interval
RA	Right atrium
RALDH2	Retinaldehyde dehydrogenase 2
RB	Retinoblastoma
ROS	Reactive oxygen species
RV	Right ventricle

SASP	Senescence-associated secretory phenotype
Sca-1	Stem cell antigen 1
SCIPIO	Stem Cell Infusion in Patients with Ischaemic cardiOmyopathy
SEC	Suspension explant culture
Shh	Sonic hedgehog
SirT1	Sirtuin 1
SMA	Smooth muscle actin
Sox-2	Sex determining region Y-box 2
SP	Side population
SSEA-4	Stage-specific embryonic antigen 4
STAT3	Signal transducer and activator of transcription 3
TAA	Thoracic aortic aneurysm
TAD	Thoracic aortic dissection
Tbx18	T-box 18
Tbx5	T-box 5
TERT	Telomerase reverse transcriptase
TG	Transgenic
TGF-β1	Transforming growth factor beta 1
Tβ4	Thymosin beta 4
VEGF	Vascular endothelial growth factor
$VO_{2\,max}$	Maximal oxygen consumption
VSELs	Very small embryonic-like stem cells
vWF	von Willebrand factor
Wnt	Wingless-type
Wt1	Wilm's tumour 1
ZEG	lacZ/enhanced green fluorescent protein

1 The Adult Myocardium as a Self-Renewing Organ

For a long time, the cardiovascular research community, focusing on the cardiomyocyte, has treated the adult mammalian heart as a post-mitotic organ without intrinsic regenerative capacity. The prevalent notion was that the >20-fold increase in cardiac mass from birth to adulthood and in response to different stimuli in the adult heart results exclusively from the enlargement of pre-existing myocytes [1–3]. It was accepted that this myocyte hypertrophy, in turn, was uniquely responsible for the initial physiological adaptation and subsequent deterioration of the overloaded heart. This belief was based on two generally accepted notions: (a) all myocytes in the adult heart, formed during fetal life or shortly thereafter, were terminally differentiated and could not be recalled into the cell cycle [4, 5]; therefore, all cardiac myocytes had to be of the same chronological age as the individual [6]; (b) the heart has no intrinsic parenchymal regenerative capacity because it lacks a stem–progenitor cell population able to generate new myocytes [7].

Despite published evidence that this prevalent view was incorrect [7–13], it is still debated and remains controversial whether the adult heart, unlike all other organs, is

self-renewing and harbours a true population of tissue-specific stem–progenitor cells. This belief is in part because the new accepted 'measured' myocyte turnover of the adult heart does not appear to be very robust [14], and therefore its physiological significance has remained in doubt. In this review we will evaluate the literature on the identification, location and potential of the stem–progenitor cells resident in the adult mammalian heart, including human. We will also assess the clinical applicability of these tissue-specific, endogenous cardiac stem–progenitor cells (referred to hereafter as eCSCs) to repair and regenerate physiologically and functionally significant new myocardium.

2 Identity and Location of Endogenous Cardiac Stem–Progenitor Cells

2.1 c-Kit^{pos}

In 2003, our group in collaboration with Piero Anversa identified the first population of eCSCs in the adult mammalian rat heart [15]. These cells express the stem cell marker c-kit (c-kit^{pos}) and are positive for Sca-1 and MDR-1 (ABCG2), yet are negative for markers of the blood cell lineage, CD31, CD34 and CD45 (described as Lin^{neg}). They are self-renewing, clonogenic and multipotent and exhibit significant regenerative potential when injected into the adult rat heart following a myocardial infarction (MI), forming new myocytes and vasculature and restoring cardiac function [15]. c-kit^{pos} eCSCs with similar properties to those originally identified in the rat have been identified and characterised in the mouse [16, 17], dog [18], pig [19] and human [16, 20–23]. These cells are present at a similar density in all species (~1 eCSC per 1,000 cardiomyocytes or 45,000 human eCSCs per gram of tissue) [24]. Similar to the rodent heart, the distribution of c-kit^{pos} eCSCs in the pig and human heart varies with cardiac chamber. Arsalan et al. [23] reported that the left (LA) and right (RA) atria harbour significantly more c-kit^{pos}, Lin^{neg} cells (5 % of mononucleated cells isolated) than the left ventricle (LV, 0.62 %) and this is also true for the pig [19]. These adult-derived c-kit^{pos} eCSCs are very similar in their characteristics and potential to a population of cardiac-specific (c-kit^{pos}/Nkx2.5^{pos}) cells identified in the mouse embryo that differentiate into cardiomyocytes and also smooth muscle cells [25]. Indeed, embryonic cardiac c-kit^{pos}/Nkx2.5^{pos} cells possessed the capacity for long-term expansion in vitro, clonogenicity and differentiation into both cardiomyocytes and smooth muscle cells from a single cell-derived colony [25].

The number of total c-kit^{pos} cardiac cells (lacking any identification/description/characterisation of the 'real' cell with stem properties and regenerative potential within this cell cohort) is significantly higher in the neonatal compared to adult heart and it has been claimed that neonatal c-kit^{pos} cardiac cells have robust regenerative properties which are lost in the adult cell cohort counterpart [26, 27]. Indeed, in the adult heart the total population of cardiac c-kit^{pos} cells (including the CD45^{pos} fraction representing cardiac mast cells and CD34^{pos} cells representing vascular progenitors;

see below) has little cardiomyogenic potential and following cryogenic injury (induced by touching a 1 mm diameter copper probe that is equilibrated in liquid nitrogen to the apex of the left ventricle) contributes predominantly through revascularisation of the damaged tissue. These cells express Flk-1 and Pecam-1 (CD34), suggesting that they are primarily vascular progenitors and bear more resemblance to the bone marrow-derived c-kit^{pos}/Sca-1^{pos}/Flk-1^{pos} cells identified by Fazel and colleagues [28], which following an MI home to the heart and contribute to the revascularisation of the infarcted/damaged area by establishing a pro-angiogenic milieu. Importantly, the c-kit^{pos} eCSCs are CD34 negative making them distinguishable from these vascular progenitor c-kit^{pos} cells [29]. Because of these findings, the role of c-kit^{pos} eCSCs in the adult mammalian heart has been questioned [30–32]. c-kit (also known as CD117) is a tyrosine kinase type III receptor, which is expressed in several cell types and plays a significant role in a variety of cell functions, including identifying haematopoietic stem cells while regulating their cell fate [33]. However, lone c-kit detection/expression does not identify stem–progenitor cells. Thus, relying on genetic labelling of c-kit^{pos} cells or quantifying c-kit^{pos} cells within any tissue, including the heart, to extrapolate their plasticity or regenerative potential is clearly a major biological and practical pitfall that brings data which are either inconclusive at best or non-interpretable overall. The latter was also the case for the first erroneous claim that c-kit^{pos} eCSCs did not exist in the adult human heart. Indeed, Pouly et al. [31] investigated c-kit-positive cells in endomyocardial, right ventricular (RV) biopsies and right atrial appendages of heart transplant recipients 73.5 months post-transplantation. Using immunohistochemistry they found that c-kit-positive cells were rare (1/mm^2 atrial tissue and 2.7/mm^2 RV tissue). None of the c-kit-positive cells identified expressed Nxk2.5 or CD105, markers of cardiomyocyte and endothelial lineages, respectively; however, all of these cells expressed CD45 and tryptase, identifying them as cardiac mast cells. It is not surprising that the authors only identified mast cells, as cardiac mast cells have previously been reported to account for ~80 % of the total number of c-kit^{pos} cells in the atria [19].

Finally, c-kit is also expressed on other populations of identified eCSCs such as cardiosphere-derived cells from mouse [16], human [16, 34] and porcine hearts [34] and those identified as side population (SP) and Sca-1^{pos} progenitor cells in the mouse [35], as shown in Table 1 and discussed below. Intriguingly, the exact role and function of c-kit in the clonogenicity, self-renewal and cardiac regenerative potential of eCSCs are yet unknown.

2.2 *Sca-1^{pos}*

As first described in 2003 [36], Sca-1^{pos} cardiac progenitor cells (CPCs) are resident non-myocyte cells from the adult murine heart that express stem cell antigen 1 (Sca-1). While the total Sca-1 CPCs express early cardiac-specific factors such as Gata-4 and MEF2C [36, 37], a fraction of them exhibit stem cell properties of self-renewal [37–39]. Sca-1^{pos} Lin^{neg} CPCs are small and round, being ~2–3 μm in length with a high nucleus to cytoplasm ratio [40].

Table 1 Characteristics of resident eCSC populations identified in the heart

Phenotype	Expression	Species	In vitro stem cell properties			In vivo cardiac regenerative potential
			Self renewal	Cardiomyocyte differentiation	Multipotency	
c-kit	Nkx2.5 [15, 19, 151] MEF2C [15] GATA4 [15, 19] Sca-1 [16, 18] MDR-1 [15, 18, 23] CD90 [19] CD133 [23] CD166 [19] Flk-1 [16, 19] Nestin [15] Nucleostemin [100] Oct3/4 [19, 23] SSEA-4 [19] Nanog [19, 23] Bmi-1 [19, 23] TERT [23]	Adult Rat [15, 151] Neonatal and Adult Mouse [16, 17, 26, 27, 100] Adult Porcine [19] Adult Dog [18] Post-natal and Adult Human [21–23]	Yes	Yes	Yes	Yes

(continued)

Table 1 (continued)

Phenotype	Expression	Species	In vitro stem cell properties			In vivo cardiac regenerative potential
			Self renewal	Cardiomyocyte differentiation	Multipotency	
Cardiosphere derived	CD105 [34, 57] CD90 [34, 57, 59] CD34 [16, 34, 57] CD31 [16, 34, 57] ckit [16, 34, 56–59, 66] MDR-1 [57] Nanog [57] Flk-1 [57] GATA4 [57] SSEA-4 [57] Pax6 5 [56] PCNA 5 [56] DDR2 [59] Oct4 [59] Sox2 [59] Klf4 [59] GATA4 [58, 59] Nkx2.5 [58, 59] Isl-1 [58]	Neonatal and Adult Rodent [16, 34, 59]	Yes	Yes	Yes	Yes
		Neonatal and adult Human [16, 34, 58]	Yes	Yes	Yes	Yes
		Adult Dog [56]	ND	Yes	Yes	ND
		Adult Primate [57]	ND	Yes	Yes	Yes
		Adult Porcine [34, 56]	ND	Yes	ND	Yes
Sca-1	CD31 [36] CD29 [41] Pdgfrα [39] CD45 [37] c-kit [37] Isl-1 [38] CD105 [45]	Neonatal and Adult Mouse [36–43]	Yes	Yes	Yes	Yes
		Foetal and Adult Human [45, 109]	ND	Yes	ND	Yes

Epicardial	c-kit [89, 90] MDR-1 [89] CD34 [89] GATA4 [80, 89] Nkx2.5 [72, 80, 89] Sca-1 [80] CD44 [85, 89] CD90 [85, 89] CD105 [85, 89] Tbx18 [90, 206] Wt1 [72, 80, 90, 206] Isl1 [72, 80]	Fetal, Adult Human [85, 89, 90]	ND	Yes	Yes	ND
		Adult Rat [205, 206]	ND	No	No	ND
		Fetal, Adult Mouse [72, 80, 90]	Yes	Yes	Yes	Yes
		Fetal Chick [83, 207, 208]	Yes	Yes	Yes	ND
Side population	Sca1 [35, 51, 210] c-kit [35] CD31 [51] Tie1/2 [35, 51] Nkx2.5 [51] Gata4 [51]	Neonatal and Adult Mouse [35, 50–52, 209]	Yes	Yes	Yes	Yes
Isl-1	CD31 [77] Sca-1 [38] CD144 [76] Flk1 [70, 76] GATA-4 [70, 77] Nkx2.5 [70, 76, 77]	Fetal [70, 77], Post-natal [70] and Adult [58, 80] Mouse	Yes	Yes	Yes	ND
		Fetal [79], Postnatal and Adult Rat [79]	ND	Yes	ND	ND
		Fetal [76] and Post-natal [58, 70, 78] Human	Yes	Yes	Yes	ND

ND denotes not determined

Isolated Sca-1^{pos} CPCs are capable of cardiomyogenic differentiation in vitro [36–39, 41, 42]. After treatment with 5-azacytidine (5-AZA) Sca1^{pos} CPCs express α-sarcomeric actinin, cardiac troponin, Nkx2.5 and α-MHC [36]. Furthermore, oxytocin treatment also induces spontaneous contraction and expression of calcium transients [37], which suggests that it is more potent in inducing cardiac differentiation. When Sca-1^{pos} Lin^{neg} CPCs were treated with a cocktail of 5-AZA, TGF-β1 and vitamin C, they expressed α-sarcomeric actin and connexion 43 [38]. Furthermore, Sca1^{pos} CD31-negative CPCs show cardiomyogenic differentiation when treated with 5-AZA, DKK-1, DMSO, BMP2, FGF4 and FGF8 [42]. Moreover, when co-cultured with neonatal rat cardiomyocytes they also went on to express troponin T and phospholamban, showing cardiomyogenic maturation [42].

Sca-1^{pos} CPCs exhibit in vivo regenerative potential. When administered intravenously after ischaemic/reperfusion injury, Sca1^{pos} CPCs home to the injured myocardium and form new cardiomyocytes [36]. Cell transplantation of a subpopulation of Sca1^{pos}/CD34-negative CPCs has been shown to attenuate adverse structural remodelling with an increase in LV ejection fraction and new cardiomyocyte formation [42]. A recent study also found that 4 weeks post-MI, animals injected intramyocardial with Sca1^{pos} CPCs showed improved cardiac function and increased wall thickness in the infarct area, with the infarct area being 28 % of the LV wall compared to 40 % in controls [41].

It is hard to compare the Sca-1 populations like for like as each research group isolates their Sca-1^{pos} CPCs using different surface markers and techniques and report different expression of other cardiac stem–progenitor cells markers, such as c-kit [35–37] and PDGFrα [39]. However, in all these subpopulations, stem cell properties and cardiomyocyte differentiation are reported. Importantly Sca1^{pos} CPCs show predominant in vitro differentiation potential into both endothelial and smooth muscle lineages, which is essential for competent heart regeneration. Sca1^{pos}/CD45-negative CPCs treated in vitro with VEGF show endothelial differentiation with ~20 % of cells expressing CD31, vWF and Flk-1 [38], as well as differentiation into smooth muscle, with ~34 % of cells expressing SMA [38]. Similarly VEGF treatment of Sca1^{pos} CD31-negative CPCs induced expression of CD31, vWF and Flk-1 [42]. Furthermore, Sca1^{pos} cells have also demonstrated ability to differentiate into endothelial cells in vivo [41], which is likely through activation of STAT3 via Pim-1 signalling pathway [43]. It is worth noting that there is also a population of Sca1^{pos} vascular progenitor cells which resides within the arterial adventitia (AdvSca1 cells) that have been shown to be regulated by sonic hedgehog signalling (Shh) [44].

It is currently disputed if adult tissue-specific stem cells possess true pluripotency; however, Sca1^{pos} CPCs have shown capability of differentiation into non-cardiac lineages in vitro and in vivo [39, 41]. Interestingly it has been reported that the level of Sca-1 expression may actually play a role in their differentiation potential with Sca-1 high CPCs having a broader differentiation potential, showing osteogenic, chondrogenic, smooth muscle, endothelial and cardiac differentiation in vitro, than Sca-1 low CPCs [41]. In vivo teratoma formation assays have also shown that while these cells alone do not form tumours, when injected alongside ESCs they differentiate into cells of the three germ layers [39], although this broad developmental plasticity is yet to be shown in tissue regeneration and repair in vivo.

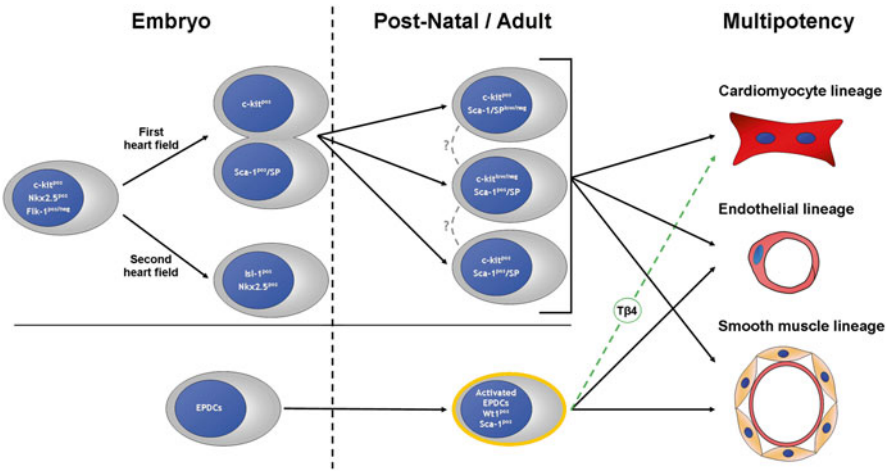

Fig. 1 A common cardiac stem–progenitor cell. The expression of markers on cardiac stem–progenitor cells is covered in this review, with their potential relationships and potencies. The *dashed green arrow* indicates that the commitment of EPDCs to a cardiomyocyte progenitor lineage is contingent upon thymosin-β-4 pre-treatment. *Tβ4* thymosin-beta-4, *EPDCs* epicardially derived cells

Although Sca-1 seems to be an ideal marker for isolating resident CPCs, its homology hasn't been confirmed in any species, other than mouse. This poses a problem when translating research to develop human regenerative therapies. Some groups have used murine Sca-1 antibodies to isolate homogenous cell populations from both fetal and adult human hearts that differentiate into mature cardiomyocytes in vitro after treatment with 5-AZA [45]. Other groups have suggested that Sca1^{pos} 'very small embryonic like stem cells' (VSELs) in mice are the same population that express CD133 in humans [46]. There is no question that Sca-1^{pos} CPCs are a potential source of cell for cardiac regeneration, and these cells have significant overlap and co-expression with other cardiac stem–progenitor cells, namely the c-kit^{pos} eCSCs (Table 1 and Fig. 1). Therefore, what now needs to be determined is whether these two cell populations represent different cell types or the same one at different developmental/differentiation stages.

2.3 *Side Population Cells*

Side population (SP) cells were first characterised as a primitive population of haematopoietic stem cells characterised by their unique ability to efflux the DNA-binding dye, Hoechst 33342 [47]. SP cells have since been isolated from extra-haematopoietic tissues, including bone marrow, skeletal muscle, liver, brain, heart and lung [48], and the ATP-binding cassette transporter (ABCG2, also referred to as MDR-1) has been identified as a molecular determinant of the SP phenotype [35, 49]. Hierlihy et al. [50]

first reported that the adult myocardium contained an endogenous cardiac side population (CSP) with stem cell-like activity and identified that this Hoechst dye-excluding population constituted ~1 % of total cardiac cells in the mouse postnatal heart [50]. These findings were supported by Martin et al. [35] who also demonstrated that the CSP isolated from adult mice could successfully differentiate into α-actinin-positive cells when co-cultured with cardiomyocytes from wild-type mice. Transcriptional profiling revealed that the CSP exhibits a Sca-1pos, c-kit^{low}, CD34^{neg}, CD45^{neg} phenotype and expresses a number of endothelial and haematopoietic transcripts ([35]; Table 1). Further interrogation of murine CSP cells revealed that 84 % express the stem cell marker, Sca-1, while 75 % express the endothelial marker, CD31. The Sca-1^{pos} CD31^{neg} population was subsequently identified as having the greatest cardiomyogenic potential and was found to represent ~10 % of the total CSP. These findings are in support of Wang and colleagues, [42]. Phenotypic analysis of Sca-1^{pos} CD31^{neg} CSP revealed that they also expressed cardiac-specific markers, such as Nkx2.5, GATA4, SMA and desmin. Furthermore, once subjected to co-culture with primary cardiomyocytes they adopt a more mature phenotype expressing α-actinin, troponin I, and connexin-43 and undergo spontaneous contraction [51]. Oyama et al. [52] demonstrated comparable findings using rat neonatal CSP cells utilising oxytocin or trichostatin A, in the absence of co-culture, to differentiate CSP cells into beating cardiomyocytes [52]. The first CSP cell transplantation experiments involved direct injection of Sca-1^{pos} CD31^{neg} cells into the infarct region of mice post-MI and this was found to improve LV ejection fraction (LVEF) and promote myocardial neo-angiogenesis consequently providing the first evidence of CSP cells regenerative potential in vivo [42]. In addition, Oyama et al. found that neonatal CSP cells were able to home to cryoinjured hearts and upon reaching the site of injury form new cardiomyocytes, fibroblasts, endothelial and smooth muscle cells [52].

2.4 Cardiosphere-Derived Cells

The ability of eCSCs to grow in suspension and generate spheres was first demonstrated in the original paper describing the discovery of c-kit^{pos} eCSCs [15]. Soon after Messina with colleagues [16] described the isolation of undifferentiated cells that grow as 'clusters' from postnatal atrial or ventricular human biopsy specimens and murine hearts, that the authors named 'cardiospheres' (CS) because they resembled the neurospheres formed by neural progenitors [16, 53]. These spheres were clonogenic, self-renewing, differentiated into cardiomyocyte, endothelial and smooth muscle lineages [15, 16, 54].

Cardiosphere-derived cells (CDCs) are putative immature and regenerative cells which spontaneously migrate and shed from small pieces of tissue, cultured as primary explants. Cells collected from the explants are further selected for their spontaneous organisation in cardiospheres [34, 55]. CDCs have been isolated successfully from various species (rodent, porcine, canine, primate and human) at different developmental stages by several groups [16, 34, 54, 56–58]. CDCs represent a

mixed cell population, consisting of cells with mostly mesenchymal markers CD105 and CD90, with a minority of CD34/CD31 endothelial cells [16, 34, 59] (Table 1). c-kit expression was also reported in CDCs isolated from all species, as c-kit^{pos} eCSCs were the first population to show CS formation [15]. However, c-kit expression within CDCs varies (0.1–30 %) depending on the species, donor age and culturing method [34, 54, 59–62], being more abundant in neonatal CDCs [58]. Interestingly, 80 % of adult primate CDCs expressed SSEA-4 [57], but other pluripotent cell markers like Oct4, Sox2 and Klf4 were rare, despite being found in neonatal rat [59] and human CDCs [58]. CDCs can be successfully isolated from all four human heart chambers [58] and the left ventricles of dog and rhesus monkey [56, 57]. However, some locations like human ventricular epicardium were less successful [62], as epicardial progenitor cells need to be activated [63]. So far, the most surgically accessible region in the human hearts, both neonatal and adult, is right atrium [58], which is also an abundant source of c-kit^{pos} eCSCs [64].

CDCs derived from human, mouse and rat hearts are clonogenic and self-renewing ([16, 54], Table 1). Multipotency and differentiation of CDCs into cardiomyocytes, smooth muscle and endothelial lineages have been demonstrated for human, rat, mouse and dog [16, 38, 54, 56, 59]. CDCs derived from primates showed differentiation in vivo towards an endothelial CD31^{pos} cell lineage [57].

It has been proposed that the growth of eCSCs in the form of cardiospheres mimics the stem cell niche microenvironment. In a recent study, when cardiospheres and monolayer CDCs were injected intramyocardially following MI in mice, only cardiosphere-treated mice showed a significant increase in LVEF after 3 weeks [60]. These effects may be due to the higher oxidative stress resistance, as shown by a fourfold increased viability of cardiospheres, compared to monolayer cultured cells, after H_2O_2 treatment [56], and twofold increase in stem cell markers Sox-2, Nanog and TERT [60]. These findings taken together lead to better survival and engraftment of cardiosphere CDCs in vivo [56, 60]. Furthermore, in a mouse model of MI, CDCs appeared superior to bone marrow-derived cells or adipose-derived mesenchymal cells [65]. These findings in small animal models have led to initiation of large animal pre-clinical studies where safety, dose and route of administration are determined in the porcine model of MI [66, 67].

Several groups have questioned the concept of CDCs, stating that their unique properties come from haematopoietic and cardiomyocyte contamination and spheres were not clonogenic, but merely formed by clumping of fibroblast-like cells [68, 69]. Their concerns were addressed by Davis and colleagues who performed careful studies of stem cell characteristics including lineage-tracing experiments on CDCs and showed that differences in methodology have led to discrepancy amongst findings [54].

2.5 Isl-1

A further population of progenitor cells resident in the heart is identified by the marker Islet-1 (Isl-1) (Table 1 and Fig. 1). This cell type was originally identified in

rodent and human postnatal myocardium by Laugwitz et al. in 2005 [70]. These cells are primarily found in the second (anterior) heart field during development, contributing to formation of the atria, outflow tract and right ventricle, a process dependent on Wnt/β-catenin signalling [71]. Islet-1-positive cells are also involved in the development of the proepicardium and endocardium [72, 73]. The neural crest also contributes to an Isl-1-expressing cardiac cell population during development [74]. Expression of Isl-1 is involved in the direction of primitive cardiac progenitors to more specific lineages of progenitor cells [75]. Indeed, when Isl-1-positive cells are derived from human ESCs, they are capable of generating cells committed to cardiomyocyte, smooth muscle or endothelial cell lineages [76].

The definite cardiac origin of these cells, their contribution to cardiac development and their multipotency [77] have been clearly defined. Successful derivation of Isl-1-positive multipotent cells has been achieved from ESCs [75, 76] or from neonatal cardiac tissue [70], but there is far less evidence of Isl-1-expressing cells playing a significant role in adult life. The scarcity of Isl-1-positive cells after embryonic development, with very few identified throughout the heart in the 1-day-old neonatal rat [70] or 2- and 6-day neonatal human tissue [78], argues against a major contribution of Isl-1 cells to cardiac cellular homeostasis in adult life.

However, there is evidence of an Isl-1-expressing cardiac cell population being present in adult cardiac tissue. A few Isl1-positive cells were seen in the adult (11–13 weeks) rat heart, although these were also all cardiac troponin-I positive, indicating their cardiomyogenic differentiation [79]. These cells have also been studied in non-physiological situations, where Isl-1-positive cells have been identified in the periphery of an infarct in the mouse heart following pre-treatment with thymosin-β-4 [80]. In addition, Isl-1-positive cells have been obtained in vitro from CDCs, which were in turn obtained from cells activated in vivo following myocardial infarction in 9-month-old mice [38]. On the other hand, Isl-1-positive cells have been recently identified in the adult heart as a novel marker of the sinoatrial node and do not serve as cardiac stem–progenitor cells [81].

2.6 Epicardial-Derived Cells

Based on their key roles in cardiac development as the source of endothelial and smooth muscle cells in coronary vasculature and adventitial, interstitial fibroblasts [82] and cardiomyocytes [83, 84], epicardial-derived progenitor cells (EPDCs) have been suggested as a population of cells with possible cardiogenic potential (Table 1 and Fig. 1).

EPDCs explanted in culture from human right atrial appendages have a fibroblastic appearance and adhere to plastic dishes [85]. Human EPDCs express the mesenchymal markers CD44, CD90 and CD105 and the cardiac transcription marker Gata-4, yet are negative for Isl-1 and Sca-1 [85]. In vitro, they undergo spontaneous epithelial to mesenchymal transition (EMT), while after infection with an adenovirus vector encoding myocardin or after treatment with transforming growth factor β1 (TGF-β1) or bone

morphogenetic protein 2 (BMP-2), they obtain characteristics of smooth muscle cells but fail to form endothelial cells [85]. After transplantation into infarcted mouse hearts, EPDCs differentiate into endothelial and smooth muscle cells but not cardiomyocytes [86, 87]. Nevertheless, they were shown to improve cardiac function, mainly through paracrine protection of the surrounding tissue, when transplanted into ischaemic myocardium on their own or combined with cardiomyocyte progenitor cells [86, 87]. It is important to note, however, that during cardiac development, EPDCs are generated from a subset of epicardial cells which delaminate from the epicardial epithelium and undergo EMT, a process which is observed to occur at the atrioventricular junction, in the ventricular epicardium, and in the epicardium at the junction between the ventricles and the outflow tract, but not in the atrial epicardium [83].

Adult mouse EPDCs induced to express Wilm's tumour 1 (Wt1; a key embryonic epicardial gene) through pre-treatment (priming) with thymosin β4 (Tβ4), an actin monomer binding protein previously shown to restore vascular potential to adult EPDCs in infarcted hearts [63, 88], can result in neovascularisation and de novo cardiogenesis, after myocardial infarction [80]. $Wt1^{pos}$ EPDCs are positive for Sca-1 (~80 %) and co-express early cardiac progenitor markers Isl-1, Nkx2.5 and Gata-4, but were negative for c-kit [80].

Identification of another two distinct adult epicardial stem cell populations has been reported by a different research team [89]. One is c-kit^{pos} and the other is $CD34^{pos}$, while both subsets are negative for CD45. These cells, found in the subepicardial compartment of human epicardium and mesothelial layer of mouse epicardium, display a nonadherent phenotype, express early and late cardiomyocyte-specific transcription factors and acquire an endothelial phenotype in vitro [89]. In a mouse model of MI, epicardial c-kit^{pos} cells mediate a regenerative response, by proliferating, migrating toward the site of injury and differentiating into myocardial and vascular cells, significantly preventing cardiac function impairment and LV remodelling [89]. This procedure is thought to involve EMT as well as activation of embryonic epicardial genes, Tbx18, Wt1 and RALDH2. Consistent with Smart et al. [80] the expression of these genes is significantly enhanced after MI, influenced by growth factor release in the pericardial fluid [80, 90].

Due to their similarities in phenotype and differentiation potency, it is argued that epicardial c-kit^{pos} cells and the c-kit^{neg} EPDCs represent the same cell population at different developmental stages, where the former exists in a more undifferentiated state and at a later stage loses expression of c-kit and gives rise to EPDCs [91].

3 Cardiac Stem–Progenitor Cell Populations as a Whole

Considering the variety of eCSC population identities that have been established throughout embryonic to postnatal and adult life, the question arises of whether and how much these populations may overlap, or whether they may in fact constitute a single stem–progenitor cell with a sequence of lineages, which alters in phenotype (and perhaps also in their degree of potency) across life (Fig. 1).

4 Origins of Resident Cardiac Stem–Progenitor Cells

4.1 'Native' to the Heart Since Development

An intriguing question concerning eCSCs resident in the heart is whether they are directly descended from lineages which have been present since early development or have possibly 'migrated' to the heart later in life. A study of Nkx2.5-positive, multipotent cardiac stem cells early in development found expression of c-kit in ~28 % of these cells, which were also negative for CD45, demonstrating that c-kit expression marks a major subset of cardiac progenitors during development [25]. Furthermore, Nkx2.5^{pos}, c-kit^{pos} cells were more proliferative and less differentiated than Nkx2.5^{pos}, c-kit^{neg} cells; this correlation was not found with Sca-1 expression levels in Nkx2.5^{pos} cells [25]. However, it has not been determined if the adult c-kitpos eCSCs are directly descended from these cells.

Lineage tracing of Isl1-expressing and Nkx2.5-expressing cells showed that these each contribute to both the first and second heart fields during development, although Isl1-expressing cells make a smaller contribution to the left ventricle than elsewhere in the heart [92].

Analysis of GFP-positive cells in the embryo of a c-kit-GFP transgenic mouse during cardiac development showed a c-kit-expressing population of progenitor cells that was resident in the heart and did not migrate from extra-cardiac tissue (although a contribution to the c-kit-positive population from extra-cardiac sources could not be excluded) and were present in the postnatal period [93]. These cells were also shown to have comparable properties to c-kit^{pos} eCSCs in adult life in terms of proliferation, multipotency and myocardial regenerative capacity [93].

4.2 Bone Marrow Origin, May Have Previously Migrated

c-kit^{pos} eCSCs do not express the haematopoietic lineage markers CD45 or CD34. Although it has been determined that resident c-kit^{pos} eCSCs are separate from circulating bone marrow-derived cells, the possibility remains that these eCSCs may have previously migrated into the cardiac tissue from another source, such as bone marrow, after cardiac development and lost their haematopoietic phenotype.

The first findings indicating a regenerative potential in the adult mammalian heart were of host cells taking up residence within transplanted cardiac tissue and developing into cardiac-specific cell types [10, 94]. Subsequent findings were made of donor cells' presence in myocardial tissue in patients following bone marrow transplant [95]. This last study did not identify any donor-derived cardiac-specific cells, and indeed it appears that these cells' presence in an infarction does not necessarily indicate cardiac lineage potential [27].

However, this does not exclude their ability to contribute to the population of resident stem–progenitor cells in the adult heart. Indeed, it has been shown that

bone marrow-derived cells can replenish the numbers of SP eCSCs following infarction [96]. It should be noted, however, that the regenerative potential of exogenous injected stem–progenitor cells post-MI can be significantly affected by the source of the cells or the selection of a subpopulation by specific phenotype [97].

It remains possible that these cells provide only a supportive function during the eCSC population's recovery after injury, rather than reconstituting its abilities. An investigation of the role of bone marrow-derived c-kit-positive cells in cardiac repair post-MI indicated that the vast majority of the c-kit-positive cells in the heart post-MI were of bone marrow origin [28]. However, this study used a model where irradiation prior to bone marrow transplant may have eliminated, or rendered dysfunctional, a significant percentage of the resident c-kit^{pos} eCSCs also. The use of a double transgenic model provided strong evidence that the role of exogenous bone marrow cells post-MI is to support the endogenous cardiac regenerative capacity and that more precise selection of the exogenous bone marrow cells to be transplanted increased the efficacy of this support [98].

It therefore appears clear that with regard to c-kit^{pos} eCSCs there is a resident population distinct from any circulating bone marrow cells. However, the possibility that circulating bone marrow stem cells take up residency post-development and then adopt a specific 'cardiac' stem–progenitor cell phenotype has not yet been conclusively confirmed or disproved. If the latter were the case, then the eCSC pool will largely depend on bone marrow-derived stem cell biology and regenerative potential. However, it should be pointed out that eCSCs isolated from the adult heart have a phenotype not present (or so far not identified) in the bone marrow. Therefore, if the eCSCs originated from the bone marrow, they either represent a specific and rare bone marrow cell population not yet identified or they have resided in the myocardium long enough to have lost the bone marrow stem cell-specific epitopes.

4.3 Evidence of Site Specificity of Stem–Progenitor Cells Within the Heart

Using genetic lineage-tracing techniques a number of eCSC populations have been characterised further to determine the origins, niche and endogenous location within the healthy adult mammalian heart. When conducting lineage-tracing studies, it is difficult to absolutely determine from where a cell has originated as many types of cells express the same markers or express them at different time points during embryogenesis and postnatal growth. Flk-1, for example, originally thought to be associated with haematopoietic and vascular lineages, is also expressed at a stage during cardio- and skeletal myocyte development [99]. Some markers can mark cells from a specific dermal layer, such as brachyury, which marks cells from the mesoderm [99]. What these studies do allow us to do is to label a population of cells at a specific time point to allow us to track them over time [26, 27, 100].

The location of a population of c-kit^{pos} cells in the embryonic and adult heart has been assessed using c-kit BAC-EGFP mice [26, 27]. Fluorescent EGFP+ cells

were observed in both atrial and ventricular walls at 14.5dpc, with the total number of EGFP^{pos} expressing cells increasing as the heart developed, peaking 2–3 days after birth. After this time numbers rapidly decline with the majority of EGFP^{pos} cells found as mononuclear cells within vascular compartments. Co-staining of c-kit^{pos} cells in 2–3 day postnatal mice shows co-expression with CD45 within the epicardial border indicating a haematopoietic lineage, while those found in the atrioventricular region express Flk-1 suggesting they are ES cell-derived and vascular progenitors [26]. As adult c-kit^{pos} eCSCs are CD45^{neg}, the EGFP cells identified by Tallini et al. [26] cannot be classified at eCSCs and have likely migrated from the bone marrow. Furthermore, this study did not characterise the EGFP^{pos} cells against other markers previously identified in adult c-kit^{pos} eCSCs [15, 16, 19–21].

A recent study by Chong et al. [39] found that a population of Sca-1/PDGFrα^{pos} eCSCs in the adult heart are found predominantly in the perivascular, adventitial niche and through Cre-lineage tracing were found to have a pro-epicardial origin [39]. These findings are in support of Smart et al. [80] who found that 80 % of EPDCs are Sca-1^{pos}.

Studies have shown a change in location and number of eCSCs between the adult and embryonic heart [39, 58] which has been implicated in differences in their regenerative potential [101].

MerCreMer-ZEG mice have been used to show if resident stem–progenitor cells are responsible for myocyte turnover in the healthy adult and/or in injured myocardium. Transgenic MerCreMer-ZEG mice were treated with tamoxifen to label 80 % of cardiomyocytes with GFP while 20 % remain β-galactosidase (β-gal) positive. After 1 year of ageing there was no reduction in the percentage of GFP^{pos} or β-gal^{pos} myocytes indicating that no new myocytes had been formed [100], although another study reported a 50 % replacement of myocytes over a human's lifetime [14]. After Injury by MI or pressure overload, there was a significant decrease in the percentage of GFP^{pos} cardiomyocytes in the border of MI and other remote areas, from 82.5 % in sham-operated animals to 67.5 % in MI and 76.6 % in pressure overload animals [100]. This was balanced with an increase in β-gal^{pos} myocytes of 34 % in MI border areas and 30 % in pressure overloaded hearts [100]. This supports the idea that adult stem–progenitor cells do contribute to cardiomyocyte renewal following injury, and it is not due to myocyte proliferation.

5 Derivation of Stem–Progenitor Cells from Cardiac Tissue

5.1 Obtaining Cardiac Stem–Progenitor Cells from Mouse and Rats

All of the eCSC populations identified above (c-kit, Sca-1, Isl-1, EPDCs, CDCs, SP) have been successfully isolated from rodents [15, 16, 35, 36, 70, 102]. The first isolation of c-kit^{pos} eCSCs from adult tissue used enzymatic digestion with selection

of the cells of interest using specific markers in magnetic activated cell sorting (MACS; Miltenyi) or FACS [15]. A broadly very similar protocol was used to isolate Sca-1^{pos} eCSCs from mice [36]. One successful method of isolating eCSCs from rodents is by enzymatic digestion through retrograde perfusion of the heart (via a cannula placed in the aorta) to remove blood and perfuse digestive enzymes through the tissue [103, 104].

The initial isolation of Isl-1^{pos} cells was carried out by enzymatic digestion of postnatal mouse heart tissue followed by the use of selective culture conditions to enrich the Isl1-expressing population [70]. The isolation of side population cells by definition involves selection based on dye exclusion, and the method for isolating these cells is based around tissue digestion and percoll gradient cell separation, followed by FACS analysis and cell selection [35].

An alternative to the use of complete tissue digestion has been to grow stem cell populations from small pieces of tissue explanted in culture to allow the cells to migrate out of the tissue and expand in culture for subsequent isolation. This method was used to obtain a population of eCSCs for the first generation of cardiosphere generating cells [16]. Optimising the culturing conditions of this technique allows expansion of CDCs [34], and a monolayer of CDCs can be formed for characterisation [55].

An important consideration when isolating stem cells using the tissue digestion method is to allow liberation of eCSCs from deep within myocardium, but without damaging these small cell populations, as eCSC surface markers can readily be affected by over-digestion [105]. Combining the advantage of the explant technique with the population specificity of subsequent MACS has been successfully used to isolate eCSC populations expressing Sca-1 [106] and c-kit [107].

The isolation and expansion in vitro of epicardium-derived progenitor cells (EPDCs) have been achieved using essentially an explant technique, which is augmented by the addition of the peptide thymosin-β-4 to activate the otherwise quiescent EPDCs and promote their outgrowth [102].

5.2 Obtaining Cardiac Stem–Progenitor Cells from Human Subjects

As stated above the isolation of eCSCs has been performed based mainly on the expression of surface or transcriptional markers. However, the first successful isolation of adult human eCSCs reported undifferentiated cells, growing from explants of postnatal atrial/ventricular human biopsy specimens, which were able to form cardiospheres in suspension culture [16]. It was first defined that these cardiospheres were composed of a heterogenous population of cells (cardiac stem–progenitor cells, differentiating progenitors, spontaneously differentiated cardiomyocytes and vascular cells).

The original culture method [16] was modified to improve efficiency with the resultant first documentation of CDCs [34]. The human endomyocardial tissue fragments were partially digested enzymatically and then cultured as explants on dishes

coated with fibronectin. After several days a layer of phase-bright cells arose from adherent explants, which expressed antigenic characteristics of stem–progenitor cells [34], including Isl-1 and c-kit [58]. The number of CDCs extracted depends on age and sample source [58], with human CDCs being most abundant during the neonatal period and decreasing in number over age. Furthermore, CDCs can also be isolated reproducibly from right atrial samples [58].

In another study, the conditions were established for the isolation and expansion of c-kit^{pos} human eCSCs from small samples of myocardium [22]. Two methodologies of isolation were used: the first consisted of the enzymatic dissociation of myocardial samples and the second where samples were cultured by the primary explant technique. c-kit^{pos} eCSCs were sorted with immunobeads (MACS; Miltenyi) and plated at low density to obtain multicellular clones from single founder cells. Recently, human c-kit^{pos} eCSCs have been successfully isolated from the left ventricle, the appendages of the right atrium and left atrium by enzymatic digestion [23]. Moreover, the concentration of c-kit^{pos} human eCSCs was higher in the atria than in the ventricle, confirming previous findings [20, 21, 24].

A new culture method that favours clinical translation by reducing the need for exogenously added factors to explants has been developed [108]. This method, identified herein as suspension explant culture (SEC), allows for derivation and enrichment of migratory human c-kit^{pos} cells and is based on modifications of the method originally described by Messina et al. [16] and later modified by Smith et al. [34]. Human right atrial appendages are placed in a higher volume of media without previous enzymatic digestion. This higher volume ensures that the tissue remains suspended and does not become adherent to the surfaces of the flask, which minimises contact-dependent fibroblast outgrowth. Furthermore, without enzymatic digestion, the addition of exogenous enzymes is avoided, which can affect cell characterisations reliant on immunoreactive surface markers [108].

Despite its homology not being confirmed in any mammalian species other than the mouse Smits et al. have isolated human Sca-1-like cardiac progenitor cells from the auricle, from appendix of the atrium that is removed during heart surgery or from fetal heart tissue [109]. It is postulated that the anti-mouse Sca-1 antibody may cross-react with an unknown protein, still leading to a homogenous cell population.

c-kit^{pos}, Sca-1^{pos}, MDR-1^{pos}, CD34^{pos}, CD45^{neg}, CD31^{neg} EPDCs have been isolated by enzymatic digestion and immunobead sorting from the fetal and adult human epicardium [91], which included the mesothelial layer and the subepicardial space containing the adipose tissue. Moreover, treatment of human epicardial explants with thymosin beta-4 stimulated extensive outgrowth of Wt1^{pos} EPDCs cells that differentiated into fibroblast, endothelial and smooth muscle cells [63].

5.3 Expansion of Cardiac Stem–Progenitor Cells In Vitro

The successful isolation of resident cardiac stem–progenitor cells from heart biopsies has allowed us to study their biological characteristics and their

application in therapeutic approaches for the repair and regeneration of the ischaemic/infarcted heart. In order to obtain a sufficient amount of cells for use in cardiac regenerative therapy, cardiac stem–progenitor cells have to be first expanded in vitro. It is generally accepted that a better understanding on how to generate large numbers of cells in a short period of time without compromising their regenerative quality and differentiation potential is needed. Improper conditions used for cardiac stem–progenitor cell isolation and expansion can adversely affect their potential; therefore, modifications to the expansion protocol may be further developed and optimised. Indeed, it has been shown that over a long period of time in culture, rat c-kit^{pos} eCSCs may upregulate GATA-4 and become more committed to the cardiomyocyte lineage, which may be beneficial for prospective therapeutic application. On the other hand, over long-term culture c-kit^{pos} eCSCs have shown unwanted adipogenic and skeletal muscle differentiation [107].

In order to perfect the in vitro expansion protocol for cardiac stem–progenitor cells, many factors can be manipulated and studied. Culture media supplemented with different growth factors and molecules have led to successful expansion of clonal c-kit^{pos} eCSCs and CDCs without affecting their phenotype, differentiation potential or genomic stability [16, 19, 22, 34, 58, 110]. Indeed, clones of pig c-kit^{pos} eCSCs, which have a ~22 h doubling time can be propagated for ~65 passages and serially subcloned every ten passages without reaching growth arrest or senescence or showing any detectable chromosomal alterations [19]. Interestingly, surface modification with amine residues improved c-kit^{pos} eCSC proliferation, expansion rate and attachment ability and improved c-kit expression maintenance, possibly by modulating the ECM complex and/or MAPK signalling [111].

Several groups have shown independently that low (physiological) oxygen has favourable effects on eCSC expansion in vitro. Tang and colleagues showed that hypoxic preconditioning in vitro increases chemokine receptor CXCR4 expression in human c-kit^{pos} Lin^{neg} eCSCs, which consequently increases migration and homing of CSCs when introduced into ischaemic myocardium [112]. Culturing in 5 % O_2 increased proliferation, and therefore, cell yields, lowered senescence marker p16^{ink4a} expression and CDCs survived and engrafted better when introduced into ischaemic myocardium after infarction [113]. When autologous CDCs were expanded for use in CADUCEUS clinical trial [114], ~1/3 of preliminary runs revealed abnormal aneuploid karyotype in atmospheric O_2, whereas in hypoxic conditions, numbers of aneuploid cells have dramatically decreased [113, 115]. Data from another study revealed that short-term hypoxia leads to increased migration of human fetal-derived Sca-1-like CPCs, whereas long-term culture in low oxygen increased proliferation and the cells decreased expression of IL-8, MCP-1 and TGF-β1, showing a more pro-inflammatory phenotype [116]. Preliminary data from our lab show that human and pig c-kit^{pos} eCSCs show increased proliferation and clonogenicity when cultured in low (2 %) O_2, compared to normoxic conditions.

6 Alterations to Stem Cell Activity and Potency in Aged Tissue or Disease

6.1 Ageing-Related Changes in Cardiac Stem–Progenitor Cells in Animal Models

Ageing, an inevitable process of life, poses the largest risk factor for cardiovascular disease [117]. Although long-term exposure to known cardiovascular risk factors strongly drives the development of cardiovascular pathologies, intrinsic cardiac ageing is considered to highly influence the pathogenesis of heart disease [118]. However, the fields of the biology of ageing and cardiovascular disease have been studied separately, and only recently their intersection has begun to receive the appropriate attention.

Over the course of ageing, the heart undergoes a number of anatomical, functional and cellular alterations. Early diastolic left ventricular (LV) filling, LV contractility and ejection fraction all decrease during ageing leading to a reduced cardiac output [119–121]. In an attempt to compensate for the reduction in cardiac output, the myocardium is triggered to increase its muscle mass by undergoing hypertrophy, which in the long term results in weakened cardiac function. Ageing of the arterial system is exemplified by increased arterial thickening and stiffness, luminal enlargement and dysfunctional endothelium with decreased responsiveness to stress and injury [122]. Arterial stiffness contributes to LV pathological hypertrophy and stimulates fibroblast proliferation causing myocardial and arterial fibrosis. Impaired heart rate is another characteristic of the ageing heart. Loss of sinoatrial node cells, together with fibrosis and hypertrophy, slows electric impulse propagation throughout the heart causing decreased maximum heart rate and arrhythmias [123]. Age-imposed anomalies of the cardiovasculature lead to the onset of a variety of age-related pathologies, including ischaemia, hypertension, atherosclerosis, age-related macular degeneration and stroke [117].

One of the hallmarks of cellular ageing is the progressive accumulation of damaged macromolecules such as DNA, proteins and lipids [124]. These become chemically modified by free radicals, which are products of normal cellular metabolism and whose generation is significantly increased with age [125]. Experimental evidence indicates that this is due to mitochondrial dysfunction occurring with ageing, leading to reduced respiratory metabolism and increased generation of reactive oxygen species (ROS) [118]. DNA damage leads to cellular dysfunction by altering gene expression and protein damage leads to protein misfolding and aggregation in the cytoplasm, nucleus and endoplasmic reticulum [125]. With the passage of time, age-related macromolecular oxidative damage leads to gradual loss of normal structure and function, particularly pronounced in long-lived post-mitotic cells, such as neurons and cardiomyocytes. For continuously dividing cells, there is the added challenge of telomere erosion and replication-associated DNA mutations that occur during the process of cell division [126]. As the DNA damage increases with age,

so does the likelihood of a cell to undergo apoptosis, malignant transformation, inflammation or senescence [127].

Mammalian ageing has been defined as a gradual loss of the capacity to maintain tissue homeostasis or to repair tissues after injury or stress [124]. It is now well known that tissue regeneration and homeostasis are controlled by the tissue-specific stem–progenitor cell compartment present in every tissue [128, 129]. Therefore, it is logical to postulate that pathological and patho-physiological conditions associated with distorted homeostasis and regenerative capacity, such as ageing, underlie impairments in the corresponding stem cell pool [130–132]. Indeed in recent years, accumulated evidence signified a direct impact of cardiac ageing and pathology on eCSC activity and potency [13, 103, 130, 131, 133–136]. Outside the cardiac context, there is an already well-established overlap between ageing and stem cell impairment, observed in a number of organs and tissues [137–144]. Tissue-specific stem cells decline with age due to several factors including telomere shortening, DNA damage and external influences affecting stem cell niche homeostasis [130]. In the heart, ageing is shown to be associated with a eCSC senescent phenotype which includes attenuated telomerase activity, telomeric erosion, high incidence of telomere-induced dysfunction foci and elevated expression of the cyclin-dependent kinase inhibitors (CDKIs) $p16^{INK4a}$ and $p21^{Cip1}$ [103, 133, 135, 136, 145].

Senescent cells are characterised by impaired proliferation, an altered gene expression profile, resistance to apoptosis and epigenetic modifications, as well as producing an altered secretome (SASP; senescence-associated secretory phenotype) which acts on adjacent as well as distant cells, causing fibrosis, inflammation and a possible carcinogenic response [127, 146–148]. Although a universal marker exclusively expressed in senescent cells has not been identified, most senescent cells express $p16^{INK4a}$, a cyclin-dependent kinase inhibitor and tumour suppressor, which is not commonly expressed by quiescent or terminally differentiated cells [147, 148]. $p16^{INK4a}$, which becomes progressively expressed with age, enforces cell cycle arrest by activating retinoblastoma (RB) tumour-suppressor protein [142, 149].

In rats, chronological age leads to telomeric shortening in c-kit^{pos} eCSCs [145]. However, in the setting of patho-physiological ageing, telomerase-competent eCSCs with normal telomerases can still be found in various cardiac regions, which have the capacity to migrate to injured zones and generate a healthy progeny partly reversing the senescent phenotype and improving cardiac performance [145].

In 22-month-old mice, c-kit^{pos} eCSCs show senescence, evidenced through impaired proliferation and differentiation potential, $p16^{INK4a}$ expression, reduced telomerase activity, telomere shortening, senescence and increased apoptosis [103]. Senescent eCSCs become largely unable to generate new functionally competent myocytes, compromising cardiomyocyte turnover and favouring the accumulation of old poorly contracting cardiomyocytes [103]. These findings show that cardiovascular ageing impairs eCSCs, leading to their decline and dysfunction, which leads to the development of cardiac dysfunction and failure. Interestingly, this progression is altered favourably in IGF-1-TG mice [103] (see below).

6.2 Ageing-Related Observations in Human Cardiac Stem–Progenitor Cells

Cardiac stem–progenitor cells govern physiologic turnover of the heart by supplying the cardiomyocytes and coronary vessels, following wear and tear [150] and physiological growth/adaptation [151]. However, eCSCs do not act effectively in response to ischaemia and in the diseased (see below) and aged organism context [133]. The ageing process in the human heart is accompanied by progressive ventricular cardiomyocyte loss and compensatory hypertrophy of remaining cells [152]. An important question that needs to be addressed is: Does the heart age because of stem–progenitor cell exhaustion or functional failure due to senescence-associated changes?

A recent study by Bergmann and colleagues using radiocarbon 14 dating found that ~50 % of cardiomyocytes are replaced during the average human life span [14]. Much higher levels of myocyte turnover were estimated in another study, where the whole myocyte pool was shown to be replaced 11 times in men and 15 times in women during life, with the rates progressively increasing with age [135]. Therefore, according to this study it's unlikely that the numbers of eCSCs is declining with age. Indeed, studies in aged animals and humans showed a twofold increase in c-kit^{pos} eCSCs in aged and diseased hearts, but among these, ~60–70 % were p16^{ink4a} positive, identifying them as putative senescent [103, 133].

Several studies have compared numbers and properties of eCSCs obtained from different aged human hearts. A study by Mishra and colleagues found that c-kit^{pos} eCSC numbers were highest in neonatal human hearts, but fell almost three times between ages 2 and 13 [58]. Proliferation rate was found to be highest in fetal cardiac progenitors [153] and neonatal-derived eCSCs and started to decline as age progressed to 13 years [58]. The ability to differentiate in vitro into the three cardiac lineages was similar in neonatal and young eCSCs, and robust functional improvements were present when young eCSCs were injected into infarcted rat hearts, compared to injection with adult fibroblasts [58]. In another study, fetal-derived eCSCs showed enhanced formation of endothelial networks, but less smooth muscle cells compared to adult c-kit^{pos} eCSCs [153]. In turn, adult-derived c-kit^{pos} eCSCs showed more mature cardiomyocyte phenotype by electrophysiological measurements [153]. Moreover, differentiation into myocytes, smooth muscle cells and endothelial cells was similar between c-kit^{pos} eCSCs isolated from young donor and old explanted hearts, but eCSCs isolated from old hearts had a lower migratory capacity [136].

Recently, a comparison study was carried out between c-kit^{pos} eCSCs obtained from donor and explanted hearts during transplantation, in an attempt to assess the properties of c-kit^{pos} eCSCs upon ageing. It was demonstrated that age and severe heart failure are the two main determinants of human eCSC decline, as shown by up to 25 % shortening of telomeres and twofold enhanced expression of senescent markers p16^{INK4a} and p21^{Cip1} [136]. Moreover, human c-kit^{pos} eCSCs obtained from explanted, diseased hearts showed ~3 times lower growth kinetics and clonogenicity, compared with c-kit^{pos} eCSCs obtained from donor hearts. However, it was still possible to expand them in vitro and obtain a significant number of cells, which could be used for autologous regenerative therapy if needed. Worthy of noting that

in clonally expanded cells from aged and diseased hearts, the levels of senescent markers were low and similar to the cells derived from donor hearts [136]. This is in agreement with our findings that single-derived c-kit^{pos} eCSC clones obtained from young and old mouse and human hearts were indistinguishable by their gene expression, proliferation, sub-clonogenicity and differentiation potential, supporting the hypothesis that eCSC ageing is a stochastic process, affecting only the 'aged/senescent' eCSCs and not the whole eCSC cohort ([64]; Ellison et al. unpublished data).

Taken collectively, evidence indicates that in the aged and diseased heart, the apparent increase in eCSC numbers does not compensate effectively for cardiomyocyte loss during ageing and disease, due to telomere attrition and, therefore, increased senescence of the eCSC pool and, consequently, their differentiated progeny. If eCSC ageing is itself an important determinant of cardiac ageing, then it may be possible to treat age-related diseases by developing strategies (see below) to prevent, attenuate or even reverse eCSC ageing and senescence.

6.3 *Potentiated Stem Cell Activity with Physiological Cardiac Growth and Adaptation*

In response to stress (hypoxia, exercise, work overload), a proportion of the resident eCSCs are rapidly activated, multiply and generate new muscle and vascular cells, contributing to physiological cardiac remodelling [154]. We have recently shown that physiological stress, such as the increased cardiac workload that accompanies exercise training, can stimulate c-kit^{pos} eCSC proliferation and differentiation in vivo leading to new cardiomyocyte formation and angiogenesis, which in conjunction with cardiomyocyte hypertrophy contributes to increased myocardial mass and enhanced cardiac function [151]. Specifically, we found that rats that underwent treadmill running at 85–90 % of maximal exercising capacity ($VO_{2\,max}$) for 30 min/day, 4 days/week, almost doubled their number of c-kit^{pos} eCSCs at 2 weeks, and this eCSC activation was governed by the growth factors neuroregulin and IGF-1, which were upregulated in the exercise stressed myocardium [151]. Similarly, swimming exercise has been found to significantly increase the number of c-kit^{pos} eCSCs isolated from the hearts of swim trained mice (~8 % of small cardiac cells isolated), compared to sedentary controls (1.5 %) [154].

6.4 *Potentiated Cardiac Stem–Progenitor Cell Activity in the Acute Injury Setting*

In comparison to chronic heart disease which develops over a long period of time, acute damage such as an acute myocardial infarction (MI; ischaemia) develops very rapidly, leading to segmental loss of myocardium. In response to MI, resident Sca1^{pos}/CD31^{neg} eCSCs significantly increase in number in the LV 7 days post-MI

[42]. Furthermore, 1 and 2 weeks after MI there is an increase in the number of CDCs generated from tissue explants, with the largest fraction within these CDCs being the Sca1^{pos}/CD45^{neg} subpopulation [38]. However this increase is attenuated 4 weeks post-MI back to baseline [38]. A study by Mouquet et al. [96] observed a decrease in cardiac SP cells upon permanent coronary occlusion, followed by reconstitution of this population to normal levels through self-proliferation and homing of bone marrow-derived cells (BMDCs) [96].

Although eCSCs can respond to myocardial infarction and segmental tissue loss with an expansion in cell number and differentiation into new cardiomyocytes and vasculature [8, 13], the response is insufficient to recover and replace the damaged tissue and restore cardiac function. In addition to this, the new cardiomyocytes that are formed are not fully matured resulting in small myocytes with reduced contractile power [19].

Recently, Emmert et al. [155] documented that the number of cardiac SP and c-kit^{pos} eCSCs was significantly elevated in the atria and ventricles samples isolated from ischaemic, compared to non-ischaemic human hearts [155]. The response of eCSCs to MI is much more potent in models of acute infarction compared to chronic damage. Urbanek and colleagues [13] reported that the increase in the number of telomerase-competent dividing c-kit^{pos} eCSCs was greater in patients with acute infarction (28 %) than those with chronic infarctions (14 %) or controls (1.5 %). Mitotic index of these cells increased 29-fold in acute damage and 14-fold in chronic damage. Differentiation of these cells into the three main myocardial lineages (cardiomyocytes, smooth muscle and endothelial) was 85- and 25-fold greater in acute and chronic infarcts, respectively. Cells expressing senescence markers (p16^{Ink4a} and p53) were more abundant in chronic damage (40 %) than acute (18 %), and apoptosis was greater in the chronic group. These studies indicate that the response of eCSCs is more potent in an acute damage setting than a chronic setting, with an increased expansion of more functional, less senescent eCSCs that show enhanced differentiation potential.

In a model that more closely resembles tissue wear and tear, we have previously shown that an acute β-adrenergic overload, thorough injection of isoproterenol (ISO; 5 mg kg^{-1}), in the rat leads to acute cardiac failure with diffuse myocardial damage killing up to 10 % of the cardiomyocytes, in the presence of a patent coronary circulation [104]. The c-kit^{pos} eCSCs are resistant to the noxious effects of this acute hyperadrenergic state and rapidly become activated, proliferate and increase in number peaking 1–3 days after the ISO-induced damage [104]. Furthermore, through their activation and ensuing differentiation into cardiomyocytes, c-kit^{pos} eCSCs participate in the spontaneous regenerative response of the myocardium and restoration of cardiac function over 28 days ([150]; Ellison et al. unpublished data).

6.5 *Alterations/Decline in Cardiac Stem–Progenitor Cells in Chronic Disease*

The effect of ageing on eCSC number has been discussed above; however, ageing is often associated with chronic heart disease, which in general stimulates an

expansion in eCSC number [13, 156–160]. Increased numbers of eCSCs have been reported in thoracic aortic aneurysm (TAA) and thoracic aortic dissection (TAD) tissue, compared to healthy control aortic tissue [156] and in hearts from patients with ischaemic cardiomyopathy, compared to controls [157].

The type of disease and treatment also appears to play a role in determining the degree of eCSC expansion. Itzhaki-Alfia et al. [110] isolated and cultured small cardiac cells from patients with valvular disease, hypertension, pulmonary hypertension and atrial dilation. They found that c-kit^{pos} eCSCs that were multipotent and able to contribute to myocardial regeneration in all four cardiac chambers were similar in number for all pathologies except those suffering from hypertension, for which the percent number of c-kit^{pos} eCSCs obtained was 10 % higher (23 %) than normotensive patients (13 %), and this increased to 31 % in patients with end-stage heart failure [110]. Gambini et al. [161] also reported that the number of c-kit^{pos} eCSCs isolated from right atrial appendages was increased in patients undergoing beta blocker treatment and statins and those with pulmonary hypertension. Interestingly, smoking, atrial fibrillation and a history of MI were significantly associated with an impairment of c-kit^{pos} eCSC proliferation [161]. Furthermore, Kubo et al. [162] reported that the number of c-kit^{pos} eCSCs is increased in patients with advanced heart failure requiring transplantation [162]. In contrast, Cesselli et al. [136] found that the number of c-kit^{pos}, Lin^{neg} eCSCs was threefold higher in the atria of hearts from healthy donors than patients with chronic heart failure (ischaemic, hypertrophic and idiopathic dilated cardiomyopathy or valvular defects and acromegaly).

As with aged patients, congenital heart disease also stimulates eCSC expansion in children [159, 160]. Sato et al. [160] found that c-kit^{pos} eCSC number was fivefold greater in the hearts of children with congenital heart disease than in controls and that the number of c-kit^{pos} CSCs detected was positively correlated to the percent number of apoptotic myocytes found [160]. Similarly, Rupp et al. [159] found that the percent number of c-kit^{pos}, tryptase^{neg}, CD45^{neg} eCSCs in the hearts of children with pressure overloaded right ventricles (0.84 % of cardiac myocyte depleted small cells) and dilated cardiomyopathy (0.22 %) was greater than in the hearts of children after transplantation (0.15 %).

Although it appears eCSCs are more abundant in the diseased heart compared to age-matched controls, as discussed above, a higher proportion of these eCSCs are senescent and dysfunctional [13, 133, 136].

7 Clinical Potential and Future Directions

7.1 Paracrine/Autocrine Repair and Regenerative Effects of Cellular Therapy

The identification of endogenous repair mechanisms in the neonatal to adult heart, where the eCSCs play a central role, has raised the question of whether endogenous cardiac repair and regeneration may be realised therapeutically.

Over the past decade, regenerative myocardial therapies have focused largely on bone marrow-derived cells (BMDCs) as contenders for cardiac repair and have thus far presented mixed results [162–164]. Many clinical trials have demonstrated improved cardiac function following BMDC transplantation; however, in most cases this has been marginal at best. A recent systematic review reported an overall improvement in LVEF of 3.96 % and a 4.03 % reduction in infarct size [165]. The underlying mechanism by which BMDCs promote regeneration is still extensively debated; however, it is now widely accepted that BMDCs secrete growth factors and cytokines positively contributing to the local regenerative milieu. Indeed, BMDCs act primarily through this paracrine mechanism, which in turn stimulates cardiac repair processes through enhanced angiogenesis, myocyte survival, decreased apoptosis and fibrosis and activation of endogenous eCSCs with ensuing new myocyte formation [7, 98, 166–168]. In support of this, MSCs have been shown to exude a broad array of cytokines, chemokines and growth factors, which may be implicated in cardiac repair [169]. Studies have also demonstrated that BMDC-associated paracrine factors are capable of stimulating cardioprotective effects via regulation of cardiac miRNAs [170]. Furthermore, BMDC conditioned media were found to improve cardiac function in rats post-MI [171].

While the scientific debate on BMDCs mode of action continues, experimental studies have nevertheless highlighted their potential as a source of autologous cells for therapeutic application due to their accessibility, ease of propagation and reparative capabilities [172].

7.2 Autologous Cardiac Stem–Progenitor Cell Transplantation

The identification of eCSCs in the adult heart has been a turning point in the quest to develop therapeutic strategies for cardiac regeneration. Initial experiments investigating the transplantation of autologous c-kit^{pos} eCSCs found that intracoronary administration stimulated functional regeneration post-MI in rats [173]. Although eCSC experimental studies are still in their infancy we have already witnessed the initiation of the first clinical trials investigating the effects of delivering autologous eCSCs to humans.

The SCIPIO clinical trial, the first man to investigate c-kit^{pos} eCSCs, reported that 16 patients with ischaemic cardiomyopathy received intracoronary infusions of $0.5–1 \times 10^6$ c-kit^{pos}, autologous eCSCs, and compared to controls these patients benefited from an 8- and 12-unit increase in LVEF, 4 and 12 months after infusion, respectively [174]. A subset of seven patients was subject to cMRI analysis, which confirmed that the infarct region had significantly decreased in size up to 12 months following c-kit^{pos} eCSC transplantation [174].

Despite the lack of information on many biological aspects of the in vitro expanded CDCs, phase I clinical trial CADUCEUS was performed in humans. In this trial, 17 patients with an LVEF ranging between 25 and 45 % were intracoronary injected with 25×10^6 CD105^{pos}, autologous CDCs and cMRI analysis revealed a

12 % decrease in infarct size, compared with controls, and viable myocardial mass increased by 13 g from baseline after 12 months; however no change in LVEF was noted following transplantation [114].

Recently, the ALCADIA clinical trial was initiated, which will focus on a hybrid biotherapy approach for treating chronic ischaemic cardiomyopathy. This translational study is focusing on the safety and efficacy of autologous clonally amplified CSCs, which have shown to be enriched for embryonic stem cell markers and have mesenchymal cell characteristics [175]. This trial is also investigating cell therapy with the controlled release of basic fibroblast growth factor (bFGF) from a gelatin hydrogel sheet.

While this innovative work has proved promising in the respect that transplantation of autologous CSCs has not resulted in any adverse health effects, we now await further studies which focus on the efficacy of eCSC-based therapies and compare these to results obtained with BMDCs. However, many questions relating to eCSC basic biology still remain unanswered, particularly their long-term effectiveness and regenerative potential. It is imperative that such issues be addressed quickly if the full potential of these cells is to be realised, manipulated and applied clinically. This information should become available before the start of clinical trials, because it can be better obtained in the laboratory and with experimental animal models before posing patients at risk.

The advent of induced pluripotent stem cell (iPSC) technology in recent years has provided another potential source of autologous cells for myocardial repair [176]. To date, functional cardiomyocytes have been successfully generated from iPSCs in vitro [177] and have been highlighted as an inexhaustible source of cardiovascular cells. Functional characterisation of iPSC-derived cardiomyocytes (iPSC-CMs) has been conducted using molecular and electrophysiological techniques in order to verify their cardiomyocyte-like phenotype and a number of studies have shown effective modelling of cardiovascular diseases, such as long-QT syndrome using iPSC-CMs [178, 179]. In moving iPSC knowledge towards the clinic, a major breakthrough came when Ieda et al. identified a triage of transcription factors, Gata4, Mef2c and Tbx5 (GMT), which together are capable of directly reprogramming mouse fibroblasts towards a cardiomyocyte fate (iCMs) [180]. This strategy has since been applied in vivo with successful reprogramming of native murine cardiac fibroblasts to cardiomyocyte-like cells by local delivery of GMT after coronary ligation [181]. Of note, these strategies have only been performed in murine models and have yet to be proven in humans. While a multitude of techniques for generating iPSCs have been developed in recent years, there are currently a number of limitations concerning the safety and efficiency of iPSCs for use in regenerative therapies and until we are able to generate iPSCs free from foreign chemical or genomic elements on a much larger scale we will need to look towards alternative sources for treatment of cardiovascular injury.

While autologous eCSCs undoubtedly hold great promise for cardiac repair their isolation and expansion prior to cell transplantation can be complex, time-consuming and costly. This has raised the question of whether it may be advantageous to target the activation and regenerative capacity of the resident eCSCs to reconstitute damaged myocardium in the absence of cell therapy.

7.3 Autologous Regeneration Without Cell Transplantation

In an attempt to move towards cell-free, protein-based therapies, various growth factors and cytokines have been identified as potential candidates for therapeutic cardiac regeneration and as this list expands so too does our awareness of growth factor-mediated regenerative potential. Vascular endothelial factor (VEGF) is one such factor, which has been identified as central in promoting neo-vascularisation post-MI [182]. Initially phase II clinical trials suggested that limited functional benefits were observed upon direct administration of VEGF post-MI [183]. However, this is now known to be due to the short half-life of VEGF and goes some way to demonstrate how important initial experimental studies are when designing clinical trials. Recent studies have focused on delivering VEGF in combination with various scaffolds and have achieved much greater success in stimulating angiogenesis and restoring cardiac function [184, 185].

Neuregulin 1 (NRG-1) is another key factor implicated in stimulating cardiac repair and regeneration [151, 186]. An Ig domain containing form of NRG-1β, also known as glial growth factor 2 (GG2), has been shown to improve LVEF and remodelling in pigs post-MI, compared to controls [187]. It is thought that NRG-1 imparts functional benefits by activating and increasing c-kit^{pos} eCSC proliferation [151], inducing cardiomyocyte replacement [188], protecting cardiomyocytes from apoptosis and improving mitochondrial function [187].

Insulin growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) are also promising targets. IGF-1 acts as an intermediate of several growth hormone responses and modulates multiple signalling cascades, resulting in a potent proliferative signal that blocks apoptosis and stimulates growth in many different cells and organs [189]. Paradoxically, experimental evidence shows that reduced IGF-1 signalling in animals is associated with life span extension, rendering an unclear image of this factor's potential [190]. Experimental studies have begun to unravel the complex function of IGF-1 with skeletal muscle-restricted expression of IGF-1 found to increase bone marrow and local stem cell pools, providing evidence for its dramatic effects on muscle mass in vivo [191]. Comparable findings have been reported in the heart with cardiac overexpression of IGF-1 found to increase the abundance of c-kit^{pos}/Sca-1^{pos} SP cells in the bone marrow and CD34^{pos} SP cells in the heart [192]. In addition, IGF-1 was found to mediate the release of cytokines involved in activating these SP cells, therefore promoting crosstalk between the heart and bone marrow and leading to an enrichment of capillaries in response to injury [193]. Kawaguchi et al. demonstrated that c-kit^{pos} GATA-4^{high} eCSCs exert a paracrine effect when co-cultured with rat cardiomyocytes, enhancing their survival and contractility through induction of the IGF signalling pathway [194]. Cardiac overexpression of IGF-1 led to restoration of cardiac function in post-infarcted mice, which was facilitated by modulated inflammatory response, increased anti-apoptotic signalling and increased proliferative activity in the ventricular tissue [192]. Furthermore, IGF-1 overexpression reduced c-kit^{pos} eCSC and cardiomyocyte senescence and death by enhancing telomerase activity in eCSCs and younger cardiomyocytes and conferring protection from telomeric shortening, oxidative

injury, growth arrest and apoptosis [103]. It is suggested that the beneficial paracrine mechanism of IGF-1 in the heart acts via JNK1/SirT1 signalling [195], through interference with ROS generation and oxidative DNA damage reversal by homologous recombination [196, 197]. Elucidating these mechanistic roles for IGF-1 will play an important part in understanding how to stimulate endogenous regeneration in a clinical setting.

HGF and its associated receptor c-met were primarily identified as mediators of liver regeneration [198]. However, since its initial discovery, HGF has been linked to various tissues and has been reported to have mitogenic and anti-apoptotic capabilities [199]. Nakamura et al. [200] have provided evidence of the role of endogenous HGF in cardioprotection by identifying the expression of both HGF and c-met in cardiomyocytes and demonstrated that plasma levels of HGF rapidly increase in response to ischaemia/reperfusion injury. Neutralisation of HGF in vivo has shown to result in the progression of cardiac dysfunction while administration of exogenous HGF has shown to reduce apoptosis, decrease the infarct size and stimulate an improvement in cardiac function, compared with controls [200, 201]. A number of experimental studies have targeted HGF for myocardial regeneration and have shown that HGF is capable of stimulating migration, proliferation and differentiation of eCSCs [19, 202]. To date in vivo evidence of HGF myocardial regeneration has largely been achieved using adult stem cells overexpressing HGF; for example, Zhu et al. [203] investigated the effects of transplantation of human adipose tissue-derived stem cells overexpressing HGF in a rat model of AMI and reported an increased LVEF along with marked improvements in fibrosis and angiogenesis after 28 days [203]. Recent advances in gene therapy have led to the first human clinical trials investigating the feasibility of delivering HGF to patients with severe coronary artery disease. Yang et al. were able to confirm the safety of delivering HGF using an adenoviral vector delivery system; however, this study provided little evidence on the efficacy of HGF in promoting functional cardiac regeneration [204].

We have recently tested the regenerative effects of intracoronary administration of IGF-1 and HGF, in doses ranging from 0.5 to 2 μg HGF and 2 to 8 μg IGF-1, just below the site of left anterior descendent occlusion, 30 min after AMI during coronary reperfusion in the pig. This growth factor cocktail triggers a regenerative response from the c-kit^{pos} eCSCs, which is potent and able to produce anatomically, histologically and physiologically significant regeneration of the damaged myocardium without the need for cell transplantation [19]. IGF-1 and HGF induced eCSC migration, proliferation and functional cardiomyogenic and microvasculature differentiation. Furthermore, IGF-1/HGF, in a dose-dependent manner, improved cardiomyocyte survival and reduced fibrosis and cardiomyocyte reactive hypertrophy. Interestingly, the effects of a single administration of IGF-1/HGF are still measurable 2 months after its application, suggesting the existence of a feedback loop triggered by the external stimuli that activates the production of growth and survival factors by the targeted cells, which explains the persistence and long duration of the regenerative myocardial response. These histological changes were correlated with a reduced infarct size and an improved ventricular segmental contractility and ejection fraction at the end of the follow-up assessed by cMRI [19].

Finally, Smart et al. [80] showed in the adult infarcted mice heart that $Wt1^{pos}$ EPDCs through pre-treatment (priming) with thymosin β4 (Tβ4) proliferated and, following migration to the peri-infarct and border regions, induced expression of vascular and cardiomyocyte proteins, giving rise to neovascularisation and structurally and functionally competent cardiomyocytes. These findings add significant knowledge on the cardiogenic potential of the EPDCs in the adult heart; however, it must be noted that Tβ4 activation only caused a very low number of EPDCs (<1 % of the total activated EPDCs) to differentiate into cardiomyocytes, marking this approach as inefficient and placing EPDCs far from being applicable in resident cell-based therapy in human cardiac disease.

Taken together these findings highlight the potential for utilising growth factors and cytokines as non-invasive strategies for cardiac regeneration and may lead the way to a therapy which is 'off-the-shelf', affordable, readily available and compatible with current clinical standard of care for AMI. Significant progress in understanding the mobilisation, proliferation and differentiation of eCSCs has been made in recent years. Nevertheless, we still require a more comprehensive understanding of eCSC biology, in particular in terms of their long-term effectiveness and regenerative potential, in order to derive maximal potential from these cells in a clinical setting. Elucidating factors that stimulate eCSCs and regulate their fate and maturation will facilitate the identification of therapeutic solutions for cardiac regeneration and enable us to develop effective, non-invasive strategies for myocardial regeneration in the future.

References

1. Soonpaa MH, Field LJ (1998) Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res 83(1):15–26
2. Hunter JJ, Chien KR (1999) Signaling pathways for cardiac hypertrophy and failure. N Engl J Med 341(17):1276–1283
3. Laflamme MA, Murry CE (2011) Heart regeneration. Nature 473(7347):326–335
4. Nadal-Ginard B (1978) Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15(3):855–864
5. Chien KR, Olson EN (2002) Converging pathways and principles in heart development and disease: CV@CSH. Cell 110(2):153–162
6. Oh H, Taffet GE, Youker KA, Entman ML, Overbeek PA, Michael LH, Schneider MD (2001) Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc Natl Acad Sci U S A 98(18):10308–10313
7. Ellison GM, Nadal-Ginard B, Torella D (2012) Optimizing cardiac repair and regeneration through activation of the endogenous cardiac stem cell compartment. J Cardiovasc Transl Res 5(5):667–677
8. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P (2001) Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 344(23):1750–1757
9. Anversa P, Nadal-Ginard B (2002) Myocyte renewal and ventricular remodelling. Nature 415(6868):240–243
10. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P (2002) Chimerism of the transplanted heart. N Engl J Med 346(1):5–15

11. Nadal-Ginard B, Kajstura J, Leri A, Anversa P (2003) Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res 92(2):139–150
12. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P (2003) Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci U S A 100(18):10440–10445
13. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F, Bolli R, Leri A, Kajstura J, Anversa P (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci U S A 102(24):8692–8697
14. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J (2009) Evidence for cardiomyocyte renewal in humans. Science 324(5923):98–102
15. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114(6):763–776
16. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95(9): 911–921
17. Fransioli J, Bailey B, Gude NA, Cottage CT, Muraski JA, Emmanuel G, Wu W, Alvarez R, Rubio M, Ottolenghi S, Schaefer E, Sussman MA (2008) Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. Stem Cells 26(5):1315–1324
18. Linke A, Müller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Böhm M, Quaini F, Urbanek K, Leri A, Hintze TH, Kajstura J, Anversa P (2005) Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A 102(25):8966–8971
19. Ellison GM, Torella D, Dellegrottaglie S, Perez-Martinez C, Perez de Prado A, Vicinanza C, Purushothaman S, Galuppo V, Iaconetti C, Waring CD, Smith A, Torella M, Cuellas Ramon C, Gonzalo-Orden JM, Agosti V, Indolfi C, Galiñanes M, Fernandez-Vazquez F, Nadal-Ginard B (2011) Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart. J Am Coll Cardiol 58(9):977–986
20. Torella D, Ellison GM, Méndez-Ferrer S, Ibanez B, Nadal-Ginard B (2006) Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration. Nat Clin Pract Cardiovasc Med 3(Suppl 1):S8–S13
21. Torella D, Ellison GM, Karakikes I, Galuppo V, De Serio D, Onorati F, Mastroroberto P, Renzulli A, Indolfi C, Nadal-Ginard B (2006) Biological properties and regenerative potential, in vitro and in vivo, of human cardiac stem cells isolated from each of the four chambers of the adult human heart. Circulation 114:87
22. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P (2007) Human cardiac stem cells. Proc Natl Acad Sci U S A 104(35):14068–14073
23. Arsalan M, Woitek F, Adams V, Linke A, Barten MJ, Dhein S, Walther T, Mohr FW, Garbade J (2012) Distribution of cardiac stem cells in the human heart. ISRN Cardiol 2012:483407
24. Torella D, Ellison GM, Karakikes I, Nadal-Ginard B (2007) Resident cardiac stem cells. Cell Mol Life Sci 64(6):661–673
25. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH (2006) Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell 127(6):1137–1150
26. Tallini YN, Greene KS, Craven M, Spealman A, Breitbach M, Smith J, Fisher PJ, Steffey M, Hesse M, Doran RM, Woods A, Singh B, Yen A, Fleischmann BK, Kotlikoff MI (2009) c-kit expression identifies cardiovascular precursors in the neonatal heart. Proc Natl Acad Sci U S A 106(6):1808–1813

27. Jesty SA, Steffey MA, Lee FK, Breitbach M, Hesse M, Reining S, Lee JC, Doran RM, Nikitin AY, Fleischmann BK, Kotlikoff MI (2012) c-kit+ precursors support postinfarction myogenesis in the neonatal, but not adult, heart. Proc Natl Acad Sci U S A 109(33): 13380–13385
28. Fazel S, Cimini M, Chen L, Li S, Angoulvant D, Fedak P, Verma S, Weisel RD, Keating A, Li RK (2006) Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. J Clin Invest 116(7):1865–1877
29. Fang S, Wei J, Pentinmikko N, Leinonen H, Salven P (2012) Generation of functional blood vessels from a single c-kit+ adult vascular endothelial stem cell. PLoS Biol 10(10):e1001407. doi:10.1371/journal.pbio.1001407
30. Passier R, van Laake LW, Mummery CL (2008) Stem-cell-based therapy and lessons from the heart. Nature 453(7193):322–329
31. Pouly J, Bruneval P, Mandet C, Proksch S, Peyrard S, Amrein C, Bousseaux V, Guillemain R, Deloche A, Fabiani JN, Menasché P (2008) Cardiac stem cells in the real world. J Thorac Cardiovasc Surg 135(3):673–678
32. Zaruba MM, Soonpaa M, Reuter S, Field LJ (2010) Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts. Circulation 121(18): 1992–2000
33. Roskoski R Jr (2005) Signaling by Kit protein-tyrosine kinase-the stem cell factor receptor. Biochem Biophys Res Commun 337(1):1–13
34. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E (2007) Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 115(7):896–908
35. Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, Goetsch SC, Gallardo TD, Garry DJ (2004) Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 265(1):262–275
36. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 100(21):12313–12318
37. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, Komuro I (2004) Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 279(12):11384–11391
38. Ye J, Boyle A, Shih H, Sievers RE, Zhang Y, Prasad M, Su H, Zhou Y, Grossman W, Bernstein HS, Yeghiazarians Y (2012) Sca-1+ cardiosphere-derived cells are enriched for Isl1-expressing cardiac precursors and improve cardiac function after myocardial injury. PLoS One 7(1):e30329
39. Chong JJ, Chandrakanthan V, Xaymardan M, Asli NS, Li J, Ahmed I, Heffernan C, Menon MK, Scarlett CJ, Rashidianfar A, Biben C, Zoellner H, Colvin EK, Pimanda JE, Biankin AV, Zhou B, Pu WT, Prall OW, Harvey RP (2011) Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell 9(6):527–540
40. Samal R, Ameling S, Wenzel K, Dhople V, Völker U, Felix SB, Könemann S, Hammer E (2012) OMICS-based exploration of the molecular phenotype of resident cardiac progenitor cells from adult murine heart. J Proteomics 75(17):5304–5315
41. Takamiya M, Haider KH, Ashraf M (2011) Identification and characterization of a novel multipotent sub-population of Sca-1$^+$ cardiac progenitor cells for myocardial regeneration. PLoS One 6(9):e25265
42. Wang X, Hu Q, Nakamura Y, Lee J, Zhang G, From AH, Zhang J (2006) The role of the sca-1+/CD31- cardiac progenitor cell population in postinfarction left ventricular remodeling. Stem Cells 24(7):1779–1788
43. Iwakura T, Mohri T, Hamatani T, Obana M, Yamashita T, Maeda M, Katakami N, Kaneto H, Oka T, Komuro I, Azuma J, Nakayama H, Fujio Y (2011) STAT3/Pim-1 signaling pathway plays a crucial role in endothelial differentiation of cardiac resident Sca-1+ cells both in vitro and in vivo. J Mol Cell Cardiol 51(2):207–214

44. Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW (2008) A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. Proc Natl Acad Sci U S A 105(27):9349–9354
45. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ (2008) Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J 16(5):163–169
46. Zuba-Surma EK, Wojakowski W, Ratajczak MZ, Dawn B (2011) Very small embryonic-like stem cells: biology and therapeutic potential for heart repair. Antioxid Redox Signal 15(7):1821–1834
47. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183(4):1797–1806
48. Asakura A, Rudnicki MA (2002) Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol 30(11):1339–1345
49. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7(9):1028–1034
50. Hierlihy AM, Seale P, Lobe CG, Rudnicki MA, Megeney LA (2002) The post-natal heart contains a myocardial stem cell population. FEBS Lett 530(1–3):239–243
51. Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R (2005) CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 97(1):52–61
52. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, Takahashi T, Goto M, Mikami Y, Yasuda N, Akazawa H, Uezumi A, Takeda S, Komuro I (2007) Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. J Cell Biol 176(3):329–341
53. Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052):1707–1710
54. Davis DR, Zhang Y, Smith RR, Cheng K, Terrovitis J, Malliaras K, Li TS, White A, Makkar R, Marbán E (2009) Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. PLoS One 4(9):e7195
55. Tan JJ, Carr CA, Stuckey DJ, Ellison GM, Messina E, Giacomello A, Clarke K (2011) Isolation and expansion of cardiosphere-derived stem cells. Curr Protoc Stem Cell Biol 16:2C.3.1–2C.3.12
56. Bartosh TJ, Wang Z, Rosales AA, Dimitrijevich SD, Roque RS (2008) 3D-model of adult cardiac stem cells promotes cardiac differentiation and resistance to oxidative stress. J Cell Biochem 105(2):612–623
57. Martens A, Gruh I, Dimitroulis D, Rojas SV, Schmidt-Richter I, Rathert C, Khaladj N, Gawol A, Chikobava MG, Martin U, Haverich A, Kutschka I (2011) Rhesus monkey cardiosphere-derived cells for myocardial restoration. Cytotherapy 13(7):864–872
58. Mishra R, Vijayan K, Colletti EJ, Harrington DA, Matthiesen TS, Simpson D, Goh SK, Walker BL, Almeida-Porada G, Wang D, Backer CL, Dudley SC Jr, Wold LE, Kaushal S (2011) Characterization and functionality of cardiac progenitor cells in congenital heart patients. Circulation 123(4):364–373
59. Carr CA, Stuckey DJ, Tan JJ, Tan SC, Gomes RS, Camelliti P, Messina E, Giacomello A, Ellison GM, Clarke K (2011) Cardiosphere-derived cells improve function in the infarcted rat heart for at least 16 weeks – an MRI study. PLoS One 6(10):e25669
60. Li TS, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, Zhang Y, Matsushita N, Smith RR, Marbán E (2010) Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. Stem Cells 28(11):2088–2098
61. Zakharova L, Mastroeni D, Mutlu N, Molina M, Goldman S, Diethrich E, Gaballa MA (2010) Transplantation of cardiac progenitor cell sheet onto infarcted heart promotes cardiogenesis and improves function. Circ Res 106(5):971–980

62. Chan HH, Meher Homji Z, Gomes RS, Sweeney D, Thomas GN, Tan JJ, Zhang H, Perbellini F, Stuckey DJ, Watt SM, Taggart D, Clarke K, Martin-Rendon E, Carr CA (2012) Human cardiosphere-derived cells from patients with chronic ischaemic heart disease can be routinely expanded from atrial but not epicardial ventricular biopsies. J Cardiovasc Transl Res 5(5): 678–687
63. Smart N, Risebro CA, Melville AA, Moses K, Schwartz RJ, Chien KR, Riley PR (2007) Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature 445(7124):177–182
64. Ellison GM, Polydorou J, Mercaldi RFC, Burdina AO, Vicinanza C, Theologou T, Oo A, Kudavalli M, Wright DJ, Field M, Nadal-Ginard B. Torella D, (2012) Aging and Senescence of Endogenous Cardiac Stem Cells (eCSCs) Determines Their Growth and Differentiation Potential. *Circulation* 124: A16327
65. Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, Marbán L, Marbán E (2012) Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. Lancet 379(9819):895–904
66. Johnston PV, Sasano T, Mills K, Evers R, Lee ST, Smith RR, Lardo AC, Lai S, Steenbergen C, Gerstenblith G, Lange R, Marbán E (2009) Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. Circulation 120(12):1075–1083
67. Lee ST, White AJ, Matsushita S, Malliaras K, Steenbergen C, Zhang Y, Li TS, Terrovitis J, Yee K, Simsir S, Makkar R, Marbán E (2011) Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction. J Am Coll Cardiol 57(4): 455–465
68. Shenje LT, Field LJ, Pritchard CA, Guerin CJ, Rubart M, Soonpaa MH, Ang KL, Galiñanes M (2008) Lineage tracing of cardiac explant derived cells. PLoS One 3(4):e1929
69. Andersen DC, Andersen P, Schneider M, Jensen HB, Sheikh SP (2009) Murine "cardiospheres" are not a source of stem cells with cardiomyogenic potential. Stem Cells 27(7):1571–1581
70. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433(7026):647–653
71. Cohen ED, Wang Z, Lepore JJ, Lu MM, Taketo MM, Epstein DJ, Morrisey EE (2007) Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. J Clin Invest 117(7):1794–1804
72. Zhou B, von Gise A, Ma Q, Rivera-Feliciano J, Pu WT (2008) Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. Biochem Biophys Res Commun 375(3): 450–453
73. Milgrom-Hoffman M, Harrelson Z, Ferrara N, Zelzer E, Evans SM, Tzahor E (2011) The heart endocardium is derived from vascular endothelial progenitors. Development 138(21): 4777–4787
74. Engleka KA, Manderfield LJ, Brust RD, Li L, Cohen A, Dymecki SM, Epstein JA (2012) Islet1 derivatives in the heart are of both neural crest and second heart field origin. Circ Res 110(7):922–926
75. Bondue A, Tännler S, Chiapparo G, Chabab S, Ramialison M, Paulissen C, Beck B, Harvey R, Blanpain C (2011) Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation. J Cell Biol 192(5):751–765
76. Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, Roberts DJ, Huang PL, Domian IJ, Chien KR (2009) Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460(7251):113–117
77. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR (2006) Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127(6):1151–1165

78. Amir G, Ma X, Reddy VM, Hanley FL, Reinhartz O, Ramamoorthy C, Riemer RK (2008) Dynamics of human myocardial progenitor cell populations in the neonatal period. Ann Thorac Surg 86(4):1311–1319
79. Genead R, Danielsson C, Andersson AB, Corbascio M, Franco-Cereceda A, Sylvén C, Grinnemo KH (2010) Islet-1 cells are cardiac progenitors present during the entire lifespan: from the embryonic stage to adulthood. Stem Cells Dev 19(10):1601–1615
80. Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT, Riley PR (2011) De novo cardiomyocytes from within the activated adult heart after injury. Nature 474(7353):640–644
81. Weinberger F, Mehrkens D, Friedrich FW, Stubbendorff M, Hua X, Müller JC, Schrepfer S, Evans SM, Carrier L, Eschenhagen T (2012) Localization of Islet-1-positive cells in the healthy and infarcted adult murine heart. Circ Res 110(10):1303–1310
82. Gittenberger-de Groot AC, Vrancken Peeters MP, Mentink MM, Gourdie RG, Poelmann RE (1998) Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. Circ Res 82(10):1043–1052
83. Wessels A, Pérez-Pomares JM (2004) The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. Anat Rec A Discov Mol Cell Evol Biol 276(1):43–57
84. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT (2008) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454(7200):109–113
85. van Tuyn J, Atsma DE, Winter EM, van der Velde-van Dijke I, Pijnappels DA, Bax NA, Knaän-Shanzer S, Gittenberger-de Groot AC, Poelmann RE, van der Laarse A, van der Wall EE, Schalij MJ, de Vries AA (2007) Epicardial cells of human adults can undergo an epithelial-to-mesenchymal transition and obtain characteristics of smooth muscle cells in vitro. Stem Cells 25(2):271–278
86. Winter EM, Grauss RW, Hogers B, van Tuyn J, van der Geest R, Lie-Venema H, Steijn RV, Maas S, DeRuiter MC, deVries AA, Steendijk P, Doevendans PA, van der Laarse A, Poelmann RE, Schalij MJ, Atsma DE, Gittenberger-de Groot AC (2007) Preservation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. Circulation 116(8):917–927
87. Winter EM, van Oorschot AA, Hogers B, van der Graaf LM, Doevendans PA, Poelmann RE, Atsma DE, Gittenberger-de Groot AC, Goumans MJ (2009) A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells. Circ Heart Fail 2(6):643–653
88. Bock-Marquette I, Shrivastava S, Pipes GC, Thatcher JE, Blystone A, Shelton JM, Galindo CL, Melegh B, Srivastava D, Olson EN, DiMaio JM (2009) Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. J Mol Cell Cardiol 46(5):728–738
89. Limana F, Zacheo A, Mocini D, Mangoni A, Borsellino G, Diamantini A, De Mori R, Battistini L, Vigna E, Santini M, Loiaconi V, Pompilio G, Germani A, Capogrossi MC (2007) Identification of myocardial and vascular precursor cells in human and mouse epicardium. Circ Res 101(12):1255–1265
90. Limana F, Bertolami C, Mangoni A, Di Carlo A, Avitabile D, Mocini D, Iannelli P, De Mori R, Marchetti C, Pozzoli O, Gentili C, Zacheo A, Germani A, Capogrossi MC (2010) Myocardial infarction induces embryonic reprogramming of epicardial c-kit(+) cells: role of the pericardial fluid. J Mol Cell Cardiol 48(4):609–618
91. Limana F, Capogrossi MC, Germani A (2011) The epicardium in cardiac repair: from the stem cell view. Pharmacol Ther 129(1):82–96
92. Ma Q, Zhou B, Pu WT (2008) Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. Dev Biol 323(1):98–104
93. Ferreira-Martins J, Ogórek B, Cappetta D, Matsuda A, Signore S, D'Amario D, Kostyla J, Steadman E, Ide-Iwata N, Sanada F, Iaffaldano G, Ottolenghi S, Hosoda T, Leri A, Kajstura J, Anversa P, Rota M (2012) Cardiomyogenesis in the developing heart is regulated by c-kit-positive cardiac stem cells. Circ Res 110(5):701–715

94. Laflamme MA, Myerson D, Saffitz JE, Murry CE (2002) Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 90(6):634–640
95. Bayes-Genis A, Muñiz-Diaz E, Catasus L, Arilla M, Rodriguez C, Sierra J, Madoz PJ, Cinca J (2004) Cardiac chimerism in recipients of peripheral-blood and bone marrow stem cells. Eur J Heart Fail 6(4):399–402
96. Mouquet F, Pfister O, Jain M, Oikonomopoulos A, Ngoy S, Summer R, Fine A, Liao R (2005) Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ Res 97(11):1090–1092
97. Gaebel R, Furlani D, Sorg H, Polchow B, Frank J, Bieback K, Wang W, Klopsch C, Ong LL, Li W, Ma N, Steinhoff G (2011) Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. PLoS One 6(2):e15652
98. Loffredo FS, Steinhauser ML, Gannon J, Lee RT (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8(4):389–398
99. Kattman SJ, Adler ED, Keller GM (2007) Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. Trends Cardiovasc Med 17(7):240–246
100. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT (2007) Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 13(8):970–974
101. Simpson DL, Mishra R, Sharma S, Goh SK, Deshmukh S, Kaushal S (2012) A strong regenerative ability of cardiac stem cells derived from neonatal hearts. Circulation 126(11 Suppl 1): S46–S53
102. Smart N, Riley PR (2009) Derivation of epicardium-derived progenitor cells (EPDCs) from adult epicardium. Curr Protoc Stem Cell Biol Chapter 2:Unit2C.2
103. Torella D, Rota M, Nurzynska D, Musso E, Monsen A, Shiraishi I, Zias E, Walsh K, Rosenzweig A, Sussman MA, Urbanek K, Nadal-Ginard B, Kajstura J, Anversa P, Leri A (2004) Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 94(4):514–524
104. Ellison GM, Torella D, Karakikes I, Purushothaman S, Curcio A, Gasparri C, Indolfi C, Cable NT, Goldspink DF, Nadal-Ginard B (2007) Acute beta-adrenergic overload produces myocyte damage through calcium leakage from the ryanodine receptor 2 but spares cardiac stem cells. J Biol Chem 282(15):11397–11409
105. Levesque JP, Hendy J, Winkler IG, Takamatsu Y, Simmons PJ (2003) Granulocyte colony-stimulating factor induces the release in the bone marrow of proteases that cleave c-KIT receptor (CD117) from the surface of hematopoietic progenitor cells. Exp Hematol 31: 109–117
106. Tang YL, Shen L, Qian K, Phillips MI (2007) A novel two-step procedure to expand cardiac Sca-1+ cells clonally. Biochem Biophys Res Commun 359(4):877–883
107. Miyamoto S, Kawaguchi N, Ellison GM, Matsuoka R, Shin'oka T, Kurosawa H (2010) Characterization of long-term cultured c-kit+ cardiac stem cells derived from adult rat hearts. Stem Cells Dev 19(1):105–116
108. Steele A, Boucek RJ Jr, Jacobs JP, Steele P, Asante-Korang A, Chamizo W, Steele J, Chai PJ, Quintessenza JA (2012) Heart cells with regenerative potential from pediatric patients with end stage heart failure: a translatable method to enrich and propagate. Stem Cells Int 2012: 452102
109. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ (2009) Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc 4(2):232–243
110. Itzhaki-Alfia A, Leor J, Raanani E, Sternik L, Spiegelstein D, Netser S, Holbova R, Pevsner-Fischer M, Lavee J, Barbash IM (2009) Patient characteristics and cell source determine the number of isolated human cardiac progenitor cells. Circulation 120(25):2559–2566

111. Choi SH, Jung SY, Yoo SM, Asahara T, Suh W, Kwon SM, Baek SH (2012) Amine-enriched surface modification facilitates expansion, attachment, and maintenance of human cardiac-derived c-kit positive progenitor cells. Int J Cardiol. doi:10.1016/j.ijcard.2012.09.065, pii: S0167-5273(12)01171-0. EPUB-ahead of print
112. Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, Kishore R, Phillips MI, Losordo DW, Qin G (2009) Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res 104(10):1209–1216
113. Li TS, Cheng K, Malliaras K, Matsushita N, Sun B, Marbán L, Zhang Y, Marbán E (2011) Expansion of human cardiac stem cells in physiological oxygen improves cell production efficiency and potency for myocardial repair. Cardiovasc Res 89(1):157–165
114. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E (2012) Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379(9819):895–904
115. Li TS, Marbán E (2010) Physiological levels of reactive oxygen species are required to maintain genomic stability in stem cells. Stem Cells 28(7):1178–1185
116. van Oorschot AA, Smits AM, Pardali E, Doevendans PA, Goumans MJ (2011) Low oxygen tension positively influences cardiomyocyte progenitor cell function. J Cell Mol Med 15(12):2723–2734
117. North BJ, Sinclair DA (2012) The intersection between aging and cardiovascular disease. Circ Res 110(8):1097–1108
118. Dutta D, Calvani R, Bernabei R, Leeuwenburgh C, Marzetti E (2012) Contribution of impaired mitochondrial autophagy to cardiac aging: mechanisms and therapeutic opportunities. Circ Res 110(8):1125–1138
119. Schulman SP, Lakatta EG, Fleg JL, Lakatta L, Becker LC, Gerstenblith G (1992) Age-related decline in left ventricular filling at rest and exercise. Am J Physiol 263(6 Pt 2): H1932–H1938
120. Fleg JL, Shapiro EP, O'Connor F, Taube J, Goldberg AP, Lakatta EG (1995) Left ventricular diastolic filling performance in older male athletes. JAMA 273(17):1371–1375
121. Lakatta EG, Levy D (2003) Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. Circulation 107(2):346–354
122. Lakatta EG, Levy D (2003) Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. Circulation 107(1): 139–146
123. Antelmi I, de Paula RS, Shinzato AR, Peres CA, Mansur AJ, Grupi CJ (2004) Influence of age, gender, body mass index, and functional capacity on heart rate variability in a cohort of subjects without heart disease. Am J Cardiol 93(3):381–385
124. Jeyapalan JC, Sedivy JM (2008) Cellular senescence and organismal aging. Mech Ageing Dev 129(7–8):467–474
125. Haigis MC, Yankner BA (2010) The aging stress response. Mol Cell 40(2):333–344
126. Liu L, Rando TA (2011) Manifestations and mechanisms of stem cell aging. J Cell Biol 193(2):257–266
127. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS (2010) The essence of senescence. Genes Dev 24(22):2463–2479
128. Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100(1):157–168
129. Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327(5965):542–545
130. Sharpless NE, DePinho RA (2007) How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol 8(9):703–713
131. Rossi DJ, Jamieson CH, Weissman IL (2008) Stems cells and the pathways to aging and cancer. Cell 132(4):681–696

132. Beltrami AP, Cesselli D, Beltrami CA (2011) At the stem of youth and health. Pharmacol Ther 129(1):3–20
133. Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A, Maseri A, Anversa P (2003) Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res 93(7): 604–613
134. Thijssen DH, Torella D, Hopman MT, Ellison GM (2009) The role of endothelial progenitor and cardiac stem cells in the cardiovascular adaptations to age and exercise. Front Biosci 14:4685–4702
135. Kajstura J, Gurusamy N, Ogórek B, Goichberg P, Clavo-Rondon C, Hosoda T, D'Amario D, Bardelli S, Beltrami AP, Cesselli D, Bussani R, del Monte F, Quaini F, Rota M, Beltrami CA, Buchholz BA, Leri A, Anversa P (2010) Myocyte turnover in the aging human heart. Circ Res 107(11):1374–1386
136. Cesselli D, Beltrami AP, D'Aurizio F, Marcon P, Bergamin N, Toffoletto B, Pandolfi M, Puppato E, Marino L, Signore S, Livi U, Verardo R, Piazza S, Marchionni L, Fiorini C, Schneider C, Hosoda T, Rota M, Kajstura J, Anversa P, Beltrami CA, Leri A (2011) Effects of age and heart failure on human cardiac stem cell function. Am J Pathol 179(1):349–366
137. Martin K, Potten CS, Roberts SA, Kirkwood TB (1998) Altered stem cell regeneration in irradiated intestinal crypts of senescent mice. J Cell Sci 111(Pt 16):2297–2303
138. Flores I, Cayuela ML, Blasco MA (2005) Effects of telomerase and telomere length on epidermal stem cell behavior. Science 309(5738):1253–1256
139. Liang Y, Van Zant G, Szilvassy SJ (2005) Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood 106(4):1479–1487
140. Nishimura EK, Granter SR, Fisher DE (2005) Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 307(5710):720–724
141. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443(7110):421–426
142. Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. Nature 443(7110):453–457
143. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ (2006) Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature 443(7110):448–452
144. Beerman I, Maloney WJ, Weissmann IL, Rossi DJ (2010) Stem cells and the aging hematopoietic system. Curr Opin Immunol 22(4):500–506
145. Gonzalez A, Rota M, Nurzynska D, Misao Y, Tillmanns J, Ojaimi C, Padin-Iruegas ME, Müller P, Esposito G, Bearzi C, Vitale S, Dawn B, Sanganalmath SK, Baker M, Hintze TH, Bolli R, Urbanek K, Hosoda T, Anversa P, Kajstura J, Leri A (2008) Activation of cardiac progenitor cells reverses the failing heart senescent phenotype and prolongs lifespan. Circ Res 102(5):597–606
146. Kuilman T, Peeper DS (2009) Senescence-messaging secretome: SMS-ing cellular stress. Nat Rev Cancer 9(2):81–94
147. Rodier F, Campisi J (2011) Four faces of cellular senescence. J Cell Biol 192(4):547–556
148. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM (2011) Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 479(7372):232–236
149. Kim WY, Sharpless NE (2006) The regulation of INK4/ARF in cancer and aging. Cell 127(2):265–275
150. Ellison GM, Torella D, Karakikes I, Nadal-Ginard B (2007) Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. Nat Clin Pract Cardiovasc Med 4(Suppl 1):S52–S59
151. Waring CD, Vicinanza C, Papalamprou A, Smith AJ, Purushothaman S, Goldspink DF, Nadal-Ginard B, Torella D, Ellison GM (2012) The adult heart responds to increased work-

load with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. Eur Heart J. EPUB-ahead of print
152. Olivetti G, Giordano G, Corradi D, Melissari M, Lagrasta C, Gambert SR, Anversa P (1995) Gender differences and aging: effects on the human heart. J Am Coll Cardiol 26(4): 1068–1079
153. van Vliet P, Smits AM, de Boer TP, Korfage TH, Metz CH, Roccio M, van der Heyden MA, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ (2010) Foetal and adult cardiomyocyte progenitor cells have different developmental potential. J Cell Mol Med 14(4):861–870
154. Ellison GM, Waring CD, Vicinanza C, Torella D (2011) Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms. Heart 98(1): 5–10
155. Emmert MY, Emmert LS, Martens A, Ismail I, Schmidt-Richter I, Gawol A, Seifert B, Haverich A, Martin U, Gruh I (2013) Higher frequencies of BCRP+ cardiac resident cells in ischaemic human myocardium. Eur Heart J 34:2830–2838
156. Shen YH, Hu X, Zou S, Wu D, Coselli JS, LeMaire SA (2012) Stem cells in thoracic aortic aneurysms and dissections: potential contributors to aortic repair. Ann Thorac Surg 93(5): 1524–1533
157. Castaldo C, Di Meglio F, Nurzynska D, Romano G, Maiello C, Bancone C, Müller P, Böhm M, Cotrufo M, Montagnani S (2008) CD117-positive cells in adult human heart are localized in the subepicardium, and their activation is associated with laminin-1 and alpha6 integrin expression. Stem Cells 26(7):1723–1731
158. Genead R, Fischer H, Hussain A, Jaksch M, Andersson AB, Ljung K, Bulatovic I, Franco-Cereceda A, Elsheikh E, Corbascio M, Smith CI, Sylvén C, Grinnemo KH (2012) Ischemia-reperfusion injury and pregnancy initiate time-dependent and robust signs of up-regulation of cardiac progenitor cells. PLoS One 7(5):e36804
159. Rupp S, Bauer J, von Gerlach S, Fichtlscherer S, Zeiher AM, Dimmeler S, Schranz D (2012) Pressure overload leads to an increase of cardiac resident stem cells. Basic Res Cardiol 107(2):252
160. Sato H, Shiraishi I, Takamatsu T, Hamaoka K (2007) Detection of TUNEL-positive cardiomyocytes and c-kit-positive progenitor cells in children with congenital heart disease. J Mol Cell Cardiol 43(3):254–261
161. Gambini E, Pesce M, Persico L, Bassetti B, Gambini A, Alamanni F, Agrifoglio M, Capogrossi MC, Pompilio G (2012) Patient profile modulates cardiac c-kit(+) progenitor cell availability and amplification potential. Transl Res 160(5):363–373
162. Kubo H, Jaleel N, Kumarapeli A, Berretta RM, Bratinov G, Shan X, Wang H, Houser SR, Margulies KB (2008) Increased cardiac myocyte progenitors in failing human hearts. Circulation 118(6):649–657
163. Schächinger V, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Süselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM, REPAIR-AMI Investigators (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355(12):1210–1221
164. Dawn B, Abdel-Latif A, Sanganalmath SK, Flaherty MP, Zuba-Surma EK (2009) Cardiac repair with adult bone marrow-derived cells: the clinical evidence. Antioxid Redox Signal 11(8):1865–1882
165. Jeevanantham V, Butler M, Saad A, Abdel-Latif A, Zuba-Surma EK, Dawn B (2012) Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: a systematic review and meta-analysis. Circulation 126(5):551–568
166. Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T (2003) Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 107(3):461–468
167. Deb A, Davis BH, Guo J, Ni A, Huang J, Zhang Z, Mu H, Dzau VJ (2008) SFRP2 regulates cardiomyogenic differentiation by inhibiting a positive transcriptional autofeedback loop of Wnt3a. Stem Cells 26(1):35–44

168. Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM (2010) Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107(7):913–922
169. Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98(5):1076–1084
170. Iekushi K, Seeger F, Assmus B, Zeiher AM, Dimmeler S (2012) Regulation of cardiac microRNAs by bone marrow mononuclear cell therapy in myocardial infarction. Circulation 125(14):1765–1773
171. Takahashi M, Li TS, Suzuki R, Kobayashi T, Ito H, Ikeda Y, Matsuzaki M, Hamano K (2006) Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. Am J Physiol Heart Circ Physiol 291(2):H886–H893
172. Janssens S (2010) Stem cells in the treatment of heart disease. Annu Rev Med 61:287–300
173. Dawn B, Stein AB, Urbanek K, Rota M, Whang B, Rastaldo R, Torella D, Tang XL, Rezazadeh A, Kajstura J, Leri A, Hunt G, Varma J, Prabhu SD, Anversa P, Bolli R (2005) Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. Proc Natl Acad Sci U S A 102(10):3766–3771
174. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P (2011) Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378(9806):1847–1857
175. Matsubara H (2012) Kyoto Prefectural University School of Medicine. AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy (ALCADIA). ClinicalTrials.gov. Available from: http://clinicaltrials.gov/ct2/show/NCT00981006
176. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
177. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104(4):e30–e41
178. Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flügel L, Dorn T, Goedel A, Höhnke C, Hofmann F, Seyfarth M, Sinnecker D, Schömig A, Laugwitz KL (2010) Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med 363(15):1397–1409
179. Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, Boulos M, Gepstein L (2011) Modelling the long QT syndrome with induced pluripotent stem cells. Nature 471(7337):225–229
180. Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142(3):375–386
181. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu JD, Srivastava D (2012) In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature 485(7400):593–598
182. Crottogini A, Meckert PC, Vera Janavel G, Lascano E, Negroni J, Del Valle H, Dulbecco E, Werba P, Cuniberti L, Martínez V, De Lorenzi A, Telayna J, Mele A, Fernández JL, Marangunich L, Criscuolo M, Capogrossi MC, Laguens R (2003) Arteriogenesis induced by intramyocardial vascular endothelial growth factor 165 gene transfer in chronically ischemic pigs. Hum Gene Ther 14(14):1307–1318
183. Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER, VIVA Investigators (2003) The VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis. Circulation 107(10):1359–1365

184. Wu J, Zeng F, Huang XP, Chung JC, Konecny F, Weisel RD, Li RK (2011) Infarct stabilization and cardiac repair with a VEGF-conjugated, injectable hydrogel. Biomaterials 32(2): 579–586
185. Formiga FR, Pelacho B, Garbayo E, Abizanda G, Gavira JJ, Simon-Yarza T, Mazo M, Tamayo E, Jauquicoa C, Ortiz-de-Solorzano C, Prósper F, Blanco-Prieto MJ (2010) Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J Control Release 147(1):30–37
186. Wadugu B, Kühn B (2012) The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. Am J Physiol Heart Circ Physiol 302(11):H2139–H2147
187. Kasasbeh E, Murphy A, Ahmad FA, Yu E, Williams P, Nunnally A, Adcock J, Caggiano AO, Parry T, Ganguly A, Sawyer DB, Cleator JH (2011) Neuregulin-1β improves cardiac remodeling after myocardial infarction in swine. Circulation 124:Abstract 15531
188. Bersell K, Arab S, Haring B, Kühn B (2009) Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138(2):257–270
189. Fontana L, Vinciguerra M, Longo VD (2012) Growth factors, nutrient signaling, and cardiovascular aging. Circ Res 110(8):1139–1150
190. Russell SJ, Kahn CR (2007) Endocrine regulation of ageing. Nat Rev Mol Cell Biol 8(9): 681–691
191. Musarò A, Giacinti C, Borsellino G, Dobrowolny G, Pelosi L, Cairns L, Ottolenghi S, Cossu G, Bernardi G, Battistini L, Molinaro M, Rosenthal N (2004) Stem cell-mediated muscle regeneration is enhanced by local isoform of insulin-like growth factor 1. Proc Natl Acad Sci U S A 101(5):1206–1210
192. Santini MP, Tsao L, Monassier L, Theodoropoulos C, Carter J, Lara-Pezzi E, Slonimsky E, Salimova E, Delafontaine P, Song YH, Bergmann M, Freund C, Suzuki K, Rosenthal N (2007) Enhancing repair of the mammalian heart. Circ Res 100(12):1732–1740
193. Santini MP, Lexow J, Borsellino G, Slonimski E, Zarrinpashneh E, Poggioli T, Rosenthal N (2011) IGF-1Ea induces vessel formation after injury and mediates bone marrow and heart cross-talk through the expression of specific cytokines. Biochem Biophys Res Commun 410(2):201–207
194. Kawaguchi N, Smith AJ, Waring CD, Hasan MK, Miyamoto S, Matsuoka R, Ellison GM (2010) c-kitpos GATA-4 high rat cardiac stem cells foster adult cardiomyocyte survival through IGF-1 paracrine signalling. PLoS One 5(12):e14297
195. Vinciguerra M, Santini MP, Martinez C, Pazienza V, Claycomb WC, Giuliani A, Rosenthal N (2012) mIGF-1/JNK1/SirT1 signaling confers protection against oxidative stress in the heart. Aging Cell 11(1):139–149
196. Kajstura J, Fiordaliso F, Andreoli AM, Li B, Chimenti S, Medow MS, Limana F, Nadal-Ginard B, Leri A, Anversa P (2001) IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. Diabetes 50(6):1414–1424
197. Yang S, Chintapalli J, Sodagum L, Baskin S, Malhotra A, Reiss K, Meggs LG (2005) Activated IGF-1R inhibits hyperglycemia-induced DNA damage and promotes DNA repair by homologous recombination. Am J Physiol Renal Physiol 289(5):F1144–F1152
198. Huh CG, Factor VM, Sánchez A, Uchida K, Conner EA, Thorgeirsson SS (2004) Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A 101(13):4477–4482
199. Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Matsumoto K, Nakamura T, Kaneda Y, Horiuchi M, Ogihara T (2001) Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. Hypertension 37(2 Part 2):581–586
200. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T (2000) Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest 106(12):1511–1519
201. Ueda H, Nakamura T, Matsumoto K, Sawa Y, Matsuda H, Nakamura T (2001) A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. Cardiovasc Res 51(1):41–50

202. Madonna R, Rokosh G, De Caterina R, Bolli R (2010) Hepatocyte growth factor/Met gene transfer in cardiac stem cells – potential for cardiac repair. Basic Res Cardiol 105(4): 443–452
203. Zhu XY, Zhang XZ, Xu L, Zhong XY, Ding Q, Chen YX (2009) Transplantation of adipose-derived stem cells overexpressing hHGF into cardiac tissue. Biochem Biophys Res Commun 379(4):1084–1090
204. Yang ZJ, Zhang YR, Chen B, Zhang SL, Jia EZ, Wang LS, Zhu TB, Li CJ, Wang H, Huang J, Cao KJ, Ma WZ, Wu B, Wang LS, Wu CT (2009) Phase I clinical trial on intracoronary administration of Ad-hHGF treating severe coronary artery disease. Mol Biol Rep 36(6):1323–1329
205. Eid H, Larson DM, Springhorn JP, Attawia MA, Nayak RC, Smith TW, Kelly RA (1992) Role of epicardial mesothelial cells in the modification of phenotype and function of adult rat ventricular myocytes in primary coculture. Circ Res 71(1):40–50
206. Wada AM, Smith TK, Osler ME, Reese DE, Bader DM (2003) Epicardial/mesothelial cell line retains vasculogenic potential of embryonic epicardium. Circ Res 92:525–531
207. Männer J (1999) Does the subepicardial mesenchyme contribute myocardioblasts to the myocardium of the chick embryo heart? A quail-chick chimera study tracing the fate of the epicardial primordium. Anat Rec 255(2):212–226
208. Perez-Pomares JM, Carmona R, González-Iriarte M, Atencia G, Wessels A, Muñoz-Chápuli R (2002) Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. Int J Dev Biol 46(8):1005–1013
209. Tomita Y, Matsumura K, Wakamatsu Y, Matsuzaki Y, Shibuya I, Kawaguchi H, Ieda M, Kanakubo S, Shimazaki T, Ogawa S, Osumi N, Okano H, Fukuda K (2005) Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 170:1135–1146
210. Martin CM, Ferdous A, Gallardo T, Humphries C, Sadek H, Caprioli A, Garcia JA, Szweda LI, Garry MG, Garry DJ (2008) Hypoxia-inducible factor-2alpha transactivates Abcg2 and promotes cytoprotection in cardiac side population cells. Circ Res 102(9):1075–1081

Adult Pituitary Stem Cells

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Abstract Underlying mechanisms leading to pituitary plasticity by which the gland adapts the number of hormone-producing cell to the continuously changing physiological requirements are still poorly understood. Adult stem cells were shown to direct homeostatic cell maintenance, regeneration, and functional plasticity in several organs and tissues. Only recently potential stem cells were identified and phenotypically characterized in adult pituitary. Multiple possible stem/progenitor cell candidates were proposed, but different studies have been only partially reconciled. Here, we critically analyzed the reports addressing the identification of adult pituitary stem cells, trying, when possible, to reunite the results of the different studies. Nonetheless, in light of the still non-complete characterization of these cells, some discrepancies among the published studies are still apparent. Importantly, long-term in vitro self-renewal, a defining feature of stem cells, remains to be unequivocally demonstrated. Finally, the potential role of adult pituitary stem (or progenitor) cells in pituitary adenoma development will be discussed.

Keywords Adult stem cells • Pituitary • Colony-forming unit • Regeneration • Cancer

Abbreviations

CSC	Cancer stem cells
FCS	Fetal calf serum
FS cells	Folliculo-stellate cells
GPS cells	GFRα2-PROP1-STEM cells
HMG	High mobility group

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MP	Main population
PASCs	Pituitary adenoma stem-like cells
Sca1	Stem cell antigen 1
SMA	Smooth muscle actin
SP	Side population
TIC	Tumor-initiating cells

1 Introduction

The existence of stem-like/progenitor cell population was hypothesized since 1969 in studies in which the transplantation of undifferentiated (chromophobe) pituitary cells in hypophysectomized rats was reported, to originate differentiated (hormone-producing) pituitary cells [1].

Moreover, indirect evidence of the presence of multipotent cells within pituitary was obtained by the adaptive responsiveness of pituitary to both physiological and pathological conditions.

In vertebrates pituitary develops from the most anterior part of the anterior neural ridge [2]. During early development oral and neural ectoderms are located in close proximity: differentiating signals start from neural ectoderm to oral ectoderm that invaginates in the Rathke's pouch. Subsequently, Rathke's pouch forms a closed epithelial structure separating from the oral ectoderm, giving rise to anterior pituitary, which is composed of several types of specialized endocrine cells able to produce and release the different hypophyseal hormones, namely, GH (somatotrophs), prolactin (lactotrophs), TSH (thyrotrophs), POMC (corticotrophs), and LH/FSH (gonadotrophs). On the other hand, from neural ectoderm originate the infundibulum and the pituitary neural lobe containing hypothalamic neuron terminal and releasing vasopressin and oxytocin [3].

Several studies analyzing cell proliferation and differentiation within Rathke's pouch identified two levels of regulation of stem/progenitor cell activity involving embryogenesis and postnatal days. In mouse embryo, between days 11.5 and 18.5, pituitary cells shift from mainly proliferating to differentiating populations, while proliferating cells can be observed only around the ridge of the Rathke's pouch, named marginal zone [4]. Marginal zone (also defined as the cleft separating anterior and intermediate pituitary lobes) has been indeed identified as putative "stem cell niche" in pituitary [5]. Functional and morphological evidence supports the assumption that cells located in this region could actually represent stem cells. For example, cells in marginal zone do not express secretory granules, as differentiated pituitary cells, and are characterized by reduced endoplasmic reticulum, by abundance of free ribosomes and polysomes, and by the expression of potential stem cell markers, such as Sox2 [3]. Importantly, while during pituitary organogenesis proliferating Sox2-expressing cells are readily observed throughout the gland, after development they are mainly localized within the marginal zone.

In the adulthood, pituitary proliferating cells are highly reduced in number, and, concomitantly, the number of differentiated cells increases. While all

hormone-producing cell types are already developed at birth, pituitary is not completely formed yet and, in rodents, its growth and maturation continue for a few weeks in postnatal period [6]. It was shown that terminally differentiated hormone-secreting cells during this period can reenter the cell cycle to increase a given hormone-producing population cell number, although current evidence proposes that the increase in pituitary cell number is mainly derived from a population of so-called "transient-amplifying cells" derived from differentiation of cells in the stem compartment that migrate into pituitary parenchyma where they start to actively proliferate [7].

The continuous generation of "transient-amplifying cells" from stem cells would also represent the mechanism by which, in normal conditions, the whole adult pituitary cell compartment is completely renewed every 5–8 weeks [3, 8, 9]. In fact, in "basal" conditions, in the absence of hypothalamic or other hormonal stimuli, most of these cells undergo apoptosis commitment, limiting the proliferation activity to the replacement of dying cells. Nevertheless, in several physiological conditions (growth, puberty, pregnancy, lactation), in which a general reorganization of pituitary structure and cell composition is required, the activity of these cells can largely increase [8, 9]. For example, after adrenalectomy or orchiectomy a great surge in the number of corticotroph or gonadotroph cells occurs in adult pituitary [10, 11]. In a more physiological setting, lactotroph number increases several folds during pregnancy and lactation to adapt the prolactin secretion required in those conditions [12].

Still three mechanisms can account for adult pituitary plasticity: (1) differentiated (hormone-producing) cells enter mitosis [13]; (2) transdifferentiation of differentiated cell populations [14]; and (3) recruitment of putative adult pituitary stem/progenitor cells [15].

The first two mechanisms, mitotic activation of differentiated/hormone-producing pituitary cells and transdifferentiation from a different pituitary cell type (mostly conversion of somatotrophs in lactotrophs), are believed to represent only a minor way of pituitary plasticity. In fact, hormonally null cells comprise more than 90 % of mitotic events observed during adrenalectomy, and only after few weeks differentiate corticotroph cells are generated (for review, see [16, 17]). Similarly, genetic lineage trace experiments demonstrated that new prolactin-secreting cells, developed in response to estrogens (for example, during pregnancy), rarely derive from differentiated somatotroph cells [18], but, again, mainly derive from a self-limiting wave of proliferation of the nonhormonal pool of pituitary cells [12]. Thus, most of basal pituitary cell turnover and cell lineage changes, occurring as dynamic adaptation in physiological or pathological conditions, are driven by recruitment of pituitary cell subpopulation endowed with stem/progenitor cell characteristics [15].

In the past years, a large effort was dedicated to the search of putative pituitary stem or progenitor cells, and only in recent years convincing data were produced to sustain this hypothesis, opening a completely novel and rapidly growing field of research. In fact, the definitive demonstration of pituitary stem cells may have significant clinical impact, for example, in adult-onset hypopituitarism in the context of regenerative medicine [19], as proposed for pancreatic β-cells in type I diabetes

or, more importantly, for pituitary tumors, in light of the cancer stem cell theory of tumor development [20].

Several cell populations in adult pituitary showing phenotypes and biological activity resembling those of stem cells (i.e., undifferentiated cells expressing stemness markers, able to self-renew, residing in specific areas of each tissue named "niches," endowed of resistance to drugs or toxins due to high activity of DNA repair mechanisms and ABC transporters, and importantly to generate all the cell populations in a given organ or tissue [21]) were identified in studies mainly involving murine models, but a definitive consensus about the characteristics of these cells has not been reached so far.

In the following paragraphs are reported the more relevant studies aimed to define adult pituitary stem cells, trying to highlight both differences and concordances among the features of the cell populations proposed.

In Table 1 are reported the main genes involved in the putative pituitary stem cell activity.

2 Side Population

Increased activity of ABC transporters has been the first stem-like cell feature exploited to identify the presence of this cell subpopulation in mouse pituitary. The activity of ABC transporters causes the extrusion of potential toxicants from the cells, representing a defining characteristic of stem cells granting their survival and protection against genotoxic insults for all the life span of an organism. In fact, due to their prolonged survival stem cells are exposed to continuous environmental injuries that require the development of efficient defensive systems, including DNA repair mechanisms and extruding pumps [22]. This characteristic was applied to identify stem cells in different tissues as the cell population able to extrude fluorescent dyes, such as Hoechst 33342, from the cytosol. Importantly, these cells can be visualized by FACS analysis as a "side population" (SP) forming a small "streak" separated from the main population (MP) that retains the dye [23].

SP cells have been identified in adult pituitary in mouse and dog, although with some difference in the phenotype among the species [24, 25].

SP cells comprise about 1.5 % of 3–8 weeks old mouse anterior pituitary cells. These cells are characterized by the overexpression of several stem cell-associated markers (nestin, Sca1, Nanog, CD133, Oct-4) and molecular effectors of self-renewal (Notch, Wnt, Shh) [24, 26], while in a small percentage (5.8 % of total SP) a phenotype resembling folliculo-stellate (FS) cells was detected [24]. Conversely, all these markers were not (or minimally) expressed in the pituitary MP [24].

SP cells are clonogenic, growing as non-adherent spheres (named following their origin as pituispheres) [24], a feature that, when retained after serial passages, is an index of active self-renewal characterizing the growth of stem/progenitor cells of multiple tissues [27, 28]. Spherogenesis activity was restricted to a subgroup of the cells in pituitary SP, representing about 0.02 % of the seeded anterior pituitary cells.

Table 1 Summary of some of the main genes potentially involved in pituitary stem cells and progenitors activity and/or their differentiation (see the text for references)

Gene name	Protein encoded	Main characteristic
Transcriptions factors (TF)		
Sox2	SRY-related HMG box TF	General stem cell marker; it is expressed in cells in the pituitary marginal zone
Sox9	SRY-related HMG box TF	Marker of the transition between pituitary stem cells/progenitors and transit-amplifying cells
Prop1	Paired-like homeodomain TF (Prophet of Pit1)	It is expressed in pituitary marginal zone by pituitary progenitors
Pouf1f1(Pit1)	POU homeodomain TF	It is involved in the differentiation of cells of somato-lactotroph and thyrotroph lineages
Octa4 (Pou5f1)	POU homeodomain TF	Expressed in stem cells of several tissue
Nanog	Homeobox TF	Involved in maintaining stem cell pluripotency and self-renewal
Hes1	Basic helix-loop-helix TF	Notch direct downstream target and effector; it is a repressor of cell cycle inhibitors, expressed in $S/G_2/M/G_1$, but not in G_0
Cell cycle regulators		
Bmi1	BMI1 polycomb ring finger oncogene	Regulates cell cycle inhibitor genes in stem cells from several tissues
Cdk4	Cyclin-dependent kinase 4	Involved in cell cycle G_1 phase progression
Intermediate filament proteins		
Nestin	Type VI intermediate filament protein	It is expressed in pituitary marginal zone as well as in progenitors from several tissues
GFAP	Glial fibrillary acidic protein (intermediate filament protein)	It is expressed in folliculo-stellate cells
Cytokeratin 8 (Krt8)	Keratin-containing intermediate filament protein	Expressed in pituitary marginal zone in adulthood, but not detected in SP
Receptors, adhesion and cell surface proteins, and other genes		
E-cadherin (Cdh1)	Calcium-dependent adhesion molecule (type 1 transmembrane protein)	It is involved in transition from proliferation to differentiation during EMT
CD90	Thy1 or CD90 (cell surface protein)	Marker of a variety of stem cells
CXCR4	Chemokine CXC-motif receptor 4	It is the receptor for the chemokine CXCL12 expressed by multiple stem cells or progenitors, including pituitary SP
Gfra2	GDNF receptor α 2	Stem cell marker in testis and ovary; it is expressed in pituitary marginal zone by the "GPS" cells
Prom1	Prominin1 (CD133)	It is expressed by multiple stem cells or progenitors, including pituitary SP
S100β	S100 calcium binding protein β	Marker of pituitary folliculostellate cells
Sca1	Stem cell antigen 1	It is expressed in SP cells and pericytes
Notch1	Notch 1	Transmembrane receptor involved in progenitor differentiation in CNS

This small cell population was phenotypically characterized as the cells that express Oct-4, CD133, and components of the Notch, Wnt, and Shh pathways, while did not contain pituitary hormones. This pattern of expression represents a molecular picture largely shared by SP cells in several tissues [24].

The differences in the clonogenicity among SP cells fostered a more detailed analysis of SP cells. In these studies two cell subpopulations were selected, accordingly to the level of expression of the stem cell antigen 1 (Sca1) that, conversely, was minimally expressed in the MP cells. In particular, it was proposed that SP cells expressing Sca1 at low level (named non-Sca1^{high} fraction) and representing about 40 % of the SP compose the putative adult pituitary progenitor population. In fact, differently from MP cells and the SP cells expressing high levels of Sca1 (Sca1^{high} population), non-Sca1^{high} cells grow as pituispheres (at least for a limited number of passages) and are able to differentiate in the pituitary hormone-secreting cell types. As compared to the Sca1^{high} SP cells, non-Sca1^{high} cells overexpress (up 25-fold more) most of the transcription factors controlling pituitary development (Hesx1, Prop1, Pax6, Lhx3, Lhx4, OTX2, PITX1, and PITX2), inhibitors of apoptosis, and components of MAP kinase, Wnt, and Notch pathways [29]. In particular, the activation of Notch pathway maintains stem cells in undifferentiated status [19], playing a relevant role in the stem cells proliferation being modulated by growth factors commonly used to select stem cells in vitro (EGF, bFGF, and LIF) [29]. Interestingly non-Sca1^{high} cells co-express pituitary transcription factors normally identified at different stages during pituitary development [19], suggesting that this population is heterogeneous including both stem-like cells and more differentiated progenitors [3].

Sox2 and Sox9 were also detected only in non-Sca1^{high} cells (about 50 % and 30 %, respectively) but were virtually absent in Sca1^{high} cells, while nestin, as well as OCT4 and Bmi-1, was equally detected in the two populations [29], suggesting that these markers (representing protein involved in self-renewal of stem cells) are not specific for pituitary stem cells. The majority of the cells in pituispheres derived from non-Sca1^{high} cells co-express Sox2 and nestin [30]. This observation suggests that self-renewal activity mainly rests in Sox2-expressing cells (representing 50 % of the non-Sca1^{high} population). After differentiation in hormone-producing cells, Sox2 expression ceased, confirming the specificity of the expression of this transcription factor in putative pituitary stem cells. Importantly, in agreement with other studies [30], Sox2^{+} cells were identified in small number in proximity of the pituitary stem cell niche (the marginal zone around the cleft) [5]. However, clusters of Sox2^{+} cells are also scattered within the anterior pituitary parenchyma [24, 30–32], being suggestive of the existence of multiple niches [5]. On the other hand, the high level of expression of nestin in Sca1^{high} cells is in agreement with data showing that nestin is also expressed in non-endocrine pituitary cells, including folliculo-stellate (FS) cells and pericytes that, as a consequence, were interpreted as subsets of the Sca1^{high} cell component. Indeed, microarray analysis showed that Sca1^{high} cells also express S100β and several angiogenesis-related genes, and thus, in consideration of the recognized role of Sca1 in endothelial development [33], it was proposed that these cells may represent a subset of FS cells and/or endothelial progenitors rather than pituitary progenitors [29].

3 SOX2$^+$/SOX9$^-$ Cells

In agreement with the studies on pituitary SP, using different approaches, several other studies focused on Sox2 as a marker for putative pituitary stem cells. Sox2, a transcription factor of the high mobility group (HMG) family, is a reasonable candidate to identify these subpopulations since it is expressed at high levels in embryos, playing a relevant role in CNS development [34], while it is downregulated during cell differentiation. Thus, it was proposed that, in adulthood, only stem cells retain Sox2 expression. Importantly, Sox2 is also required for normal pituitary development [35].

In murine postnatal pituitary, the pattern of Sox2 expression is similar to that of developing embryonic gland, being localized in about 3 % of the postnatal anterior pituitary cells localized in the marginal zone between anterior and intermediate lobe and occasionally scattered throughout the adenohypophysis [29, 30, 36]. Sox2$^+$ cells often co-express E-cadherin, suggesting that epithelial to mesenchymal transition might be required to Sox2-expressing cells to become rapidly dividing and differentiate [30]. In postnatal pituitary, Sox2$^+$ cells were also reported to express PROP1, a transcription factor involved in pituitary organogenesis, but their number rapidly decreases at postnatal day 15, suggesting that a significant qualitative transition occurs during this early phase of development [36]. As far as the meaning of the Sox2$^+$ cells scattered throughout the anterior pituitary parenchyma, it was hypothesized either a transition to a "niche-independent" state or that supporting cells, such as FS, could represent a sort of diffuse "mini-niche".

In the adult, Sox2-expressing cells represent less than 5 % of total anterior lobe cell population, but, differently from embryonic cells, only 1 % of the Sox2$^+$ cells do not express Sox9, another transcription factor involved in embryo development for sex determination and later for chondrogenesis [37, 38]. Sox9 is also involved in pituitary development, but its expression in Rathke's pouch occurs much later than Sox2. Indeed, there is a temporal modification in the expression of these transcription factors during embryogenesis, with a progression during development from a Sox2$^+$/Sox9$^-$ phenotype to a double positivity and only a minority of the cells remain Sox2$^+$/Sox9$^-$. Similar, but quantitatively different, results were obtained by SP studies [29] (see above), with non-Sca1^{high} cells also being Sox2$^+$, and only a low percentage of these cells was reported to be also Sox9 positive [30]. Importantly, in BrdU labeling experiments, Sox2$^+$/Sox9$^+$ cells were showed to retain a significant proliferation rate, suggesting that this population may represent transit-amplifying cells. In fact, the proliferation wave observed in pituitary shortly after birth is mainly represented by Sox2$^+$/Sox9$^+$ cells. Conversely, Sox2$^+$/Sox9$^-$ showed persistence of the staining during label-retaining experiments [30], an index of slow cell division activity, as often observed in adult stem cells. This observation suggests that, in adult pituitaries, Sox2$^+$/Sox9$^-$ cells represent the reserve of quiescent multipotent cells for organ maintenance, evolving into Sox2$^+$/Sox9$^+$ cells in case of tissue loss or in response to physiological adaptive requirements [3].

This hierarchical order in pituitary stem cells was confirmed in vitro. Pituispheres generated in vitro by potential postnatal murine pituitary stem cells using selective

culture conditions (medium containing growth factors without fetal calf serum, FCS) are composed by cells showing $Sox2^+/Sox9^-$ phenotype, while Sox9 expression occurs only after at least 1 week in culture [30]. FACS analysis of $Sox2^+$ cells isolated from pituitaries of Sox2-EGFP mice [39] confirmed that Sox2 expression is required for the formation of spheres that originated only from GFP-positive cells [17]. However, it was not possible to propagate these spheres for more than two passages, suggesting that these $Sox2^+$ cells could represent multipotent progenitors rather than "real" stem cells, for which at least five in vitro passages are considered a defining requirement [15]. Alternatively, the in vitro culture conditions used might still lack some relevant factors required to retain stemness [3]. Again, in agreement with SP studies [29], $Sox2^+$ cells within pituispheres express also E-cadherin and are completely hormone negative [30]. However, co-expression of Sca1 was also reported [30], an observation discordant with the characteristics identified in the stem/progenitor cells within SP (non-$Sca1^{high}$ phenotype) [29].

Prolonged (1 week) in vitro culture of $Sox2^+$ cells induced, besides Sox9, also the expression of nestin and S100, while Sca1 is downregulated, but no spontaneous differentiation (for pituitary hormone expression) occurred. The shift to a medium containing FCS, as reported for stem cell cultures from several tissues, caused the disaggregation of spheres and the adherence of the cells to the culture substrate inducing a FS-like phenotype. To obtain endocrine differentiation this pituitary cell population has to be cultured as cellular aggregates on matrigel without growth factors [30]. In these latter experimental conditions all the pituitary hormones were detected with, sometime, the expression of multiple hormones in same sphere, confirming the multipotentiality of these cells. Altogether these observations allowed the proposal of $Sox2^+$/E-$cadherin^+/Sox9^-/S100^-$ as putative pituitary stem/progenitor cells, while $Sox2^+/Sox9^+/S100^+$/E-$cadherin^-$ cells could represent FS cells with transit-amplifying properties, already committed to differentiation [17].

More recently, a substantial evidence for the role of $Sox2^+$ cells as stem cell component was demonstrated in a transgenic mouse model of pituitary regeneration [40, 41]. To conditionally destroy selective pituitary cell types (i.e., GH- or prolactin-secreting cells) a transgene was constructed to have a conditional expression of diphtheria toxin receptor driven by the promoters for GH (GHCre/iDTR mice) [40] or PRL (PRLCre/iDTR mice) [41]: the expression of the toxin receptor leads to the disruption of the cells after activation induced by administration of diphtheria toxin, thus representing a model of selective adult pituitary cell ablation. The treatment of GHCre/iDTR mice for 3 days with diphtheria toxin causes, 1 week later, the obliteration of about 90 % of the GH cells. In these conditions a rapid surge of Sox2-expressing cells, as well as of FS cells, was detected. In situ analysis showed that $Sox2^+$ cell expansion occurred mainly in the marginal layer. Concomitantly, double labeled Sox2/GH cells appeared throughout the gland. These data clearly support the regenerative potential of adult pituitary and that $Sox2^+$ cells represent a main component of the subpopulation endowed with this activity. Similar results were obtained in the PRL-ablation model [41]. Throughout the regeneration period (2–6 weeks), $Sox2^+$, as well as double $Sox2^+/PRL^+$, cells continue to be more abundant than in pituitaries of control mice. Moreover, surviving or newborn lactotrophs

increase their proliferative activity, and bi-hormonal PRL^+/GH^+ cells become detectable, suggesting somatotroph to lactotroph transdifferentiation [41].

In conclusion, these studies provided a significant evidence that adult pituitary is able to regenerate both somatotroph and lactotroph compartments after destruction, through the activation of $Sox2^+$ adult stem cells [40, 41].

4 Nestin-Expressing Cells

Similarly to studies in other tissues, the possible existence of stem cells in pituitary relies on the identification of stem markers in subpopulations of cells in adult tissues, and nestin is one of the most commonly studied. In rat pituitary, nestin-expressing cells were identified all over the gland [42], mainly within the marginal zone lining the cleft, the possible pituitary stem cell niche [15]. These cells do not show features of hormone-secreting nor FS cells, but in vitro cultures, established from nestin-expressing cells, demonstrated characteristics of cells possessing a mesenchymal phenotype [42].

Using transgenic mice expressing nestin-GFP fluorescent cells, this cell population was detected in the Rathke's pouch in mouse embryos, but fluorescence was also detected in high number of cells in postnatal pituitary, mainly localized in the proximity of the cleft and showing Sox2 co-expression [43].

Cell lineage studies using nestin-Cre mice, in which the progeny of nestin-expressing cells are permanently GFP positive, demonstrated that about 2 % of fluorescent cells are located in the pituitary of newborn mice, but this percentage increases up to 20 % in adults [43]. While nestin-expressing cells can originate all pituitary endocrine cells, they are heterogeneous for Pit1 expression, suggesting that only a subpopulation of nestin-expressing cells could represent pituitary progenitors. However, the discrepancy between the lower percentage of nestin-positive cells in embryos than in adult endocrine cells allows to hypothesize that adult pituitary stem cells may be formed by a different pool of cells with respect to the embryonic progenitors. In agreement with this hypothesis it was demonstrated that nestin-expressing cells originate from the differentiation of $Sox2^+$ embryonic progenitors [30], possibly representing "transient-amplifying" population from which endocrine cells may derive.

Nestin-GFP cells were reported to be clonogenic in vitro and, when grown in "differentiation culture medium" (additioned with FCS or high concentration of cAMP induced by forskolin treatment), initially co-express nestin and Sox2 and, after time, differentiate in cells expressing all pituitary hormones [43].

A similar observation was obtained in autoptic human pituitary specimens. Nestin-expressing cells identified in the perivascular space of pituitary capillaries did not express pituitary hormones or FS and endothelial cell markers (as also demonstrated in rat and mouse pituitaries [42, 43]). Conversely, nestin was co-expressed with smooth muscle actin (SMA), suggesting a possible differentiation in pericytes. Human $nestin^+$ pituitary cells grown on fibroblast feeder layer allowed the

identification of the existence of two independent populations, of which the first was characterized as differentiated pericytes (phenotypically nestin$^+$ and SMA$^+$) and the second (only nestin$^+$) was proposed as human pituitary progenitor cells accordingly to self-renewal ability (spherogenesis) and differentiative potential (induction of prolactin expression in the presence of high cAMP levels) [44]. However, since a direct derivation of colonies from nestin$^+$ human cells was still not reported and long-term self-renewal never evaluated, further studies are required to definitively confirm these data.

Although nestin-GFP cells having a clonogenic pattern of growth in vitro do not express Sca1, nestin expression was identified in non-Sca1^{high} pituitary SP [29]. This discrepancy may depend on the different timings of analysis (soon after explant for the SP or after few days of in vitro culture in the nestin-GFP experiments), possibly confirming that progenitors express differential markers at various development stages.

However, recent studies, raising the possibility that ectopic expression of the nestin transgene may occur in a group of cells in the Rathke's pouch [45], cast doubts on the interpretation of these data. In fact, ectopic activity in the embryonic pituitary and, as observed with the nestin-GFP transgene in Rathke's pouch progenitors, would cause a significant staining in postnatal anterior pituitary cells that however does not reflect the actual expression in adult pituitary. Since nestin$^+$ cells mainly divide after birth, it was proposed that these cells are quiescent progenitors required for the initial wave of pituitary cell proliferation occurring after birth, and to maintain pituitary function in adults, but not the cells responsible of embryonic pituitary development. In particular, to define nestin-expressing cells as actual stem cells, it would be required to show exclusive co-localization of Cre and nestin [45]. In any case, from these studies it was hypothesized that nestin is expressed by different cell populations during pituitary development and in the adulthood, all labeled by nestin-GFP and including both stem/progenitor cells (Sox2$^+$ and LHX3$^+$), supportive (FS) cells, or vascular progenitors. In this line, it is to note that nestin is expressed in both pituitary SP groups, classified as non-Sca1^{high} (believed to represent pituitary progenitors) and Sca1^{high} (interpreted as vascular-endothelial progenitors) [29].

Thus, to date no definitive evidence has been provided about different studies on nestin$^+$ putative pituitary stem/progenitor cell origin, and further studies will be required to establish their role in pituitary development.

5 GFRα2-PROP1-Stem Cells

Another potential stem-like cell population in rat adult pituitary was identified according to the expression of GFRα2, the GDNF co-receptor (altogether with the tyrosine kinase receptor c-Ret) [31].

GFRα2-expressing cells represent about 0.9 % of all pituitary cells, as expected for adult stem cells [31]. All these cells also express E-cadherin, β-catenin, and

stem cell markers such as OCT4 and SSEA4, about 90 % of them express Sox2 and Sox9, and about 50 % is $S100^+$. On the contrary, nestin was not detected in these cells, at odds with the studies described above [29, 42, 43]. Interestingly, GFRα2-expressing cells also express PROP1, a transcription factor required to induce pituitary progenitors to differentiate in Pit1-positive cells [46]. In embryos, Sox2 co-localizes with PROP1 (but not with Pit1), while about 10 % of the cells express both PROP1 and Pit1. In adults, differentiation commitment makes hormone-expressing cells retaining Pit1 but not PROP1 [47]. This complex phenotype (expression of Sox9 and S100, co-localization of GFRα2 and PROP1) allowed the hypothesis that GFRα2-PROP1-stem (GPS) cells may represent transit-amplifying cells committed to differentiation rather than multipotent stem cells [17], very similar to the $Sox2^+/Sox9^+$ described by Fauquier [30], which retain the short-term ability to form spheres. GPS cell niche was identified in the periluminal zone in both rodents and humans, in which GPS cells are organized in an oriented manner, as single cell layer bordering the cleft [31]. Thus, the most concordant evidence from all the studies looking for pituitary stem cells is that the region of the cleft could represent pituitary niche.

Neurturin, a GFRα2 ligand, is expressed in anterior pituitary but not in the niche, suggesting that the secretion of this growth factor may regulate the activity of the GPS cells and their directional migration from the niche to the anterior pituitary [31]. GPS cells, isolated by FACS sorting, generate spheroids when grown in the absence of FCS, also without the addition of EGF and bFGF, growth factors commonly used to select stem cells, while neurturin, likely acting on GFRα2, increased the sphere-forming efficiency, producing a trophic effect on the survival of these cells [31]. The pituispheres express OCT4, E-cadherin, and PROP1 and, when dissociated, can differentiate in cells expressing all pituitary hormones and/or β-III-tubulin (a neuronal marker), when grown in monolayer onto collagen IV- or poly-L-lysine-coated plates. The expression of pituitary hormones required the incubation in specific media, each one able to induce the expression of one pituitary hormone. Differentiated cells showed a downregulation of GFRα2, OCT-4, and PROP1 expression and cell growth arrest [31]. More recently, cells expressing the same phenotype as rat GPS cells were identified in human pituitary, further supporting the relevance of this subpopulation as putative adult pituitary stem cells [48].

6 Pituitary Colony-Forming Cells/Folliculo-Stellate Cells

A different approach to identify stem/progenitor cells from adult pituitary was the colony-forming assay: pituitary cells isolated from Rathke's pouch can clonally expand in vitro as colonies, representing 0.2 % of the total pituitary cell number [49]. Not surprisingly, some of these embryonic clones are multipotent and give origin to all pituitary hormonal cell types, both in vitro and in vivo, after hypothalamic transplantation in hypophysectomized rats [50, 51]. More recently, pituitary colony-forming cells were shown to belong to the FS compartment accordingly to

their ability to internalize the fluorescent dipeptide β-Ala-Lys-Nε-AMCA [52]. It was calculated that about 12 % of mouse pituitary FS cells (phenotypically characterized as $S100^+$ and $GFAP^+$) was clonogenic and in about 40 % of the cells also Sca1 was expressed. Within the colonies formed, few GH- or prolactin-expressing cells were detected, suggesting the differentiation from pluripotent progenitors [49].

Using a more sophisticated in vivo approach, about 3.3 % of the colony-forming FS cells was demonstrated to be able to differentiate in GH-expressing cells [53]. It was also detected that the FS cell marker S100β was expressed in 80 % of the $Sox2^+$ pituitary cells, identified as putative pituitary stem cells (see above), and that after in vitro treatment with retinoic acid and bFGF, a small number of them can differentiate in Pit1-expressing or GH-secreting cells [54].

These studies provided evidence that FS are composed of several subpopulations, also comprising putative pituitary progenitors [53]. Conversely, other cells within this population may overlap, at least phenotypically, with the $Sca1^{high}$ SP (high expression of S100 and Sca1) [29]. Importantly, the clonogenic population is localized in the marginal zone of the pituitary cleft (the marginal zone between intermediate and anterior lobes), the proposed pituitary stem cell niche [55]. Thus, some postnatal pituitary FS cells exhibit stem cell-associated features such as the in vitro expansion as adherent colonies. However, given the limited differentiation capacity observed (mostly somatotrophs), these cells were interpreted as already committed progenitor cells.

7 Adult Pituitary Stem Cells and Tumor Development: The Role of Cancer Stem Cells

According to the current view of carcinogenesis, tumors possess a heterogeneous cell type patterning, including a large number of “tumor NON-initiating cells” (or “differentiated tumor cells”) constituting the tumor mass, and a small fraction of phenotypically distinct “tumor-initiating cells” (TIC), comprising the so-called “cancer stem cells” (CSCs). Similarly to what was observed in normal tissues, these tumor cell populations are biologically distinct as (1) slowly dividing stem cells (CSCs) from which all the other tumor cells are originated, (2) precursor cells (rapidly dividing) and transit-amplifying cells, and (3) differentiated cells that form the mass of the tumoral tissue [56].

In this model, CSCs are named after their ability to develop tumors (in the same way by which normal stem cells are responsible of organ development) and do not necessarily represent the malignant transformation of stem cells. In fact, different theories were provided on this issue including some evidence in support of the actual origin of CSCs from the oncogenic transformation of normal stem cells and other data demonstrating the possibility of a redifferentiation/dedifferentiation of committed progenitors or differentiated cells; likely both mechanisms can be active, in different conditions, to give origin of CSCs [57]. Independently from their origin, CSCs may arise from normal stem or progenitor cells by

alterations of proto-oncogenes that, as a result of accumulative oncogenic events, may grant a deregulated self-renewal ability to transform normal cells into CSCs. On the other hand, cancer development and progression may derive from modification of the microenvironment surrounding the stem cells (for example, the niche), leading to loss of extrinsic proliferation control of normal stem cells or progenitors, and the development of CSCs. Several genes and intracellular signaling pathways, representing important regulators of normal stem cell self-renewal and proliferation, are deregulated in cancer: Sox2, Notch1, Hedgehog, Wnt, and nestin, among others [58, 59].

Many biological features of normal stem cells are retained in CSCs: long life span, including the capacity of self-renewal, the expression of common markers, the possibility to differentiate into different cell types, and the strong resistance to chemotherapeutic drugs. The high self-renewal activity renders CSCs, differently from all other tumor cells, to be capable of a potentially unlimited proliferation activity that allows the maintenance and expansion of the tumor, although they themselves are often slow growing. Conversely, differentiated tumor cells proliferate at high rate, but for a limited number of divisions. Thus, CSCs represent a reservoir of tumor cells necessary to sustain tumor development. In light of CSC theory, a reconsideration of pharmacological approaches for tumors is ongoing, since the elimination of the differentiated and rapidly dividing tumor cells, as occurring with the classical chemotherapeutic agents, could fail to obtain a successful long-term disease remission if CSCs are not eliminated. Most of the currently used cytotoxic drugs are not able to affect the survival of undifferentiated and slow proliferating CSCs that, surviving to the treatment, represent a cell reservoir able to rapidly repopulate the tumor. Drug resistance of CSCs was ascribed to the expression at high levels of DNA repair enzyme that can elude genotoxic effects of antitumoral drugs, and of ABC-family transporters that can pump chemotherapeutic agents out of the cell. For these and other cellular properties, distinct from the rest of tumor cell populations CSCs often escape the traditional cancer therapy that becomes insufficient to clear up the "tumor-initiating cells" from the organism [60].

Recent studies focused on the role of CSCs in pituitary tumor development.

Mice bearing mutations that alter pituitary β-catenin proteolysis, leading to constitutive activation of the WNT/β-catenin pathway (obtained by crossing a Hesx1-CRE knock-in strain to a β-catenin strain that produces degradation-resistant β-catenin mutant, upon recombination), spontaneously develop tumors histologically and phenotypically resembling the human adamantinomatous craniopharyngiomas [61]. Importantly, pituitary tumorigenesis in these mice was dependent on the selective expression of the β-catenin mutant in pituitary cells endowed with progenitor/stem cells features. These cells were described as $Sox2^+$ and colony-forming cells, both characteristics proposed for adult pituitary stem cells (see above). In these trangenic mice, $Sox2^+$ cells were increased in number and showed a higher proliferation rate than the *w.t.* counterpart. Importantly, when cultured in vitro these cells showed a long-lasting (at least eight passages) clonogenic activity [61], clearly indicating a powerful, likely deregulated, self-renewal activity [15]. Moreover, in this study, a significant support to the origin of CSC from oncogenic

transformed normal adult stem cells was provided, since the expression of the same proteolysis-resistant β-catenin isoform in differentiated pituitary cells was not tumorigenic [61]. Accordingly, it was reported that a novel mutation in *SOX2* gene did not impair transactivation or DNA binding, but failed to repress β-catenin-mediated target activation, resulting in WNT/β-catenin increased activity [62]. Thus, the β-catenin pathway, normally involved in the control of the balance between self-renewal and differentiation of stem cells, is inactivated in normal adult pituitary, and when this downregulation does not occur sustained progenitor proliferation is induced, leading, eventually, to tumor development [63]. Moreover, mice, in which a conditional deletion of the tumor suppressor retinoblastoma gene is induced, developed silent corticotroph pituitary adenomas originating from $Pax7^+$ progenitors. In normal pituitary $Pax7^+$ cells are located in the Rathke's pouch cleft and derive from $nestin^+$ progenitors, not only in mice but also in primate pituitaries and were identified in both human functioning and silent ACTH-secreting adenomas, although a direct involvement of CSC in these tumors was not studied [64].

Another recent study showed the expression of GPS cell markers in human adamantinomatous craniopharyngiomas suggesting a common origin from these putative adult pituitary stem cells, with the only difference in the lack of expression of GFR2α that was interpreted as a way of deregulation of growth in the tumor cells [48].

Thus, although this is a still open issue, several evidence supports that, at least as far as some pituitary tumor is concerned, CSC might originate from genetic or epigenetic alterations in adult pituitary stem cells or progenitors.

Much less clear are the role of CSCs in pituitary adenoma development and the origin of these cells from normal adult pituitary stem cells. Although still debated, a growing bulk of evidence is now supporting the role of CSC as tumor-initiating cells also in benign tumors [20].

To date, molecular and cellular determinants of pituitary adenoma pathogenesis are still largely unknown [65], and although not definitively proved, the hypothesis that the formation of CSC subpopulation from stem or progenitor cells may also cause the formation of pituitary adenomas is currently under investigation.

Several indirect evidence was provided. For example, it was shown that CXCR4, a chemokine receptor identified as a marker in several stem cell populations [66] including pituitary stem/progenitors during development and in SP cells in adulthood [5], is expressed in subpopulations of human normal pituitary (about 30 % of the cells) with its expression shared by subpopulations of GH-, prolactin-, and ACTH-secreting cells, strongly suggesting a lineage derivation from common precursor cells [67]. In several $CXCR4^+$ cells also its ligand (CXCL12) was expressed, suggesting a possible autocrine/paracrine mechanism of activation, and also a few $CXCR4^+$/hormone negative cells, scattered throughout the anterior pituitary, were identified [67]. Although the co-expression of CXCR4/CXCL12 with stemness marker (Sox2, nestin) was not evaluated in these studies, it could be hypothesized that these hormone negative cells could be pituitary stem cells (see before). CXCR4 and CXCL12 expression was also analyzed in a large series of human secreting and nonfunctioning) pituitary adenomas, and both molecules were found highly

overexpressed, with all tumoral cells positive for both CXCR4 and CXCL12. Moreover, in vitro studies showed that CXCL12 activation is a mitogen for human pituitary adenoma cells [67]. Thus, it was proposed that putative CSCs for human pituitary adenomas may derive from $CXCR4^{+}/CXCL12^{+}$ cells in normal pituitary and that, due to the proliferative advantage granted by the autocrine activation of CXCR4 via the constitutive CXCL12 secretion, these cells could be one of the cell populations that clonally expand during pituitary tumorigenesis [68].

In another study, the expression of stem cell/progenitor markers (Sox2, nestin, Lhx3), but not pituitary hormones, was identified in cells composing hyperplastic pituitary nodules developed in nestin-GFP/$Rb^{+/-}$ mice. Moreover, in adult mice, the tumors contained twofold more nestin/Sox2-expressing cells than normal tissues, although the real nature of these masses was not really characterized [43].

However, to date, only one study formally analyzed the possible role of CSCs in human pituitary adenomas pathogenesis [69]. In this study, putative CSCs, called "pituitary adenoma stem-like cells" (PASCs), were obtained from one GH-secreting and one clinically nonfunctioning human pituitary adenomas, culturing dispersed postsurgical specimens in stem cell permissive medium (DMEM/F12, enriched with B27 supplement, bFGF, and EGF). After 1 week in vitro, few pituitary adenoma cells generated spheroids that were able to generate secondary spheres [69]. Spheroid cells were nestin and CD133 (prominin-1) positive but did not release GH, when the single GH-secreting adenoma was analyzed. Quantitative RT-PCR analysis performed in one PASC spheroid culture showed high expression of stem cell-associated genes (CD90, OCT-4, Musashi-1, NOTCH4, JAG2, and DLL-1). Conversely, culturing the cells in differentiation medium (containing FCS, without growth factors) several neural markers were induced (β-tubulin III, GFAP). Cells from dissociated spheroids, cultured for 2 weeks in differentiation medium (additioned with FCS, but devoid of growth factors), were able to release pituitary hormones in response to hypothalamic peptides [69]. In particular, the cells derived from the GH-secreting tumor did not express GH or LH before differentiation, but these hormones were detected in significant amounts after treatment with GHRH and GnRH, respectively, in differentiation medium. On the contrary, undifferentiated cells released PRL and TSH in response to PRL-releasing peptide and TRH, while the secretory activity was reduced after differentiation. This unexpected result allowed to hypothesize that in the spheroids generated by PASCs are also present differentiated cells originated by spontaneous differentiation also in stem-permissive culture conditions [69].

However, although phenotype characterization was still not completely defined and totally convincing, the most important evidence shown in this study was that spheroid-derived cells (1×10^4 cells), but not the adherent/differentiated cells (1×10^5), were able to reproduce the tumors when implanted into the forebrain of immunodeficient mice, since in vivo tumorigenicity is still the best feature to define bona fide CSCs. After 6 months from the transplant, tumor cells were harvested and dissociated giving rise to new spheroids that again were tumorigenic when reinjected into the brain of the NOD/SCID mice. Cell masses were immunopositive to human antigens, and some of them also expressed human GH [69].

While to date this is the best evidence about the existence of CSC in pituitary adenomas, inconclusive answers and few inconsistencies are still present (see [20]), and in particular, since only two human tumors were analyzed, the reproduction of these data from more specimens is required to definitely prove the CSC existence in pituitary adenomas, as well as their derivation from adult pituitary stem cells.

8 Conclusions and Future Perspectives

In this chapter we report the main studies proposing the existence of multipotent stem/progenitor cells in adult pituitary and the possibility that a deregulation of the activity of these cells may result in pituitary tumor development. Although not all the studies are completely concordant, several features, including Sox2 expression, SP nature, and a niche-like configuration, seem to characterize the phenotype of these cells in all the studies to date available. The main limit of almost all the evidence reported to characterize pituitary stem cells is that they are performed in murine models. A necessary step ahead will be the reproduction of these conclusions in human tissues.

The role of stem/progenitor cells in adult pituitary cell homeostasis, regeneration after injury, genetic endocrine deficits, and tumor pathogenesis will have significant clinical relevance, and the possibility of isolation and functional analysis of this cell population will provide important information to define these issues. Importantly, the characterization of pituitary stem/progenitor cells could also allow a better understanding of the biological basis of some pituitary pathologies including hypopituitarism and adenoma tumorigenesis also allowing the identification of potential novel pharmacological targets for pituitary tumors.

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References

1. Yoshimura F, Harumiya K, Ishikawa H, Otsuka Y (1969) Differentiation of isolated chromophobes into acidophils or basophils when transplanted into the hypophysiotrophic area of hypothalamus. Endocrinol Jpn 16:531–540
2. Kawamura K, Kouki T, Kawahara G, Kikuyama S (2002) Hypophyseal development in vertebrates from amphibians to mammals. Gen Comp Endocrinol 126:130–135
3. Castinetti F, Davis SW, Brue T, Camper SA (2011) Pituitary stem cell update and potential implications for treating hypopituitarism. Endocr Rev 32:453–471
4. Bilodeau S, Roussel-Gervais A, Drouin J (2009) Distinct developmental roles of cell cycle inhibitors p57Kip2 and p27Kip1 distinguish pituitary progenitor cell cycle exit from cell cycle reentry of differentiated cells. Mol Cell Biol 29:1895–1908
5. Vankelecom H (2010) Pituitary stem/progenitor cells: embryonic players in the adult gland? Eur J Neurosci 32:2063–2081

6. Carbajo-Perez E, Watanabe YG (1990) Cellular proliferation in the anterior pituitary of the rat during the postnatal period. Cell Tissue Res 261:333–338
7. Levy A (2008) Molecular and trophic mechanisms of tumorigenesis. Endocrinol Metab Clin North Am 37:23–50, vii
8. Levy A (2002) Physiological implications of pituitary trophic activity. J Endocrinol 174: 147–155
9. Levy A (2008) Stem cells, hormones and pituitary adenomas. J Neuroendocrinol 20:139–140
10. Nolan LA, Kavanagh E, Lightman SL, Levy A (1998) Anterior pituitary cell population control: basal cell turnover and the effects of adrenalectomy and dexamethasone treatment. J Neuroendocrinol 10:207–215
11. Nolan LA, Levy A (2006) The effects of testosterone and oestrogen on gonadectomised and intact male rat anterior pituitary mitotic and apoptotic activity. J Endocrinol 188:387–396
12. Nolan LA, Levy A (2009) The trophic effects of oestrogen on male rat anterior pituitary lactotrophs. J Neuroendocrinol 21:457–464
13. Kominami R, Yasutaka S, Taniguchi Y, Shinohara H (2003) Proliferating cells in the rat anterior pituitary during the postnatal period: immunoelectron microscopic observations using monoclonal anti-bromodeoxyuridine antibody. Histochem Cell Biol 120:223–233
14. Frawley LS, Boockfor FR (1991) Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. Endocr Rev 12:337–355
15. Vankelecom H (2007) Stem cells in the postnatal pituitary? Neuroendocrinology 85:110–130
16. McNicol AM, Carbajo-Perez E (1999) Aspects of anterior pituitary growth, with special reference to corticotrophs. Pituitary 1:257–268
17. Rizzoti K (2010) Adult pituitary progenitors/stem cells: from in vitro characterization to in vivo function. Eur J Neurosci 32:2053–2062
18. Castrique E, Fernandez-Fuente M, Le Tissier P, Herman A, Levy A (2010) Use of a prolactin-Cre/ROSA-YFP transgenic mouse provides no evidence for lactotroph transdifferentiation after weaning, or increase in lactotroph/somatotroph proportion in lactation. J Endocrinol 205: 49–60
19. Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC, Dattani MT (2009) Genetic regulation of pituitary gland development in human and mouse. Endocr Rev 30:790–829
20. Florio T (2011) Adult pituitary stem cells: from pituitary plasticity to adenoma development. Neuroendocrinology 94:265–277
21. Furusawa C, Kaneko K (2012) A dynamical-systems view of stem cell biology. Science 338:215–217
22. Kim M, Morshead CM (2003) Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci 23:10703–10709
23. Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. Stem Cells 24: 3–12
24. Chen J, Hersmus N, Van Duppen V, Caesens P, Denef C, Vankelecom H (2005) The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. Endocrinology 146:3985–3998
25. van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC, Meij BP (2012) Identification and characterisation of side population cells in the canine pituitary gland. Vet J 192:476–482
26. Chen J, Crabbe A, Van Duppen V, Vankelecom H (2006) The notch signaling system is present in the postnatal pituitary: marked expression and regulatory activity in the newly discovered side population. Mol Endocrinol 20:3293–3307
27. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253–1270
28. Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115–1124

29. Chen J, Gremeaux L, Fu Q, Liekens D, Van Laere S, Vankelecom H (2009) Pituitary progenitor cells tracked down by side population dissection. Stem Cells 27:1182–1195
30. Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC (2008) SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci USA 105:2907–2912
31. Garcia-Lavandeira M, Quereda V, Flores I, Saez C, Diaz-Rodriguez E, Japon MA, Ryan AK, Blasco MA, Dieguez C, Malumbres M, Alvarez CV (2009) A GRFa2/Prop1/stem (GPS) cell niche in the pituitary. PLoS One 4:e4815
32. Gremeaux, L, Fu QV, Van Duppen V, Van den broeck A, Wouters J, van Loon J, Bex M, and Vankelecom H (2009) Cancer stem cells in human pituitary adenoma: identification and characterization of a tumor 'side population'. In: Abstract, Oncoforum 2009. Leuven, Belgium
33. Cherqui S, Kurian SM, Schussler O, Hewel JA, Yates JR 3rd, Salomon DR (2006) Isolation and angiogenesis by endothelial progenitors in the fetal liver. Stem Cells 24:44–54
34. Pevny LH, Nicolis SK (2010) Sox2 roles in neural stem cells. Int J Biochem Cell Biol 42:421–424
35. Alatzoglou KS, Kelberman D, Dattani MT (2009) The role of SOX proteins in normal pituitary development. J Endocrinol 200:245–258
36. Yoshida S, Kato T, Yako H, Susa T, Cai LY, Osuna M, Inoue K, Kato Y (2011) Significant quantitative and qualitative transition in pituitary stem / progenitor cells occurs during the postnatal development of the rat anterior pituitary. J Neuroendocrinol 23:933–943
37. Kawakami Y, Rodriguez-Leon J, Izpisua Belmonte JC (2006) The role of TGFbetas and Sox9 during limb chondrogenesis. Curr Opin Cell Biol 18:723–729
38. Sekido R (2010) SRY: a transcriptional activator of mammalian testis determination. Int J Biochem Cell Biol 42:417–420
39. Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH (2006) SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev 20: 1187–1202
40. Fu Q, Gremeaux L, Luque RM, Liekens D, Chen J, Buch T, Waisman A, Kineman R, Vankelecom H (2012) The adult pituitary shows stem/progenitor cell activation in response to injury and is capable of regeneration. Endocrinology 153:3224–3235
41. Fu Q, Vankelecom H (2012) Regenerative capacity of the adult pituitary: multiple mechanisms of lactotrope restoration after transgenic ablation. Stem Cells Dev 21(18):3245–3257
42. Krylyshkina O, Chen J, Mebis L, Denef C, Vankelecom H (2005) Nestin-immunoreactive cells in rat pituitary are neither hormonal nor typical folliculo-stellate cells. Endocrinology 146:2376–2387
43. Gleiberman AS, Michurina T, Encinas JM, Roig JL, Krasnov P, Balordi F, Fishell G, Rosenfeld MG, Enikolopov G (2008) Genetic approaches identify adult pituitary stem cells. Proc Natl Acad Sci USA 105:6332–6337
44. Weiss S, Siebzehnrubl FA, Kreutzer J, Blumcke I, Buslei R (2009) Evidence for a progenitor cell population in the human pituitary. Clin Neuropathol 28:309–318
45. Galichet C, Lovell-Badge R, Rizzoti K (2010) Nestin-Cre mice are affected by hypopituitarism, which is not due to significant activity of the transgene in the pituitary gland. PLoS One 5:e11443
46. Ward RD, Raetzman LT, Suh H, Stone BM, Nasonkin IO, Camper SA (2005) Role of PROP1 in pituitary gland growth. Mol Endocrinol 19:698–710
47. Yoshida S, Kato T, Susa T, Cai LY, Nakayama M, Kato Y (2009) PROP1 coexists with SOX2 and induces PIT1-commitment cells. Biochem Biophys Res Commun 385:11–15
48. Garcia-Lavandeira M, Saez C, Diaz-Rodriguez E, Perez-Romero S, Senra A, Dieguez C, Japon MA, Alvarez CV (2012) Craniopharyngiomas express embryonic stem cell markers (SOX2, OCT4, KLF4, and SOX9) as pituitary stem cells but do not coexpress RET/GFRA3 receptors. J Clin Endocrinol Metab 97:E80–E87
49. Lepore DA, Roeszler K, Wagner J, Ross SA, Bauer K, Thomas PQ (2005) Identification and enrichment of colony-forming cells from the adult murine pituitary. Exp Cell Res 308: 166–176

50. Bowie EP, Ishikawa H, Shiino M, Rennels EG (1978) An immunocytochemical study of a rat pituitary multipotential clone. J Histochem Cytochem 26:94–97
51. Shiino M, Ishikawa H, Rennels EG (1977) In vitro and in vivo studies on cytodifferentiation of pituitary clonal cells derived from the epithelium of Rathke's pouch. Cell Tissue Res 181:473–485
52. Otto C, tom Dieck S, Bauer K (1996) Dipeptide uptake by adenohypophysial folliculostellate cells. Am J Physiol 271:C210–C217
53. Lepore DA, Thomas GP, Knight KR, Hussey AJ, Callahan T, Wagner J, Morrison WA, Thomas PQ (2007) Survival and differentiation of pituitary colony-forming cells in vivo. Stem Cells 25:1730–1736
54. Osuna M, Yako H, Yoshida S, Sonobe Y, Inoue K, Kato T, Kato Y (2011) S100b-expressing folliculo-stellate cells are found in SOX2-positive population in the anterior pituitary lobe and show multiple differentiation capacities in the defined culture conditions. Endocr Rev 32:P1–P386
55. Lepore DA, Jokubaitis VJ, Simmons PJ, Roeszler KN, Rossi R, Bauer K, Thomas PQ (2006) A role for angiotensin-converting enzyme in the characterization, enrichment, and proliferation potential of adult murine pituitary colony-forming cells. Stem Cells 24:2382–2390
56. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
57. Soltysova A, Altanerova V, Altaner C (2005) Cancer stem cells. Neoplasma 52:435–440
58. Cerdan C, Bhatia M (2010) Novel roles for Notch, Wnt and Hedgehog in hematopoesis derived from human pluripotent stem cells. Int J Dev Biol 54:955–963
59. Krupkova O Jr, Loja T, Zambo I, Veselska R (2010) Nestin expression in human tumors and tumor cell lines. Neoplasma 57:291–298
60. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB (2009) Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 8:806–823
61. Gaston-Massuet C, Andoniadou CL, Signore M, Jayakody SA, Charolidi N, Kyeyune R, Vernay B, Jacques TS, Taketo MM, Le Tissier P, Dattani MT, Martinez-Barbera JP (2011) Increased Wingless (Wnt) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans. Proc Natl Acad Sci USA 108:11482–11487
62. Alatzoglou KS, Andoniadou CL, Kelberman D, Kim HG, Botse-Baidoo E, Pedersen-White JR, Layman LC, Martinez-Barbera JP, Dattani MT (2011) Clinical manifestations of a novel SOX2 mutation may result from failure to repress β-catenin-mediated target activation: suggestion for a new mechanism for the interaction between SOX2 and β-catenin. Endocr Rev 32:P3–P758
63. Camper SA (2011) Beta-catenin stimulates pituitary stem cells to form aggressive tumors. Proc Natl Acad Sci USA 108:11303–11304
64. Hosoyama T, Nishijo K, Garcia MM, Schaffer BS, Ohshima-Hosoyama S, Prajapati SI, Davis MD, Grant WF, Scheithauer BW, Marks DL, Rubin BP, Keller C (2010) A postnatal Pax7 progenitor gives rise to pituitary adenomas. Genes Cancer 1:388–402
65. Melmed S (2011) Pathogenesis of pituitary tumors. Nat Rev Endocrinol 7:257–266
66. Miller RJ, Banisadr G, Bhattacharyya BJ (2008) CXCR4 signaling in the regulation of stem cell migration and development. J Neuroimmunol 198:31–38
67. Barbieri F, Bajetto A, Stumm R, Pattarozzi A, Porcile C, Zona G, Dorcaratto A, Ravetti JL, Minuto F, Spaziante R, Schettini G, Ferone D, Florio T (2008) Overexpression of stromal cell-derived factor 1 and its receptor CXCR4 induces autocrine/paracrine cell proliferation in human pituitary adenomas. Clin Cancer Res 14:5022–5032
68. Barbieri F, Bajetto A, Pattarozzi A, Gatti M, Würth R, Porcile C, Thellung S, Corsaro A, Villa V, Nizzari M, Florio T (2011) The chemokine SDF1/CXCL12: a novel autocrine/paracrine factor involved in pituitary adenoma development. Open Neuroendocrinol J 4:64–76
69. Xu Q, Yuan X, Tunici P, Liu G, Fan X, Xu M, Hu J, Hwang JY, Farkas DL, Black KL, Yu JS (2009) Isolation of tumour stem-like cells from benign tumours. Br J Cancer 101:303–311

Toward Translating Molecular Ear Development to Generate Hair Cells from Stem Cells

Azel Zine, Hubert Löwenheim, and Bernd Fritzsch

Abstract The ear develops through the transformation of embryonic ectoderm into the labyrinth and, through a stepwise molecular restriction of cell fate options, into cells neurosensory cells (hair cells, neurons) and non-neurosensory cells. Hair cells can degenerate because of age, ototoxic drugs, acoustic trauma or genetic predisposition. Two principle approaches have been developed to restore hair cells that do not regenerate: a cell-based therapy and a gene-based therapy. One approach recapitulates developmental steps to transform embryonic stem cells into hair cells. Alternatively, gene therapy uses the molecular basis of hair cell development to transform remaining cells into hair cells. We review the molecular basis of normal neurosensory development, the state of cell and gene-based approaches, and indicate future improvements to increase the yield from either adult stem cells or embryonic- or adult-induced pluripotent stem cells (ES and iPS).

Keywords Hair cells • Age-related hearing loss • Regeneration • Hearing restoration • Gene therapy

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Abbreviations

Abcg2	ATP-binding cassette sub-family G member 2
Ascl1	Achaete-scute complex homolog 1
Atoh1	Atonal homolog 1
bHLH	Basic helix–loop–helix transcription factors
Bmp	Bone morphogenetic protein
Cdk	Cyclin-dependent kinase
Cki	Cyclin-dependent kinase inhibitors
Delta	Delta-like (Dll)
Dicer1	Dicer 1, ribonuclease type III
E2f	E2F transcription factor
EGF	Epidermal growth factor
Eya1	Eyes absent homolog 1
FACS	Fluorescence-activated cell sorting
Fgf	Fibroblast growth factor
Foxg1	Forkhead box G1
Gata3	GATA-binding protein 3
Gdp	Guanosine diphosphate
GER	Greater epithelial ridge
GFP	Green fluorescent protein
Hes	Hairy and enhancer of split
Hey	Hairy/enhancer-of-split related with YRPW motif
iPS cells	Induced pluripotent stem cells
Jag1	Jagged 1
Kcc4	Solute carrier family 12 (potassium/chloride transporter), member 7
Kir4.1	Potassium inwardly rectifying channel, subfamily J, member 10
Klf4	Kruppel-like factor 4
LCC	Light coat and circling
LER	Lesser epithelial ridge
Lgr5	Leucine-rich repeat containing G protein-coupled receptor 5
Lmo4	LIM domain only 4
Lmx1a	LIM homeobox transcription factor 1 alpha
Mib	Mind bomb
miR	Micro RNA
Nanog	Nanog homeobox
Neur	Neuralized
Neurod1	Neurogenic differentiation 1
Neurog1	Neurogenin 1, a proneural transcription factor
Nicd	Notch intracellular domain
N-Myc	v-myc Avian myelocytomatosis viral oncogene neuroblastoma-derived homolog
Nop1	Nasal and otic placode 1
NSCs	Neural stem cells

OC	Organ of Corti
OCSCs	Organ of Corti stem cells
Oct4	aka Pou5f1, POU class 5 homeobox 1
p21	Cyclin-dependent kinase inhibitor 1A (Cdkn1a)
p27	Cyclin-dependent kinase inhibitor 1B (Cdkn1b)
p53	Tumor protein p53
p75	Nerve growth factor receptor (TNFR superfamily, member 16)
Pax2	Paired box gene 2
pRb	Retinoblastoma protein
Prox1	Prospero homeobox 1
Rbpj	Recombination signal-binding protein for immunoglobulin kappa J region
Shh	Sonic hedghog
Six1	Sine oculis-related homeobox 1
Sox2	SRY (sex-determining region Y)-box 2
Swi/Snf	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1
Tbx1	T-box 1
Wnt	Wingless-related MMTV integration site
YSB	Yellow submarine

1 Introduction

Like all vertebrate neurons, the ear develops from a population of ectodermal cells that undergo otic placode transformation and clonal expansion through differential proliferation. The cells derived from this proliferating population are transformed from an epidermal to an inner ear progenitor cell, followed by progressive restriction in their multipotency to become the non-sensory and neurosensory cells of the ear (Fig. 1). Non-sensory cells of the ear continue the transformation of the flat otic placode, past the otic vesicle, into the complex labyrinth. The adult mammalian ear consists of three semicircular canals interconnected by the utricle, the saccular recess, and the cochlear duct with the sensory organ of Corti (OC). This ear labyrinth consists mostly of non-sensory cells and is needed to direct physical stimulation associated with hearing; angular and linear acceleration of vestibular sensation to the specific endorgans. This allows the ear to decode gravity-related stimuli (utricle and saccule), head shaking (three canal cristae), and various aspects of sound (organ of Corti).

In mammals, neurosensory precursors form all the sensory neurons of the ear first, followed by the formation of the sensory epithelia consisting of the clonally related hair cells and supporting cells (Fig. 1). For this review, we concentrate on the molecular basis of neurosensory cell formation. These cells are lost in various forms of neurosensory hearing loss. We will define critical molecular steps in the transformation of neurosensory precursors into neurosensory cells that can restore hearing and vestibular sensation. To achieve this goal, various strategies aiming at the

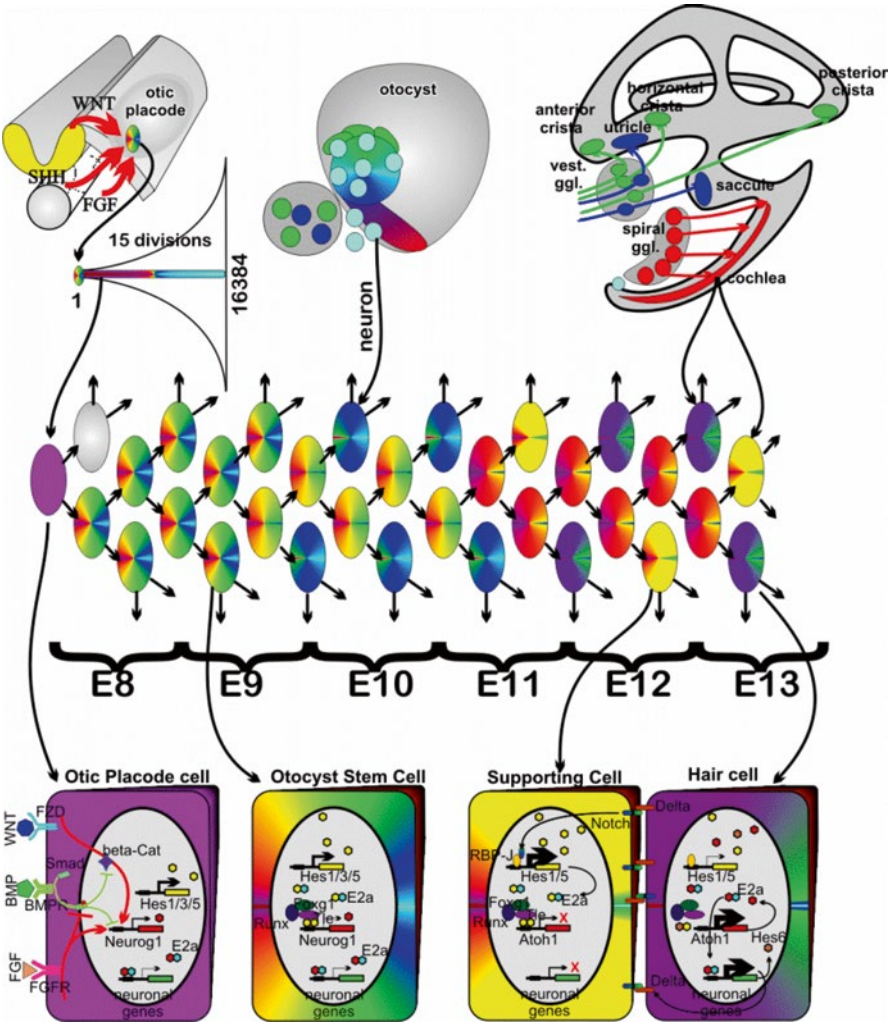

Fig. 1 In mice development of the inner ear begins at approximately E8 with the induction of ectodermal cells adjacent to the hindbrain, the otic placode. Wnt, Shh, and Fgf diffuse from the neural tube and hindbrain to induce the formation of the placode. Upon induction, the previously defined ectodermal cells become neuroectoderm-otic placode cells. At this stage, otic placode cells are relatively undefined and small in number. At this point, proto-oncogene levels are high and proliferation is rampant, with no differentiation occurring. During this time of rapid proliferation comes growth and morphological development, that is, the invagination of the newly forming otocyst, polarization of the axes, and eventually the tightly controlled three-dimensional shaping of the inner ear, occurring around E10. Further along the development of the inner ear, the cell cycle is likely lengthening. This may result from reduced levels of proto-oncogenes and increased levels of tumor suppressor. This shifting in the balance results in a reduced rate of proliferation and increased cell cycle exit, as such, differentiation begins. During this timeframe, some degree of asymmetric division occurs, with the production of neurons and neurosensory precursor cells or otocyst stem cells from otocyst stem cells. At this point, these neurons may be destined for targeting specific portions of the ear and are subsequently defined as either inferior (*blue*) or superior (*green*) vestibular

regeneration of sensory hair cells are currently employed (summary in Fig. 2). These include (1) the transplantation of stem cells into the damaged organ of Corti (OC) to restore lost hair cells (see Sect. 4), (2) the direct conversion of supporting cells into hair cells via gene delivery of hair cell-specific transcription factors (see Sect. 6) and (3) manipulation of the cell cycle (see Sect. 7.2) and (4) stimulation of putative endogenous stem/progenitor cells (Fig. 2). These various approaches will be detailed in the following sections.

In the ear, there is variable evidence of the clonal relationship of sensory neurons and hair cells indicating that at least three populations of neurosensory cells are set aside early in development: (a) a population giving rise to only neurons through the upregulation of the bHLH transcription factor *Neurog1*; (b) a bifunctional population giving rise to first neurons, followed by hair cells; and (c) a third population giving rise only to hair cells [1, 2]. As it turns out, mammals are unique among vertebrates in that they cannot spontaneously regenerate lost hair cells. Hair cell loss can be a consequence of loud sound, exposure to ototoxic drugs, genetic predisposition, and/or as a result of aging. This leaves mostly elderly people without basic communication with the environment they have become accustomed to over their lifetime. Vestibular hair cell loss, slightly later in onset, adds to this problem by causing various ataxias that result in increased frequency of falling. In fact, this ataxia-related problem coincides with a time when bone healing is compromised through a number of negative indicators, particularly in postmenopausal women. Given that about half of people over the age of 70 will suffer from some form of hearing deficit [3], hearing deficit is the most common sensory disorder facing the fast growing population of elderly worldwide. Finding a durable solution to the problem of age-dependent hearing and vestibular hair cell loss is urgently needed. Currently, only electrical devices can be used to generate signals that stimulate the remaining primary neurons in the ear to restore some resemblance of hearing [4]. Vestibular implants are even less developed with no clear indication of when they will be ready.

Using animal models, attempts are currently made to bring the insights gained from other sensory systems to the ear. In fact, notable success has been reported (mostly with cell but also gene-based therapies) in another sensory system, the retina. One major approach relates to technical adaptation of existing cell-based therapy protocols to generate hair cells either out of ear-derived adult stem cells, embryonic stem cells, or iPS cells. The basic idea of this approach is to take transformable cells

Fig. 1 (continued) ganglion neurons or cochlear (*red*) ganglion neurons. These cells are typically marked by their sequential responses to bHLH transcriptions Neurog1 and NeuroD1. Cells that remain in the cell cycle or are otherwise unresponsive to these proneural genes will later form other neurosensory cells in the ear. Neurosensory precursor cells that have not yet undergone differentiation into neurons will form the sensory components of the inner ear, consisting of hair cells and supporting cells. Identical to the balance between neurons and glial cells, hair cells and supporting cells form due to the presence of Atoh1 and the repression of hair cell fate through the Delta/Notch system, respectively. Once again, neither proper neuronal formation nor sensory formation can occur in the disruption of balance between proliferation and differentiation.Modified after [183]

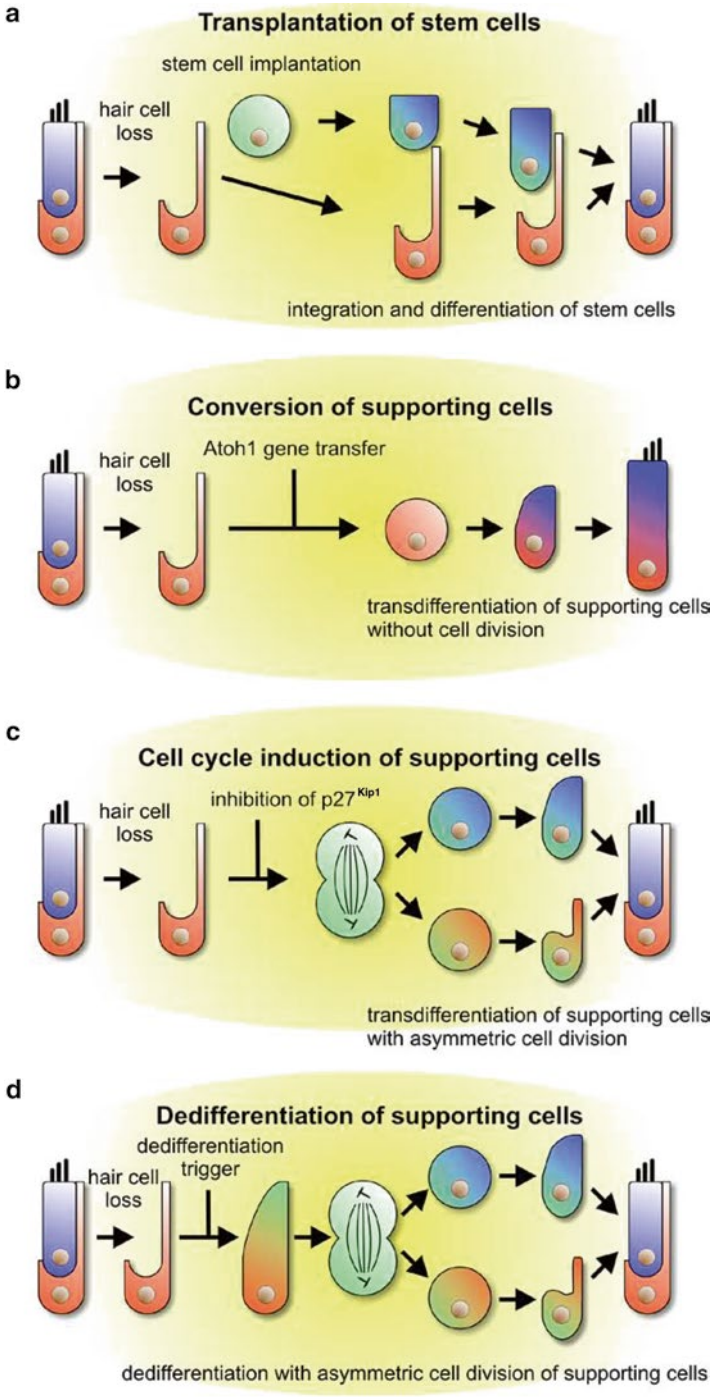

Fig. 2 Strategies for mammalian hair cell regeneration. (**a**) Replacement of lost hair cells (HC) by implantation and differentiation of exogenous stem cells. (**b**) Transdifferentiation of remaining supporting cells (SC) into HC by gene therapy forcing the expression of the HC inducer Atoh1. (**c**) Induction of asymmetric SC division by targeted inhibition of cell cycle inhibitors, e.g., $p27^{Kip1}$. (**d**) Induction of dedifferentiation in SCs by an exogeneous trigger allowing to asymmetric cell division and subsequent redifferentiation

in the Petri dish and transform through specific treatments into whatever cells are needed, mostly following known molecular steps of development [5]. These treatments can reprogram existing cells into neurosensory precursors to generate hair cells. The ultimate goal is to insert such stably transformed hair cells into the topographically correct position, so they can tie into the mechanical stimuli generated by frequency-specific sound waves to transduce sound into electrical signals. Regrown nerve fibers can conduct the information to the brain for processing [6–8].

A second approach is to transform in situ remaining cells into hair cells by overexpressing, with viral vectors, genes that play a crucial role in normal development of hair cells or elicit the remaining potential of supporting cells to transdifferentiate [9]. Such therapeutic approaches work well in embryos, newborns, and juveniles, but have had limited effect on older ears where the replacement of hair cells is most needed. The progressive reduction in the ability of experimental animals to respond to this treatment will require additional work to prepare the remaining cells to respond properly to gene therapy [10, 11], possibly by reinitiating stem cell-like conditions in cells through induced proliferation [12] and upregulation of stem cell-inducing genes such as Sox2 [13]. Indeed, it is possible that recently reported success to transform some supporting cells into outer hair cells will be limited to younger stages [9].

Both the cell- and gene-based therapies are still in search of the appropriate molecular sequence to induce a stem cell-like behavior of remaining ear cells in vivo or to set up the in vitro conditions to differentiate, with higher efficiency and homogeneity, stem cells into hair cells. Below, we will highlight some of the sequences of gene expressions known during development. We will also indicate how this information may be combined with the knowledge on iPS cells to enhance the outcome of either approach.

2 The Otic Placode as a Transformer of Ectoderm into Otic Stem Cells

2.1 Generalized Markers of Stem Cells: Oct4, Sox2, N-Myc

The work of many people culminated in the Nobel prize winning 2006 paper that showed once and for all that reprogramming of general cells into pluripotent stem cells (iPS cells) is possible with just four factors, Oct4, Sox2, Klf4, and c-Myc [14]. More recent work has narrowed down those factors and further refined the technical approach to induce pluripotency in cells. Using a sophisticated single cell gene expression analysis, a recent paper identified two distinct phases in this transformation process: a first phase in which stochastic upregulation of genes happens without any clear prediction of the outcome [15] and a second phase characterized by the upregulation of Sox2. After Sox2 expression is achieved, cells are transformed into iPS cells and these cells can then be initiated to differentiate into various other cell types in a deterministic way by exposing them to specific transcription factors.

Obviously, this specific case of transforming cells into iPS cells followed by directed differentiation can be sidestepped, and direct transformation of one cell type into another is possible using micro-RNA. For example, expression of micro-RNA 9 and 124 alone in fibroblasts suffices to transform them at a low rate into neurons [16], which can be significantly enhanced using neuron specifying bHLH transcription factors. Unfortunately, it is unclear if this approach will also work in iPS cells, where miRs other than miR-124 are expressed. For example, a highly conserved miR-183 is expressed in hair cells during development across phyla [17] that is essential for normal hair cell development [18]. This miR may, in part, define the molecular context within which other transcription factors can act to generate hair cells [8]. No matter the starting point, an approach that generates pluripotent stem cells out of embryonic or adult stem cells, or induces pluripotent stem cells or even directly transforms existing cell types into the required cell type (the basis of all attempts in cell-based regenerative medicine today [19]), can benefit from miRs. Following this induced pluripotency are attempts to direct the differentiation into the needed cell type ready for cell transplantation [5].

How do these insights relate to ear development? The stepwise transformation of general ectoderm into general placodal ectoderm and eventually otic placodes has been described in some detail, including the emerging hierarchy of gene interactions [20, 21]. Obviously, not all genes have yet been identified and how they cross-regulate each other requires further experimental verification; nevertheless, both general markers and otic placode-specific markers have been identified and they seem to be fairly conserved across vertebrates [22]. This indicates an ancient conserved developmental module that is functionally meaningful.

Within this molecular network are the so-called Yamanaka factors. For example, Oct4 has been identified in the otic placode of zebrafish [1] and Sox2 [23] as well as N-Myc [24] have been functionally characterized in neurosensory development of mutant mice. Only a transient early expression exists for c-Myc [25]. Klf4 has not been functionally characterized in ear development [20] although it appears to be a non-differentially regulated gene during development [13]. As previously suggested [1], several factors act together to antagonize Bmp and Wnt signaling while at the same time increasing Fgf signaling [20]. Cooperation of these factors results in the sustained expression of Six1/4 and Eya1/2, essential genes for neurosensory development [26, 27]. Transient expression changes of Fgf signaling followed by alteration of Wnt signaling appear to be necessary to stabilize expression of genes needed to specify the otic placode, the Pax2/8 and Eya1/Six1 genes. Co-expression of Pax2/8 with Eya1/Six1 and Gata3 seems to consolidate the otic placode fate, but how they interact and regulate downstream genes is not completely clear [28]. Ultimately, Sox2 needs to be co-expressed with Pax2 and Eya1/Six1 to induce expression of genes associated with neurosensory precursors such as Sox2 [23] and the downstream proneural bHLH genes that execute neurosensory differentiation.

Genes expressed in the otic placode can be grouped as follows: (1) Genes that result in limited or no neurosensory development at all when knocked out, (2) genes that lead only to loss of neurons and hair cells in the cochlea, and (3) genes that disturb overall development with limited and largely indirect effects on neurosensory development. A further subdivision is into genes that are only transiently

expressed in contrast to long-term expressed genes. Obviously, the transiently expressed genes, while certainly important for early placode and otocyst formation, are difficult to use at later stages. Many of these transient genes that belong to the Wnt, Fgf, Shh, and BMP signaling pathways impact the overall ear development in varying degrees and neurosensory development by setting up gene expression associated with neurosensory precursor fate commitment [20]. Obviously, genes in the first category with long-term expression are the most relevant for an understanding of the molecular basis of all neurosensory development, whereas those in the second category are particularly important for cochlear development. However, it should be pointed out that it may be that in some genes, the existing mutations may not reveal the full phenotype due to delayed recombination or effects prior to the gene being deleted. For example, Pax2-cre-mediated recombination of the miR generating enzyme Dicer1 leads to formation of patches of hair cells in the cochlear duct [18], whereas Foxg1-cre-induced recombination of the same enzyme leads to loss all of hair cells and neurons in the entire cochlea duct [29].

Genes with long lasting expressions and specific effects on all or subsets of neurosensory cells are Eya1/Six1, Pax2/8, Gata3, and Sox2. Null mutants of Eya1/Six1 lack most or all neurosensory formation in a dose-dependent manner [30]. More recent work indicates that this may come about by interacting with the SWI/SNF chromatin-remodeling complex and by binding with Sox2 to the promoter region of neurosensory decision-making genes of the bHLH type [27]. The effect of Pax2 has long been described in ear defects [20]. However, different groups reported different overall effects in the cochlea neurosensory development, possibly related to mouse strains and redundancy of signaling with Pax8 [21]. In fact, Pax8 is in mice expressed prior to Pax2 in the otic placode but also disappears before Pax2 is expressed. As a consequence, Pax8 cannot rescue Pax2, but Pax2 can compensate for loss of Pax8, which has no embryonic phenotype on its own [21]. Mice null for both Pax2 and 8 do not develop an ear past an otocyst stage [21]. Similar effects have been reported in chickens after the removal of Pax2. Since chickens have no Pax8 gene, this also confirms the interpretation of redundancy of both genes in mammalian ear development [31]. Indeed, a hypomorph of Pax2 shows a graduated defect of neurosensory development of the organ of Corti, suggesting that Pax2 is acting in a dosage- and timing-dependent fashion at various stages in both early and late development [32] much like Eya1/Six1 [30].

Gata3 is another early expressed gene that has also a lasting expression. Going beyond the obvious massive effects on all neurosensory development [33], but in particular of the cochlea [34], requires delayed deletion of Gata3 in specific cell types to fully assess its function. However, the simple fact that haploinsufficiency suffices in humans to cause deafness [35], indicates the importance of this gene with early, but also long-term expression. Data in other developing systems suggest that Pax2/8 may regulate Gata3 expression [36], but this has not yet been assessed in the ear. Unfortunately, data on conditional deletion of hair cells result in dedifferentiation of the organ of Corti and also in a loss of Gata3 expression [37]. For regeneration to be successful it might be necessary to restore Gata3, Sox2, Eya1/Six1, and Pax2 expression prior to either cell or gene therapy to prime otic epithelial cells covering the former organ of Corti for the intended regeneration.

While these genes have a dose- and/or time-dependent preferential effect on cochlear neurosensory development, Sox2 has a more pervasive effect on all neurosensory precursors [23, 38]. Even incomplete loss as in a hypomorph of Sox2 is incompatible with neurosensory precursor formation. Sox2 has a sophisticated interaction with downstream genes such as the bHLH gene Atoh1. Sox2 is required for Atoh1 expression but will also be downregulated upon expression of Atoh1 in hair cells [39], possibly through methylation of nasal and otic placode-specific enhancer elements [13].

Other genes important for normal neurosensory development are related to the Delta/Notch signaling. This signaling system may not be essential for very early prosensory definition but is required for neurosensory specification and maturation [40]. A more detailed analysis of the Delta/Notch system is provided below. For now, it is important to note that expression of certain genes of the Delta/Notch signaling pathway are necessary for continued proliferation of neurosensory precursors and affect the level of Sox2 expression [41].

Consistent with this function, Jag1 misexpression can induce competent nonsensory cells of the ear to assume a prosensory fate [42]. Adopting alternate fates of such cells seems to be suppressed normally by the action of factors limiting the proneurosensory capacity such as Lmx1a [43, 44], Tbx1 [45], and Lmo4 [46]. Numerous other factors may fall into the category of proneurosensory-promoting factors based on their expression pattern such as Islet1 [47], but no detailed data exist on mutant mice to substantiate these suggestions yet. Other factors that have been proposed to play a role in neurosensory differentiation such as Prox1 [48] have later been shown in null mutant not to be as important as suggested based on expression data [49].

Jag1 and Sox2 are important factors for proliferating pro-neurosensory precursors. They do so by regulating other genes that are necessary to expand the neurosensory precursor population to the full complement of cells needed for function. First among those are genes needed for proliferation control such as N-Myc and l-Myc [24, 50], Foxg1 [51], cyclin kinases and their inhibitors [52], and pocket proteins [53] such as pRb [54]. Obviously, the regulation of the proliferation of proneurosensory cells with proto-oncogenes and tumor suppressors will have to be tightly controlled (Fig. 3). Loss of one or more regulators of proliferation of a given family of

Fig. 3 (continued) number of pathways, including the *Wnt*/β-CATENIN pathway, *TGF-β* or *BMP/SMAD*, *Fgf/Erk1-2*, and the *Shh/Smo* pathways. In short, the Fgfs ligands diffuse to their receptors which bind the Fgfs at the surface of the cell and signal through second messengers, the RAS intracellular pathway to influence Myc signaling. The Bmps signal through the SMAD pathway intracellularly to act indirectly on Myc function. Shh which binds with Patched and interacts with Smoothened at the cell surface influences Myc. Lastly, B-Catenin is constitutively active in the presence of Wnt through disinhibition of B-Catenin breakdown. Together, Myc is an integral node integrating both upstream and downstream pathways. Furthermore, the downstream regulation by Myc shows the highly complex, interconnected, and essential balance between proliferation and differentiation. The *larger circle* represents the cell as a whole, while the *smaller circle* is the nucleus. *Arrows* indicate upregulation, while *blunted lines* represent blocking. *Dashed lines* represent a reduction to endogenous protein levels. *Red* indicates pathways favoring differentiation, *blue* indicates pathways favoring proliferation. Modified after [183]

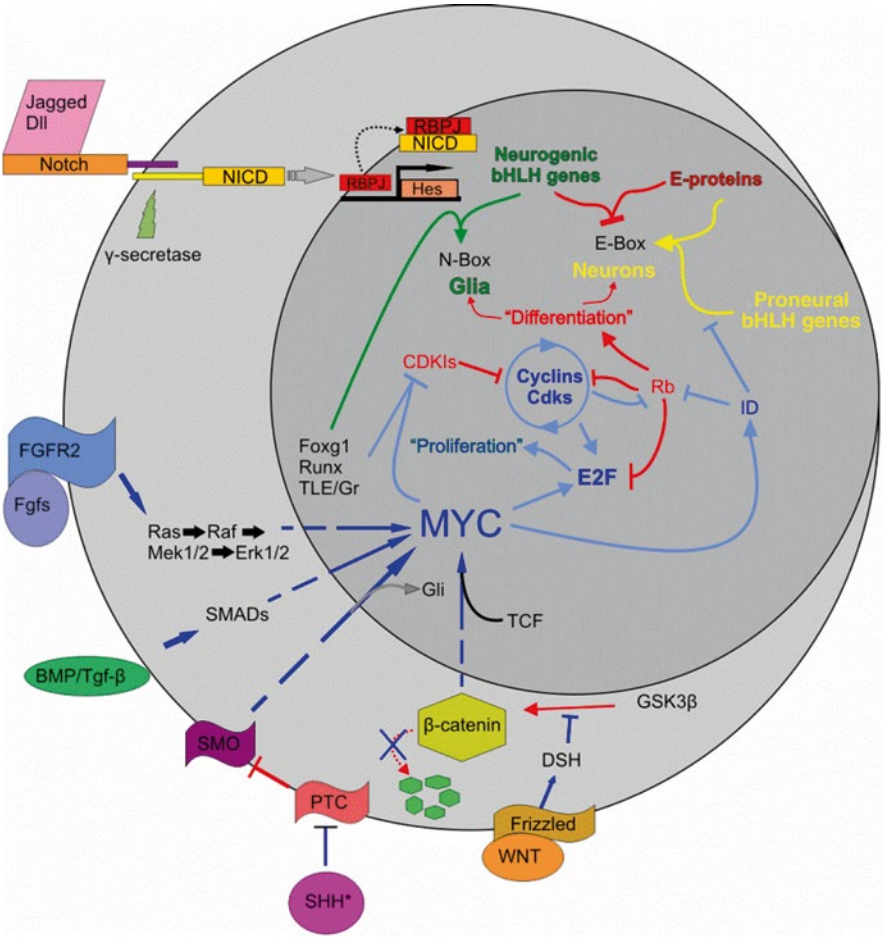

Fig. 3 In the presence of nearby neurons, Jag1 binds to Notch1, gamma-secretase is free to cleave the *N*otch *I*ntra*C*ellular *D*omain (NICD). NICD translocates to the nucleus to form a complex with RBPJ to positively regulate the transcription of the neurogenic *Hes/Hey* family of bHLH transcription factors. Hes/Hes in combination with E-proteins inhibit binding to the E-box and promote binding to the N-Box, resulting in shifting the balance from differentiation of neurons to differentiation of glial cells within the system. In the absence of Delta/Notch signaling and Hes/Hey transcription factors, proneural bHLH transcription factors bind to E-proteins to promote the formations of neurons. However, in the system exists a balance not only between glial cell fate and neuronal cell fate but there is also a balance between proliferation and differentiation. Retinoblastoma (pRB) in its unphosphorylated state binds the E2F family members. When pRB is bound to E2F, cells cannot pass the G1/S checkpoint, exit the cell cycle, and undergo differentiation either into neurons or glial cells as described above. However, both cyclins and the IDs can free E2F from pRB allowing E2F to bind to S-phase promoting genes and allowing the cell to continue to DNA replication. ID, or Inhibitor of Differentiation and DNA binding, had an additional function that promotes proliferation; ID can directly bind proneural bHLH transcription factors and inhibit their binding to the promoter regions of target genes. This inhibits differentiation and promotes proliferation, illustration of the tight regulation, and importance of the balance between proliferation and differentiation. On the other hand, the cyclin-dependent kinase inhibitors (CDKIs) inhibit the CDKs, resulting in net proliferation. Upstream of the IDs, cyclins, E2Fs, and CDKIs is Myc. The proto-oncogene Myc is an important node in this pathway, as illustrated by the many developmental defects and tumorigenetic defects caused by Myc deregulation. MYC is regulated by a

factors leads to predictable changes in the number of cells. Among the least understood aspects of ear development is the connection between cell cycle exit of neurosensory precursor cells and initiation of differentiation at the molecular level basis [55, 56]. This important aspect will be discussed below in the context of hair cell-specific differentiation, an essential step to generate a functional organ of Corti.

3 Lineage Restriction and Fate Determination of Neurosensory Precursors

3.1 Determining Neurons, Hair Cells, and Mixed Clones: Neurog1, Neurod1, Atoh1 Interactions

Proliferating neurosensory precursor cells eventually exit the cell cycle and initiate differentiation into neurosensory cells such as inner ear neurons, hair cells, and supporting cells around hair cells. The best characterized candidates mediating directly the neurosensory cell fate decisions are basic helix–loop–helix proteins such as the proneural Neurog1, Neurod1, and Atoh1 for neurosensory cells and Hes/Hey genes for supporting cells. The proneural bHLH transcription factors form an interacting network whereby Neurog1 and Atoh1 regulate the expression of Neurod1 in neurons and hair cells, whereas Neurod1 in turn limits the expression of Neurog1 in neurons and of Atoh1 in hair cells [57]. Null mutations have contributed to our understanding of the functional importance of these genes in neuron and hair cell development. As it turns out, loss of Neurog1 eliminates all neuron development from the otocyst [58], whereas loss of Atoh1 eliminates all hair cell differentiation [59]. In addition, loss of Neurog1 affects hair cell formation in two ways: it causes not only loss in some neurosensory areas but also gain in non-sensory areas [60].

Unfortunately, the case of Neurod1 adds to this complexity. While initially thought of as a simple gene downstream of Neurog1 [58] to maintain neurons [61], follow-up work painted a different picture [57, 62]. Neurod1 suppresses Atoh1 in differentiating neurons and in the absence of Neurod1, these neurons express more profoundly Atoh1 and turn these cells into hair cells inside the inner ear ganglion [57]. Neurod1 expression in hair cells requires Atoh1 for upregulation [63], but the level of expression in hair cells is increased after Neurog1 loss [60]. Similar to neurons, loss of Neurod1 also affects hair cell development. However, instead of turning neurons into hair cells as in ganglia, it turns outer hair cell-like cells into inner hair cell-like cells [57]. The simplest explanation for these interactions is that all three proneural factors interact within a given neurosensory precursor (Fig. 4) to define the outcome of bifunctional cells as either neurons or hair cells [1, 2]. However, how big this bifunctional population is in embryos and how much it changes as development progresses, remains unclear [6, 64].

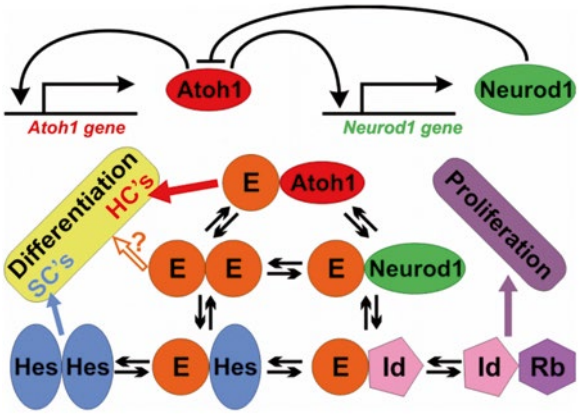

Fig. 4 Various basic helix–loop–helix proteins form complex interactions in a given cell. Data in mice and flies suggest that all proneural factors compete for the E-proteins to form heterodimers for proper binding. However, E-proteins can also interact with Hes/Hey factors and the inhibitors of DNA binding (Ids). In essence, the binding properties and frequency of the binding partners will determine whether a cell is differentiating as a neuron/hair cell, a supporting/glial cell, or is continuing proliferation as a prosensory precursor

3.2 Separating Hair Cells from Supporting Cells: Delta/Notch; Hes, Hey Factors

In addition to the proneural class of bHLH genes, the ear requires, like all other developing neuronal systems, the expression of the class of neurogenic genes downstream to the Delta/Notch signal. Given its profound function in ear development, we will provide an in depth analysis of its expression and function.

3.2.1 Notch Basic Aspects: Receptors and Ligands

The Notch signaling pathway controls tissue formation and homeostasis during embryonic and adult life. It achieves this through local cell-to-cell interactions that occur via Notch receptors, of which there are four in mammals (Notch1–4). Notch ligands are the Delta-like (Dll1, 3, and 4) and Jagged (Jag1 and Jag2) proteins [65]. Notch receptor proteins are first glycosylated by the GDP-fucose protein O-fucosyltransferase 1 and three b1,3-GlcNAc-transferases (lunatic fringe, manic fringe, and radical fringe), which are necessary for the generation of functional receptors and can impact their response to the ligands [66].

During maturation and trafficking to the cell surface membrane, Notch receptors undergo proteolytic cleavage by furin at site 1 (S1), which converts the Notch polypeptide into a heterodimer, composed of the Notch extracellular domain and the Notch transmembrane/intracellular domains [67]. A wide range of Notch ligands

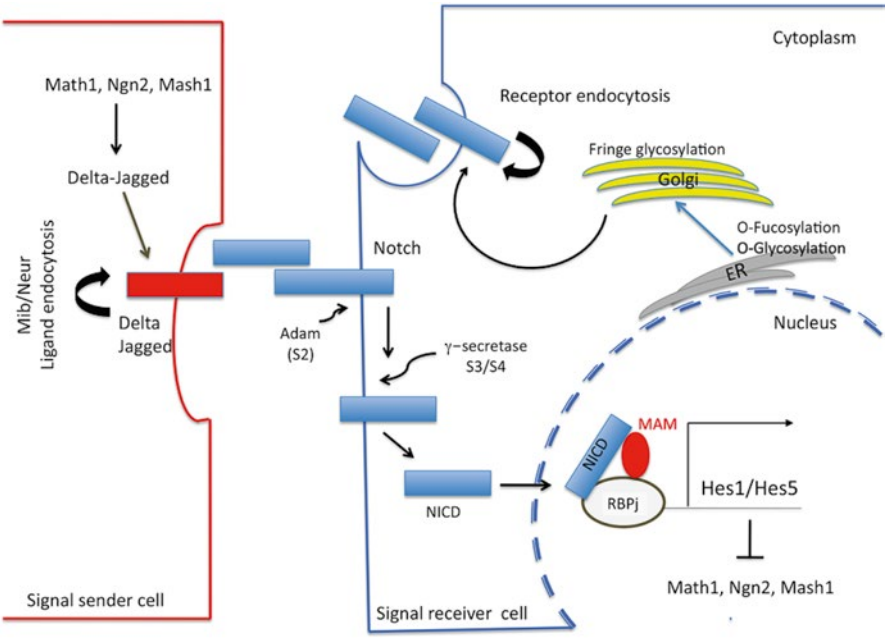

Fig. 5 The core canonical signal transduction pathway of Notch-dependent cellular processes is shown. Notch receptor and ligands are transmembrane proteins. The ligand-induced activation of Notch signaling is regulated by endocytic trafficking, which can be modulated by the different ubiquitin ligases, such as Mind bomb and Neuralized. The proneural genes Mash1, Ngn2, and Math1 induce expression of Notch ligands such as Dll1, which activate Notch signaling in neighboring cells. Upon activation, the notch intracellular domain (NICD) is released from the transmembrane region after successive proteolytic cleavages and transferred into the nucleus, where it will associate with RBPj and MAM to form a transcriptional complex and activate the expression of the Notch target genes such as Hes1 and Hes5. The Hes genes in turn, repress proneural gene expression. The Notch receptor is glycosylated in the ER and the Golgi complex. Both ligand and receptor are regulated by endocytosis

bind to and activate the Notch receptor. The activity of Notch receptors and ligands are also regulated by endocytic trafficking, which can be modulated by various ubiquitin ligases, such as Mind bomb (Mib) and Neuralized (Neur) [68]. Upon ligand activation of the Notch receptors on neighboring cells, Notch receptors are cleaved by ADAM family metalloproteases at site 2 (S2). The truncated transmembrane/intracellular domains are subjected to further proteolytic events by presenilin proteases complex known as γ-secretase (Fig. 5).

3.2.2 Activation of the Notch Receptor by Its Ligands

The cleaved intracellular domain of the Notch receptor (NICD) is released from the cell membrane and translocated to the nucleus, where it combines with the

DNA-binding protein RBPj/CBF-1 and other transcriptional co-activators, such as Mam, to bind and activate downstream target genes [65]. The best characterized Notch targets are the Hes and related Hey genes, which encode a family of basic helix–loop–helix (bHLH) transcriptional repressors [69, 70]. The Hes/Hey proteins inhibit transcription of their target genes, such as Ascl1/Mash1, Atoh1, and Neurogenins, preventing undifferentiated progenitor cells from progressing toward more differentiated states (Fig. 4) except for a continued differentiation as supporting cells [71].

3.2.3 Notch Transcriptional Targets

Different outputs of Notch signaling pathway can lead to the transcription of specific target genes that often differs between cell types and tissues. The most expressed and best characterized Notch target genes encode bHLH proteins of the Hes/Hey family that are transcriptional repressors [70, 72]. The transcriptional activity of the Hes/Hey genes is dynamic, temporally and spatially regulated as the expression of the corresponding mRNAs can be detected as early as 30 min after Notch activation and their encoded proteins are thought to be rapidly degraded [70, 73]. One major function of the Hes/Hey transcription factors is to repress the proneural factors of the Achaete-scute, Atoh, and Neurogenin family of bHLH transcriptional activators, possibly by competing for a set of bHLH factors (Figs. 3, 4, and 5) needed to form heterodimers for proneural bHLH gene signaling [1]. In addition, the Hes proteins have been shown to repress their own transcription [74], resulting in oscillatory expression in neural progenitor cells [75]. The Hes/Hey genes are not the only targets of Notch activation. In many tissues the loss of function of Hes/Hey genes has not been shown to correlate with the phenotypes of the loss of Notch activity suggesting additional target genes [76]. It has been reported that in cell types in which the Notch activation enhances proliferation, Myc and CyclinD act as direct target genes of Notch [77, 78]. In cell types where Notch promotes exit from the cell cycle and differentiation, cyclin-dependent kinase inhibitors (from the Cdnk1-family) such as p21Cip1 (Cdkn1a) and $p27^{Kip1}$ (Cdkn1b) act as targets of Notch activation [79, 80].

3.2.4 Notch Pathway in Inner Ear Sensory Patterning

The inner ear regionalization is the result of consecutive inductive signals emanating from neighboring tissues. Several lines of evidence support that the Notch1/Jag1 signaling initially helps specifying sensory versus non-sensory epithelium within the ear, building the limits by a lateral inductive mechanism [1, 81–85]. Subsequently, the Notch1/Jag2-Dll1 pathway inhibits hair cell differentiation and establishes a mosaic of cell fate by a lateral inhibition mechanism [85–87].

Among the many ligands (Table 1) Delta-like1 (Dll1), Delta-like3 (Dll3), Jag1 and Jag2, and the Notch receptors Notch1 and Notch3 expressed in the inner ear

Table 1 Notch receptors and ligands expression in the developing mouse inner ear

Notch pathway protein	Expression patterns	References
Notch1	Prosensory area and supporting cells	[80, 85, 86]
Notch2	Outside the sensory epithelia	[89]
Notch3	Epithelium of the otic vesicle	[88]
Notch4	Outside the sensory epithelia	[89]
Dll1	Hair cells	[90, 92]
Dll3	Hair cells	[93]
Dll4	Not determined	–
Jag1	Prosensory area and supporting cells	[41, 86]
Jag2	Hair cells	[85, 98]

[85, 88–93], Notch1 and Jag1 are expressed in patterns consistent with a role in otic prosensory specification. Although they are, together with Lfng, initially expressed in more diffuse patterns in the otic cup, each relocates to areas within the developing prosensory regions [81, 94–96]. Jag1 expression is first localized to the prosensory domain, while later on, its expression is confined to the supporting cell subtypes.

Recent results have provided new data regarding the role for Notch signaling in the specification of prosensory domains. In particular, analysis of inner ears from mice in which Jag1 has been specifically deleted at the early otocyst stage using a Foxg1-dependent Cre-expressing mouse line, reveals that most of the vestibular organs, with the exception of the saccular maculae, are absent, and within the cochlea, a reduced number of mis-patterned hair cells are restricted to apical portions of the cochlear duct [41, 97, 98]. Moreover, misexpression of Jag1 can induce ectopic sensory epithelia formation [42].

Consistent with this essential need of this signaling pathway, deletion of RBPj, a transcriptional repressor that is required for Notch function [99], has been reported to lead to a complete absence of all vestibular epithelia while the sensory epithelium in the cochlea is restricted to the most apical area [100]. However, other data suggest that the initial prosensory formation may progress up to a point in the absence of RBPj but that prosensory formation is only transient [40] becoming rapidly destabilized. Consistent with the later data, inhibition of γ-secretase activity, a component of the Notch signaling pathway, is needed for maintenance but not initial formation of prosensory formation in the chick otocyst [101]. Conversely, overexpression of an activated form of chicken Notch1, cNotch1-intracellular domain (NICD), in non-sensory regions of the chick otocyst leads to the formation of ectopic sensory patches [87]. All these results are consistent with a role for Jag1-dependent Notch activation in early steps of the specification of prosensory domains throughout the ear including the cochlear duct.

Additional factors are required for sensory specification in the cochlear system, since all these mutants showed only a reduction instead of a complete loss of the sensory hair cell area. In contrast, inner ear deletion of Notch1, by the use of Foxg1-Cre line as a driver, resulted in an overproduction of hair cells in both the vestibular and cochlear epithelia [98]. The mechanism for this effect is most likely related to

the role of Notch signaling in the determination of individual cell fates within prosensory domains by a lateral inhibition mechanism. Consistent with a role of Notch in cell fate specification in the inner ear sensory epithelium, nuclear localization of NICD, Hes1, Hes5, and Jag1 expression are detected in supporting cells, while Jag2 and Dll1 expressions are restricted to the hair cells [81, 83, 94, 102].

Null mutations for Jag2, Hes1, Hes5, and/or Dll1 in mouse embryos and mind bomb (E3 ubiquitin involved in ubiquitinylation and endocytosis of Delta required for Notch function) in zebrafish embryos result in overproduction of hair cells at the expense of supporting cells, indicating that Notch signaling regulates sensory cell fate specification by a mechanism of lateral inhibition [85, 86, 91, 94]. Thus, Jag2/Dll1 ligands induce the activation of Notch pathway and Hes5 in adjacent cells that inhibits hair cell fate determination. Notch activity is then required for sensory development to first, make cells competent to form a prosensory patch conferring them a prosensory identity and, subsequently, inhibit hair cell differentiation and establish a regular sensory mosaic pattern of hair cells and supporting cells [87]. This interpretation parallels interpretations derived from Sox2 manipulations that indicates the need to molecularly define neurosensory precursors/stem cells [13, 39].

The mechanism by which Notch activation can play dual roles for early steps of prosensory specification and later functions in lateral inhibition aspects to sort out supporting cells and hair cells is not clear. These two distinct functions are likely to require other downstream effectors for converting Notch activation into distinct transcriptional outputs. The Hes-related genes, Hey1 and Hey2, are expressed in the developing cochlea and treatment of cochlear explant cultures identified Hes and Hey factors as downstream effectors of the prosensory Jag1 signal in the cochlea [103]. Another study showed that the expression of Hey2 overlaps with that of Hes1 and Hes5 [104]. The patterning defects observed in Hes1 or Hes5 null mutant mice [105] were increased in Hes1 and Hey2, and Hes5 and Hey2 compound mutants suggesting cooperative functions of Hes/Hey genes to regulate the complex patterning in the mammalian cochlea [71, 106]. Co-expression of Hes and Hey factors, each regulated by different means, is important to avoid differentiation of supporting cells that express transiently Atoh1 [60, 107] into hair cells [71]. Unfortunately it is unclear how much of a co-expression of Atoh1 and downstream effectors of the Delta/Notch signaling happens in early neurosensory specification. The fact that at least some specification of hair cell precursors can occur without the RBPj-mediated Delta/Notch signaling leaves open that this signal pathway plays only a crucial, but nevertheless secondary role in the initial neurosensory specification [40].

4 Stem Cells in the Adult Ear, Their Identification, and Localization

While nonmammalian species can repair their damaged inner ear sensory epithelia, neither the hair cells nor the neurons have the potential to regenerate in the mammalian cochlea [108, 109]. In avians and lower vertebrates, supporting cells

can be triggered by dying hair cells to replace them by either proliferating or transdifferentiating modes [110]. Similar to birds [111], mammalian supporting cells may share a common cell progenitor with hair cells during development. However, supporting cells of the mammalian organ of Corti fail to show any regenerative response to hair cell loss either via direct transdifferentiation or mitosis [112]. The vestibular organ (including the utricle, the saccule, and the cristae ampullaris) is also a mechanosensory structure located in the inner ear that conveys information on position and gravitational acceleration. In contrast to the cochlea, limited regeneration of hair cells has been observed in the vestibular sensory epithelia in guinea pigs following ototoxic drug treatment at different time-points [113] or after hair cells have been eliminated by molecular means in neonatal animals [114, 115].

A study on the murine utricle has confirmed evidence that vestibular sensory hair cells can spontaneously regenerate after ototoxic drug exposure. A large number of immature hair cells could be seen as early as 2 weeks after the lesion. However, neither the regenerated cell numbers nor their morphological appearance was normal [116]. Recently, a population of putative stem cells (about 0.025 % of the cells within the utricle) have been isolated from the adult mouse utricle [117], and a subset of them proliferate and form cellular spheres in culture. Moreover, these spheres were shown to contain multipotent stem cells with the ability to give rise to hair cell and supporting-like cells under differentiating culture conditions and when transplanted into a permissive environment, such as the developing chick otocyst.

Following the first isolation of vestibular stem cells, many studies were conducted to check whether similar cells are present in the mouse cochlea. Using a similar culture procedure, sphere-forming cells with the capacity to generate hair cell and supporting-like cells were isolated from the neonatal cochlea of mice [118, 119]. However, this population of stem/progenitor cells cannot be isolated from the mouse cochlea beyond the third week of age [118]. This decline in the sphere forming capacity of cells derived from the cochlear epithelium could indicate that these stem/progenitor cell types either cease to exist or to a loss of their stem/progenitor properties in the adult cochlea. Indeed, a recent expression study using immunocytochemistry and qPCR (Fig. 6) revealed a decrease in the expression levels of Sox2 and Jagged1, two known potent stem cell markers from the mature cochlea [120]. This implies intrinsic changes leading to a loss of stem/progenitor properties with maturation.

It remains open what the potential structural "niches" are and which stem cells can reside and maintain their identity in the inner ear environment. Previous studies with postnatal rat cochlea have proposed the lesser epithelial ridge (LER) and

Fig. 6 (continued) of Sox2 and Jagged1 quantified by qRT-PCR (**a**) in the adult cochlea, their pattern of expression is almost similar as in the P3 cochlea. In the adult cochlea, Abcg2 is downregulated in the supporting cells within the sensory area, while its expression is upregulated in the medial (i.e., inner sulcus) and lateral regions (i.e., Hensen's cells) of the sensory area. In addition, Abcg2 colocalized with GFAP within the interdental cells in the apical portion of the adult cochlea. *IHC* inner hair cell, *OHCs* outer hair cells, *DCs* Deiter's cells, *HeCs* Hensen's cells, *IDCs* interdental cells, *IRC* inner rod cell, *ORC* outer rod cell, *BC* border cell, *IPC* inner phalange cell, *IS* inner sulcus, *GER* greater epithelial ridge, *BM* basilar membrane

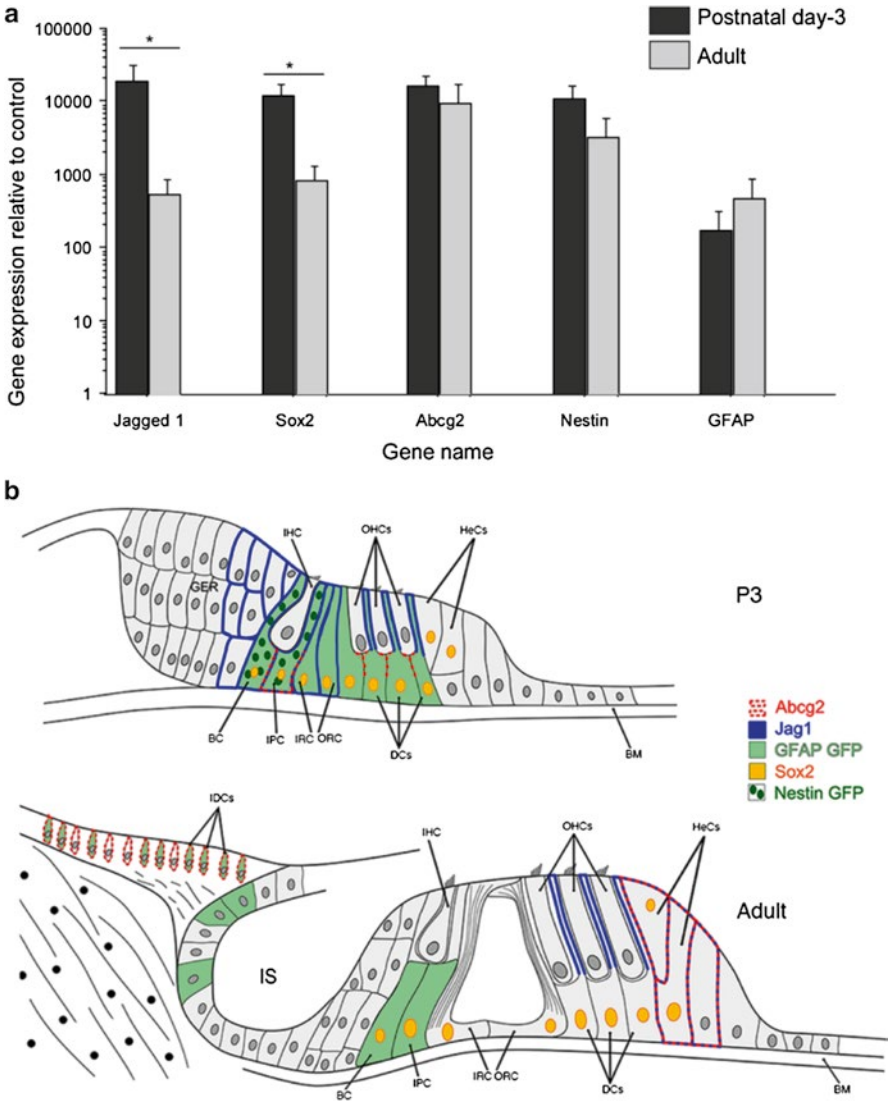

Fig. 6 Expression of some stem/progenitor markers in the postnatal day-3 (P3) and adult mouse cochlear epithelia. (**a**) Differential expression of Sox2, Jagged1, GFAP, Nestin, and Abcg2. Transcripts were quantified by qRT-PCR, and data were analyzed using the $2^{-A_{Ct}}$ method. GAPDH was used as endogenous housekeeping control gene. Gene expression is represented as $2^{-A_{Ct}} \times 10^6$ on a $\log_{10}$ scale. *Error bars* indicate standard deviations. *Asterisks* indicate differences in the $2^{-A_{Ct}}$ between P3 and adult stages (student's *T*-test; $P \leq 0.05$). Real-time RT-PCR analysis revealed a significant transcript declines by approximately 18 % for Sox2 and 12 % for Jagged1 in the cochlear epithelia between P3 and adult ($P \leq 0.05$). Although insignificant, the GFAP was the only gene that showed a slight increased transcript expression in the adult cochlea when compared to the P3 cochlea. (**b**) Schematic summary of the expression patterns of Sox2, Jagged1, GFAP, Nestin, and Abcg2 within P3 and adult cochlear epithelia. Despite the decrease in the expression

greater epithelial ridge (GER), located respectively laterally and medially to the organ of Corti as two potential "niches" for hair cell progenitors [121, 122]. These studies demonstrated that isolated LER and GER cells proliferate and form spheres when grown in vitro in the presence of EGF and differentiate hair cell-like and supporting-like cells when co-cultured with utricular mesenchymal cells. This hypothesis was supported by a demonstration that both of these cochlear epithelium locations (i.e., LER and GER) can give rise to ectopic hair cells when forced to express Atoh1 [123, 124]. However, these transient embryonic cells disappear in the adult cochlea.

An interesting population of tissue-specific stem cells was isolated from cochleae of 9–11-week-old human fetuses [64, 125]. These human fetal progenitor cells were capable of undergoing long-term in vitro expansion (i.e., for at least up to 1 year) and kept the potential to differentiate into hair cells-like and neuron-like cells that displayed electrophysiological properties similar to those of developing cochlear hair cells and auditory neurons in vivo.

Also relevant are the nestin-positive neural stem/progenitors isolated from the adult guinea pig and human spiral ganglia [126]. These stem/progenitor cells formed spheres and differentiate in vitro into neurons that express neurotrophin receptors (i.e., TrkB and TrKC). These findings suggest that the mammalian auditory nerve has the capability for self-renewal and replacement.

An unexpected source of adult stem cells was reported within the mouse inner ear as being bone marrow-derived cells that may constitutively exist in the adult cochlea [127, 128]. The results of these studies suggest that mesenchymal cells in the adult inner ear continuously form from bone marrow and are replaced slowly over a period of months. However, the majority of these stem cells within the inner ear differentiate into fibrocytes or resident/infiltrating macrophages. Still, these cells raise the intriguing possibility whether they could be receptive to exogenous signals that may induce hair cell replacement in the injured adult cochlea.

It has also been hypothesized that hair cell progenitor cells in early postnatal cochlea might in fact be the supporting cells themselves, as it has been shown that supporting cells isolated from neonatal mouse cochlea can proliferate and give a rise to both hair cell and supporting-like cells [129]. We will develop these findings with more details in the next paragraphs.

5 Markers for Adult Stem Cells of the Organ of Corti: Isolation/Sorting Through FACS

Data from different groups suggested that hair cell stem/progenitor cells in the postnatal period might in fact be the supporting cells, as it has been shown that isolated supporting cells from postnatal mice can proliferate and transdifferentiate into hair cell-like cells in vitro [119, 129]. For instance, White et al. [129] used a transgenic mouse expressing GFP under control of the Cdkn1b ($p27^{Kip1}$) promoter to isolate the supporting cell population from postnatal mouse cochleae by

fluorescence-activated cell sorting (FACS). When these cells were co-cultured with periotic mesenchyme, a subset was able to reenter the cell cycle and give rise to daughter cells that expressed some hair cell markers. This suggests that some isolated/sorted postnatal mammalian supporting cells can divide and transdifferentiate into hair cell-like cells by both mitotic and non-mitotic events. In addition, the same study reported that supporting cells isolated from P14 mouse generated a low number of cells transdifferentiating into hair cells as compared to cultures of supporting cells isolated from neonatal mice. This decrease in the capacity of supporting cells to transdifferentiate into hair cells under culture conditions was associated with the inability of supporting cells isolated from P14 mouse cochleae to downregulate the $p27^{Kip1}$ at this stage of maturation and beyond.

Interestingly, an Abcg2-expressing side population (SP) of progenitor cells has been identified within the supporting cells in the P3 mouse cochlea and can be isolated by FACS based on their ability to exclude Hoechst dye [119]. These FACS-isolated cells can divide and express markers of stem/progenitor cells such as Abcg2, a determinant of the SP phenotype, and Musashi1, a neural stem/progenitor cell marker (Fig. 6). Further, these cells can give rise to hair cell-like cells under certain culture conditions. However, both purified neonatal cochlear supporting cells from $p27^{Kip1}$-GFP and those isolated through a side population analysis displayed a very limited capacity for self-renewal. This contrasts with the supporting cells isolated from bird inner ear sensory tissue that can be propagated for extended periods in a monolayer culture [130]. Altogether, these studies suggest that dissociated supporting cells from the early postnatal mammalian cochlea behave as hair cell progenitor cells in vitro, but the phenotypic identity of the supporting cell subtype that retains stem/progenitor features is unknown. Recent studies from different groups have taken advantage of cell surface markers routinely used to characterize hematopoietic cells to fractionate the organ of Corti by FACS into different single cell populations [8, 131–133]. These studies demonstrate differences in the proliferative ability of different populations, but more significantly show that the subpopulations vary significantly in their ability to produce hair cells in sphere cultures.

Of interest, Sinkkonen [131] used a battery of cell surface markers (CD271, CD326, and CD146) to prospectively identify and purify different non-sensory cell populations from the neonatal mouse cochlea and to determine their capacity to proliferate and form hair cells. In this study, four different non-sensory cell populations have been isolated and shown to display proliferative potential, but only lesser epithelial ridge (LER) and supporting cells robustly give rise to hair cell marker-positive cells. These results suggest that cochlear supporting cells and cells of the LER area show robust potential to dedifferentiate, proliferate, and differentiate epithelial cell patches harboring hair cell- and supporting cell-like cells.

Indeed, two recent papers [132, 133] have used the expression of the Wnt-responsive receptor Lgr5 to prospectively identify, characterize and purify subpopulations of supporting cells from the mouse cochlea. In culture, these Lgr5-positive cells displayed progenitor cell ability, formed clonal colonies, and gave rise to myosin VIIa positive hair cells, which display additional hair cell-specific markers when

compared to other purified supporting cell populations. Together, these data indicate that Lgr5, a Wnt target gene, marks Wnt-regulated sensory progenitor cells in the postnatal cochlea.

The identification of a potent-dividing supporting cell population and a necessary triggering signal that can activate this division are an important step in establishing the identity of cell populations for mammalian inner ear replacement or repair. Certainly, figuring out the role of the Delta/Notch signaling pathway not only in the initiation of supporting cell differentiation and suppression of neurosensory cell fate needs to be expanded to understand its role in supporting proliferation of neurosensory precursor cells. Below we will expand on this issue.

6 Direct Transformation of Supporting Cells to Hair Cells: A Viable Strategy for the Cochlea?

From the above it follows that supporting cells are turned away from a hair cell or general neurosensory differentiation mostly through the activation of Delta/Notch downstream effects of the Hes/Hey family of bHLH genes. In fact, even limited expression of Atoh1 reported in supporting cells [60] can be counteracted by Hes/Hey to maintain a supporting cell differentiation during normal development [60, 71, 107]. This suggests that not only simple distribution of various transcription factors but their quantitative mix is important for the cell fate decision making process. Simply speaking, it will be the sustained expression of either a proneural bHLH factor or neurosensory bHLH factor that will in the end determine the future fate of a precursor cell. What remains unclear is how long co-existence of both factors last and at which level co-expression can be sustained. Also unclear is for how long the initial decision making process can be reverted through elevated expression of the opposing bHLH factor. Recent data suggest that in mice this may last until about 4 weeks of age [9]. This theoretical framework is important to understand the initial success and the apparent limitations of Atoh1 replacement therapy or Delta-Notch suppressor approaches, detailed below.

6.1 Success with Atoh1 Gene Therapy and Its Limitations

Shortly after the mutational analysis demonstrated that Atoh1 was an essential gene for hair cell differentiation [59], Atoh1 transfection showed that embryonic inner ear cells can be converted to hair cells [123]. In fact, more recent data have dramatically expanded this initial finding but also indicated its limitations. For example, almost the entire developing ear can be converted into a mosaic of hair cells/supporting cells using an inducible overexpression of Atoh1 [134]. However, such hair cells have a limited viability. Furthermore, neither induction of hair cells nor induction of proliferation of supporting cells using Atoh1 is possible in older postnatal

mice [135]. Some transformation of supporting cells seems to be possible under very limited circumstances with this approach in older animals [136] but certainly not after the organ of Corti has dedifferentiated due to the absence of hair cell-mediated signals [11]. Since the latter will be the likely situation in humans with variable time delays after hair cell loss-related neurosensory deafness, it is unclear how the embryonic and early neonatal success with Atoh1 therapy can be expanded to the nearly completely degenerated organ of Corti in the elderly, possibly after years of loss of hair cells.

More recent data indicate that indeed there is a limited expression of Atoh1 in the adult organ of Corti [60] and this expression is apparently used to drive transdifferentiation of supporting cells by inhibiting γ-secretase [9]. Unfortunately, the most profound effects in terms of hearing restoration coincided in these experiments with areas that showed no supporting cell transformation. How long these transformed cells remain and how long after loss of hair cells this approach will work is unclear.

Obviously, the work on hair cell loss in mind bomb mutants where all supporting cells turn into hair cells [94] suggests that simple conversion of the remaining cells of the organ of Corti after hair cells have been lost will not suffice to restore hearing. Therefore, attempts should be made to initiate proliferation of the remaining cells before conversion. It is entirely possible that postmitotic cells will be easier to be reprogrammed with proper molecular treatment into hair cells. While conceptually appealing, the attempts to initiate proliferation in the adult cochlea deprived of all hair cells has not been very successful as yet [10]. Only limited attempts have been made to use more powerful stimulators of proliferation such as the various tumor suppressor or proto-oncogenes expressed in the developing ear-like knocking-out of retinoblastoma [54, 137, 138], N-Myc [50, 55] or combining these approaches with knocking down of cyclin kinase inhibitors such as p21 [52] and other factors. Obviously, controlled and transient overexpression of proto-oncogenes would be easier to shut down to initiate secure differentiation of postmitotic cells with gene therapy. Future work certainly should explore the possibility to use inducible, transient expression of N-Myc to safely initiate proliferation in the ear [50].

6.2 Can Hair Cells Survive Long Term Without Supporting Cells? The Need for Proliferation Prior to Attempts of In Vivo Transformation

As stated above, proliferation is not only essential to restore potentially reprogrammable cells in the fully degenerated cochlea but also is essential for any attempt to transform supporting cells into hair cells. Data on the zebrafish mind bomb mutation show clearly that hair cells that form without supporting cells are not maintained and degenerate fast [94]. This insight puts a limitation on any attempt to simply convert remaining supporting cells after hair cell loss into hair cells. Obviously, such hair cells, deprived of supporting cells, will not have the desired long-term viability. Therefore, it would be important to initiate proliferation prior to attempts to transform with molecular treatment the supporting cells into hair cells.

In conclusion, no matter the starting point in terms of remaining degree of differentiation of supporting cells, it will be essential to initiate proliferation of remaining cells in the organ of Corti before attempts are initiated to transform remaining cells into hair cells. In fact, it is possible that the newly postmitotic cells, in particular when induced to express prosensory markers such as Sox2, Eya1/Six1, Gata3, and Pax2, are less committed to a specific phenotype. These cells may be more likely to be reprogrammed molecularly into a specific type of neurosensory cell using the information partially outlined above on early development of the organ of Corti [28, 139].

7 Turning Back the Clock of Differentiation: Adult Induction of Stem Cell Properties In Situ Using Molecular Therapy

As outlined above and inferred from a loss in regenerative potential, stem cells seem to disappear in the OC in mice during postnatal maturation. At this time, it remains undetermined what kind of stemness exists in the OC, whether (1) the adult OC harbors true stem/progenitors cells in a dormant state or if (2) such stem/progenitor cell properties may potentially be induced by dedifferentiation of differentiated supporting cells by exogenous stimuli. This "true oxymoron" of inner ear stem cells has been discussed in a recent review [140].

In the adult auditory sensory epithelium of nonmammalian vertebrates, transdifferentiation of supporting cells with or without proliferation can replace lost hair cells. This regenerative potential is lost in the mammalian cochlea. A central question around the pursuit to regenerate hair cells in the OC therefore is how endogenous cells in the mammalian auditory epithelium may be stimulated to regain the regenerative potential observed in the nonmammalian counterparts. From the two concepts of stemness as described above arise two concepts to restore the regenerative potential, (1) the potential activation of dormant resident stem cells or (2) the potential dedifferentiation of differentiated supporting cells. For both approaches, the search for specific cells with potential stem/progenitor cell features within the mature cochlea is critical.

In search for stem/progenitor cells in the mouse inner ear, Nestin promoter-green fluorescent protein (GFP) transgenic mice have been investigated to determine the presence of Nestin-positive cells in the OC. It was shown that the expression of Nestin in the mouse inner ear became developmentally downregulated, but a small, albeit persisting population of Nestin-positive Deiter's cells was retained in the adult inner ear [141]. The presence of Nestin-positive cells in the mature cochlea was recently confirmed using Nestin-β-gal transgenic mice. Interestingly, both the number of Nestin-positive cells and the expression of the Nestin mRNA increased after a noise induced hair cell loss [142] (Fig. 7c, d). If Nestin is accepted as a stem cell marker these results indeed are in support of both hypotheses that (1) a small persisting population of resident cells with dormant stem/progenitor cell features exist in the mature cochlea and (2) that an additional subset of differentiated

supporting cells dedifferentiates towards a stem/progenitor cell phenotype upon a damaging stimulus. Since the further steps of a full regenerative response such as proliferation and re-differentiation are lost in the mature OC, an additional set of signals appears needed to trigger dormant stem cells and/or dedifferentiated supporting cells to regenerate hair cells in the mature OC. Nevertheless the identification of potentially responsive endogenous progenitor cells represents a promising, emerging cellular target for further molecular intervention. Toward the hypothesis of a dormant stem/progenitor cell population it is important to note that in vitro cultures of isolated p75-NTR+ [129, 131] and Lgr-5+ cells [132, 133] demonstrated the generation of hair cell-like cells after mitotic cell devision (Fig. 7a, b). Furthermore, the results from in vivo lineage tracing experiments suggest that Lgr-5+ cells can give rise to hair cells in the neonatal cochlea [132, 133].

In further support of the hypothesis of a dedifferentiation response are data on supporting cells of the (OC) of ototoxic drug treated guinea pigs and mice. In guinea pigs damaged by aminoglycosides supporting cells showed an increase in proteins involved in Notch activation such as Jag1 in pillar and Deiter's cells, Notch1 signal in surviving supporting cells, and the absence of Jag2 and Dll1. Furthermore, the proneural bHLH protein Atoh1 was absent, while the repressor bHLH transcription factors Hes1 and Hes5 were detected in surviving supporting cell nuclei (Fig. 7g, h). Studies conducted in C57BL/6 and CBA/Ca mice after treatment with an aminoglycoside-diuretic combination produced a loss of all outer hair cells within 48 h in both strains. A very limited dedifferentiation response was seen in specialized columnar-supporting cells with downregulation of Kir4.1, but KCC4, GLAST, microtubule bundles, connexin expression patterns and pathways of intercellular communication were retained (Fig. 7e, f) [185]. The inconsistency of a dedifferentiation response amongst species and supporting cell populations clearly indicates that this step toward regeneration is incomplete or rudimentary. Thus, any attempt to promote endogenous hair cell regeneration will depend on further exogenous stimulation.

Further steps needed toward a full regenerative response such as proliferation and re-differentiation have recently been demonstrated by molecular manipulation of the cell cycle or by isolation and explantation of cells from the OC in vitro. The artificial isolation of cells from the OC reveals an intrinsic stemness or "turning back the clock" as indicated by the presence of multipotent stem cells that in an otic sphere formation assay can be induced to self-renew and differentiate into supporting and hair cell lineages [117, 118, 131, 143, 144]. Such stem cell-like properties have been linked to the supporting cell population of the postnatal OC by multiple approaches. As detailed above (Sect. 4) postnatal supporting cells purified by fluorescence-activated cell sorting using approaches such as $p27^{Kip1}$-GFP transgenic mice [129], side population analysis [119], selective surface markers on supporting cells [131] or Lgr5-GFP transgenic mice [132, 145], acquire stem cell-like properties similar to progenitor cells in the early embryonic OC. However, after the second postnatal week this sphere forming capacity of isolated cells from the OC is lost in the functionally mature wild-type OC [118]. Interestingly, studies using transgenic mice deficient for the cyclin-dependent kinase inhibitor *CDKN1B* ($p27^{Kip1}$) showed ongoing proliferation of supporting cells in the postnatal OC [146, 147] and adult [147] OC in vivo and in vitro at the p14 developmental time point [129]. These studies provided

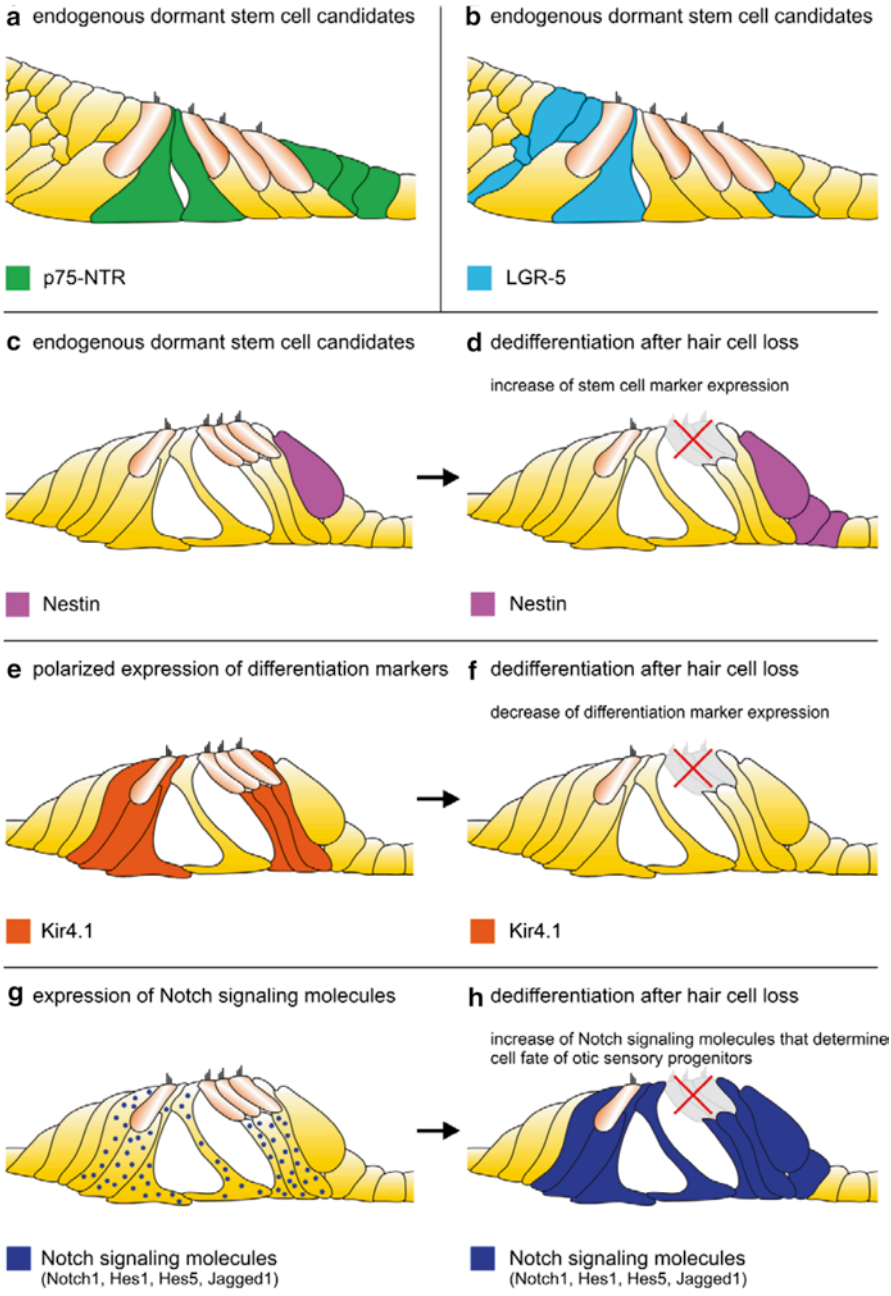

Fig. 7 Potential markers of endogenous dormant stem cell candidates and induction of dedifferentiation within the mammalian organ of Corti. (**a**) The differentiated mammalian OC harbors endogenous dormant progenitor cells [120] that can be isolated by their surface-marker expression, i.e., p75-neurotrophine receptor (NTR) [129, 131] Pillar cells and Hensen's cells express the p75-neurotrophine receptor (NTR) at early postnatal stages; (**b**) Endogeneous progenitor marker Lgr-5 is expressed in cells of the greater epithelial ridge (GER), inner pillar cells, and the third row of Deiter's cells [132, 133]; (**c**) Nestin+ supporting cells in the mature OC; (**d**) After hair cell loss Nestin expression is increased in the apical turn of the mature mammalian cochlea [142].

a "proof of principle" for future strategies that aim to circumvent or reverse the cell cycle exit within the postnatal mammalian OC that is established during the period of terminal mitosis during embryogenesis [60, 148–151] (Fig. 2a, b). Following the discovery of $p27^{Kip1}$ in the supporting cell population of mammalian auditory epithelium and its function in otic cell cycle control [146, 147], several other cell cycle regulators and their spatiotemporal expression patterns in the developing OC have been identified. These constitute putative targets for small molecule interventions aiming at the induction of proliferative properties in the adult mammalian OC. Section 7.2 summarizes current knowledge about the expression and the functional significance of cell cycle regulators for a regenerative response, in particular the cyclin-dependent kinase inhibitors CDKN1, CDKN2, and the pocket protein family.

7.1 Re-creating Inner Ear Stem Cells Using Ubiquitous Stem Cell Markers: Oct4, Nanog, and Sox2

The pluripotent state of ESCs is governed by a core of transcription factors Oct4, Sox2, and Nanog which are essential for establishing and maintaining the pluripotent ground state (i.e. [152]. Since stem cells have been isolated from the vestibular portion of the inner ear and phenotypically characterized as pluripotent stem cells [117], it is reasonable to include the key pluripotency factors Oct4, Nanog, and Sox2 in current analyses and characterization of inner ear stem cells including the OC. This discovery of stem cells in the vestibular portion of the inner ear in the adult mouse utricle [117] has triggered the search for stem cells in the OC. Stem cells in the mouse OC have been also isolated and characterized from the OC. As outlined above, the isolation of stem cells from the OC has been limited to postnatal developmental stages and isolation from the adult OC has not been achieved [118]. It has been shown that organ of Corti SCs (OCSCs)—isolated from the postnatal OC—can self-renew and differentiate into supporting and hair cell-like cells, which is consistent with a multipotent stem cell state. A systematic comparative analysis of pluripotent ESCs, multipotent NSCs, and OCSCs from the same genetic background aimed at the definition of the molecular signature of OCSCs as related to a pluripotent stem cell state. While Sox2 was maintained in all three cell populations, Oct4 and Nanog were downregulated in OCSCs at the epigenetic, transcriptional, and translational level, which was also observed in multipotent NSCs but not in the pluripotent ESCs [13]. Similarly, in vestibular inner ear stem cells Sox2 expression is maintained, while transcription of Nanog and Oct4 is downregulated [153].

Fig. 7 (continued) (**e**, **f**) Dedifferentiation response in ototoxic drug treated mice. Limited de-differentiation response in columnar supporting cells with down-regulation of Kir4.1 [185]; (**g**, **h**) Dedifferentiation response in ototoxic drug treated guinea pigs. Supporting cells showed an increase in proteins involved in Notch activation [10]

Therefore, OCSCs show no pluripotent molecular signature but similar to NSCs endogenously retain the expression of the pluripotency factor Sox2 as well as c-Myc and Klf4. Interestingly, single factor reprogramming by the exogenous expression of Oct4 with or without the endogenously expressed factors Sox2, c-Myc, and Klf4 is sufficient to generate pluripotent stem cells from adult mouse NSCs [154] and fetal human NSCs [155]. These mouse and human one-factor-induced pluripotent stem cells (1F iPSCs) are similar to respective ESCs in vitro and in vivo. Based on the similar endogenous expression profile of NSCs and OCSCs, it appears likely that OCSCs may be a valuable cellular target for single-factor iPSCs generation. Interestingly, in auditory epithelial cells derived from the human fetal cochlea at developmental stages corresponding to the postnatal mouse cochlea, a pool of Oct4+ and Sox2+ stem cells has been identified [125]. These Oct4+/Sox2+ stem cells have retained their in vitro capacity to differentiate into sensory hair cells and neurons, even after long-term expansion [125]. Therefore single factor iPSCs cell generation using Oct4 may also be a viable option to generate otic derived iPCSs.

Furthermore, it has been demonstrated in genetic mouse models in vivo that the absence or reduced expression of Sox2 in inner ear progenitors resulted in a severe malformation of the auditory sensory epithelium with disorganized and fewer sensory hair cells [23], indicating that the pluripotency factor Sox2 is an important determinant of stemness in the OC. Furthermore, it has recently been shown that the self-renewal potential of Sox2-expressing OCSCs is negatively correlated with the epigenetic methylation status of the inner ear-specific Sox2 enhancers NOP1 and NOP2 [13]. In ESCs, Sox2 functions as a molecular rheostat as the level of Sox2 expression appears to control the expression of critical subsets of genes, thereby shifting its function from self-renewal to differentiation [156]. This functional dualism is also seen for NSCs. In NSCs Sox2 is essential to maintain “stemness” [157], but it also controls the differentiation of neurons in the brain [158] and retinal cells in the eye [159]. These examples indicate that Sox2 has multiple dose- and context dependent functions in pluripotent and multipotent stem cells, as well as differentiated cells [1, 160].

This functional dualism of Sox2 is further extended to the OC as Sox2 appears to serve a pivotal role in establishing progenitors in the prosensory domain [23] and the subsequent differentiation of supporting cells [39]. It has been suggested that Sox2 plays a dose-dependent role in the inversely correlated phenomena of stemness and differentiation [39]. Further evidence for a dose-dependent Sox2 rheostat-like function in the OC is provided by concomitant changes in Sox2 mRNA levels during OC development and the progressive methylation of inner ear selective Sox2 enhancer elements NOP1 and NOP2 [13]. Likewise, Sox2 has also been described to function in both sphere formation and differentiation of inner ear stem cells derived from the vestibular part of the inner ear [161].

The role of the inner ear selective Sox2 enhancer elements NOP1 and NOP2 as a critical factors in establishing the prosensory domain of the OC is further indicated by observations in two allelic Sox2 mouse mutants, Lcc and Ysb [23]. In these mutants neither the protein-coding region nor the promoter of Sox2 was affected. However, sequence analysis revealed that the evolutionarily conserved avian NOP1

and NOP2 sequences [162] map to the murine genomic locus affected by the Lcc mutation [13, 23]. Similarly, in the Ysb mutation identified, chromosomal rearrangement might interfere with the NOP1 and NOP2 sequences [23]. Therefore, both Lcc and Ysb phenotypes appear to be correlated with a compromise in function of the murine NOP1 and NOP2 enhancer elements of Sox2.

As mentioned above, there is increasing evidence that Sox2 plays a dose-dependent role in both stemness and differentiation [39]. Increasing Sox2 levels during late embryonic development may activate an autoregulatory loop mediated by Sox2-binding sites in the NOP1 and NOP2 enhancer elements [13], which may contribute to the maintenance of the supporting cell phenotype, consistent with the reciprocal antagonistic relationship between Sox2 in the differentiating supporting cells and Atoh1 in the differentiating hair cells [39]. The transcriptional downregulation of Sox2 in late postnatal development can be explained by an increase in NOP1 and NOP2 enhancer methylation. Sox2 expression in proliferating prosensory precursors provides a link to early Notch signaling. Sox2 has been identified as a target gene of the Notch ligand Jag1, which is strongly expressed in the proliferating prosensory precursors before cell differentiation [41, 105] and bHLH transcription factors are known to interact with E-box motifs [163, 164], which have been identified in the NOP1 and NOP2 sequences [13]. As the bHLH transcription factors act as transcriptional repressors [70], the binding of Hes1 to NOP1 and NOP2 could interfere with Sox2 at prosensory precursor stage (see Sect. 3). Hes1 expression is downregulated at the time period when the precursor cells exit the cell cycle and undergo cell fate decisions toward hair cell and supporting cell phenotypes [105]. Simultaneously, transcriptional silencing of NOP1 and NOP2 may be released and result in an upregulation of Sox2 expression potentially contributing to the differentiation and maintenance of supporting cells [13].

7.2 Reinitiation of Proliferation of Stem Cell Clones: CKIs and pRb

The organ of Corti is a terminally differentiated organ and the definition of terminal differentiation implies (1) permanent withdrawal from the cell cycle and (2) phenotypic differentiation. These two phenomena are usually irreversible and interdependent. During the development of the organ of Corti progenitor cells proliferate until the time point of "terminal mitosis" at which they leave the cell cycle and differentiate into hair and supporting cells. For both hair cells and supporting cells, terminal mitosis peaks around embryonic day 13.5 (Fig. 8) [56, 148]. This permanent cell cycle withdrawal may be of advantage to the maintenance of a complex cytoarchitecture and functionally relevant for the micromechanical properties which form the basis for sensory transduction. However, if all supporting cells in the organ of Corti have undergone terminal mitosis and terminal differentiation, the reinitiation of the cell cycle in the context of proliferation-based regeneration of hair cells appears outside the biological setting.

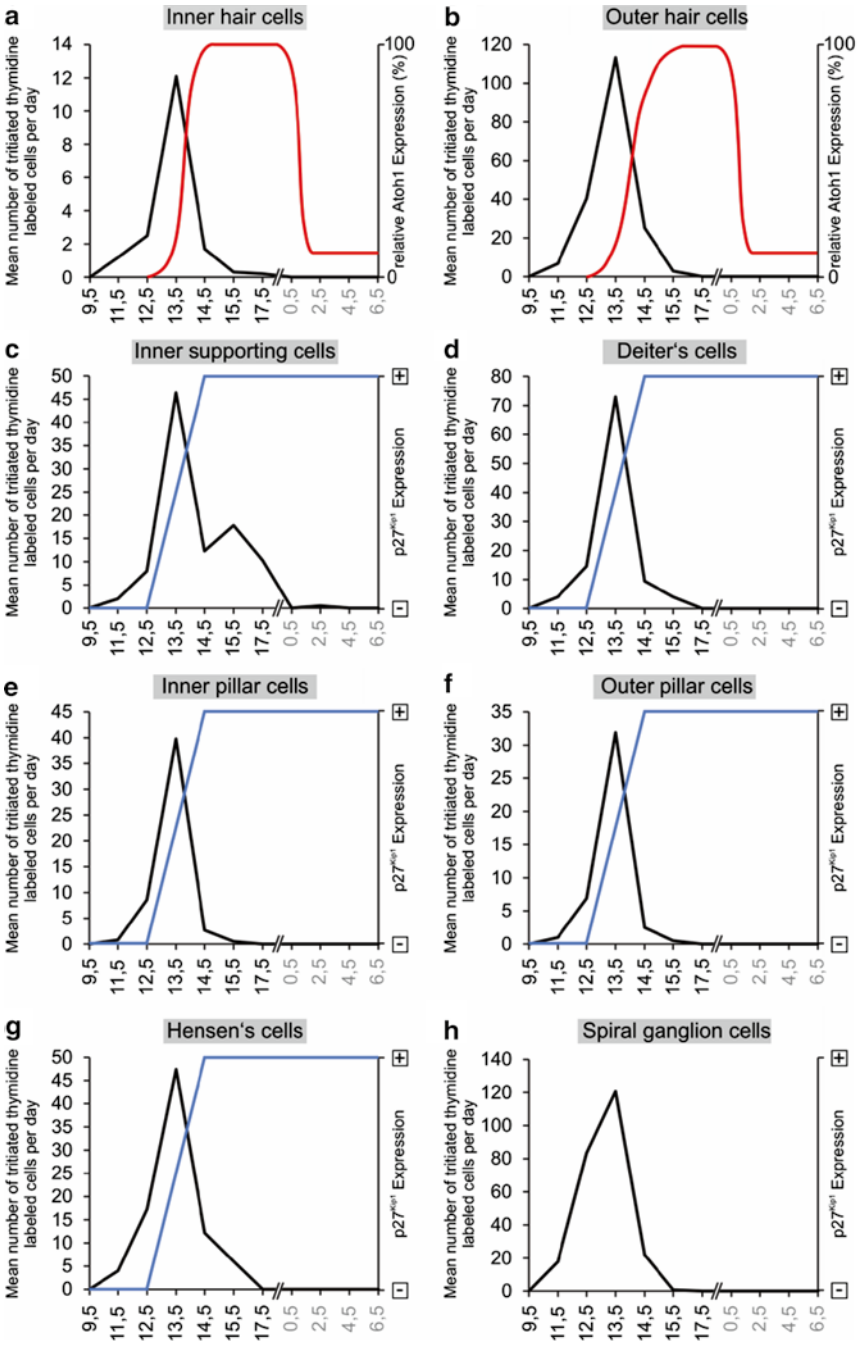

Fig. 8 Hypothetical correlation of labeling activity of different cell types in the developing mouse cochlea with tritiated thymidine (from [148]) and onset of Atoh1 expression in inner (**a**) and outer (**b**) hair cells (from [63]), and $p27^{Kip1}$ expression (from [151, 184]) in different supporting cell types (**c**–**g**), respectively. The highest number of thymidine-labeled cells (*black lines*, **a**–**h**) was observed at embryonic day (**e**) 13.5 in all cochlear cell types, indicating that, dependent on their

The decision to determine if a cell divides or not is taken during the G1 phase of the cell cycle at the restriction point. The restriction point divides the G1 phase into an early/mid phase and a late phase. After cells pass the restriction point, they act mitogen independent and follow through the cell cycle without the need of exogenous stimulation. If a cell exits the cell cycle reversibly and enters a state of quiescence (also termed the G0 state), the restriction point may be passed by application of appropriate exogenous stimuli as observed in avian auditory hair cell epithelium in the event of hair cell loss. However, terminally differentiated cells leave the cell cycle irreversibly and become refractory to exogenous stimuli, implying that the restriction point cannot be passed despite the application of stimulating factors. Consequently, if the supporting cells of the (OC) were terminally differentiated, conceptually reinitiation of the cell cycle is not a simple biological problem.

Therefore, it appears necessary to elucidate and understand the molecular control of the exit from the cell cycle during development and in particular the inhibitory molecular signals that control the restriction point in the OC that may prevent the supporting cells from reinitiating the cell cycle.

At the transcriptional level, the activation of the E2F transcription factor family is essential to S-phase entry. The transcription factor E2F interacts with the cell cycle via the pocket protein of the retinoblastoma protein (pRb) family [53]. In brief, the tumor suppressor pocket proteins exist in phosphorylated and dephosphorylated states. In the dephosphorylated and hence active state, pRb binds to and thereby inhibits E2F binding to target genes. This prevents passage through the restriction point and hence entry into the cell cycle. In a phosphorylated and inactive state, pRb is released from E2F binding which allows initiation of E2F mediated transcription and initiation of S-phase. Therefore, the phosphorylation state of pRb acts like a switch whether a cell enters the cell cycle or not. This pRb-related switch in turn is regulated by cyclin-dependent kinases (CDKs). The CDKs can either be activated by their catalytic subunits, the cyclins, or inhibited by cyclin-dependent kinase inhibitors (CKIs), which are divided into two protein families (Fig. 10a). The ratio of activating cyclin/CDK complexes and inhibiting CKIs will therefore determine the phosphorylation status of the pocket proteins of the pRb family and balance cell division versus cell arrest (Fig. 11a). This cell cycle machinery is also active in the supporting cells and the hair cells of the organ of Corti. Expression studies (Table 2) followed by targeted deletion of the particular regulators (Table 3), have elucidated at least in part their selective roles for these different cell types.

Fig. 8 (continued) spatial distribution in the cochlear duct, the cells underwent terminal mitosis around this time point [148]. Onset and increase of Atoh1 expression in inner (**a**) and outer (**b**) hair cells with a hair cell type-specific slope (*red lines*, data from the basal cochlear turn, adapted from [63]) was observed around the time point of terminal mitosis. Expression of $p27^{Kip1}$ in supporting cells (**c**–**g**) (*blue lines*) followed a steep wave between E12.5 and E14.5 [151, 184] that preceded the wave of cell cycle exit in an apical to basal direction (*X*-axis: *black numbers* indicate days of gestation, *gray numbers* indicate days post-partum; Due to a different conventions regarding the determination of age post-conception in the study by [148], data points derived from this study were dated back by 0.5 day)

Table 2 Expression pattern of cell cycle regulators in the cochlea

Class of CDK inhibitors		Expression pattern in cochlea cell types			Developmental stage in which expression was confirmed	Supplemental information	References
		IHC	OHC	SC			
Cdkn1	Cdkn1A (p21Cip1)	+	+	+	E14.5, pn6, pn60	In early developmental stages in cells of the stria vascularis and the spiral ligament; at pn60 only in Deiter's cells	[52, 54]
	Cdkn1B ($p27^{Kipl}$)	–	–	+	E12, E14, E16, pn2, pn4, pn14, pn21	At E12-E14 in dorsal region of the otocyst; postnatally in $Sox2^+$ inner phalangeal cells, pillar cells, Deiter's cells, and Hensen's cells; at pn2 in cochlear ganglion neurons	[13, 129, 146, 147, 151]
	Cdkn1C (p57Kip2)	(+)	+	+	pn0, pn2, pn3, pn7	At pn0 in cells of the inner sulcus (limbus) and some IHCs; no expression after pn3	[52, 180, 181]
Cdkn2	Cdkn2A (p16Ink4a)	–	–	+	pn14	Co-expression with Sox2; at pn4 only in a subset of $Sox2^+$ cells	[13]
	Cdkn2B (p15Ink4b)	n.d.	n.d.	n.d.	n.d.	n.d.	–
	Cdkn2C (p18Ink4c)	–	–	+	pn2	In mesenchymal cells	[52]
	Cdkn2D (p19Ink4d)	+	+	–	E15.5, pn0, pn10, pn15, pn60	At pn15, pn60 expression decreased in OHCs	[52, 146]

Pocket proteins (pRbs)	Rb1 (p105)	+	+	+	E12.5, E15.5, E18.5, pn6, pn14, pn35	At E12.5 expression in all cells of the otocyst; at E18.5 and pn6 expression only in cells of the GER; in adulthood only in IHC; increasing quantitative expression in postnatal development of the organ of Corti	[54, 168, 169, 182]
	Rbl1 (p107)	–	+	–	pn21	Decreasing quantitative expression in postnatal development of the organ of Corti	[53, 169]
	Rbl2 (p130)	(+)	(+)	+	pn0, pn14, pn30, pn35	At pn0 expression only in SC; at later developmental stages (starting at pn14) expression also in IHC and OHC with an apex-to-base gradient; quantitative increase of expression during postnatal development of the OC	[169]

Cdk cyclin-dependent kinase, *Cdkn* cyclin-dependent kinase inhibitor, *GER* greater epithelial ridge, *IHC* inner hair cell, *n.d.* not analyzed, *OHC* outer hair cell, *SC* supporting cells, *Rb* retinoblastoma protein

Table 3 Inner ear phenotypes of mice deficient for cell cycle regulator genes

Gene name	Function in inner ear development	Phenotypic defects in the inner ear of knockout mice	References
Cdkn2A (p16Ink4a)	n.d.	n.d.	–
Cdkn2B (p15Ink4b)	n.d.	n.d.	–
Cdkn2C (p18Ink4c)	n.d.	n.d.	–
Cdkn2D (p19Ink4d)	Control of the postmitotic state of HCs	Increase in ABR threshold (~50 dB compared to WT animals) starting between 7 and 15 weeks of age; induction of proliferation and apoptosis in HC; progressive HC loss	[54, 167, 180]
Cdkn1A (p21Cip1)	Control of the postmitotic state of HCs	No phenotypic inner ear defects between E16.5 and pn60; no morphological abnormalities; no induction of proliferation	[54, 167, 180]
$Cdkn2D^{-/-}/Cdkn1A^{-/-}$	(See corresponding genes)	Cell cycle reactivation in cochlear HC; HC proliferation and apoptosis	[180]
Cdkn1B ($p27^{Kip1}$)	Cell cycle arrest in SC	Postnatal hyperplasia of SC and HC; progessive loss of HC; and a severe hearing impairment in adulthood	[129, 147]
Cdkn1C (p57Kip2)	n.d. ($p57^{KIP2}$ mediates the differentiation- and survival-promoting functions of Gfi1 on cochlear HCs)	n.d. ($p57^{Kip2}$ expression was downregulated in cochlear HCs of Gfi1-deficient ($^{-/-}$) mice; HC degeneration occurred around pn0 in $Gfi1^{-/-}$ mice)	[181]
Rb1 (p105)	Maintenance of differentiated HCs (and SCs) in a postmitotic state	GER showed an increase in thickness and high numbers of mitotic nuclei at E18.5; excessive numbers of supernumerary HCs and SCs (Deiter's cells and pillar cells); increased apoptosis of HCs; multinucleated HCs;	[54, 168, 182]
Rbl1 (p107)	n.d.	n.d.	–
Rbl2 (p130)	Maintenance of cell cycle arrest and quiescence of postmitotic SCs and HCs	Supernumerary IHCs, OHCs, and $Sox2^{+}$ SCs (particularly Deiter's cells) in the apical and upper middle cochlear turns; ectopic HCs in the apical turn; elevated ABR thresholds at frequencies <12 kHz compared to WT animals; DPOAEs were frequency-specific decreased, but not statistically different to WT animals	[169]
		n.d.	–

ABR auditory brainstem response, *DPOAE* distortion product otoacoustic emissions, *Cdk* cyclin-dependent kinase, *Cdkn* cyclin-dependent kinase inhibitor, *GER* greater epithelial ridge, *IHC* inner hair cell, *n.d.* not determined, *OHC* outer hair cell, *Rb* retinoblastoma protein, *SC* supporting cells, *WT* wild type

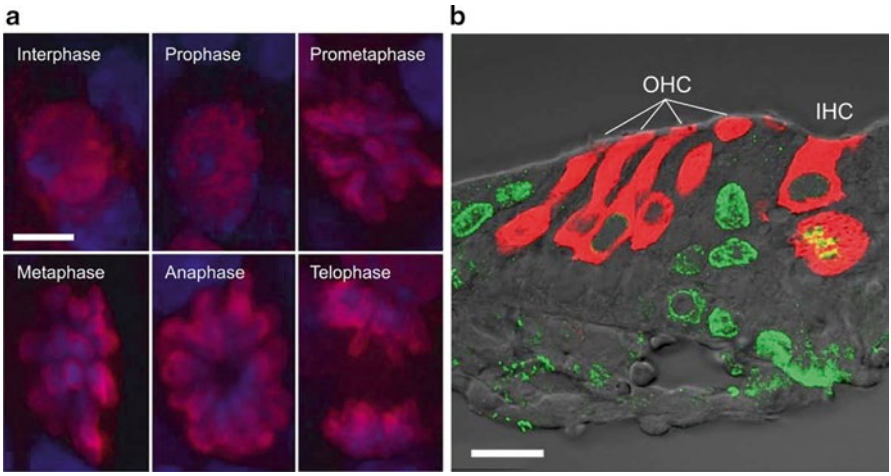

Fig. 9 Ongoing proliferation in the organ of Corti (OC) of $p27^{Kip1}$ deficient mice at postnatal day 6. (**a**) Proliferation in cells within the OC is demonstrated by BrdU-labeling (*red*) that reveals all mitotic stages. (Dapi, *blue*). (**b**) BrdU-labeling (*green*) of a Myosin VII + cell (*red*) next to the inner hair cell (IHC) indicates the generation of a new hair cell based on proliferation in the $p27^{Kip1}$ deficient organ of Corti. (OHC, outer hair cells; Scale bar = 10 μm). (Figure by courtesy of J. Waldhaus and H. Löwenheim)

Based on expression pattern investigations it was found that the CKI $p27^{Kip1}$ from the *CDKN1B* family is selectively expressed in the supporting cells of the OC. The expression appears simultaneous with the onset of differentiation following the terminal mitosis during embryonic development and is maintained throughout adult life [146, 147]. Further elucidation of the functional role of $p27^{Kip1}$ for the proliferative status of supporting cells in the organ of Corti in a knockout mouse revealed cellular hyperplasia in the OC. More importantly, the supporting cells within the normally postmitotic OC continued to divide indicating an intact intrinsic cell cycle machinery that drives mitotic cell division (Fig. 9a) [146, 147] and allows subsequent differentiation into hair cells (Fig. 9b). Complementary in vitro studies using $p27^{GFP}$-transgenic mice showed that isolated $p27^{GFP}$-positve supporting cells from constitutive knock-out of $p27^{Kip1-/-}$ mice divided and gave rise to myosin-VI^{+} hair cell-like cells in vitro even when isolated at mature developmental stages at postnatal day 14. In the $p27^{Kip1-/-}$ knockout situation, this is apparently based on proliferation of supporting cells, as opposed to wild-type $p27^{Kip1}$ which generated hair cells only based on direct conversion of supporting cells into hair cells without a proliferative step. A further study used gene silencing of the $p27^{Kip1}$-encoding gene *CDKN1B* in the in vitro-cultured postnatal OC. The siRNA-induced knockdown of $p27^{Kip1}$ reinitiated the cell cycle re-entry of postmitotic supporting cells, as confirmed by BrdU incorporation in vitro [165]. This finding was expanded to the in vivo situation in a recent study using a tamoxifen-inducible mouse model to delete $p27^{Kip1}$ expression [166]. This study demonstrated that supporting cells in the

postnatal as well as in the adult mammalian cochlea are still responsive to changes in $p27^{Kip1}$ expression.

In summary, expression of $p27^{Kip1}$ protein is initiated at the time point of terminal mitosis and remains present in the functionally mature cochlear supporting cells. Deletion studies for $p27^{Kip1}$ imply that the strong inhibition on cell division may be circumvented by such an approach but may not be sufficient and may have to take other factors into consideration that may act redundant to $p27^{Kip1}$ (Figs. 10b and 11b). How CDK inhibitors are regulated downstream of N-Myc and other transcription factors remains unclear [50].

Moving to the *CDKN1A* family of cyclin-dependent kinase inhibitors $p19^{Ink4d}$ is selectively expressed in hair cells as determined by in situ hybridization and FACS analysis. The targeted deletion of $p19^{Ink4d}$ led to a disruption of the postmitotic state of the HCs. In $p19^{Ink4d}$ knockout mice, HCs were observed to aberrantly re-enter the cell cycle and subsequently undergo apoptosis, resulting in progressive hearing loss [167]. The co-deletion of $p19^{Ink4d}$ and $p21^{Cip1}$ led to cell cycle re-entry of HCs during a restricted period in early postnatal life and to the transient appearance of supernumerary HCs followed by p53-mediated apoptosis. This example demonstrates that maintenance of the postmitotic state can be cooperatively regulated by several CKIs and is critical for survival of hair cells (Figs. 10c and 11c).

Downstream of the CDKs acts the tumor suppressor family of the Retinoblastoma protein (pRb) including the family members p107 and p130. The conditional ablation

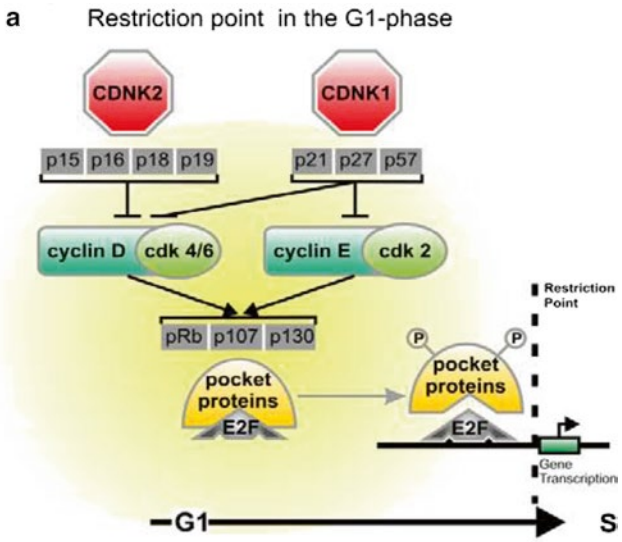

Fig. 10 Ongoing proliferation in the organ of Corti (OC) of $p27^{Kip1}$ deficient mice at postnatal day 6. (**a**) Proliferation in cells within the OC is demonstrated by BrdU-labeling (*red*) that reveals all mitotic stages. (Dapi, *blue*). (**b**) BrdU-labeling (*green*) of a Myosin VII + cell (*red*) next to the inner hair cell (IHC) indicates the generation of a new hair cell based on proliferation in the $p27^{Kip1}$ deficient organ of Corti. (OHC, outer hair cells; Scale bar = 10 μm). (Figure by courtesy of J. Waldhaus and H. Löwenheim)

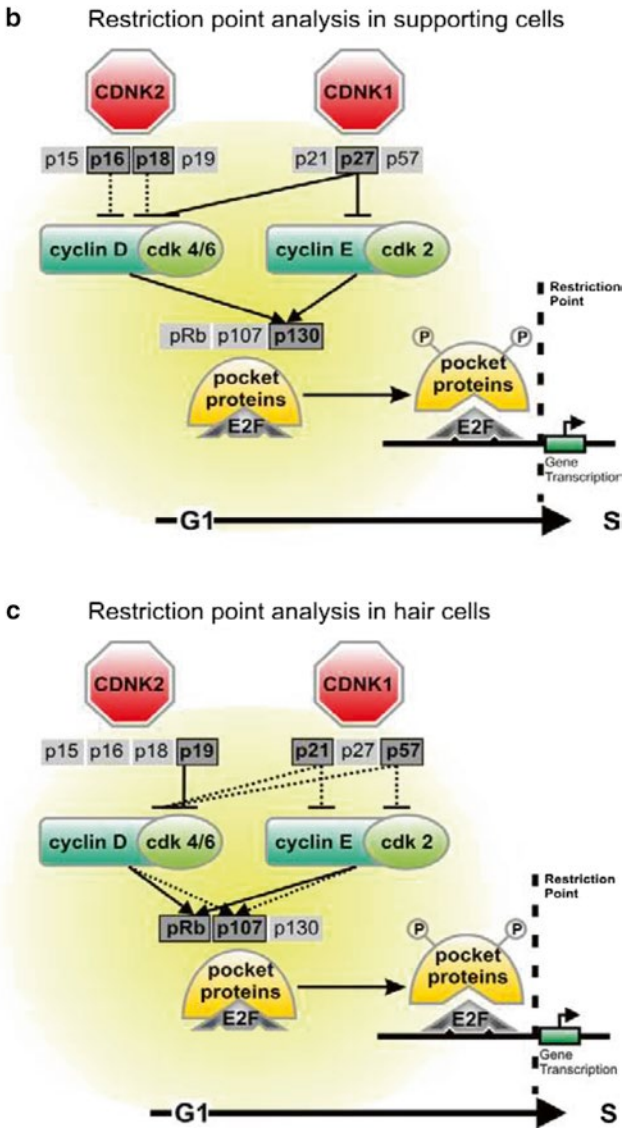

Fig. 10 (continued)

of pRb also generated a phenotype of supernumerary hair cells and supporting cells as well as proliferating supporting cells and hair cells [54, 168]. Targeting hair cells by using an Atoh1-Cre inducible system, deletion of pRb led hair cells to re-enter the cell cycle but they failed to differentiate and underwent cell death leading to complete deafness [137]. Targeting supporting cells by a Prox1-CreERT2 line deletion of pRb led supporting cells to proliferate but they failed to differentiate into hair cells and also underwent cell death with some delay. Finally, the knock-out of the pRb family member p130 mice resulted in hyperplasia of HCs and SCs in the

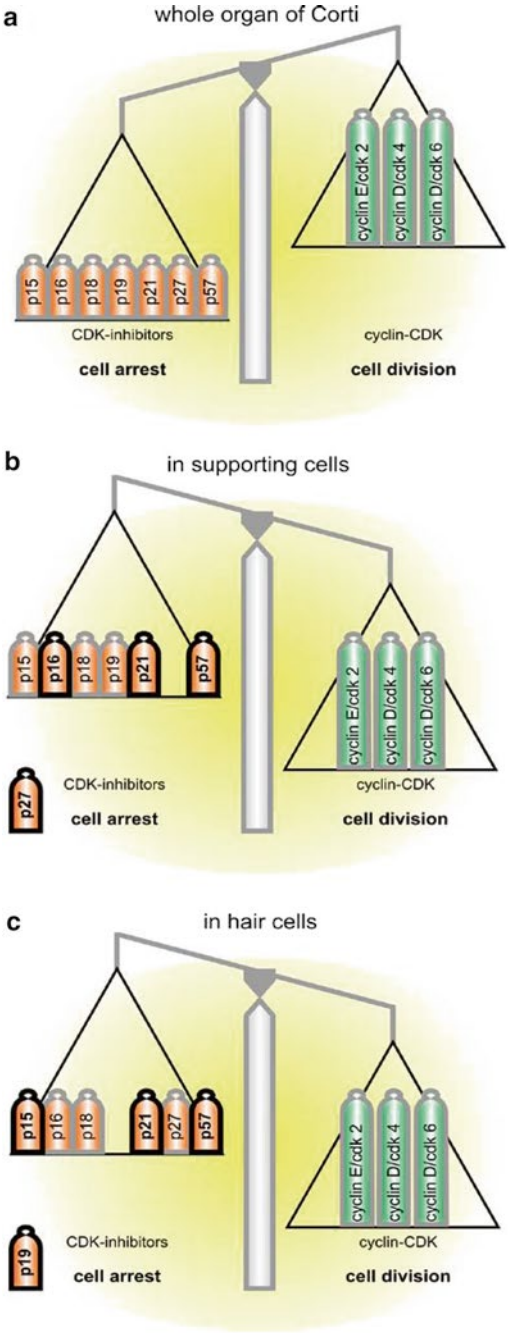

Fig. 11 Hypothetical stoichiometric regulation of the cell cycle transition from G1- to S-phase in cochlear supporting (SC) and hair cells (HC) by cyclins, cyclin-dependent kinases (Cdk) and Cdk-inhibitors. (**a**) The balanced actions of Cdk-inhibitors on one side and cyclin/Cdk-complexes on the other side determine between cell cycle arrest or cell cycle progression. (**b**) In cochlear SCs the balance is shifted towards cell division by inactivation of $p27^{Kip1}$. Other potential Cdk-inhibitors, whose function in cell cycle controls of SCs is not determined experimentally, but which are selectively expressed in SCs are labeled by fat outline. (**c**) In cochlear hair cells the balance is shifted towards cell division by inactivation of p19Ink4d. Other potential Cdk-inhibitors selectively expressed in HCs are labeled by fat outline

apical regions of the cochlea [169]. Interestingly, there was no evidence of transdifferentiation of these supernumerary SCs into HCs. These mice exhibited nearly normal peripheral auditory sensitivity (Fig. 10b, c).

More recently, one Yamanaka factor that is an upstream regulator of several CDK and CDKIs has been investigated in the ear, N-Myc [24, 25, 50]. As expected based on the known position of this transcription factor (Fig. 3), profound effects on proliferation and differentiation were found in these mutants. Unfortunately, all data collected with these proto-oncogenes thus far are based on loss of function studies. What is needed now are studies that demonstrate the gain in proliferation possible with a regulated overexpression of N-Myc, in particular in late stage differentiation when N-Myc is expressed only in hair cells with seemingly no function [55].

8 Non-otic Stem Cells Can Generate Hair Cells and Neurons

The above details show that the transformation of remaining cells in the organ of Corti suffering from various degrees of hair cell loss will require a two step approach: (1) initiating proliferation followed by (2) initiation of differentiation into supporting cells and hair cells. For reasons detailed above, it is essential to generate novel, partially or uncommitted precursors as attempts to transform fully differentiated supporting cells or adult non-sensory otic epithelium seems to be extremely difficult if not impossible [11, 136]. How far these proliferative cells will need to be molecularly transformed to resemble developing neurosensory precursor cells through expression of Sox2, Pax2, Eya1, Gata3, and other factors associated with the neurosensory precursors remains to be elucidated. Following the work on iPS cells, it may be possible to reduce the multitude of genes currently characterized into a smaller essential set but work needs to be done to define such a smaller set of genes.

At the face of it, transformation of cells in a dish toward a hair cell precursor will follow somewhat similar approaches. No matter the origin of these cells, ultimately they need to be turned into an otic neurosensory precursor that has a high yield of hair cell, currently just marginally better than the proof of principle that this is possible [6, 7, 170]. Where these stem cells are derived from may eventually prove to be an issue [171] but, certainly there appears to be no clear-cut bias at the moment. Even mesenchymal-derived stem cells can be converted to hair cell-like cells in vitro [8, 172] using powerful transcription factors [26, 27], even though they do not transform cells along the normal ectodermal-derived placodal developmental pathway [28, 50]. Obviously, generating neurons out of the various precursor cells that can be incorporated into the cochlea seems to be the easier task [6, 153]. These can further be increased by notch signal manipulation and upregulation of the neuron-specific transcription factor Neurog1 [161]. However, manipulating both the Delta/Notch-signaling pathway as well as the Wnt-, Shh-, and retinoic acid-signaling pathways can enhance the commitment toward a hair cell phenotype [132, 161, 173] to over 10 %. Clearly this is progress over the 1 % or less transformation of various stem cells into hair cells possible only a few years ago [6]. Nevertheless, how stable

this commitment toward a hair cell fate will be when these cells are inserted into the adult organ of Corti remains to be seen.

As we learn more about the molecular basis of precursor specification and transformation into hair cells, it is possible that this process can be further optimized. Such optimization will not only provide a higher yield but also a more stable predisposition toward hair cell differentiation. Such robust predisposition is needed to ensure that these cells form fully mature and lasting hair cells in the variously degenerated organs of Corti. In humans, we will be faced with different stages of degeneration based on genetic and epigenetic differences causing unique patterns and progression of hair cell loss. Nevertheless, at the moment the in vitro approach to transform variously derived stem cells into a hair cell precursor shows slightly more success compared to the in vivo approach of molecularly guided transformation of non-sensory otic cells into a functional organ of Corti. However, while successful at the level of inducing hair cell-like cells more reliably, the ex vivo approach has a build-in problem for which there is no apparent solution, namely how to seed the hair cell precursors into the highly ordered pattern of inner and outer hair cells needed for the cochlear function [171]. In fact, we are just beginning to understand the molecular basis of outer and inner hair cell differentiation [56] and the detailed organization of the many different supporting cells that is essential for the normal cochlear function [174] remains to be established quantitatively.

8.1 Theoretical Limitations of the Approach of the Stem Cell Insertion to Restore an Organ of Corti

No matter the source and in vitro success rate of converting various stem cells into hair cell precursors suitable for in vivo differentiation, the proper insertion of such precursors into the appropriate topology of the organ of Corti will offer a unique challenge for which we have, at the moment, no solution. Inserting hair cells prespecified to differentiate along the inner and outer hair cell-specific pathway [56] into the right position and have them survive under the unusual ionic conditions seems to be nearly impossible. This problem becomes even more acute when combined with the need to have these cells differentiate in the correct position and correct numerical ratio relative to the various supporting cells. While supporting cell differentiation clearly depend on both Delta/Notch interaction and thus hair cells [1, 71, 105] and diffusible factors such as Fgfs and BMPs [139, 175] to initiate expression of proper supporting cell differentiation, the details of the topology of this interaction remain unclear.

Indeed, a simple model of Delta/Notch interaction would require that each differentiating hair cell be surrounded by five to six supporting cells which is neither the case for the single row of inner hair cells nor for the three rows of outer hair cells [174]. In fact, outer hair cells seem to have a one-to-one ratio with adjacent outer phalangeal (Deiter's) supporting cells, being separated from each other at the reticular lamina by single phalangeal processes of outer pillar/Deiter's cells in a checkerboard pattern [176]. The quantitative relationship of inner hair cells to surrounding

supporting cells is even less clear due to the fact that boundaries between inner phalangeal cells and border cells as well as the distribution of these cells medial and lateral to inner hair cells is not fully quantitatively established (Fig. 6). Moreover, it is well known that inner hair cells form large areas of contact with each other, but it is unclear at which stage those areas of contact form, and how this relates to the apparent lack of lateral inhibition via the Delta/Notch system between adjacent inner hair cells. It remains speculative how the initial pattern of hair cell and supporting cell distribution appears at the time cell specification is manifested by the progressive upregulation of Atoh1 in postmitotic hair cell precursors [56]. It is also unclear how this original pattern is translated through convergent extension movements [177] into the adult pattern. What is clear is that there is no easy fit in ratios and distribution of hair cells and supporting cells that would be the result of a simple lateral inhibition model of a hair cell completely surrounded by supporting cells.

If indeed the function of the organ of Corti hinges on this delicate adult structure as implied by numerous papers, there will be several equally difficult ways to resolve this during attempts of restoration. One approach could be the sequential seeding of individual hair cell precursors primed to differentiate as inner and outer hair cells into their respective position along the basilar membrane. Such a process would require surgical approaches beyond the comparatively easy insertion of the cochlear implant into the scala tympani and would instead require inserting hair cell precursors topographically correct underneath the tectorial membrane. A variation of this theme would be to induce differentiation of hair cell types in the topographically correct position using a yet to be defined molecular means.

Given this obstacle, perhaps an easier approach would be to print different kinds of hair cells in the appropriate configuration using 3 D printers and insert the entire in vitro assembled OC into the scala media. Provided the matrix holding the hair cells in place can be resorbed over time, such an approach would at least allow inserting the properly reconstituted organ topologically correct under the tectorial membrane. Clearly, even after the transformation of stem cells into hair cell-like cells, the reconstitution of the fully functional OC will not be immediately obtainable due to the complicated interlaced cellular assembly needed for the function, let alone reconstructing the complicated innervation pattern of two types of afferents and efferents to innervate the functionally distinct two types of hair cells [56]. How much of a regeneration that conforms to the normal organization of the OC is needed to establish significant hearing gains remains to be established. This could potentially reduce or aggravate this problem.

9 Defining Novel Differentiation Trajectories to Regenerate a Mammalian Organ of Corti

Thus far we have highlighted the molecular basis of neurosensory development and the potential use of this information to induce dedifferentiation and proliferation of the remaining cells in the organ of Corti that can be transformed into hair cells. The biggest conceptual obstacle seems to be the ordered restoration of the organ of Corti

after loss of all hair cells. This seems to be exceedingly difficult to accomplish from a flat epithelium composed of uniform featureless cells left after long-term degeneration of hair cells. However, this end stage of complete loss of all morphological features seems to be associated only with extremely long deafness typically associated with embryonic or neonatal loss of hair cells. In fact, even slightly delayed loss of hair cells in neonates results in retention of some Pillar cells that are distributed along the organ of Corti [63, 178]. The remaining partially differentiated cells could serve as anchor points for attempts to reconstitute the organ. In most human hearing loss, the progression is slow and topographically distinct, starting typically in the high frequency end of the basal turn and progressing over years to the apex. It seems that the most logical way to sidestep some of the problems outlined above is to start the regeneration process at a time when the remaining cells of the organ of Corti could serve as a scaffold to guide restoration, in other words before hearing loss is profound and has extended over multiple years, wiping out all features of an OC. Recent data suggest that the use of scaffolds may contribute to recovery after acoustic trauma [9].

No matter the starting point (in vitro transformation of stem cells to hair cell precursors or in vivo induced proliferation followed by molecularly induced differentiation as hair cells), mouse models that mimic closely the base-to-apex progression of hair cell loss without any additional damage to the system are needed. Such mouse models could allow testing novel approaches toward restoration of an incompletely lost organ of Corti at various progression stages. In fact, a conditional deletion of Atoh1 using the Atoh1 enhancer mediated expression of Cre seems to achieve exactly this kind of rapid progression of hair cell loss with retention of some features of the organ of Corti suitable to serve as a scaffold for attempts to reconstitute the entire organ [63]. Modern approaches could reinsert the conditionally deleted Atoh1 gene near the remaining LoxP side using the recently developed TALEN technique [179]. Inserting an Atoh1 gene at various times after it was excised by Cre near the remaining LoxP side could allow us to pinpoint exactly how long a window of opportunity exist to reconstitute an organ of Corti after severe and early loss of hair cells and dedifferentiation of the organ of Corti.

All said, such approaches would at the moment favor in vivo molecular over ex vivo approaches. Both approaches are essential to fine-tune our ability to combat the inevitable outcome of hair cell loss, deafness, particularly in the elderly. Recent data showing limited transformation of supporting cells to generate outer hair cell-like cells indicates that indeed an early intervention rather than a long delay might be the best approach to restore hearing.

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References

1. Fritzsch B, Beisel KW, Hansen LA (2006) The molecular basis of neurosensory cell formation in ear development: a blueprint for hair cell and sensory neuron regeneration? Bioessays 28(12):1181–1193
2. Sapède D, Dyballa S, Pujades C (2012) Neurod1 regulates hair cell and neuron development in the zebrafish ear: Cell lineage analysis reveals three different progenitor pools for neurosensory elements in the otic vesicle. J Neurosci 32(46):16424–16434.
3. Stevens G, Flaxman S, Brunskill E, Mascarenhas M, Mathers CD, Finucane M (2011) Global and regional hearing impairment prevalence: an analysis of 42 studies in 29 countries. Eur J Public Health 23(1):146–152.
4. Eppsteiner RW, Shearer AE, Hildebrand MS, Deluca AP, Ji H, Dunn CC, Black-Ziegelbein EA, Casavant TL, Braun TA, Scheetz TE, Scherer SE, Hansen MR, Gantz BJ, Smith RJ (2012) Prediction of cochlear implant performance by genetic mutation: the spiral ganglion hypothesis. Hear Res 292(1–2):51–58
5. Menendez L, Kulik MJ, Page AT, Park SS, Lauderdale JD, Cunningham ML, Dalton S (2013) Directed differentiation of human pluripotent cells to neural crest stem cells. Nat Protoc 8(1):203–212
6. Chen W, Jongkamonwiwat N, Abbas L, Eshtan SJ, Johnson SL, Kuhn S, Milo M, Thurlow JK, Andrews PW, Marcotti W, Moore HD, Rivolta MN (2012) Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. Nature 490(7419):278–282
7. Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S (2010) Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell 141(4):704–716
8. Lin Z, Perez P, Sun Z, Liu JJ, Shin JH, Hyrc KL, Samways D, Egan T, Holley MC, Bao J (2012) Reprogramming of single-cell-derived mesenchymal stem cells into hair cell-like cells. Otol Neurotol 33(9):1648–1655
9. Mizutari K, Fujioka M, Hosoya M, Bramhall N, Okano HJ, Okano H, Edge AS (2013) Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. Neuron 77(1):58–69
10. Batts SA, Shoemaker CR, Raphael Y (2009) Notch signaling and Hes labeling in the normal and drug-damaged organ of Corti. Hear Res 249(1–2):15–22
11. Izumikawa M, Batts SA, Miyazawa T, Swiderski DL, Raphael Y (2008) Response of the flat cochlear epithelium to forced expression of Atoh1. Hear Res 240(1–2):52–56
12. Kopecky B, Fritzsch B (2011) Regeneration of hair cells: making sense of all the noise. Pharmaceuticals (Basel) 4(6):848–879
13. Waldhaus J, Cimerman J, Gohlke H, Ehrich M, Muller M, Lowenheim H (2012) Stemness of the organ of Corti relates to the epigenetic status of Sox2 enhancers. PLoS One 7(5):e36066
14. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
15. Buganim Y, Faddah DA, Cheng AW, Itskovich E, Markoulaki S, Ganz K, Klemm SL, van Oudenaarden A, Jaenisch R (2012) Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. Cell 150(6):1209–1222
16. Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-Messer C, Dolmetsch RE, Tsien RW, Crabtree GR (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476(7359):228–231
17. Pierce ML, Weston MD, Fritzsch B, Gabel HW, Ruvkun G, Soukup GA (2008) MicroRNA-183 family conservation and ciliated neurosensory organ expression. Evol Dev 10(1):106–113
18. Soukup GA, Fritzsch B, Pierce ML, Weston MD, Jahan I, McManus MT, Harfe BD (2009) Residual microRNA expression dictates the extent of inner ear development in conditional Dicer knockout mice. Dev Biol 328(2):328–341
19. Goldman SA, Nedergaard M, Windrem MS (2012) Glial progenitor cell-based treatment and modeling of neurological disease. Science 338(6106):491–495

20. Grocott T, Tambalo M, Streit A (2012) The peripheral sensory nervous system in the vertebrate head: a gene regulatory perspective. Dev Biol 370(1):3–23
21. Bouchard M, de Caprona D, Busslinger M, Xu P, Fritzsch B (2010) Pax2 and Pax8 cooperate in mouse inner ear morphogenesis and innervation. BMC Dev Biol 10:89
22. Schlosser G (2010) Making senses development of vertebrate cranial placodes. Int Rev Cell Mol Biol 283:129–234
23. Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, Tease C, Lovell-Badge R, Steel KP, Cheah KS (2005) Sox2 is required for sensory organ development in the mammalian inner ear. Nature 434(7036):1031–1035
24. Kopecky B, Santi P, Johnson S, Schmitz H, Fritzsch B (2011) Conditional deletion of N-Myc disrupts neurosensory and non-sensory development of the ear. Dev Dyn 240(6):1373–1390
25. Dominguez-Frutos E, Lopez-Hernandez I, Vendrell V, Neves J, Gallozzi M, Gutsche K, Quintana L, Sharpe J, Knoepfler PS, Eisenman R, Trumpp A, Giraldez F, Schimmang T (2011) N-myc controls proliferation, morphogenesis and patterning of the inner ear. J Neurosci 31(19):7178–7189
26. Ahmed M, Xu J, Xu PX (2012) EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear. Development 139(11):1965–1977
27. Ahmed M, Wong YM, Sun J, Xu J, Wang F, Xu P-X (2012) Eya1-Six1 interaction is sufficient to induce hair cell fate in the cochlea by activating Atoh1 expression in cooperation with Sox2. Dev Cell 22(2):377–390
28. Schimmang T (2013) Transcription factors that control inner ear development and their potential for transdifferentiation and reprogramming. Hear Res 297:84–90
29. Kersigo J, D'Angelo A, Gray BD, Soukup GA, Fritzsch B (2011) The role of sensory organs and the forebrain for the development of the craniofacial shape as revealed by Foxg1-cre-mediated microRNA loss. Genesis 49(4):326–341
30. Zou D, Erickson C, Kim EH, Jin D, Fritzsch B, Xu PX (2008) Eya1 gene dosage critically affects the development of sensory epithelia in the mammalian inner ear. Hum Mol Genet 17(21):3340–3356
31. Christophorou NA, Mende M, Lleras-Forero L, Grocott T, Streit A (2010) Pax2 coordinates epithelial morphogenesis and cell fate in the inner ear. Dev Biol 345(2):180–190
32. Cross SH, McKie L, West K, Coghill EL, Favor J, Bhattacharya S, Brown SD, Jackson IJ (2011) The Opdc missense mutation of Pax2 has a milder than loss-of-function phenotype. Hum Mol Genet 20(2):223–234
33. Karis A, Pata I, van Doorninck JH, Grosveld F, de Zeeuw CI, de Caprona D, Fritzsch B (2001) Transcription factor GATA-3 alters pathway selection of olivocochlear neurons and affects morphogenesis of the ear. J Comp Neurol 429(4):615–630
34. Duncan JS, Lim KC, Engel JD, Fritzsch B (2011) Limited inner ear morphogenesis and neurosensory development are possible in the absence of GATA3. Int J Dev Biol 55(3):297–303
35. Ali A, Christie PT, Grigorieva IV, Harding B, Van Esch H, Ahmed SF, Bitner-Glindzicz M, Blind E, Bloch C, Christin P, Clayton P, Gecz J, Gilbert-Dussardier B, Guillen-Navarro E, Hackett A, Halac I, Hendy GN, Lalloo F, Mache CJ, Mughal Z, Ong AC, Rinat C, Shaw N, Smithson SF, Tolmie J, Weill J, Nesbit MA, Thakker RV (2007) Functional characterization of GATA3 mutations causing the hypoparathyroidism-deafness-renal (HDR) dysplasia syndrome: insight into mechanisms of DNA binding by the GATA3 transcription factor. Hum Mol Genet 16(3):265–275
36. Grote D, Souabni A, Busslinger M, Bouchard M (2006) Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. Development 133(1):53–61
37. Pan N, Jahan I, Kersigo J, Kopecky B, Santi P, Johnson S, Schmitz H, Fritzsch B (2011) Conditional deletion of Atoh1 using Pax2-Cre results in viable mice without differentiated cochlear hair cells that have lost most of the organ of Corti. Hear Res 275(1–2):66–80
38. Puligilla C, Dabdoub A, Brenowitz SD, Kelley MW (2010) Sox2 induces neuronal formation in the developing mammalian cochlea. J Neurosci 30(2):714–722

39. Dabdoub A, Puligilla C, Jones JM, Fritzsch B, Cheah KS, Pevny LH, Kelley MW (2008) Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. Proc Natl Acad Sci U S A 105(47):18396–18401
40. Basch ML, Ohyama T, Segil N, Groves AK (2011) Canonical Notch signaling is not necessary for prosensory induction in the mouse cochlea: insights from a conditional mutant of RBPj{kappa}. J Neurosci 31(22):8046–8058
41. Kiernan AE, Xu J, Gridley T (2006) The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. PLoS Genet 2(1):e4
42. Pan W, Jin Y, Stanger B, Kiernan AE (2010) Notch signaling is required for the generation of hair cells and supporting cells in the mammalian inner ear. Proc Natl Acad Sci U S A 107(36):15798–15803
43. Koo SK, Hill JK, Hwang CH, Lin ZS, Millen KJ, Wu DK (2009) Lmx1a maintains proper neurogenic, sensory, and non-sensory domains in the mammalian inner ear. Dev Biol 333(1):14–25
44. Nichols DH, Pauley S, Jahan I, Beisel KW, Millen KJ, Fritzsch B (2008) Lmx1a is required for segregation of sensory epithelia and normal ear histogenesis and morphogenesis. Cell Tissue Res 334(3):339–358
45. Raft S, Nowotschin S, Liao J, Morrow BE (2004) Suppression of neural fate and control of inner ear morphogenesis by Tbx1. Development 131(8):1801–1812
46. Deng M, Pan L, Xie X, Gan L (2010) Requirement for Lmo4 in the vestibular morphogenesis of mouse inner ear. Dev Biol 338(1):38–49
47. Radde-Gallwitz K, Pan L, Gan L, Lin X, Segil N, Chen P (2004) Expression of Islet1 marks the sensory and neuronal lineages in the mammalian inner ear. J Comp Neurol 477(4):412–421
48. Bermingham-McDonogh O, Oesterle EC, Stone JS, Hume CR, Huynh HM, Hayashi T (2006) Expression of Prox1 during mouse cochlear development. J Comp Neurol 496(2):172–186
49. Fritzsch B, Dillard M, Lavado A, Harvey NL (2010) Canal cristae growth and fiber extension to the outer hair cells require Prox1 activity. PLoS One 5(2):1–12
50. Kopecky B, Fritzsch B (2012) The Myc road to hearing restoration. Cells 1:667–698
51. Pauley S, Lai E, Fritzsch B (2006) Foxg1 is required for morphogenesis and histogenesis of the mammalian inner ear. Dev Dyn 235(9):2470–2482
52. Laine H, Sulg M, Kirjavainen A, Pirvola U (2010) Cell cycle regulation in the inner ear sensory epithelia: role of cyclin D1 and cyclin-dependent kinase inhibitors. Dev Biol 337(1):134–146
53. Rocha-Sanchez SM, Beisel KW (2007) Pocket proteins and cell cycle regulation in inner ear development. Int J Dev Biol 51(6–7):585–595
54. Mantela J, Jiang Z, Ylikoski J, Fritzsch B, Zacksenhaus E, Pirvola U (2005) The retinoblastoma gene pathway regulates the postmitotic state of hair cells of the mouse inner ear. Development 132(10):2377–2388
55. Kopecky BJ, Jahan I, Fritzsch B (2013) Correct timing of proliferation and differentiation is necessary for normal inner ear development and auditory hair cell viability. Dev Dyn 242(2):132–147
56. Jahan I, Pan N, Kersigo J, Fritzsch B (2013) Beyond generalized hair cells: molecular cues for hair cell types. Hear Res 297:30–41
57. Jahan I, Pan N, Kersigo J, Fritzsch B (2010) Neurod1 suppresses hair cell differentiation in ear ganglia and regulates hair cell subtype development in the cochlea. PLoS One 5(7):e11661
58. Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ (1998) neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20(3):469–482
59. Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY (1999) Math1: an essential gene for the generation of inner ear hair cells. Science 284(5421):1837–1841

60. Matei V, Pauley S, Kaing S, Rowitch D, Beisel KW, Morris K, Feng F, Jones K, Lee J, Fritzsch B (2005) Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. Dev Dyn 234(3):633–650
61. Liu M, Pereira FA, Price SD, Chu MJ, Shope C, Himes D, Eatock RA, Brownell WE, Lysakowski A, Tsai MJ (2000) Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems. Genes Dev 14(22):2839–2854
62. Kim WY, Fritzsch B, Serls A, Bakel LA, Huang EJ, Reichardt LF, Barth DS, Lee JE (2001) NeuroD-null mice are deaf due to a severe loss of the inner ear sensory neurons during development. Development 128(3):417–426
63. Pan N, Jahan I, Kersigo J, Duncan J, Kopecky B, Fritzsch B (2012) A novel Atoh1 'self-terminating' mouse model reveals the necessity of proper Atoh1 expression level and duration for inner ear hair cell differentiation and viability. PLoS One 7(1):e30358
64. Chen W, Cacciabue-Rivolta DI, Moore HD, Rivolta MN (2007) The human fetal cochlea can be a source for auditory progenitors/stem cells isolation. Hear Res 233(1–2):23–29
65. Kopan R, Ilagan MXG (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137(2):216–233
66. D'Souza B, Miyamoto A, Weinmaster G (2008) The many facets of Notch ligands. Oncogene 27(38):5148–5167
67. Fortini ME, Bilder D (2009) Endocytic regulation of Notch signaling. Curr Opin Genet Dev 19(4):323–328
68. Fortini ME (2009) Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 16(5):633–647
69. Kageyama R, Nakanishi S (1997) Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 7(5):659–665
70. Kageyama R, Ohtsuka T, Kobayashi T (2007) The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 134(7):1243–1251
71. Doetzlhofer A, Basch ML, Ohyama T, Gessler M, Groves AK, Segil N (2009) Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. Dev Cell 16(1):58–69
72. Bray S, Bernard F (2010) Notch targets and their regulation. Curr Top Dev Biol 92:253–275
73. Krejčí A, Bray S (2007) Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers. Genes Dev 21(11):1322–1327
74. Cooper MTD, Tyler DM, Furriols M, Chalkiadaki A, Delidakis C, Bray S (2000) Spatially restricted factors cooperate with Notch in the regulation of Enhancer of split genes. Dev Biol 221(2):390–403
75. Shimojo H, Ohtsuka T, Kageyama R (2008) Oscillations in Notch signaling regulate maintenance of neural progenitors. Neuron 58(1):52–64
76. Krejčí A, Bernard F, Housden BE, Collins S, Bray SJ (2009) Direct response to notch activation: signaling crosstalk and incoherent logic. Sci Signal 2(55):ra1
77. Palomero T, Wei KL, Odom DT, Sulis ML, Real PJ, Margolin A, Barnes KC, O'Neil J, Neuberg D, Weng AP, Aster JC, Sigaux F, Soulier J, Look AT, Young RA, Califano A, Ferrando AA (2006) NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A 103(48):18261–18266
78. Ronchini C, Capobianco AJ (2001) Induction of cyclin D1 transcription and CDK2 activity by Notchic: implication for cell cycle disruption in transformation by Notchic. Mol Cell Biol 21(17):5925–5934
79. Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 20(13):3427–3436
80. Murata K, Hattori M, Hirai N, Shinozuka Y, Hirata H, Kageyama R, Sakai T, Minato N (2005) Hes1 directly controls cell proliferation through the transcriptional repression of p27 Kip1. Mol Cell Biol 25(10):4262–4271

81. Adam J, Myat A, Le Roux I, Eddison M, Henrique D, Ish-Horowicz D, Lewis J (1998) Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. Development 125(23):4645–4654
82. Lewis J (1998) Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol 9(6):583–589
83. Eddison M, Le Roux I, Lewis J (2000) Notch signaling in the development of the inner ear: lessons from Drosophila. Proc Natl Acad Sci U S A 97(22):11692–11699
84. Zine A (2003) Molecular mechanisms that regulate auditory hair-cell differentiation in the mammalian cochlea. Mol Neurobiol 27(2):223–238
85. Lanford PJ, Lan Y, Jiang R, Lindsell C, Weinmaster G, Gridley T, Kelley MW (1999) Notch signalling pathway mediates hair cell development in mammalian cochlea. Nat Genet 21(3):289–292
86. Zine A, Van de Water TR, de Ribaupierre F (2000) Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. Development 127(15):3373–3383
87. Daudet N, Lewis J (2005) Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. Development 132(3):541–551
88. Lindsell CE, Boulter J, DiSibio G, Gossler A, Weinmaster G (1996) Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol Cell Neurosci 8(1):14–27
89. Lewis AK, Frantz GD, Carpenter DA, De Sauvage FJ, Gao WQ (1998) Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. Mech Dev 78(1–2):159–163
90. Morrison A, Hodgetts C, Gossler A, Hrabé De Angelis M, Lewis J (1999) Expression of Delta1 and Serrate1 (Jagged 1) in the mouse inner ear. Mech Dev 84(1–2):169–172
91. Kiernan AE, Ahituv N, Fuchs H, Balling R, Avraham KB, Steel KP, De Angelis MH (2001) The Notch ligand Jagged1 is required for inner ear sensory development. Proc Natl Acad Sci U S A 98(7):3873–3878
92. Brooker R, Hozumi K, Lewis J (2006) Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. Development 133(7):1277–1286
93. Hartman BH, Hayashi T, Nelson BR, Bermingham-McDonogh O, Reh TA (2007) Dll3 is expressed in developing hair cells in the mammalian cochlea. Dev Dyn 236(10):2875–2883
94. Haddon C, Jiang YJ, Smithers L, Lewis J (1998) Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. Development 125(23):4637–4644
95. Cole LK, Le Roux I, Nunes F, Laufer E, Lewis J, Wu DK (2000) Sensory organ generation in the chicken inner ear: contributions of bone morphogenetic protein 4, Serrate1, and lunatic fringe. J Comp Neurol 424(3):509–520
96. Zhang N, Martin GV, Kelley MW, Gridley T (2000) A mutation in the Lunatic fringe gene suppresses the effects of a Jagged2 mutation on inner hair cell development in the cochlea. Curr Biol 10(11):659–662
97. Tsai H, Hardisty RE, Rhodes C, Kiernan AE, Roby P, Tymowska-Lalanne Z, Mburu P, Rastan S, Hunter AJ, Brown SDM, Steel KP (2001) The mouse slalom mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti. Hum Mol Genet 10(5):507–512
98. Kiernan AE, Cordes R, Kopan R, Gossler A, Gridley T (2005) The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. Development 132(19):4353–4362
99. Mizutani T, Taniguchi Y, Aoki T, Hashimoto N, Honjo T (2001) Conservation of the biochemical mechanisms of signal transduction among mammalian notch family members. Proc Natl Acad Sci U S A 98(16):9026–9031
100. Yamamoto N, Chang W, Kelley MW (2011) Rbpj regulates development of prosensory cells in the mammalian inner ear. Dev Biol 353(2):367–379

101. Daudet N, Ariza-McNaughton L, Lewis J (2007) Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear. Development 134(12):2369–2378
102. Lanford PJ, Kelley MW (2005) Notch signaling and cell fate specification. In: Kelley MW et al (eds) Development of the inner ear. Springer, New York, pp 122–157
103. Hayashi T, Kokubo H, Hartman BH, Ray CA, Reh TA, Bermingham-McDonogh O (2008) Hesr1 and Hesr2 may act as early effectors of Notch signaling in the developing cochlea. Dev Biol 316(1):87–99
104. Li S, Mark S, Radde-Gallwitz K, Schlisner R, Chin MT, Chen P (2008) Hey2 functions in parallel with Hes1 and Hes5 for mammalian auditory sensory organ development. BMC Dev Biol 8:20
105. Zine A, Aubert A, Qiu JP, Therianos S, Guillemot F, Kageyama R, de Ribaupierre F (2001) Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. J Neurosci 21(13):4712–4720
106. Tateya T, Imayoshi I, Tateya I, Ito J, Kageyama R (2011) Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development. Dev Biol 352(2):329–340
107. Yang H, Xie X, Deng M, Chen X, Gan L (2010) Generation and characterization of Atoh1-Cre knock-in mouse line. Genesis 48(6):407–413
108. Raphael Y (2002) Cochlear pathology, sensory cell death and regeneration. Br Med Bull 63:25–38
109. Warchol ME (2011) Sensory regeneration in the vertebrate inner ear: differences at the levels of cells and species. Hear Res 273(1–2):72–79
110. Morest DK, Cotanche DA (2004) Regeneration of the inner ear as a model of neural plasticity. J Neurosci Res 78(4):455–460
111. Fekete DM, Muthukumar S, Karagogeos D (1998) Hair cells and supporting cells share a common progenitor in the avian inner ear. J Neurosci 18(19):7811–7821
112. Forge A, Li L, Nevill G (1998) Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. J Comp Neurol 397(1):69–88
113. Forge A, Li L, Corwin JT, Nevill G (1993) Ultrastructural evidence for hair cell regeneration in the mammalian inner-ear. Science 259(5101):1616–1619
114. Burns JC, Cox BC, Thiede BR, Zuo J, Corwin JT (2012) In vivo proliferative regeneration of balance hair cells in newborn mice. J Neurosci 32(19):6570–6577
115. Golub JS, Tong L, Ngyuen TB, Hume CR, Palmiter RD, Rubel EW, Stone JS (2012) Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. J Neurosci 32(43):15093–15105
116. Kawamoto K, Izumikawa M, Beyer LA, Atkin GM, Raphael Y (2009) Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. Hear Res 247(1):17–26
117. Li HW, Liu H, Heller S (2003) Pluripotent stem cells from the adult mouse inner ear. Nat Med 9(10):1293–1299
118. Oshima K, Grimm CM, Corrales CE, Senn P, Moneder RM, Geleoc GSG, Edge A, Holt JR, Heller S (2007) Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. J Assoc Res Otolaryngol 8(1):18–31
119. Savary E, Hugnot JP, Chassigneux Y, Travo C, Duperray C, Van De Water T, Zine A (2007) Distinct population of hair cell progenitors can be isolated from the postnatal mouse cochlea using side population analysis. Stem Cells 25(2):332–339
120. Smeti I, Savary E, Capelle V, Hugnot JP, Uziel A, Zine A (2011) Expression of candidate markers for stem/progenitor cells in the inner ears of developing and adult GFAP and nestin promoter-GFP transgenic mice. Gene Expr Patterns 11(1–2):22–32
121. Zhai SQ, Shi L, Wang BE, Zheng GL, Song W, Hu YY, Gao WQ (2005) Isolation and culture of hair cell progenitors from postnatal rat cochleae. J Neurobiol 65(3):282–293
122. Zhang Y, Zhai S-q, Shou J, Song W, Sun J-h, Guo W, Zheng G-l, Hu Y-y, Gao W-Q (2007) Isolation, growth and differentiation of hair cell progenitors from the newborn rat cochlear greater epithelial ridgele. J Neurosci Methods 164(2):271–279
123. Zheng JL, Gao WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3(6):580–586

124. Shou JY, Zheng JL, Gao WQ (2003) Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. Mol Cell Neurosci 23(2):169–179
125. Chen W, Johnson SL, Marcotti W, Andrews PW, Moore HD, Rivolta MN (2009) Human fetal auditory stem cells can be expanded in vitro and differentiate into functional auditory neurons and hair cell-like cells. Stem Cells 27(5):1196–1204
126. Rask-Andersen H, Bostrom M, Gerdin B, Kinnefors A, Nyberg G, Engstrand T, Miller JM, Lindholm D (2005) Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. Hear Res 203(1–2):180–191
127. Lang HN, Ebihara Y, Schmiedt RA, Minamiguchi H, Zhou DH, Smythe N, Liu LY, Ogawa M, Schulte BA (2006) Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: mesenchymal cells and fibrocytes. J Comp Neurol 496(2):187–201
128. Okano T, Nakagawa T, Kita T, Kada S, Yoshimoto M, Nakahata T, Ito J (2008) Bone marrow-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the mouse cochlea. J Neurosci Res 86(8):1758–1767
129. White PM, Doetzlhofer A, Lee YS, Groves AK, Segil N (2006) Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. Nature 441(7096):984–987
130. Hu Z, Corwin JT (2007) Inner ear hair cells produced in vitro by a mesenchymal-to-epithelial transition. Proc Natl Acad Sci U S A 104(42):16675–16680
131. Sinkkonen ST, Chai R, Jan TA, Hartman BH, Laske RD, Gahlen F, Sinkkonen W, Cheng AG, Oshima K, Heller S (2011) Intrinsic regenerative potential of murine cochlear supporting cells. Sci Rep 1:26
132. Shi F, Kempfle JS, Edge ASB (2012) Wnt-responsive Lgr5-expressing stem cells are hair cell progenitors in the cochlea. J Neurosci 32(28):9639–9648
133. Chai R, Kuo B, Wang T, Liaw EJ, Xia A, Jan TA, Liu Z, Taketo MM, Oghalai JS, Nusse R, Zuo J, Cheng AG (2012) Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. Proc Natl Acad Sci U S A 109(21):8167–8172
134. Kelly MC, Chang Q, Pan A, Lin X, Chen P (2012) Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. J Neurosci 32(19):6699–6710
135. Liu Z, Dearman JA, Cox BC, Walters BJ, Zhang L, Ayrault O, Zindy F, Gan L, Roussel MF, Zuo J (2012) Age-dependent in vivo conversion of mouse cochlear pillar and Deiters' cells to immature hair cells by Atoh1 ectopic expression. J Neurosci 32(19):6600–6610
136. Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, Brough DE, Raphael Y (2005) Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat Med 11(3):271–276
137. Weber T, Corbett MK, Chow LM, Valentine MB, Baker SJ, Zuo J (2008) Rapid cell-cycle reentry and cell death after acute inactivation of the retinoblastoma gene product in postnatal cochlear hair cells. Proc Natl Acad Sci U S A 105(2):781–785
138. Yu Y, Weber T, Yamashita T, Liu Z, Valentine MB, Cox BC, Zuo J (2010) In vivo proliferation of postmitotic cochlear supporting cells by acute ablation of the retinoblastoma protein in neonatal mice. J Neurosci 30(17):5927–5936
139. Fritzsch B, Jahan I, Pan N, Kersigo J, Duncan J, Kopecky B (2011) Dissecting the molecular basis of organ of Corti development: where are we now? Hear Res 276(1–2):16–26
140. Ronaghi M, Nasr M, Heller S (2012) Concise review: inner ear stem cells – an oxymoron, but why? Stem Cells 30(1):69–74
141. Lopez IA, Zhao PM, Yamaguchi M, de Vellis J, Espinosa-Jeffrey A (2004) Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. Int J Dev Neurosci 22(4):205–213
142. Watanabe R, Morell MH, Miller JM, Kanicki AC, O'Shea KS, Altschuler RA, Raphael Y (2012) Nestin-expressing cells in the developing, mature and noise-exposed cochlear epithelium. Mol Cell Neurosci 49(2):104–109
143. Malgrange B, Belachew S, Thiry M, Nguyen L, Rogister B, Alvarez ML, Rigo JM, Van De Water TR, Moonen G, Lefebvre PP (2002) Proliferative generation of mammalian auditory hair cells in culture. Mech Dev 112(1–2):79–88

144. Oshima K, Senn P, Heller S (2009) Isolation of sphere-forming stem cells from the mouse inner ear. Methods Mol Biol 493:141–162
145. Chai R, Xia A, Wang T, Jan TA, Hayashi T, Bermingham-McDonogh O, Cheng AG (2011) Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. J Assoc Res Otolaryngol 12(4):455–469
146. Chen P, Segil N (1999) p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development 126(8):1581–1590
147. Löwenheim H, Furness DN, Kil J, Zinn C, Gultig K, Fero ML, Frost D, Gummer AW, Roberts JM, Rubel EW, Hackney CM, Zenner HP (1999) Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. Proc Natl Acad Sci U S A 96(7):4084–4088
148. Ruben RJ (1967) Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol Suppl 220:1–44
149. Roberson DW, Rubel EW (1994) Cell division in the gerbil cochlea after acoustic trauma. Am J Otol 15(1):28–34
150. Chardin S, Romand R (1995) Regeneration and mammalian auditory hair cells. Science 267(5198):707–711
151. Lee YS, Liu F, Segil N (2006) A morphogenetic wave of p27Kip1 transcription directs cell cycle exit during organ of Corti development. Development 133(15):2817–2826
152. Young RA (2011) Control of the embryonic stem cell state. Cell 144(6):940–954
153. Martinez-Monedero R, Yi E, Oshima K, Glowatzki E, Edge AS (2008) Differentiation of inner ear stem cells to functional sensory neurons. Dev Neurobiol 68(5):669–684
154. Kim JB, Sebastiano V, Wu G, Arauzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, Ehrich M, van den Boom D, Meyer J, Hubner K, Bernemann C, Ortmeier C, Zenke M, Fleischmann BK, Zaehres H, Scholer HR (2009) Oct4-induced pluripotency in adult neural stem cells. Cell 136(3):411–419
155. Kim JB, Greber B, Arauzo-Bravo MJ, Meyer J, Park KI, Zaehres H, Scholer HR (2009) Direct reprogramming of human neural stem cells by OCT4. Nature 461(7264):649–653
156. Kopp JL, Ormsbee BD, Desler M, Rizzino A (2008) Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 26(4):903–911
157. Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007) In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. Cell Stem Cell 1(5):515–528
158. Cavallaro M, Mariani J, Lancini C, Latorre E, Caccia R, Gullo F, Valotta M, DeBiasi S, Spinardi L, Ronchi A, Wanke E, Brunelli S, Favaro R, Ottolenghi S, Nicolis SK (2008) Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. Development 135(3):541–557
159. Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH (2006) SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev 20(9):1187–1202
160. Pevny LH, Nicolis SK (2010) Sox2 roles in neural stem cells. Int J Biochem Cell Biol 42(3):421–424
161. Jeon SJ, Fujioka M, Kim SC, Edge AS (2011) Notch signaling alters sensory or neuronal cell fate specification of inner ear stem cells. J Neurosci 31(23):8351–8358
162. Uchikawa M, Ishida Y, Takemoto T, Kamachi Y, Kondoh H (2003) Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. Dev Cell 4(4):509–519
163. Chaudhary J, Skinner MK (1999) Basic helix-loop-helix proteins can act at the E-box within the serum response element of the c-fos promoter to influence hormone-induced promoter activation in Sertoli cells. Mol Endocrinol 13(5):774–786
164. Fritzsch B, Eberl DF, Beisel KW (2010) The role of bHLH genes in ear development and evolution: revisiting a 10-year-old hypothesis. Cell Mol Life Sci 67(18):3089–3099
165. Ono K, Nakagawa T, Kojima K, Matsumoto M, Kawauchi T, Hoshino M, Ito J (2009) Silencing p27 reverses post-mitotic state of supporting cells in neonatal mouse cochleae. Mol Cell Neurosci 42(4):391–398

166. Oesterle EC, Chien WM, Campbell S, Nellimarla P, Fero ML (2011) p27(Kip1) is required to maintain proliferative quiescence in the adult cochlea and pituitary. Cell Cycle 10(8):1237–1248
167. Chen P, Zindy F, Abdala C, Liu F, Li X, Roussel MF, Segil N (2003) Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. Nat Cell Biol 5(5):422–426
168. Sage C, Huang M, Vollrath MA, Brown MC, Hinds PW, Corey DP, Vetter DE, Chen ZY (2006) Essential role of retinoblastoma protein in mammalian hair cell development and hearing. Proc Natl Acad Sci U S A 103(19):7345–7350
169. Rocha-Sanchez SM, Scheetz LR, Contreras M, Weston MD, Korte M, McGee J, Walsh EJ (2011) Mature mice lacking Rbl2/p130 gene have supernumerary inner ear hair cells and supporting cells. J Neurosci 31(24):8883–8893
170. Oshima K, Suchert S, Blevins NH, Heller S (2010) Curing hearing loss: patient expectations, health care practitioners, and basic science. J Commun Disord 43(4):311–318
171. Jongkamonwiwat N, Zine A, Rivolta MN (2010) Stem cell based therapy in the inner ear: appropriate donor cell types and routes for transplantation. Curr Drug Targets 11(7):888–897
172. Duran Alonso MB, Feijoo-Redondo A, Conde de Felipe M, Carnicero E, Garcia AS, Garcia-Sancho J, Rivolta MN, Giraldez F, Schimmang T (2012) Generation of inner ear sensory cells from bone marrow-derived human mesenchymal stem cells. Regen Med 7(6):769–783
173. Kondo T, Johnson SA, Yoder MC, Romand R, Hashino E (2005) Sonic hedgehog and retinoic acid synergistically promote sensory fate specification from bone marrow-derived pluripotent stem cells. Proc Natl Acad Sci U S A 102(13):4789–4794
174. Zetes DE, Tolomeo JA, Holley MC (2012) Structure and mechanics of supporting cells in the Guinea pig organ of corti. PLoS One 7(11):e49338
175. Groves AK, Fekete DM (2012) Shaping sound in space: the regulation of inner ear patterning. Development 139(2):245–257
176. Lim DJ (1986) Functional structure of the organ of Corti: a review. Hear Res 22:117–146
177. Chacon-Heszele MF, Ren D, Reynolds AB, Chi F, Chen P (2012) Regulation of cochlear convergent extension by the vertebrate planar cell polarity pathway is dependent on p120-catenin. Development 139(5):968–978
178. Pauley S, Kopecky B, Beisel K, Soukup G, Fritzsch B (2008) Stem cells and molecular strategies to restore hearing. Panminerva Med 50(1):41–53
179. Pennisi E (2012) The tale of the TALEs. Science 338(6113):1408–1411
180. Laine H, Doetzlhofer A, Mantela J, Ylikoski J, Laiho M, Roussel MF, Segil N, Pirvola U (2007) p19(Ink4d) and p21(Cip1) collaborate to maintain the postmitotic state of auditory hair cells, their codeletion leading to DNA damage and p53-mediated apoptosis. J Neurosci 27(6):1434–1444
181. Kirjavainen A, Sulg M, Heyd F, Alitalo K, Yla-Herttuala S, Moroy T, Petrova TV, Pirvola U (2008) Prox1 interacts with Atoh1 and Gfi1, and regulates cellular differentiation in the inner ear sensory epithelia. Dev Biol 322(1):33–45
182. Sage C, Huang M, Karimi K, Gutierrez G, Vollrath MA, Zhang DS, Garcia-Anoveros J, Hinds PW, Corwin JT, Corey DP, Chen ZY (2005) Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. Science 307(5712):1114–1118
183. Fritzsch B, Kopecky B (2012) Neurosensory specification and development. In: Brenner's Encyclopedia of Genetics, 2nd Edition, Ed by S. Maloy and K Hughes, released 2013. Elsevier, Amsterdam, pp 345–354
184. Chen P, Johnson JE, Zoghbi HY, Segil N (2002) The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. Development 129(10):2495–2505
185. Taylor RR, Jagger DJ, Forge A. Defining the cellular environment in the organ of Corti following extensive hair cell loss: a basis for future sensory cell replacement in the Cochlea. PLoS One. 2012;7(1):e30577.

Adult Human Corneal Epithelial Stem Cells

Nick Di Girolamo

Abstract The cornea's accessibility and visualization with minimally invasive techniques renders it an ideal organ to study SC and their differentiated progeny. The limbus houses, nurtures, and protects a rare population of epithelial stem cells which play a critical role in the long-term maintenance of the cornea. Despite the absence of an exclusive marker that identifies these cells with pinpoint accuracy, significant advances have been made towards identifying, isolating, cultivating, and transplanting limbal epithelial stem cells (LESC) for corneal reconstruction. However, determining the signals, factors, and mechanisms that maintain their "stemness" in vitro and in situ has proven major obstacles in progressing the field. Knowledge of the key molecules that comprise the niche and the signaling pathways and genetic programs that dictate LESC quiescence and differentiation is essential to improve current and develop effective next-generation cell-based therapies. This chapter will highlight limitations and controversies in the field and summarize the key concepts and experimental paradigms that have inspired researchers to develop cell therapies for patients with blinding corneal disease.

Keywords Cornea • Epithelia • Regeneration • Injury • Adult stem cells

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Abbreviations

BM	Basement membrane
CK	Cytokeratins
ECM	Extracellular membrane
ESC	Epithelial stem cells
FACS	Fluorescent-activated cell sorting
HAM	Human amniotic membrane
iPSC	Induced pluripotent stem cells
LESC	Limbal epithelial stem cells
LSCD	Limbal stem cell deficiency
SC	Stem cells
SP	Side population
TAC	Transient amplifying cells
TDC	Terminally differentiated cells
VN	Vitronectin

1 Introduction

The eye is a highly specialized, light-sensitive organ, often referred to as the "*window to the world*." Light travels through its anterior structures (cornea and lens) and fluid-filled chambers (anterior and posterior) and is eventually adsorbed by the retinal photoreceptors that convert light energy into nerve impulses for the brain to interpret. The outermost covering of the eye comprises an epithelium which spans the cornea and conjunctiva and forms the ocular surface. Since the cornea is the first light-receptive ocular structure, abnormalities on its surface could be detrimental to light processing and vision perception. The corneal epithelium provides a protective barrier against infection and desiccation and, like most epithelia, is constantly renewed. This regenerative capacity was conceptualized by Ida Mann [1] and later realized through the work of Davanger and Everson [2] who proposed that somatic SC were located within the peripheral cornea. It is now recognized that SC within this zone are nurtured and protected within deep stromal invaginations known as the Palisades of Vogt [3, 4]. Damage to these anatomical structures and/or depletion of resident SC can result in catastrophic vision-threatening diseases collectively termed limbal stem cell deficiency (LSCD), a disease characterized by painful, non-healing corneal epithelial defects that have and continue to pose a major challenge for clinicians. Over two decades ago, pioneering studies by Kenyon and Tseng [5] saw patients with LSCD treated with large autologous or allogeneic limbal grafts. With technological advances in tissue engineering and in identifying and cultivating limbal ESC (LESC), patients with the same disease are now treated with new cell-based therapies which have dramatically improved patient outcomes [6–14].

2 Ocular Surface: Structure and Function

The adult cornea has three distinctly recognizable layers; from anterior to posterior they include (1) a multilayered non-keratinizing squamous epithelium, (2) a keratocyte containing stroma which occupies approximately 90 % of the cornea and serves as a structural support, and (3) a monolayer of specialized endothelial cells which pump fluids across the cornea. Each cellular compartment is segregated by a basement membrane (BM), anteriorly by Bowman's Layer which separates the epithelium from the underlying stroma, and posteriorly by Descemet's membrane, which partitions the stroma from the endothelium. The corneal epithelium typically comprises 5–6 layers of morphologically distinct cells, with columnar cells forming the basal layer and wing-shaped and flattened cells comprising the intermediate and superficial layers, respectively (Fig. 1a). The limbal epithelium begins where the corneal epithelium terminates. This region is evident in histological sections, since the consistency of the substantia propria changes to form a natural angle and the stromal blood vessel becomes obvious. The epithelium is rippled (Fig. 1b), much like the keratinocyte containing rete pegs of the epidermis.

The conjunctiva, the third and most expansive of the specialized ocular surface epithelia, extends from the posterior aspect of the eyelids (tarsal conjunctiva), drapes the sclera (bulbar conjunctiva), and then merges with the limbal epithelium adjacent to the cornea. The region between the tarsal and bulbar conjunctiva is referred to as the fornix. Mucin-secreting goblet cells are a characteristic feature of this secretory epithelium and are mainly confined to the basal epithelium within this zone. Conjunctival ESC are enriched in the bulbar and forniceal region, but unlike LESC, are not confined to a defined niche [15, 16].

3 Homeostatic Activity of the Corneal Epithelium and Its Stem Cells

The prevailing notion is that corneal epithelial self-renewal occurs through a small reservoir of LESC found within the basal limbal epithelium. These SC are regarded unipotent as they only give rise to cells of corneal lineage. However, once removed from their residence and cultured in the presence of appropriate mitogens, they display neuronal properties [17, 18], implying they have multipotential activity. LESC most likely divide asymmetrically, with one daughter cell retained in the SC pool, while the other detaches from its BM, migrates away from the niche, differentiates into a transient amplifying cell (TAC), undergoes several rounds of cell division as it makes its way through the epithelial conveyer belt, and eventually terminally differentiates (TDC) and desquamates from the corneal surface. The rate at which this cycle occurs is species dependent and is generally complete within 7–12 days. Thoft and Friend [19] developed the currently accepted model for corneal homeostasis termed the X, Y, Z hypothesis, postulating that proliferation of

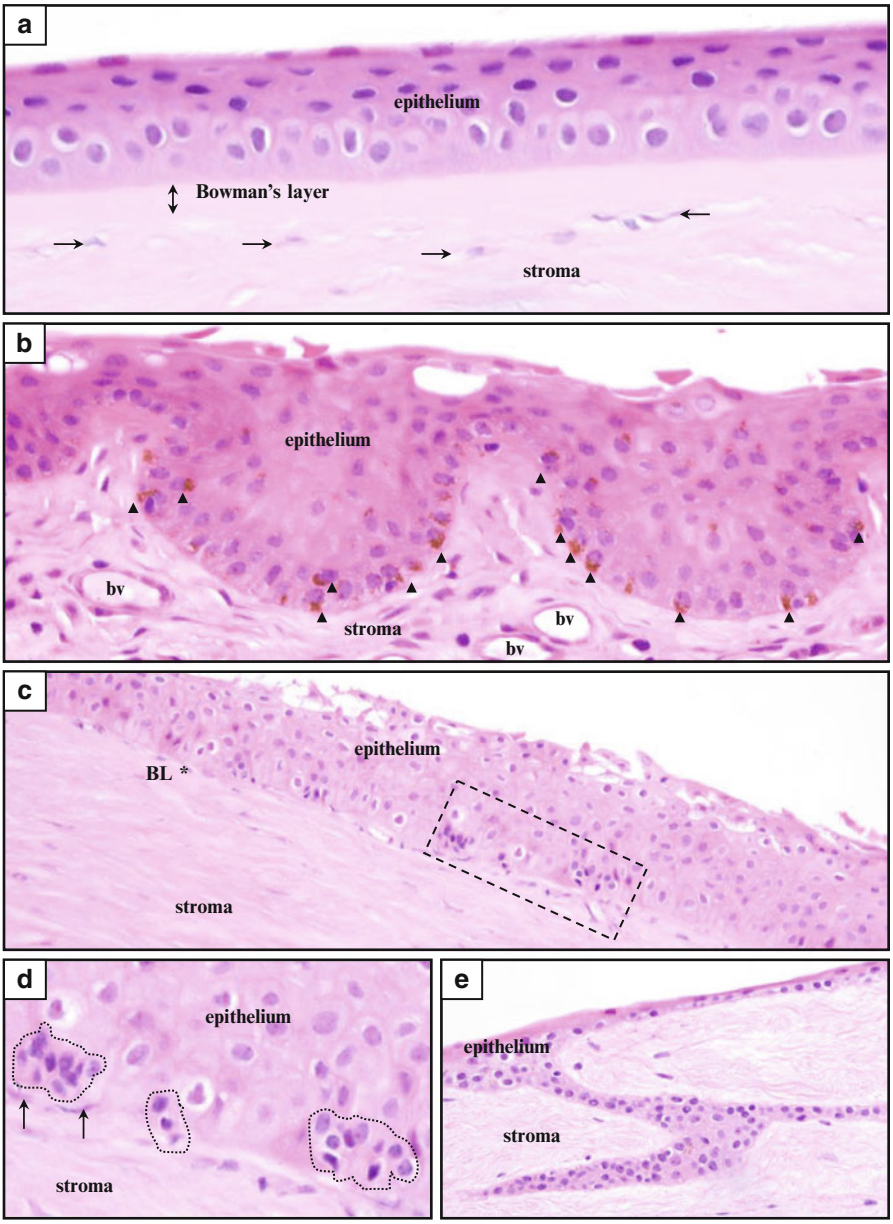

Fig. 1 Histological features of the adult human corneo-limbal region. The central cornea is composed of a multilayered epithelium, separated from the stroma by a thick BM-like structure known as Bowman's Layer (**a**, *double headed arrow*). *Arrows* in panel (**a**) point to quiescent keratocytes which are scattered throughout the corneal stroma. The adult limbus (**b–e**) has many unique features, including a corrugated epithelium known as the Palisades of Vogt (**b**). Some basal limbal epithelial cells are pigmented (*arrowheads*); the adjacent stroma has irregular-shaped mesenchymal cells and is enriched with a prominent vascular supply (BV). Longitudinal section through the

basal epithelial cells (X) and their centripetal migration (Y) equal corneal epithelial cell shedding (Z). Buck [20] tested Thoft's hypothesis by tattooing the normal mouse corneal epithelium with India ink, while noting centripetal migration at a rate of ~17 μm/day and loss of labeled cells by desquamation by 2 weeks. Later, Sharma and Coles [21] developed a mathematical model for corneal epithelial maintenance, in principle similar to Thoft's theory; however, it took into account limbal width, mitotic rate, and centripetal velocity. According to this modified model, the rabbit corneal epithelium is replaced within 12 months.

4 Early Concepts from Animal and Human Studies

Friedenwald and Buschke [22] and Hanna and O'Brien [23] were among the first to notice a high mitotic index within clusters of epithelial cells in the peripheral cornea. Pioneering work by Ida Mann [1] on rabbit corneal epithelial regeneration demonstrated pigmented cells moved from the periphery during corneal wound healing. Later, Hanna [24] corroborated these findings and proposed corneas healed by the centripetal migration of progenitor-like cells from the limbus. Davanger and Eversen [2] then visualized the movement of pigmented cells from the human limbus, recognizing this as the generative zone for the corneal epithelium. In 1979, Srinivasan and Eakins [25] provided convincing in vivo evidence that chronic corneal epithelial defects could not be induced in rabbits without significant damage to the limbus. Likewise, Chen and Tseng [26] and Huang and Tseng [27] confirmed that partial and complete removal of the rabbit limbus resulted in impaired corneal healing, a finding that supported the limbus as the regenerative zone. Cotsarelis and coworkers [28] used tritiated-thymidine incorporation to define a minor population (~10 %) of murine basal limbal epithelial cells which retained DNA label over a prolonged chase period under homeostatic conditions. However, after wounding or under the influence of a tumor promoter, these cells proliferated to the extent that 90 % of basal limbal epithelial cells retained label. Finally, Shermer [29] and Kurpakus [30] demonstrated the presence of the corneal-specific 64-kDa keratin in basal epithelial cells of the rabbit and guinea pig central cornea (but not limbus), suggesting that basal limbal epithelia were less differentiated and likely to represent ESC of the cornea.

Fig. 1 (continued) cornea provides an alternative perspective of the limbus which begins where Bowman's Layer terminates (BL*) and clusters of smaller basal limbal epithelial cells become apparent (**c**). The boxed region in panel (**c**) is magnified in (**d**). Cells within these clusters (**d**, *dashed lines*) are believed to represent LESC which are in close proximity to underlying mesenchymal niche cells (**d**, *arrows*). Other SC bearing structures including crypts have been identified emanating from the base of a limbal palisade and extending deep into the stroma (**e**). All sections were stained with H&E. Original magnification ×400 (**a**, **b**, **d**, **e**) and ×200 (**c**)

Through these early animal studies, investigators began to recognize the clinical importance of the limbus, not only for maintaining corneal health under homeostatic conditions, but also for corneal re-epithelialization in patients with severe corneal epithelial defects. Kinoshita and associated [31] were among the first to provide clinical evidence in humans for the regenerative activity of the limbus by showing corneal epithelial could be replaced by host cells after lamellar keratoplasty. Kenyon and Tseng [5] were next to show significant improvements in visual acuity and corneal health in patients with LSCD who received large limbal segments from the healthy or less affected contralateral eye. Due to the technically challenging task of identifying and isolating LESC at the time, large sectors of limbal tissue were used as the SC source and carrier.

Indirect evidence supporting the limbus, the repository for corneal SC, comes from studying the prevalence of ocular surface tumors. The limbus is a common site for congenital (dermoid), benign (pterygium) [32], precancerous (dysplasia), and malignant (squamous cell carcinoma and melanoma) neoplasms [33, 34] as are other epithelial SC harboring transition zones throughout the human body [35]. In addition, mutagenic damage to LESC through radiotherapy [36] or chemotherapy [37] can induce a temporary or permanent state of LESC failure.

5 Limbal Epithelial Stem Cell Niche Architecture and Function

The "niche" hypothesis for SC was conceptualized in 1983 by Schofield [38] who proposed that SC are dependent on the immediate microenvironment for their safety and for ensuring the turnover of the tissue they are programmed to support throughout life. The limbus, which has both anatomical and functional dimensions, is the proposed niche for LESC. The most striking anatomical feature is its corrugated topography as indicated by the Palisades of Vogt (Fig. 1b), where early evidence suggested that basal epithelial cells within the undulated epithelium constituted a population of putative LESC [28]. Reasons for the folded appearance are not known for certain. However, it is thought that the deep stromal invaginations provide protection from sheering forces, trauma and environmental insults, and more LESC can be confined to a narrow band of tissue. Moreover, the superior and inferior limbus contains the highest concentrations of palisades [3, 4], and this may represent another level of protection which is offered by the upper and lower eyelids. The limbus is in a dynamic state throughout life as these structures diminish with age [39], concomitant with loss of SC activity [40–42]. Other distinguishing features with functional implications include the presence of basal melanocytes [43] (Fig. 1b), transporters of melanin pigments into neighboring epithelial cells providing a further level of protection from damaging ultraviolet radiation [44], and the presence of lymphocytes and Langerhan's cells [45] which partake in local immune surveillance. Basal limbal epithelial cells can be observed as clusters of 5–10 cells which are purported to include putative LESC [46, 47] (Fig. 1c, d).

Many of the niche's nurturing factors are derived from the adjacent substantia propria. Firstly, this collagenous structural support harbors an array of cells including an elaborate vascular and lymphatic network [48] that provides gaseous exchange, nutrients, and immunological signals. Secondly, the stroma contains a heterogenous population of mesenchymal cells including keratocytes (quiescent fibroblasts), which are in close proximity to (Fig. 1d, arrows) and may protect LESC from differentiation and apoptotic signals, and a more recently discovered stromal SC population [49]. Thirdly, the limbal stroma is highly innervated [50] and it has been suggested that centripetal migration of LESC follows the course of radial nerves within the cornea [51, 52].

In addition to the Palisades of Vogt, three other niche-like structures have been identified. Dua and colleagues [53, 54] discovered limbal epithelial crypts. Serial sections through an entire human cornea disclosed seven crypts, which resembled solid cords of epithelial cells that extend from the periphery of the lower aspect of a palisade (Fig. 1e). However, unlike the Palisades of Vogt, crypts were more abundant in the nasal limbus [53]. Independent researchers identified two alternative niche structures; the first were also called limbal epithelial crypts (these are yet to be confirmed as being identical to Dua's crypts), and the second, called focal stromal projections, described as finger-like projections of stroma which contain a central blood vessel, surrounded by small tightly packed epithelial cells [55]. Immunolocalization with specific markers confirmed these novel formations harbored limbal epithelial progenitor cells.

Despite the strong evidence supporting the limbus as the residence for LESC, several recent reports have tested this long-standing notion. In 2008, Majo and associates [56] identified ESC in the central cornea of several mammalian species, which, when extracted and cultured, displayed holoclone activity and p63/CK-3 expression. Curiously, adult human specimens were not included in their study, and their data on corneas from young children were not so convincing. Others [57] have shown that central human corneal and limbal epithelium can be cultured into spheres which possess SC properties and p63 expression. Furthermore, islands of normal epithelial cells can persist (>60 months) in the cornea of patients with LSCD, suggesting that they are supported by SC derived from the central cornea [58]. Chang and colleagues [59] provided corroborating short-term (12 h) evidence of corneal regeneration after laser ablating the human limbus in an organ culture model. Overall these ex vivo and clinical data suggest that LESC play a limited role in corneal homeostasis.

6 Corneal and Limbal Development

The cornea is one of the last ocular structures to develop [60–62], a process highly dependent on interactions between the lens vesicle and the overlying surface ectoderm. The morphogenetic programs and signaling pathways involved in its formation are not well understood, nor are they properly characterized in man. However, morphological studies in fish [63], birds [64], rodents [65], and humans [60–62, 66]

have provided important insights into this exquisitely tuned process. Molecular studies have also demonstrated the dominant role of the master oculogenic gene *Pax6* [67] and members of the Wnt signaling pathway [68] in corneal genesis. After detaching from the surface ectoderm, the lens vesicle invaginates into the optic cup and the void between these two structures is replaced by rapidly invading mesenchymal cells of neural crest origin. In humans, two cycles of migration are through to occur; the first contributes to the formation of the corneal endothelium and the second to corneal keratocytes [69]. In rodents, a single wave of mesenchymal migration occurs and these cells eventually differentiate into fibrocytes and endothelial cells within their respective compartments [70]. The human fetal corneal epithelium (8–22 weeks gestation) comprises a layer of basal cuboidal and a layer of superficial squamous cells [66] (Fig. 2a, b). However, when eyelids separate (~25 weeks gestation), the epithelium proliferates to 4–6 layers [62].

The developing human limbus is apparent at 8–9 WG as indicated by morphological changes in ocular surface epithelium and stromal architecture [60]. The limbal corrugations present in adulthood are not apparent during fetal development. Instead, a ridge-like structure (Fig. 2a, c), postulated to represent the rudimentary LESC niche, circumscribes the peripheral cornea [66]. This ridge comprises 5–6 layers of epithelial cells compared to the bi-layered central cornea (Fig. 2b). Precisely, when the ridge regresses is not clearly defined, but probably occurs at or around the time of eyelid opening and subsequent exposure to amniotic fluid. Furthermore, it is not known when the Palisades of Vogt form; these anatomical changes likely occur well into the postnatal period [66].

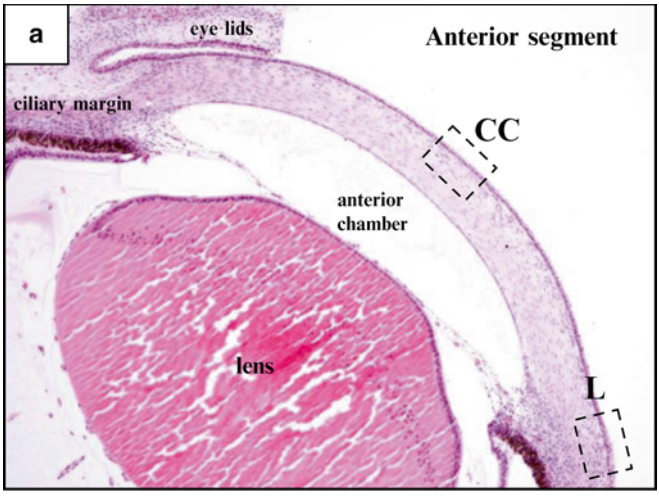

Fig. 2 Histological features of the developing human cornea. At 12 WG the human iris, ciliary body, lens, and cornea are clearly visibly but incompletely developed (**a**). The *boxed regions* in panel (**a**) are magnified and displayed in panels (**b**; CC, central cornea) and (**c**; L, limbus). *Arrows* in (**c**) point to a raised multilayered epithelial structure which is the proposed rudimentary LESC niche. Note the dense cellular stroma within each ocular surface region. All sections were stained with H&E. Original magnification ×100 (**a**), ×1,000 (**b**, **c**)

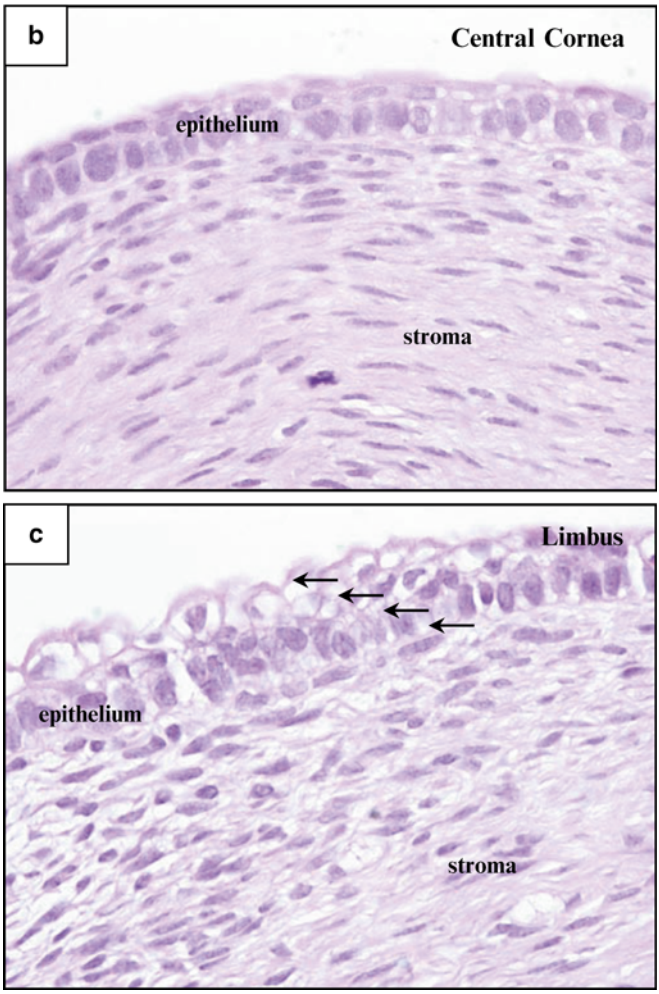

Fig. 2 (continued)

7 Protein Components of the Niche and the Molecular Pathways That Control LESC Fate

A major challenge for researchers studying LESC has been defining the precise constituents of the limbal niche and the molecular signals and pathways that ensure its SC remain in a quiescent state with lifelong self-renewal activity. Once adult SC move out this protective and privileged microenvironment, they lose "stemness," meaning that they differentiate as they replenish the organ they are

programmed to renew [38, 71]. Knowledge of the proteins that comprise the LESC niche is important, not only for understanding the biology of these cells and their selective enrichment, but also for identifying better culture conditions for their expansion and clinical use.

A good starting point is to look at differences in BM composition between the limbus and central cornea, after all, epithelial cells in both regions are in direct contact with these structures, yet putative LESC probably only exist in the basal limbal epithelium. BM are not just structural barriers and attachment sites for epithelial cells, they are also effective modulators of cell proliferation and differentiation as they sequester cytokines and growth factors through their proteoglycan content [72]. The importance of the corneo-limbal BM is exemplified by studies which show modulation of keratin expression due to its presence [73]. Early studies in man and rabbit demonstrated differences in distribution of laminin and type-VII collagen between the corneal and limbal BM [74, 75]. Others have noted type-IV collagen on the limbal but not the central corneal BM [76, 77]. BM heterogeneity was comprehensively studied by two independent groups, who immunostained over 60 BM and extracellular matrix (ECM) proteins [78, 79]. Limbal-specific proteins included tenascin C, vitronectin (VN), BM40/SPARC, laminin α1, α2, β1 chain, and agrin, with some of these factors co-localizing to clusters of presumed LESC [78]. A recent investigation from our laboratory also identified abundant immunoreactivity for vitronectin along the limbal but not corneal BM (Fig. 3). This factor supported cultured human limbal epithelial holoclones [80] and has been shown by others to enhance the self-renewal activity of human embryonic SC [81].

As well as cell-to-cell and cell-to-matrix interactions, LESC-niche cross talk also involves autocrine and paracrine factors and their receptors which impact intracellular signaling cascades relevant to SC survival and function. Differential expression patterns for TGFβ1, IL-1β, IGF1, bFGF, KGF/FGF-7, GDNF, NT-3, NT-4, and NGF [82, 83] as well as their corresponding receptors [46, 82–85] have been identified between epithelial and stromal cells of the cornea and limbus. These data also suggest that Wnt/β-catenin, Notch, TGF-β/BMP, and FGF-7 signaling pathways are involved in supporting LESC and are exemplified in *Dickkopf2* (DKK2; a Wnt inhibitor) null mice which lose corneal fate decision. Consequently, Wnt/β-catenin pathway is induced in the limbal but not the corneal stroma, and the cornea transdifferentiates into an epidermal phenotype [86]. These data provide evidence that the mesenchymal niche also plays a critical role in LESC differentiation.

8 The Stromal Niche Controls Plasticity

The importance of the limbal stromal niche as a LESC support has been studied through in vitro models. For example, when rabbit corneal epithelial cells were cultured on corneal stromal fibroblasts, they maintained a corneal phenotype, whereas a limbal epithelial phenotype persisted when either corneal or limbal epithelial cells were nurtured on limbal stromal fibroblasts [87]. Likewise, limbal

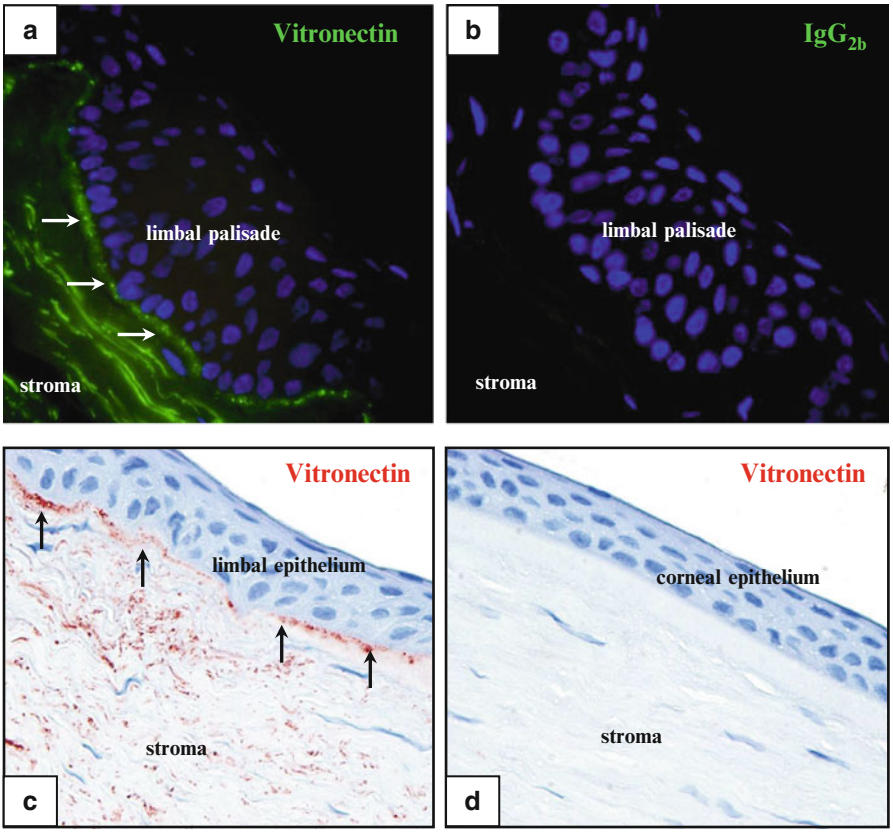

Fig. 3 Localization of vitronectin within the human limbus. Immunofluorescence (**a**, **b**) and immunohistochemistry (**c**, **d**) was performed on formalin-fixed paraffin-embedded adult human corneas. Sections were incubated with a monoclonal antibody against human vitronectin (HV2; **a**, **c**, **d**) or a control IgG (**b**). Vitronectin reactivity (*green* and *red*) developed along the BM adjacent to a limbal palisade as well as within the stromal connective tissue (**a**) and along the basal limbal epithelium (**c**), but was absent from the central cornea (**d**). Sections were counterstained with DAPI (**a**, **b**) or hematoxylin (**c**, **d**). Original magnification ×1,000 (**a**–**d**)

epithelial progenitors were maintained when human limbal epithelial cells were recombined with mitotically active limbal fibroblasts, [88], and when LESC and stromal niche cells were disaggregated and reunited in culture, they formed large spheres and holoclones [89]. Also, when human embryonic SC were cultured on type-IV collagen (a limbal BM protein), in medium conditioned by limbal fibroblasts, they lost pluripotency and differentiated into limbal epithelial-like cells [90], and when murine hair follicle keratinocyte SC were cultured on laminin 5 in the presence of conditioned media from limbal stomal fibroblasts, they stratified into epithelial sheets that expressed corneal-specific cytokeratin (CK)-12 and the oculogenic *PAX6* gene [91]. In animal models of tissue recombination, corneal epithelial

cells developed sebaceous glands and hair follicles when placed in direct contact with the dermal bed [92, 93]. Overall, these studies provide solid evidence that the stromal microenvironment can dictate cell phenotype and that lineage committed epithelial cells can be reprogrammed depending on stromal signals they receive.

9 Limbal Stromal Niche Stem Cells

In addition to quiescent keratocytes, the limbal stroma also harbors a population of SC that is less well characterized than their epithelial counterparts. These cells can be isolated by enzymatic dissociation and sub-cultivated to express keratogenic, chondrogenic, and neurogenic markers [94]. This tri-lineage potential has recently been confirmed to be derived from mesenchymal SC [49, 95, 96]. Other researchers have isolated neural crest-derived precursors from the same region; these cells form spheres in culture and express neural SC markers [97] but were not bone marrow derived, although bone marrow-derived SC have been identified in the corneal stroma [98]. The precise function of limbal stromal SC is not completely understood, but it is tempting to speculate that mesenchymal and neural crest-derived SC serve to maintain the corneal stroma and regulate LESC activity, while bone marrow-derived SC play a role in wound healing [99].

10 Physical and Biochemical Properties of Limbal Epithelial Stem Cells

Basal limbal epithelial cells are small (~10 μm), have a high nuclear-to-cytoplasm content, have a heterochromatin dense nucleus with no obvious nucleoli, contain few cytoplasmic granules, and are tightly packed along their BM [46, 47] (Fig. 1c, d). Smaller cells preferentially express higher levels of LESC antigens, whereas larger cells are associated with markers of corneal differentiation [100]. Smaller cells (10–16 μm) represent ~11 % of the total limbal epithelial cell population; they contain more BrdU label-retaining cells and possess the greatest colony-forming efficiency and highest proliferative capacity compared to their central corneal equivalents [101].

The literature is flooded with reports of candidate antigens that have been used to identify putative LESC. However, an exclusive marker that unequivocally identifies these cells remains elusive. One reason could be the subtle differences between a true LESC and an early progenitor. Apart from the physical properties (described above), a phenotypic signature based on positively and negatively expressed genes is used to discriminate LESC from their differentiated progeny. These markers include epithelial-specific structural proteins, enzymes, transcription factors, adhesion molecules, and growth factor receptors.

Suprabasal and superficial epithelial cells of the corneolimbal junction are regarded as having undergone differentiation and as such express corneal-specific

intermediate filament proteins including CK-3 [29, 30] and CK-12 [102], the structural cytosolic protein involucrin [47] and the calcium-dependent S100A12 protein [103] which are absent from basal limbal epithelial cells. In the developing and adult cornea, the same basal cells lack the gap junction protein connexin-43 [104–106] as well as aldehyde dehydrogenase-1 (an enzyme that protects cells from toxic peroxidic aldehydes) [107]. The same study also identified the hyaluronan receptor (RHAMM/HMMR) as being absent from basal limbal epithelial cells, suggesting that it too is more specific for differentiated cells [107].

In terms of abundantly expressed genes, the list seems to be ever growing. However, a useful marker needs to be specific enough to identify a rare population of SC and sufficiently robust to facilitate their isolation. If the second criteria is taken into account, then cell surface molecules are choice, as they allow for tethering and sorting of viable cells for subsequent expansion in culture. Clues for potential markers of putative LESC have come from studies that have searched for unique somatic SC antigens in the hematopoietic and other epithelial-containing organs such as the skin. A prime example is the use of Hoechst 33342; this DNA-binding dye has been used to identify multiple mammalian SC, including those derived from the bone marrow [108]. Since Hoechst 33342 is effluxed by cells that express the ATP-binding cassette family of cell surface transporter proteins, particularly ABCG2, these cells can be identified by flow cytometry as "side population" (SP) cells [109]. SP cells are generally not present in the central corneal epithelium but have been identified and isolated from the limbal epithelia of humans [110–112] and rabbits [113] with a frequency of 0.2–0.64 % and 0.4–1.2 %, respectively. Contrary to these results, SP cells have also been found in the central corneal (4.6 %) and limbal (0.4 %) epithelia of the rat. However, higher ABCG2 expression was observed in limbal-derived rat epithelial cell [114]. These results indicate that in some species, either alternative transporter proteins are actively involved in effluxing Hoechst 33342 or the central cornea of the rat harbors an additional ESC population [56]. Moreover, a heterogeneous population of cells, including $ABCG2^{+}$/MHC class II^{+} antigen-presenting, non-epithelial SP cells [115] as well as intraepithelial lymphocytes, has been isolated from human and rabbit ocular tissue [116]. The precise biological role for ABCG2 in the limbus has not been fully elucidated; however, it could be a chemo-protective transporter of toxins induced following oxidative stress [117] or play a photoprotective role as indicated by the phototoxic lesions that develop in ABCG2-null mice [118].

The glycolytic enzyme α-enolase was originally found to be restricted to the limbus [119] and selective for LESC [120]. However, it was later identified in migrating basal limbal epithelial cells after corneal wounding, suggesting that it was also expressed by TAC [121]. More recently it was detected in suprabasal limbal as well as occasional basal cells within the cornea [46, 47], implying that it is not an exclusive marker of LESC.

Cytokeratins are also regarded semi-reliable markers of LESC since their expression can be restricted across the ocular surface. CK-14 [122–124], CK-15 [123–125], and CK-19 [122, 126] are mainly expressed by basal limbal epithelial cells. Depending on how the limbus is bisected, strings of individual cells or clusters of

basal cells can be visualized with antibodies against all three proteins (Fig. 4a); however, these antigens can also be found in other ocular surface epithelia [127], implying that identification of LESC based on cytokeratins alone is not sufficiently inaccurate. Therefore, co-localization with other putative SC antigens should be performed to more specifically disclose their location [124]. However, caution must be exercised when interpreting keratin staining results as there are species-specific differences in keratin types and patterns of expression between corneal epithelial cells [128] and limbal progenitors [125].

The transcription factor p63 (p53 homologue) is critical for the formation of stratified epithelia. This is evident in p63-null mice which have severe ectodermal developmental issues since their SC cannot be sustained [129, 130]. Pellegrini and colleagues [131] were among the first to identify keratinocyte stem-like cells from TAC in the human skin and cornea, based on p63 distribution. Subsequent studies from independent laboratories refuted the specificity of p63 as marker for LESC, since peripheral and central corneal as well as suprabasal epithelial cells within the limbus were illuminated and co-expressed markers of cell proliferation [132, 133]. It is important to mention that these inconsistent and controversial results probably arose from the use of the A4A antibody clone for p63 which identifies all isoforms of the differentially spliced *p63* gene. Di Iorio and coauthors [134] subsequently demonstrated that the truncated species of p63 (ΔNp63α) was a better marker for SC in the resting limbus (Fig. 4b) since a small proportion (~8 %) of the total basal epithelial cells were immunoreactive, while in an activated limbus, ~36 % of the basal cells expressed ΔNp63α. These data indicate that after wounding or exposure to chemical promoter, ΔNp63α is not suitable to discriminate stem from early TAC, so other candidate LESC antigens such as CK-14/CK-19 [135] and ABCG2 [111] are used in co-localization studies.

Integrins are a heterodimeric superfamily of bifunctional cell surface receptors required for cell-to-cell or cell-to-matrix interactions [136] and transmission of signals to the cytoskeleton [137]. The heterodimeric repertoire is extensive given the possible combinations (24 α and 9 β) of subunits [136]. Integrins are highly expressed in basal corneal and limbal cells and mediate cell attachment to ECM glycoproteins including fibronectin, VN [138], collagens, and laminins [137]. In the developing mouse, α9-integrin is widespread across the entire ocular surface, but with increasing gestational age, it becomes confined to the limbus [139], suggesting that it could be a candidate LESC marker. Its expression is remarkably similar to that of tenascin C [140], the ligand for α9 integrin. However in later studies, high β1 and β4-integrins and little or no α9-integrin were observed in slow-cycling limbal epithelial cells, suggesting that β-integrins promote retention of SC properties, while α9-integrin facilitates SC differentiation and departure from the niche [141]. α6-integrin$^+$ keratinocytes have been isolated from the epidermis [142, 143] and esophagus [144] and shown to possess SC qualities. Hayashi and colleagues [145] determined that a similar cell population existed in cornea. These cells were relatively small, had high clonogenic capacity, expressed ABCG2, CK-15, and ΔNp63α, and had appropriate cell cycle kinetics for SC. Immunofluorescence on tissue

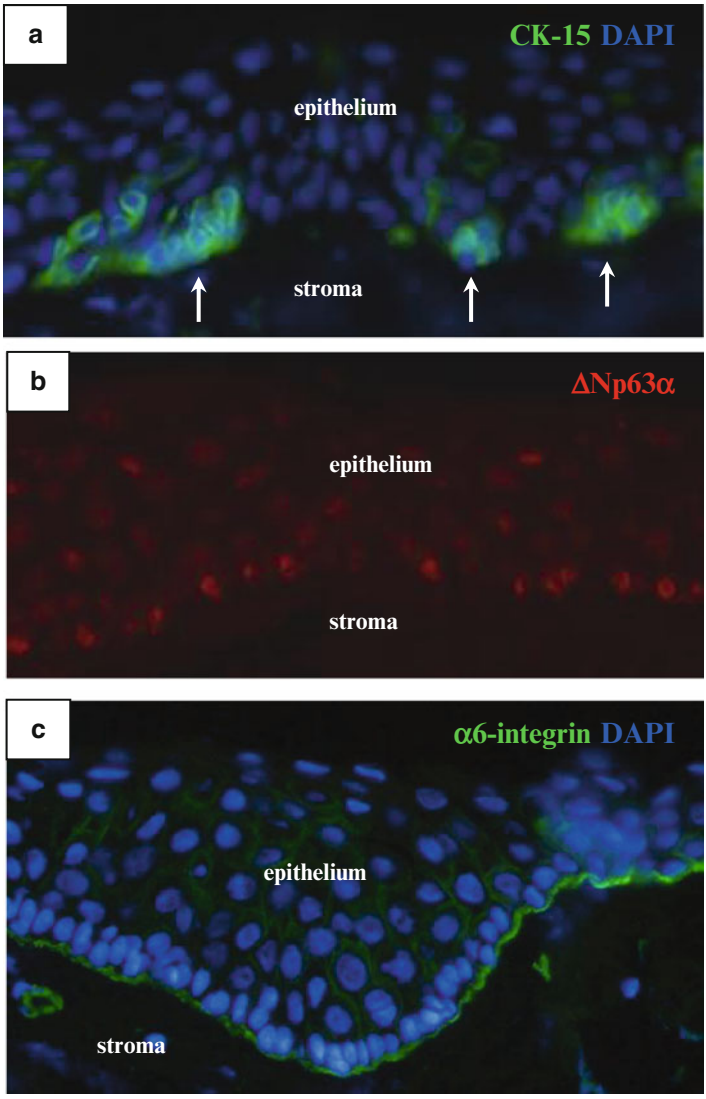

Fig. 4 Limbal epithelial stem cell marker expression. Immunofluorescence (**a**–**c**) and immunohistochemistry (**d**, **e**) was performed on fresh frozen (**a**–**c**) or formalin-fixed paraffin-embedded (**d**, **e**) adult human corneas. Sections were incubated with antibodies against human CK-15 (**a**), ΔNp63α (**b**), α6-integrin (**c**), p75 (**d**), and DKK3 (**e**) and then counterstained with DAPI (**a**, **c**) or hematoxylin (**d**, **e**). *Arrows* in panel (**a**) point to CK-15 immunoreactive basal cell clusters. Original magnification ×400 (**a**–**c**) and ×1,000 (**d**, **e**)

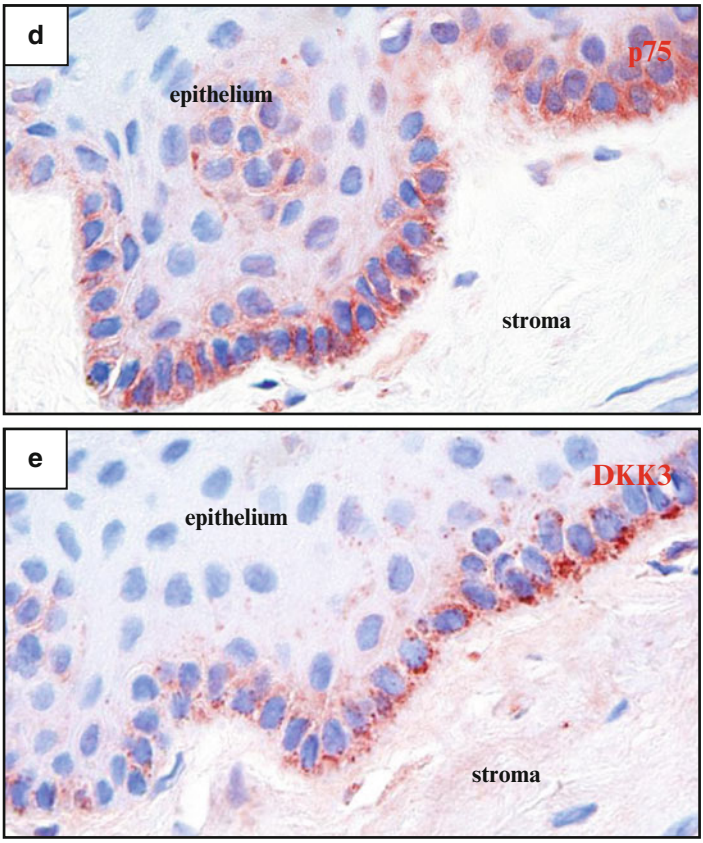

Fig. 4 (continued)

sections revealed that these cells were mainly confined to the basal limbal epithelia (Fig. 4c), but were occasionally present in the central cornea [145], implying that this is not an exclusive LESC marker. The cell adhesion molecule N-cadherin is another marker expressed by limbal progenitor cells, but also illuminates niche melanocytes, rendering it nonselective for LESC [146]. Higa and colleagues [147] elaborated on the specificity of N-cadherin as a LESC marker and found that it stained clusters of CK-15^{+} basal limbal epithelial cells. Furthermore, when N-cadherin^{+} limbal epithelial cells were isolated and cocultured with murine 3T3 feeder cells, the multilayered epithelial sheet that formed comprised CK-15^{+} basal and CK-12^{+} suprabasal cells, suggesting a pivotal role for this protein as a LESC support factor [147].

Given that both limbal and neuroepithelium are ectodermally derived, it is not surprising that LESC can be differentiated into neuronal-like cells [17, 18, 148].

Furthermore, a number of neural SC markers are expressed by putative LESC including the high (TrkA) and low (p75) affinity nerve growth factor receptors [83, 85] (Fig. 4d). Notably, however, the specificity of these markers for LESC is somewhat controversial, as some investigators found no expression in the limbus [149], while others reported expression in basal conjunctival epithelial cells [85]. Musashi-1, an RNA-binding protein involved in asymmetric division of progenitors within sensory organs of *Drosophila* [150] and mediator of Notch signaling in SC [151], was also distributed in a restricted pattern along the basal limbal epithelium [152]. Likewise, the transmembrane receptor Notch-1, essential for maintenance, but not generation of mammalian neural SC [153], was profoundly expressed in conjunction with ABCG2 and α6- and β1-integrins, in discrete cell clusters within the Palisades of Vogt but was absent from dividing cells in culture, implying a role in preventing differentiation [154]. Hes1, the downstream Notch-signaling protein and regulator or retinal neurons [155], was shown to regulate corneal development and sustain limbal stem/progenitor cell function in mice [156]. Our laboratory recently identified DKK3, a member of the dickkopf family of Wnt signaling antagonists in the basal limbal region (Fig. 4e; unpublished data), a relevant finding since deletion of a related member (DKK2) results in the loss of corneal SC fate decision and suppression of PAX6 in mutant mice [86]. Others found the Wnt-associated transcription factor TCF4 in the same cells [157], confirming the importance of the Wnt signaling pathway in supporting LESC. Both Notch and Wnt signaling pathways are thought to act in concert to control LESC self-renewal [158]. Bmi-1, another neuronal SC self-renewal factor [159], stained slow-cycling limbal epithelial SP cells [113] and co-localized with the transcription factor C/EBPδ (a controller of cell-cycle arrest) in mitotically quiescent LESC [160].

Many of the above-mentioned markers were discovered after extracting cells from the limbal niche, either by enzymatic exposure or after expanding cells from limbal tissue explants. While there are many advantages and disadvantages of these extraction and expansion methods (see below), SC phenotype and function are likely to be modulated by these procedures, thereby impacting marker expression. To overcome this problem, investigators have used a mechanical approach to scrape cells directly from corneal and limbal regions and then perform Serial Analysis of Gene Expression on extracted RNA to identify a list of differentially expressed genes [161]. Others have used a more precise approach by procuring LESC from their niche through the use of Laser Capture Microdissection [162, 163]. This technique uses a microscope to identify a region of interest on a 10–12 μm thick frozen tissue section and a laser beam to cut and collect a group of cells. The main advantage of LCMD is the assessment of global gene expression profile of cells that have been harvested directly from their niche without any manipulation in culture and with minimal contamination from adjacent cells. The main disadvantages of laser capturing SC from fresh tissue are the uncertainty of knowing precisely where these cells reside without the use of a specific marker and the small amount of nucleic acids that can be extracted from these cells for molecular studies.

11 Isolating Limbal Epithelial Stem Cells

Two goals should be met when devising culture models for limbal epithelial cells. The first is to expand our knowledge on LESC biology and the second is to develop better clinical grade cell grafts for patients with LSCD. Before briefly assessing the different culture techniques that have been developed, one must be mindful that the quality of LESC isolated and expanded is heavily reliant on the quality of acquired human donor limbal tissue [164]. There are three main sources of limbal tissue, allogeneic (living relative and cadaveric donor) and autologous (self). Cadaveric donor tissue is mainly used for research purposes, but can also be used as a clinical bioresource. Human donor corneas can be stored in Optisol-GS (Bausch and Lomb; Irvine, CA) at 2–8ºC. However, eye banks world-wide are rapidly moving towards a conventional organ culture system for longer term storage at 31–34 °C. Irrespective of the storage method, issues with epithelial integrity and apoptosis have been reported [165, 166]. Other variables that need to be considered include (1) the post-mortem delay and age of donor, (2) whether cells are expanded from a limbal biopsy or enzymatically dissociated, (3) the location of the biopsy, (4) whether cells are co-cultured with growth-arrested murine 3T3 feeder fibroblasts in suspension or as sheets, (5) the basal media used and its chemical and mitogenic content, (6) the level of oxygen exposure and whether cells are submerged or air-lifted, and (7) the scaffold used to support cells.

Although the presumed residence for LESC has been suspected for decades, only recently have more efficient methods for isolating these cells been developed. Techniques used to enrich LESC include removal of epithelial sheets from limbal tissue by enzymatic dissociation with dispase [87], trypsin/EDTA [167], or combination of enzymes [168]. Isolated cells can then be used immediately to confirm phenotype or expanded in culture for further analysis. However, issues can arise following the use of enzymes. Firstly, dissociated cells are likely to represent a mixed population of progenitor and differentiated cells. Secondly, it remains to be confirmed whether dispase and trypsin effectively remove the entire basal limbal epithelium, especially in relation to cells residing within the limbal invaginations and crypts that characterize the niche [89, 169]. Thirdly, temporary or permanent alterations in cell phenotype can arise from denaturing cell surface molecules, which may in turn impact LESC enrichment protocols.

Since it is known that the composition of the BM differs significantly between regions of the ocular surface (see above) and that LESC are in direct contact with their BM, novel methods for isolating these cells have been developed based on this knowledge. Rapid adhesion to type-IV collagen was used to isolate a minor cell fraction (~10 % of the total limbal epithelial cells) which retained SC properties and phenotype compared to slow and non-adherent cells [170]. However, only 16 % of the collagen-adherent cells displayed slow-cycling kinetics, suggesting that this is an inefficient protocol for purifying LESC.

Another common method used to extract viable LESC-like cells is fluorescent-activated cell sorting (FACS). This strategy has been used to successfully isolate SP cells based on their unique ability to efflux the Hoechst 33342 dye. While SP cells

represent a minor fraction of the total cells and are LESC-like in function and phenotype, they do not always represent a homogenous population [115, 116]. The cell surface markers ABCG2, α6-integrin, and N-cadherin have also been used to isolate viable LESC by FACS [111, 145, 146]. Separation of limbal epithelial cells based on size by flow cytometry has shown that the smallest cells expressed the highest level of LESC markers [101]. Percoll density gradient was used to distinguish two cell populations: the lightest fraction representing ~12 % of the total cells contained CK-12^{+} proliferating cells, while the densest fraction (~7 %) comprised p63^{+}/CK-12^{-} cells [171].

12 Expanding Limbal Epithelial Stem Cells

Much of the groundwork performed in corneal culture models comes from preceding work in skin. In the mid-1970s Rheinwald and Green [172] successfully cultured human epidermal keratinocytes long term on growth-arrested 3T3 murine embryonic fibroblasts [173]. Under these conditions keratinocytes formed discrete colonies and colony number was inversely proportional to donor age [172]. These colonies were morphologically classified as either holoclones, large smooth colonies with greatest growth potential, a likely reservoir of SC; paraclones, smaller colonies with restricted proliferative activity, likely to represent TDC; or merclones, intermediate colonies with wrinkled edges and significant growth potential, likely to harbor TAC [174].

Stocker [175] and Newsome [176] were among the first to successfully grow short-term cultures of corneal epithelial cells. Long-term propagation was achieved by Sun and Green [177], a procedure which is the basis for current cultures (Fig. 5a). In 1993, Wei et al. [178] showed that limbal epithelial cells could be sub-cultivated more effectively on 3T3 feeders compared to corneal epithelial equivalents. In addition, cells harvested from the limbus produced more holoclone-like colonies compared to those from the central cornea [6]. In the absence of 3T3 feeders, increased serum enhanced colony-forming efficiency and induced the development of larger colonies, suggesting that serum-derived mitogens are important for LESC activity [179]. More recently, human limbal epithelial cells have been cultured into spheres which possessed SC properties [57] (Fig. 5b).

There is still much debate as to whether cell suspension cultures are better than those derived from limbal tissue explants. An obvious drawback of the explant system is the length of time required to establish a reasonable number of early generation cells. The longer explants remain in culture, the greater the risk of stromal fibroblast contamination, and there is no guarantee that all LESC will migrate out from the explanted tissue. Advantages include the ease with which the sample is prepared, the minimal physical and chemical (enzymes) trauma endured by cells, and the fact that the niche and its signals are temporally preserved throughout the initial culture period. In our hands, no visible morphological differences are noted between cells expanded from either technique (Fig. 5c, d), nor have we observed differences in the number of cell generation that can be propagated (data not shown).

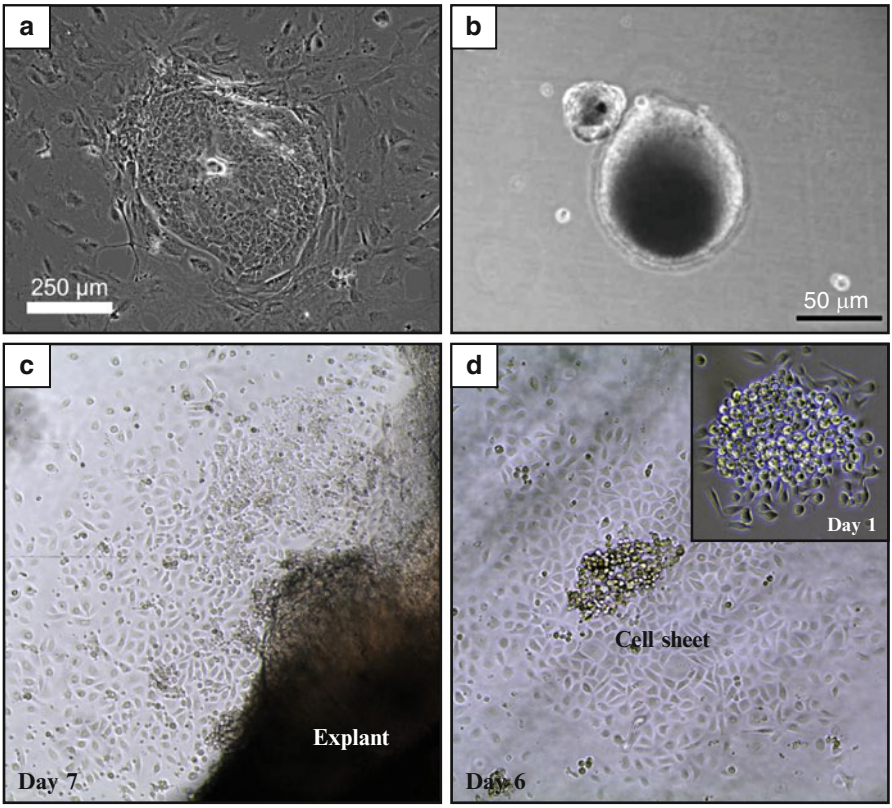

Fig. 5 Primary cultures of human limbal epithelial cells. An island of human limbal epithelial cells cocultured with growth arrested murine 3T3 feeder cells. This morphology is typical of the first week in culture (**a**). Isolated epithelial cells from human limbal rims and their subsequent culture in Neurobasal A medium reveal the formation of cell spheres, varying size, and capable of self-maintenance over a prolonged period (10–12 weeks) (**b**). Biopsies from the human limbus (**c**) or dispase-dissociated sheets of epithelial cells (**d**) were harvested, placed in culture, submerged in media, and allowed to expand. Within 6–7 days, ample epithelial cell outgrowth was noted, and irrespective of the method used, emerging cells were morphologically identical (**c**, **d**; original magnification ×100). Inset panel (**d**) shows an epithelial sheet that has settled onto the plastic substratum with cells beginning to emerge

Aside from the different methodologies employed for propagating cells in the laboratory, if these cells are to be considered for grafting, then transferring loose limbal epithelial sheets can be technically challenging [6, 180]. The most universally accepted bio-substrate for culturing LESC is human amniotic membrane (HAM). This semi-transparent scaffold consists of a monolayer of epithelial cells, a thick BM, and an avascular stroma. These properties, along with the myriad of proteins within its BM (some common to the limbal BM) [181], the host of mitogens sequestered within its connective tissue [182], and its anti-inflammatory, anti-angiogenic, and hypoimmunogenic activity [183], render it the most suitable substrate for growing cells for the purpose of ocular surface reconstruction. Notably, the method of

preparation (intact versus denuded) can impact the quality and phenotype of expanded limbal epithelial cells [184]. Variations on the use of HAM with limbal tissue explants have been developed and used to successfully treat patients with LSCD [7, 8, 12–14]. However, a universally accepted protocol for preparing HAM has not yet been established. Emerging literature suggests that LESC potential is equally effective on HAM, irrespective of whether limbal epithelial cells are derived from explant or suspension cultures [185]. However, several undesirable activities have been recorded; firstly limbal epithelial cells growing on HAM can decline in growth potential and secondly they can transition into mesenchymal-like cells as they invade into the HAM matrix [186]. Meyer-Blazejewska and colleagues [187] recently assessed a host of culture variables and found that limbal epithelial cells grown from a superior limbal biopsy, dissociated with dispase/trypsin–EDTA, and cultured in low-to-medium calcium (0.03–0.4 mM) in 10 % FBS with EGF and FGF yielded the highest clonal growth of undifferentiated cells. Interestingly, the same authors identified fibrin as the scaffold which preserved holoclones. Fibrin is composed of fibrinogen and thrombin, a solution deemed nontoxic, transparent, and readily degradable over 2 weeks [188], rendering it a highly attractive substrate that has been used for ocular surface reconstruction in animals [189] and patients [9, 10] with LSCD.

Although HAM is purported to act as a surrogate niche for LESC, there are significant expenses associated with screening, preparing, and storing it. Moreover, it can vary from batch to batch, winkle during transfer procedure, and remodel soon after transplantation [190]. Given these limitations, there is a pressing need for alternative biocompatible substrate carriers. Recently trialed biomaterials include fish-scale [191], human cross-linked [192, 193], and compressed [194] collagens, silk fibroin [195], and human anterior lens capsule [196] as well as synthetic electrospun nanofibers [197]. Some researchers have even bypassed the need to incorporate native scaffolds and trialed non-intergradable synthetic substrates for growing and delivering LESC including standard [198, 199], surface-modified [200], and Chitosan-coated [201] contact lenses. While many of the above-mentioned studies demonstrate efficacy of these biological and synthetic carriers for supporting LESC, the act of extracting cells from their natural microenvironment and subsequent cultivation can artificially induce SC marker expression, which may misinform evaluations of their composition and phenotype [202]. Furthermore, persistence of foreign antigens, infection from disease transmission, and graft rejection are all valid concerns, particularly for protocols that incorporate foreign biologicals such as HAM, murine fibroblasts, FBS, and bovine pituitary extract [203–205].

13 Clinical Outcomes of Cell Therapies for Patients with LSCD

LSCD can be acquired or hereditary, unilateral or bilateral, total or partial. Total LSCD is the most severe form and is characterized by a compromised ocular surface, which commonly arises from an acute injury (chemical or thermal burn) or from chronic autoimmune-like disease that affects the mucous membranes

(Stevens Johnson's Syndrome and ocular cicatricial pemphigoid). Consequently, the niche is damaged, LESC reserves are depleted or become dysfunctional, and the cornea is enveloped with a vascularized, inflamed conjunctival pannus or is prone to chronic epithelial defects which are painful and non-healing and ultimately result in permanent visual impairment [5, 206]. Aniridia is the genetic form of LSCD where LESC fail as a consequence of mutations in the *PAX6* gene [207] or from deficiencies arising from the niche and its mesenchymal cells [208]. Because of the various causes, the global incidence and burden of LSCD are rarely reported, although it has been estimated that 240 new cases arise each year in the UK alone [209].

A successful long-term outcome in patients with LSCD transplanted with LESC is the ultimate functional test that these cells have been properly isolated, propagated, and transplanted in sufficient numbers to rehabilitate a severely damaged corneal surface. Since Kenyon and Tseng's [5] seminal work on transplanting autologous limbal tissue grafts (a procedure now regarded too risky) and Pellegrini's [6] landmark study on transplanting LESC (a technique regarded too cumbersome due to difficulties in manipulating fragile cell sheets), modified protocols that utilize a bio-carrier have been developed, trialed, and shown to be efficacious. However, clinical results seem to vary between studies that employ the same cell therapy. Not unexpectedly, patient outcomes also vary in studies that compare between techniques [7, 8, 10–14], and the mechanisms of transfer and integration and reasons for success or failure (which mainly occur in the first 2 years) are only partially understood. Therefore, there is a pressing need to standardize the entire procedure, including the criteria for diagnosing and categorizing patients and their posttreatment review. In addition, a unifying process for collecting, expanding, phenotyping, and transplanting LESC is needed. In principle this is achievable, but due to disease heterogeneity, the quality of donor cells available for grafting, and the facilities and expertise available to researchers, practically it may be difficult to establish and implement on a global scale.

Approximately, 700 eyes of patients with LSCD have been treated with limbal epithelial cell-based therapies since Pellegrini's original report [7, 8, 10–14]. From Baylis's recent review [13], the overall success rate stands at 76 % (range 59–100 %; 77 % for autologous and 73 % for allografts). Given this rate is remarkably similar to that reported in Shortt's 2007 review [7], it seems we have done little over the past 4 years to improve clinical outcomes for our patients. Future challenges will include determining why an LESC transplant in 20–30 % of patients fails. Due to the short follow-up recorded by many investigators, it is likely that the true failure rate is much higher than reported. It is also likely that grafted cells fail because of the hostile environment they encounter once transplanted or due to the quality of tissue they are collected from and how they are prepared and nurtured ex vivo. This is exemplified by Rama's study [10] which showed that when cultures contained >3 % ΔNp63α expressing cells prior to transplantation, a positive patient outcome was recorded (78 %). This contrasted with an 11 % success rate for cultures that supported <3 % ΔNp63α expressing cells. These data suggest that the number of LESC transferred may be just as relevant as the method of transfer. Other obvious factors that impact clinical outcome of a cell therapy include where the cells are sourced,

how the cells are delivered and protected while on the ocular surface, how the corneal bed is prepared, the postoperative medication (which can vary particularly for patients with cicatricial disease) [210], and postoperative follow-up. The field is in desperate need of strict set of inclusion/exclusion criteria which take into account disease etiology, whether a patient has had previous surgeries, and confirmation of LSCD by immunohistology or immunocytology and/or confocal microscopy [7–9]. Notably, without a unique LESC marker, determining whether a patient has partial or total LSCD may be just as difficult as determining the success of a treatment. An objective and globally accepted pre- and posttreatment grading system for patients with LSCD is also warranted. Visual acuity is a standard objective measure and an improvement in vision of $\geq$ two lines on a Snellen chart is generally considered a successful outcome. To date ~50 % of eyes that have received a LESC transplant have gained this or better level of vision [13]. The Cornea Society has recently recognized the need for standardizing the nomenclature for ocular surface rehabilitative procedures [211] and it is hoped that a similar initiative is instigated for standardizing therapeutic protocols for patients with LSCD.

14 Mechanism of LESC Transplantation

In addition to the factors which interfere with success of LESC therapies (see above), only circumstantial evidence exists in humans on how these therapies work. Questions that remain unresolved include does this therapy anatomically replace LESC or does it instruct dormant SC to become functionally active, does the niche require repair prior to LESC transplantation, and how ocular surface inflammation is suppressed after engrafting of these cells. Answers to these questions would certainly improve our current understanding of the mechanisms behind these cell-based therapies. Evidence for long-term survival of transplanted LESC has come from studies that have excised recipient corneas from patients with LSCD because keratoplasty was required to improve vision due to other complications; in these instances, an intact multilayered corneal epithelium was evident. Others have identified allograft-derived donor limbal epithelial cells in the central cornea 12 weeks postoperatively; however, these were replaced by recipient cells 20 weeks after transplantation [212]. Using DNA analyses, Daya and coauthors [213] identified transplanted donor cells 9 months post-grafting. Likewise, donor cells were detected in immunosuppressed [214] and non-immunosuppressed [215] patients with LSCD, 30 months and 3 years after engraftment, respectively. Notably, the above-mentioned studies include patients who underwent an allotransplant. Ideally, tracking the fate of autologous LESC is desirable as this would disclose their destiny and more accurately define their longevity. However, ex vivo tagging of cells could modify their function and phenotype and raise ethical issues as clinical outcomes may be adversely affected. Although such protocols have not been devised for humans, rabbit LESC have been transduced with a LacZ-producing retroviral vector for monitoring cell proliferation, differentiation, and survival in recipient animals [216].

In addition to the irreplaceable role in corneal homeostasis, it is likely that LESC have developed mechanisms which protect them from inflammatory and immune-reactions and increased their resistance to apoptotic-inducing factors. Evidence in support of this hypothesis has recently come to light, whereby murine LESC were shown to dose dependently suppress mitogen or T-cell receptor-mediated proliferation and cytokine production by lymphocytes more so than mesenchymal SC and regulatory T cells. Furthermore, murine LESC expressed high Fas ligand and other anti-apoptotic molecules [217]. Using an animal model of ocular surface damage, the same authors showed that allografted LESC significantly attenuated inflammatory mediators at the transplant site [196]. LESC can also attract stromal/mesenchymal niche cells through chemokine signaling [89]. This may be a critical role for preventing their differentiation under homeostasis; however, after transplantation they may be involved in the recruitment of mesenchymal SC, which themselves are highly immunosuppressive [218]. This data confirms an immunoregulatory role for LESC and has important implications on the success of cell therapies for patients with LSCD.

15 Alternative Stem Cells for Ocular Surface Reconstruction

Over the past 3 years, the quest to improve current SC therapies for patients with LSCD has gained momentum especially for patients with bilateral disease where healthy autologous LESC might not be available for extraction, expansion, and implantation. Perhaps the cell type most closely related to the corneal epithelium is the adjacent conjunctival epithelium. Autologous conjunctival cells have been harvested, expanded on HAM, and successfully used to treat animals with experimentally induced [219] and patients [220] with LSCD. Longer follow-up is however required to gauge success in patients receiving these cells. Perhaps the most convincing evidence that autologous cells integrate, survive long term, maintain the SC pool, and restore the ocular surface of patients with LSCD has come from studying transplanted autologous oral mucosal epithelium [221–224]. Notably, peripheral corneal neovascularization is a common adverse complication after transferring these cells and is thought to arise from suppression of local anti-angiogenic factors [225]. The only other non-corneal epithelial cell transfer technique trialed in humans has been nasal mucosa, a treatment which may be more beneficial for patients with cicatricial ocular surface disease [226]. Knowing the multipotential nature of SC, researchers are rapidly recognizing the usefulness of lineage unrelated cells including umbilical cord-lining SC [227], mesenchymal SC [228, 229], and embryonic SC [230–232] as potential future cell therapies for patients in need of corneal rehabilitation. This is exemplified by Hayashi and colleagues [233] who recently pushed the boundaries of regenerative medicine to new levels by deriving induced pluripotent SC (iPSC) from primary human limbal epithelial cells following transfection with the Yamanaka 4 transcription factors (Oct3/4, Sox 2, c-Myc, and Klf4). Three cell lines were derived, which when injected into SCID mice formed teratomas

composed of all three germ layers (neural/retinal, gut, and cartilage). These cells were differentiated into cornea-like cells as they expressed CK-3/12, CK-14, ΔNp63, and Pax6. In contrast, dermal fibroblast-derived iPSC transfected with the same transcription factors expressed less corneal differentiation markers, suggesting that iPSC probably retain properties including the epigenomic signature of the original cell type.

16 Conclusion

Therapies for patients with LSCD have certainly come a long way since Kenyon and Tseng first described their limbal tissue transplantation technique in the late 1980s [5]. However, there is scope to modify modern cell-based technologies to further improve their safety and efficacy. Studying the mechanisms that promote ocular health and visual acuity in patients who receive LESC transplants will undoubtedly contribute to a better understanding of how current treatment options work and how to develop more successful future technologies. Moreover, comprehensive assessments of the biological factors within the niche and the pathways and signaling molecules that actively partake in maintaining LESC quiescence will be necessary for identifying the best conditions for expanding these cells for clinical use. We already have solid evidence that LESC therapy works, but our challenges for the future will be to understand why some grafts fail and to eliminate xenogeneic components and minimize the use of allogeneic cells as strict regulatory requirements for cell-based therapies are becoming ever increasing [234]. The other major challenge will be to standardize LESC therapy; this should include patient diagnosis, graft preparation, and a defined set of endpoints and outcome measures. Notably, without a specific LESC marker these challenges will be difficult to address.

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References

1. Mann I (1944) A study of epithelial regeneration in the living eye. Br J Ophthalmol 28:26–40
2. Davanger M, Evensen A (1971) Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 229:560–561
3. Goldberg MF (1982) Limbal of Vogt. Trans Am Ophthalmol Soc 80:155–171
4. Townsend WM (1991) The limbal palisades of Vogt. Trans Am Ophthalmol Soc 89:721–755
5. Kenyon KR, Tseng SCG (1989) Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96:709–723

6. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349:990–993
7. Shortt AJ, Secker GA, Notara MD et al (2007) Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. Surv Ophthalmol 52:483–502
8. Shortt AJ, Tuft SJ, Daniels JT (2010) Ex vivo cultured limbal epithelial transplantation. A clinical perspective. Ocul Surf 8:80–90
9. Rama P, Bonini S, Lambiase A et al (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 72:1478–1485
10. Rama PP, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G (2010) Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med 363:147–155
11. Di Iorio E, Ferrari S, Fasolo A, Bohm E, Ponzin D, Barbaro V (2010) Techniques for culture and assessment of limbal stem cell grafts. Ocul Surf 8:146–153
12. Burman S, Sangwan V (2008) Cultivated limbal stem cell transplantation for ocular surface reconstruction. Clin Ophthalmol 3:489–502
13. Baylis O, Figueirdo F, Henein C, Lako M, Ahmad S (2011) 13 years of cultured epithelial cell therapy: a review of the outcomes. J Cell Biochem 112:993–1002
14. Sangwan VS, Basu S, Vemuganti GK et al (2011) Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. Br J Ophthalmol 95:1525–1529
15. Wei ZG, Cotsarelis G, Sun TT, Lavker RM (1995) Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis. Invest Ophthalmol Vis Sci 36:236–246
16. Pellegrini G, Golisano O, Paterna P et al (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol 145:769–782
17. Seigel GM, Sun W, Salvi R, Campbell LM, Sullivan S, Reidy JJ (2003) Human corneal stem cells display functional neuronal properties. Mol Vis 9:159–163
18. Zhao X, Das AV, Bhattacharya S et al (2008) Derivation of neurons with functional properties from adult limbal epithelium: implications in autologous cell therapy for photoreceptor degeneration. Stem Cells 26:939–949
19. Thoft RA, Friend J (1983) The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 24:1442–1443
20. Buck RC (1985) Measurement of centripetal migration of normal corneal epithelial cells in the mouse. Invest Ophthalmol Vis Sci 26:1296–1299
21. Sharma A, Coles WH (1989) Kinetics of corneal epithelial maintenance and graft loss. Invest Ophthalmol Vis Sci 30:1962–1971
22. Friedenwald JS, Buschke W (1944) Some factors concerned in the mitotic and wound-healing activities of the corneal epithelium. Trans Am Ophthalmol Soc 42:371–383
23. Hanna C, O'Brien JE (1960) Cell production and migration in the epithelial layer of the cornea. Arch Ophthalmol 64:536–539
24. Hanna C (1966) Proliferation and migration of epithelial cells during corneal wound repair in the rabbit and rat. Am J Ophthalmol 61:55–63
25. Srinivasan BD, Eakins KE (1979) The reepithelialization of rabbit cornea following single and multiple denudation. Exp Eye Res 29:595–600
26. Chen JJY, Tseng SCG (1991) Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. Invest Ophthalmol Vis Sci 32:2219–2233
27. Huang AJW, Tseng SCG (1991) Corneal epithelial wound healing in the absence of limbal epithelium. Invest Ophthalmol Vis Sci 32:96–105
28. Cotsarelis G, Cheng S-Z, Dong G, Sun T-T, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57:201–209
29. Schermer A, Galvin S, Sun T-T (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62

30. Kurpakus MA, Stock EL, Jones JCR (1990) Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. Invest Ophthalmol Vis Sci 31:448–456
31. Kinoshita S, Friend J, Thoft RA (1981) Sex chromatin of donor corneal epithelium in rabbits. Invest Ophthalmol Vis Sci 21:434–441
32. Chui J, Coroneo MT, Crouch R, Wakefield D, Di Girolamo N (2011) Ophthalmic pterygia: a stem cell disorder with pre-neoplastic features. Am J Pathol 178:817–827
33. Grossniklaus HE, Green WR, Luckenbach M, Chan CC (1987) Conjunctival lesions in adults: a clinical and histopathological review. Cornea 6:78–116
34. Garner A (1989) The pathology of tumours at the limbus. Eye 3:210–217
35. McNairn AJ, Guasch G (2011) Epithelial transition zones: merging microenvironments, niches, and cellular transformation. Eur J Dermatol 21(Suppl 2):21–28
36. Smith GT, Deutsch GP, Cree IA, Liu CSC (2000) Permanent corneal limbal stem cell dysfunction following radiotherapy for orbital lymphoma. Eye 14:905–907
37. Ellies P, Anderson DF, Touhami A, Tseng SCG (2001) Limbal stem cell deficiency arising from systemic chemotherapy. Br J Ophthalmol 85:373–374
38. Schofield R (1983) The stem cell system. Biomed Pharmacother 37:375–380
39. Zheng T, Xu J (2008) Age-related changes of human limbus on in vivo confocal microscopy. Cornea 27:782–786
40. Mort RL, Ramaesh T, Kleinjan DA, Morley SD, West JD (2009) Mosaic analysis of stem cell function and wound healing in the mouse corneal epithelium. BMC Dev Biol 9:4
41. Notara M, Shortt AJ, O'Callaghan AR, Daniels JT (2013) The impact of age on the physical and cellular properties of the human limbal stem cell niche. Age (Dordr) 35(2):289–300
42. Douvaras P, Webb S, Whitaker DA et al (2012) Rare corneal clones in mice suggest an age-related decrease of stem cell activity and supports the limbal epithelial stem cell hypothesis. Stem Cell Res 8:109–119
43. Higa K, Shimmura S, Miyashita H, Shimazaki J, Tsubota K (2005) Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. Exp Eye Res 81:218–223
44. Coroneo MT, Muller-Stolzenburg NW, Ho A (1991) Peripheral light focusing by the anterior eye and the ophthalmohelioses. Ophthalmic Surg 22:705–711
45. Vantrappen L, Geboes K, Missotten L, Maudgal PC, Desmet V (1985) Lymphocytes and Langerhans cells in the normal human cornea. Invest Ophthalmol Vis Sci 26:220–225
46. Schlotzer-Schrehardt U, Kruse FE (2005) Identification and characterization of limbal stem cells. Exp Eye Res 81:247–264
47. Chen Z, De Paiva CS, Luo L, Kretzer FL, Pfugfelder SC, Li D-Q (2004) Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22:355–366
48. der Merwe V, Kidson SH (2010) Advances in imaging the blood and aqueous vessels of the outer limbus. Exp Eye Res 91:118–126
49. Pinnamaneni N, Funderburgh JL (2012) Stem cells in the corneal stroma. Stem Cells 30:1059–1063
50. Marfurt CF, Cox J, Deek S, Dvorscak L (2010) Anatomy of the human corneal innervation. Exp Eye Res 90:478–492
51. Auran JD, Koester CJ, Kleiman NJ et al (1995) Scanning slit confocal microscope observation of cell morphology and movement within the normal human anterior cornea. Ophthalmology 102:33–41
52. Chui J, Di Girolamo N, Coroneo MT, Wakefield D (2007) The role of substance P in the pathogenesis of pterygia. Invest Ophthalmol Vis Sci 48:4482–4489
53. Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A (2005) Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. Br J Ophthalmol 89:529–532
54. Shanmuganathan VA, Foster T, Kulkarni BB et al (2007) Morphological characteristics of the limbal epithelial crypt. Br J Ophthalmol 91:514–519
55. Shortt AJ, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT (2007) Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. Stem Cells 25:1402–1409
56. Majo F, Rochat A, Nicolas M, Jaoude GA, Barrandon Y (2008) Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature 456:250–254

57. Chang CY, McGhee JJ, Green CR, Sherwin T (2011) Comparison of stem cell properties in cell populations isolated from human central and limbal corneal epithelium. Cornea 30:1155–1162
58. Dua HS, Miri A, Alomar T, Yeung AM, Said DG (2009) The role of limbal stem cells in corneal epithelial maintenance. Ophthalmology 116:856–863
59. Chang CY, Green CR, McGhee JJ, Sherwin T (2008) Acute wound healing in the human central corneal epithelium appears to be independent of limbal stem cell influence. Invest Ophthalmol Vis Sci 49:5279–5286
60. Duke-Elder S, Cook C (1963) Normal and abnormal development. Part 1: Embryology. In: Duke-Elder S (ed) System of ophthalmology. Henry Kimpton, London
61. Mann I (1964) The development of the human eye. British Medical Association, London
62. Sevel D, Isaacs R (1988) A re-evaluation of corneal development. Trans Am Ophthalmol Soc 86:178–207
63. Zhao XC, Yee RW, Norcom E et al (2006) The zebrafish cornea: structure and development. Invest Ophthalmol Vis Sci 47:4341–4348
64. Hay ED (1997) Development of the vertebrate cornea. Int Rev Cytol 63:263–322
65. Zieske JD (2004) Corneal development associated with eyelid opening. Int J Dev Biol 48:903–911
66. Davies S, Chui J, Madigan MC, Provis JM, Wakefield D, Di Girolamo N (2009) Stem cell activity in the developing human cornea. Stem Cells 27:2781–2792
67. Dora N, Ou J, Kucerova R, Parisi I, West JD, Collinson JM (2008) Pax6 dosage effects on corneal development, growth, and wound healing. Dev Dyn 237:1295–1306
68. Fokina VM, Frolova EI (2006) Expression patterns of wnt genes during development of anterior part of the chicken eye. Dev Dyn 235:496–505
69. O'Rahilly R (1975) The prenatal development of the human eye. Exp Eye Res 21:93–112
70. Graw J (2010) Eye development. Curr Top Dev Biol 90:343–386
71. Watt FM, Hogan BLM (2000) Out of Eden: stem cells and their niches. Science 287:1427–1430
72. Iozzo RV, Zoeller JJ, Nystrom A (2009) Basement membrane proteoglycans: modulators par excellence of cancer growth and angiogenesis. Mol Cells 27:503–513
73. Kurpakus MA, Stock EL, Jones JC (1992) The role of the basement membrane in differential expression of keratin proteins in epithelial cells. Dev Biol 150:243–255
74. Gipson IK (1989) The epithelial basement membrane zone of the limbus. Eye 3(Pt 2):132–140
75. Ljubimov AV, Burgeson RE, Butkowski RJ, Michael AF, Sun T-T, Kenney MC (1995) Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab Invest 72:461–473
76. Cleutjens JPM, Havenith MG, Kasper M, Vallinga M, Bosman FT (1990) Absence of type IV collagen in the centre of the corneal epithelial basement membrane. Histochem J 22:688–694
77. Tuori A, Uusitalo H, Burgeson RE, Terttunen J, Virtanen I (1996) The immunohistochemical composition of the human corneal basement membrane. Cornea 15:286–294
78. Schlotzer-Schrehardt U, Dietrich T, Saito K et al (2007) Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. Exp Eye Res 85:845–860
79. Kabosova A, Azar DT, Bannikov GA et al (2007) Compositional differences between infant and adult human corneal basement membranes. Invest Ophthalmol Vis Sci 48:4989–4999
80. Echevarria T, Chow S, Watson S, Wakefield D, Di Girolamo N (2011) Vitronectin: a matrix support factor for human limbal epithelial progenitor cells. Invest Ophthalmol Vis Sci 52:8138–8147
81. Braam SR, Zeinstra L, Litjens S et al (2008) Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cells self-renewal via αvβ5 integrin. Stem Cells 26:2257–2265
82. Li D-Q, Tseng SCG (1995) Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. J Cell Physiol 163:61–79

83. Qi H, Chuang EY, Yoon K-C et al (2007) Patterned expression of neurotrophic factors and receptors in human limbal and corneal regions. Mol Vis 13:1934–1941
84. Touhami A, Grueterich M, Tseng SCG (2002) The role of NGF signaling in human limbal epithelium expanded by amniotic membrane culture. Invest Ophthalmol Vis Sci 43:987–994
85. Di Girolamo N, Sarris M, Chiu J, Cheema H, Coroneo MT, Wakefield D (2008) Localization of the low affinity nerve growth factor receptor p75 in human limbal epithelial cells. J Cell Mol Med 12:2799–2811
86. Mukhopadhyay M, Gorivodsky M, Shtrom S et al (2006) DKK2 plays and essential role in the corneal fate of the ocular surface epithelium. Development 133:2149–2154
87. Espana EM, Kawakita T, Romano A et al (2003) Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. Invest Ophthalmol Vis Sci 44:5130–5135
88. Notara M, Shortt AJ, Galatowicz G, Calder V, Daniels JT (2010) IL-6 and the human limbal stem cell niche: a mediator of epithelial-stromal interaction. Stem Cell Res 5:188–200
89. Xie H-T, Chen S-Y, Li G-G, Tseng SCG (2011) Limbal epithelial stem/progenitor cells attract stromal niche cells by SDF-1/CXCR4 signaling to prevent differentiation. Stem Cells 29:1874–1885
90. Amad S, Stewart R, Yung S et al (2007) Differentiation of human embryonic stem cells into corneal epithelial like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells 25:1145–1155
91. Blazejewska EA, Schlotzer-Schrehardt U, Zenkel M et al (2009) Corneal limbal microenvironment can induce transdifferentiation of hair follicle stem cells into corneal epithelial-like cells. Stem Cells 27:642–652
92. Ferraris C, Chevalier G, Favier B, Jahoda CAB, Dhouailly D (2000) Adult corneal epithelium basal cells possess the capacity to activate epidermal, pilosebaceous and sweat gland genetic programs in response to embryonic dermal stimuli. Development 127:5487–5495
93. Pearton DJ, Ferraris C, Dhouailly D (2004) Transdifferentiation of corneal epithelium: evidence for a linkage between the segregation of epidermal stem cells and the induction of hair follicles during embryogenesis. Int J Dev Biol 48:197–201
94. Du Y, Funderburgh ML, Mann MM, SundarRaj N, Funderburgh JL (2005) Multipotent stem cells in human corneal stroma. Stem Cells 23:1266–1275
95. Li GG, Zhu YT, Xie HT, Chen SY, Tseng SC (2012) Mesenchymal stem cells derived from human limbal niche cells. Invest Ophthalmol Vis Sci 53:5686–5697
96. Branch MJ, Hashmani K, Dhillon P, Jones DRE, Dua HS, Hopkinson A (2012) Mesenchymal stem cells in the human corneal limbal stroma. Invest Ophthalmol Vis Sci 53:5109–5116
97. Yoshida S, Shimmura S, Nagoshi N et al (2006) Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea. Stem Cells 24:2714–2722
98. Nakamura T, Ishikawa F, Sonoda K-h et al (2005) Characterization and distribution of bone marrow-derived cells in mouse cornea. Invest Ophthalmol Vis Sci 46:497–503
99. Ye J, Lee SY, Hoon K, Yao K (2008) Bone marrow-derived progenitor cells promote corneal wound healing following alkali injury. Graefes Arch Clin Exp Ophthalmol 246:217–222
100. Kim HS, Song XJ, De Paiva CS, Chen Z, Pfugfelder SC, Li D-Q (2004) Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explants and single cell cultures. Exp Eye Res 79:41–49
101. De Paiva CS, Pflugfelder SC, Li D-Q (2006) Cell size correlates with phenotype and proliferative capacity in human corneal epithelial cells. Stem Cells 24:368–375
102. Boehlke CS, Yuan C, Kao WW, Huang AJ (2004) Cytokeratin 12 in human ocular surface epithelia is the antigen reactive with a commercial anti-Galpha q antibody. Mol Vis 10:867–873
103. Sun L, Sun T-T Lavker RM (2000) CLED: a calcium-linked protein associated with early epithelial differentiation. Exp Cell Res 259:96–106
104. Matic M, Petrov IN, Chen S, Wang C, Dimitrijevich SD, Wolosin JM (1997) Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. Differentiation 61:251–260
105. Wolosin JM, Schutte M, Zieske JD, Budak MT (2002) Changes in connexin 43 in early ocular surface development. Curr Eye Res 24:430–438

106. Chen Z, Evans WH, Pflugfelder SC, Li D-Q (2006) Gap junction protein connexin 43 serves as a negative marker for a stem cell-containing population of human limbal epithelial cells. Stem Cells 24:1265–1273
107. Ahmad S, Kolli S, Li D-Q et al (2008) A putative role for RHAMM/HMMR as a negative marker of stem cell-containing population of human limbal epithelial cells. Stem Cells 26:1609–1619
108. Kim M, Turnquist H, Jackson J et al (2002) The multidrug resistance transported ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin Cancer Res 8:22–28
109. Zhou S, Schuetz JD, Bunting KD et al (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7:1028–1034
110. Watanabe K, Nishida K, Yamato M et al (2004) Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett 565:6–10
111. De Pavia CS, Chen Z, Corrales RM, Pflugfelder SC, Li D-Q (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem Cells 23:63–73
112. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MAM, Wolosin JM (2005) Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. J Cell Sci 118:1715–1724
113. Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, Okano T (2006) Limbal epithelial side-population cells have stem cell-like properties, including quiescent state. Stem Cells 24:86–94
114. Umemoto T, Yamato M, Nishida K et al (2005) Rat limbal epithelial side population cells exhibit expression of stem cell markers that are lacking in side population cells from the central cornea. FEBS Lett 579:6569–6574
115. Chen W, Hara K, Tian Q, Zhao K, Yoshitomi T (2007) Existence of small slow-cycling Langerhans cells in the limbal basal epithelium that express ABCG2. Exp Eye Res 84:626–634
116. Wolosin JM, Akinci M, Taveras M, Turner H (2006) Intraepithelial lymphocytes are a major component of ocular surface epithelial side populations. ARVO Abstr 47:3954
117. Kubota M, Shimmura S, Miyashita H, Kawashima M, Kawakita T, Tsubota K (2010) The anti-oxidative role of ABCG2 in corneal epithelial cells. Invest Ophthalmol Vis Sci 51:5617–5622
118. Jonker JW, Buitelaar M, Wagenaar E et al (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protophorphyria. Proc Natl Acad Sci U S A 99:15649–15654
119. Zieske JD, Bukusoglu G, Yankaukas MA, Wasson ME, Keutmann HT (1992) Alpha-enolase is restricted to basal cells of stratified squamous epithelium. Dev Biol 151:18–26
120. Zieske JD (1994) Perpetuation of stem cells in the eye. Eye 8:163–169
121. Chung E-H, DeGregorio PG, Wasson ME, Zieske JD (1995) Epithelial regeneration after limbus-to-limbus debridement. Invest Ophthalmol Vis Sci 36:1336–1343
122. Barnard Z, Apel AJG, Harkin DG (2001) Phenotypic analysis of limbal epithelial cell cultures derived from donor corneoscleral rims. Clin Exp Ophthalmol 29:138–142
123. Figueira EC, Di Girolamo N, Coroneo MT, Wakefield D (2007) The phenotype of limbal epithelial stem cells. Invest Ophthalmol Vis Sci 48:144–156
124. Nieto-Miguel T, Calonge M, de la Mata A et al (2011) A comparison of stem cell-related gene expression in the progenitor-rich limbal epithelium and the differentiating central corneal epithelium. Mol Vis 17:2102–2117
125. Yoshida S, Shimmura S, Kawakita T et al (2006) Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. Invest Ophthalmol Vis Sci 47:4780–4786

126. Kasper M (1992) Patterns of cytokeratins and vimentin in guinea pig and mouse eye tissue; evidence for regional variations in intermediate filament expression in limbal epithelium. Acta Histochem 92:319–332
127. Lauweryns B, van den Oord JJ, De Vos R, Missotten L (1993) A new epithelial cell type in the human cornea. Invest Ophthalmol Vis Sci 34:1983–1990
128. Lu H, Zimek A, Chen J et al (2006) Keratin 5 knockout mice reveal plasticity of keratin expression in the corneal epithelium. Eur J Cell Biol 85:803–811
129. Yang A, Schweitzer R, Sun D et al (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714–718
130. Mills AA, Zheng B, Wang X-J, Vogel H, Roop DR, Bradley A (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398:708–713
131. Pellegrini G, Dellambra E, Golisano O et al (2001) p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 98:3156–3161
132. Dua HS, Joseph A, Shanmuganathan VA, Jones RE (2003) Stem cell differentiation and the effects of deficiency. Eye 17:877–885
133. Chee KYH, Kicic A, Wiffen SJ (2006) Limbal stem cells: the search for a marker. Clin Exp Ophthalmol 34:64–73
134. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M (2005) Isoforms of ΔNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc Natl Acad Sci U S A 102:9523–9528
135. Harkin DG, Barnard Z, Gillies P, Ainscough SL, Apel AJG (2004) Analysis of p63 and cytokeratin expression in a cultivated limbal autograft used in the treatment of limbal stem cell deficiency. Br J Ophthalmol 88:1154–1158
136. Carter RT (2009) The role of integrins in corneal wound healing. Vet Ophthalmol 12(Suppl 1):2–9
137. Stepp MA (2006) Corneal integrins and their functions. Exp Eye Res 83:3–15
138. Braam SR, Zientra L, Litjens S et al (2008) Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via αvβ5 integrin. Stem Cell 26:2257–2265
139. Pajoohesh-Ganji A, Ghosh SP, Stepp MA (2004) Regional distribution of α9β1 integrin within the limbus of the mouse ocular surface. Dev Dyn 230:518–528
140. Maseruka H, Ridgway A, Tullo A, Bonshek R (2000) Developmental changes in patterns of expression of Tenascin-C variants in the human cornea. Invest Ophthalmol Vis Sci 41:4101–4107
141. Pajoohesh-Ganji A, Ghosh SP, Simmens SJ, Stepp MA (2006) Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. Stem Cells 24:1075–1086
142. Li A, Simmons PJ, Kaur P (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci U S A 95:3902–3907
143. Tani H, Morris RJ, Kaur P (2000) Enrichment for murine keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci U S A 97:10960–10965
144. Croagh D, Phillips WA, Redvers R, Thomas RJS, Kaur P (2007) Identification of candidate murine esophageal stem cells using a combination of cell kinetics studies and cell surface markers. Stem Cells 25:313–318
145. Hayashi R, Yamato M, Saito T et al (2008) Enrichment of corneal epithelial stem/progenitor cells using cell surface markers, integrin α6 and CD71. Biochem Biophys Res Commun 367:256–263
146. Hayashi R, Yamato M, Sugiyama H et al (2007) N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human epithelial stem cell niche. Stem Cells 25:289–296
147. Higa K, Shimmura S, Miyashita H et al (2009) N-cadherin in the maintenance of human corneal limbal epithelial progenitor cells in vitro. Invest Ophthalmol Vis Sci 50:4640–4645
148. Mimura T, Yamagami S, Uchida S et al (2010) Isolation of adult progenitor cells with neuronal potential from rabbit corneal epithelial cells in serum- and feeder layer-free culture conditions. Mol Vis 16:1712–1719

149. Grueterich M, Espana EM, Tseng SCG (2003) Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 48:631–646
150. Nakamura M, Okano H, Blendy JA et al (1994) Musashi, a neural RNA-binding protein required for Drosophila adult external sensory organ development. Neuron 13:67–81
151. Sakakibara S, Nakamura Y, Yoshida T et al (2002) RNA-binding protein Musashi family: roles in CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation. Proc Natl Acad Sci U S A 99:15194–15199
152. Chen Y-T, Li W, Hayashida Y et al (2007) Human amniotic epithelial cells as novel feeder layers for promoting ex vivo expansion of limbal epithelial progenitor cells. Stem Cells 25:1995–2005
153. Hitoshi S, Alexson T, Tropepe V et al (2002) Notch pathway molecules are essential for the maintenance, but not the generation of mammalian neural stem cells. Gene Dev 16:846–858
154. Thomas PB, Liu Y-H, Zhuang FF et al (2007) Identification of notch-1 expression in the limbal basal epithelium. Mol Vis 13:337–344
155. Tomita K, Ishibashi M, Nakahara K et al (1996) Mammalian hairy and enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16:723–734
156. Nakamura T, Ohtsuka T, Sekiyama E et al (2008) Hes1 regulates corneal development and the function of corneal epithelial stem/progenitor cells. Stem Cells 26:1265–1274
157. Lu R, Qu Y, Ge J, Zhang L, Su Z, Pflugfelder SC, Li D-Q (2012) Transcription factor TCF4 maintains the properties of human corneal epithelial stem cells. Stem Cells 30:753–761
158. Bian F, Liu W, Yoon K-C et al (2010) Molecular signatures and biological pathway profiles of human corneal epithelial progenitor cells. Int J Biochem Cell Biol 42:1142–1153
159. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R (2003) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathway. Genes Dev 19:1432–1437
160. Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M (2007) C/EBPδ regulates cell cycle and self-renewal of human limbal stem cells. J Cell Biol 177:1037–1049
161. Adachi W, Ulanovsky H, Li Y, Norman B, Davis J, Piatigorsky J (2006) Serial analysis of gene expression (SAGE) in the rat limbal and central corneal epithelium. Invest Ophthalmol Vis Sci 47:3801–3810
162. Zhou M, X-m L, Lavker RM (2006) Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells. J Biol Chem 281:19600–19609
163. Kulkarni BB, Tighe PJ, Mohammed I et al (2010) Comparative transcriptional profiling of the limbal epithelial crypt demonstrates its putative stem cell niche characteristics. BMC Gemonics 11:526–544
164. Baylis O, Rooney P, Figueirdo F, Lako M, Ahmad S (2013) An investigation of donor and culture parameters which influence epithelial outgrowth from human cadaveric limbal explants cultured on amniotic membrane. J Cell Physiol 228(5):1025–1030
165. Crewe JM, Armitage WJ (2001) Integrity of epithelium and endothelium in organ-cultured human corneas. Invest Ophthalmol Vis Sci 42:1757–1761
166. Raeder S, Utheim TP, Utheim OA et al (2007) Effect of organ culture and Optisol-GS storage on structural integrity, phenotypes, and apoptosis in cultured corneal epithelium. Invest Ophthalmol Vis Sci 48:5484–5493
167. Espana EM, Romano AC, Kawakita T, Di Pascuale M, Smiddy R, Tseng SCG (2003) Novel enzymatic isolation of an entire viable human limbal epithelial sheet. Invest Ophthalmol Vis Sci 44:4275–4281
168. Arpitha P, Prajna NV, Srinivasan M, Muthukkaruppan V (2008) A method to isolate human limbal basal calls enriched for a subset of epithelial cells with a large nucleus/cytoplasm ration expressing high levels of p63. Microsc Res Tech 71:469–476
169. Chen S-Y, Hayashida Y, Vhen M-Y, Xie HT, Tseng SCG (2011) A new isolation method of human limbal progenitor cells by maintaining close association with their niche. Tissue Eng Part C 117:537–548

170. Li D-Q, Chen Z, Song XJ, De Paiva CS, Kim H-S, Pflugfelder SC (2005) Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp Eye Res 80:581–590
171. Krulova M, Pokorna K, Lencova A et al (2008) A rapid separation of two distinct populations of mouse corneal epithelial cells with limbal stem cell characteristics by centrifugation on percoll gradient. Invest Ophthalmol Vis Sci 49:3903–3908
172. Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–344
173. Todaro GJ, Green H (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17:299–313
174. Barrandon Y, Green H (1987) Three clonal types of keratinocytes with different capacities for multiplication. Proc Natl Acad Sci U S A 84:2302–2306
175. Stocker FW, Eiring A, Georgiade MSR, Georgiade N (1958) A tissue culture technique for growing corneal epithelial, stromal, and endothelial tissues separately. Am J Ophthalmol 46:294–298
176. Newsome DA, Takasugi M, Kenyon KR, Stark WF, Opelz G (1974) Human corneal cells in vitro: morphology and histocompatibility (HL-A) antigens of pure cell populations. Invest Ophthalmol 13:23–32
177. Sun T-T, Green H (1977) Cultured epithelial cells of cornea, conjunctiva and skin: absence of marker intrinsic divergence of their differentiated states. Nature 269:489–492
178. Wei ZG, Wu R-L, Lavker RM, Sun T-T (1993) In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia: implications on conjunctival epithelial transdifferentiation and stem cells. Invest Ophthalmol Vis Sci 34:1814–1828
179. Kruse FE, Tseng SCG (1993) Serum differentiation modulates the clonal growth and differentiation of cultured limbal and corneal epithelium. Invest Ophthalmol Vis Sci 34:2976–2989
180. Nishida K, Yamato M, Hayashida Y et al (2004) Functional bioengineered cornea epithelial sheet grafts from corneal stem cells expanded ex vivo on temperature-responsive cell culture surface. Transplantation 77:379–385
181. Cooper LJ, Kinoshita S, German M et al (2005) An investigation into the composition of amniotic membrane used for ocular surface reconstruction. Cornea 24:722–729
182. Koizumi NJ, Inatomi TJ, Sotozono CJ et al (2000) Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20:173–177
183. Kenyon KR (2005) Amniotic membrane: mother's own remedy for ocular surface disease. Cornea 24:639–642
184. Grueterich M, Tseng SC (2002) Human limbal progenitor cells expanded on intact amniotic membrane ex vivo. Arch Ophthalmol 120:783–790
185. Koizumi N, Cooper LJ, Fullwood NJ et al (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. Invest Ophthalmol Vis Sci 43:2114–2121
186. Li W, Hayashida Y, He H, Kuo C-L, Tseng SCG (2007) The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane. Invest Ophthalmol Vis Sci 48:605–613
187. Meyer-Blazejewska EA, Kruse FE, Bitterer K et al (2010) Preservation of the limbal stem cell phenotype by appropriate culture techniques. Invest Ophthalmol Vis Sci 51:765–774
188. Gosain AK, Lyon VB (2002) The current status of tissue glues: Part II. For adhesion of soft tissues. Plast Reconstr Surg 110:1581–1584
189. Talbot M, Carrier P, Giasson CJ et al (2006) Autologous transplantation of rabbit limbal epithelia cultured on fibrin gels for ocular surface reconstruction. Mol Vis 12:65–75
190. Shortt AJ, Secker GA, Lomas R et al (2009) The effect of amniotic membrane preparation method on its ability to serve as a substrate for the ex-vivo expansion of limbal epithelial cells. Biomaterials 30:1056–1065
191. Krishnan S, Seka S, Katheem MF, Krishnakumar S, Sastry TP (2012) Fish scale collagen – a novel material for corneal tissue engineering. Artif Organs 36:829–835

192. Dravida S, Gaddipati S, Griffith M et al (2008) A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. J Tissue Eng Regen Med 2:263–271
193. Mi S, Chen B, Wright B, Connon CJ (2010) *Ex vivo* construction of an artificial ocular surface by combination of corneal limbal epithelial cells on a compressed collagen scaffold containing keratocytes. Tissue Eng Part A 16:2091–2110
194. Levis HJ, Brown RA, Daniels JT (2010) Plastic compressed collagen as a biomimetic substrate for human limbal epithelial culture. Biomaterials 31:7726–7737
195. Bray LJ, George KA, Ainscough SL, Hutmacher DW, Chirila TV, Harkin DG (2011) Human corneal epithelial equivalents constructed on Bombyx mori silk fibroin membranes. Biomaterials 32:5086–5091
196. Albert R, Vereb Z, Csomos K et al (2012) Cultivation and characterization of corneal limbal epithelial stem cells on lens capsule in animal material-free medium. PLoS One 7:e47187
197. Zajicova A, Pokorna K, Lencova A et al (2010) Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant 19:1281–1290
198. Di Girolamo N, Chui J, Wakefield D, Coroneo MT (2007) Cultured human ocular surface epithelium on therapeutic contact lenses. Br J Ophthalmol 91:459–464
199. Di Girolamo N, Bosch M, Zamora K, Coroneo MT, Wakefield D, Watson S (2009) A contact lens-based technique for expansion and transplantation of autologous epithelial progenitors for ocular surface reconstruction. Transplantation 87:1571–1578
200. Deshpande P, Notara M, Bullett N, Daniels JT, Haddow DB, MacNeil S (2009) Development of a surface-modified contact lens for the transfer of cultured limbal epithelial cells to the cornea for ocular surface disease. Tissue Eng Part A 15:1–14
201. Tan XW, Tan D, Beuerman RW, Mehta JS (2012) Study of novel Chitosan-coated contact lens as an equivalent substrate for the therapeutic delivery of rabbit limbal epithelium. ARVO Abstr 6124/D94
202. Vascotto SG, Griffith M (2006) Localization of candidate stem and progenitor cell markers within the human cornea, limbus, and bulbar conjunctiva in vivo and in cell culture. Anat Rec A Discov Mol Cell Evol Biol 288:921–931
203. Duffy P, Wolf J, Collins G et al (1974) Possible person-to-person transmission of Creutzfeldt-Jakob disease. N Engl J Med 290:692–693
204. Schwab IR, Johnson NT, Harkin DG (2006) Inherent risks associated with manufacture of bioengineered ocular surface tissue. Arch Ophthalmol 124:1734–1740
205. Johnen S, Wickert L, Meier M, Salz AK, Walter P, Thumann G (2011) Presence of xenogenic mouse RNA in RPE and IPE cells cultures on mitotically inhibited 3T3 fibroblasts. Invest Ophthalmol Vis Sci 52:2817–2829
206. Holland EJ, Schwartz GS (1996) The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. Cornea 15:549–556
207. Nishida K, Kinoshita S, Ohashi Y, Kuwayama Y, Yamamoto S (1995) Ocular surface abnormalities in Aniridia. Am J Ophthalmol 120:368–375
208. Ramaesh K, Ramaesh T, Dutton GN, Dhillon B (2005) Evolving concepts on the pathogenic mechanisms of aniridia related keratopathy. Int J Biochem Cell Biol 37:547–557
209. Shortt AJ, Tuft SJ, Daniels JT (2011) Corneal stem cells in the eye clinic. Br Med Bull 100:209–225
210. Shimazaki J, Higa K, Morito F et al (2007) Factor influencing outcomes in cultivated limbal epithelial transplantation for chronic cicatricial ocular surface disease. Am J Ophthalmol 143:945–953
211. Daya SM, Chan CC, Holland EJ (2011) Cornea Society nomenclature for ocular surface rehabilitative procedures. Cornea 30:1115–1119
212. Williams KA, Brereton HM, Aggarwal R et al (1995) Use of DNA polymorphisms and the polymerase chain reaction to examine the survival of a human limbal stem cell allograft. Am J Ophthalmol 120:342–350
213. Daya SM, Watson A, Sharpe JR et al (2005) Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. Ophthalmology 112:470–477

214. Shimazaki J, Kaido M, Shinozaki N, Shimmura S et al (1999) Evidence of long-term survival of donor-derived cells after limbal allograft transplantation. Invest Ophthalmol Vis Sci 40:1664–1668
215. Stenevi U, Hanson C, Claesson M, Corneliusson E, Ek S (2002) Survival of transplanted human corneal stem cells: case report. Acta Ophthalmol Scand 80:105–108
216. Bradshaw JJ, Obritsch WF, Cho BJ, Gregerson DS, Holland EJ (1999) Ex vivo transduction of corneal epithelial progenitor cells using a retroviral vector. Invest Ophthalmol Vis Sci 40:230–235
217. Holan V, Pokorna K, Prochazkova J, Krulova M, Zajicova A (2010) Immunoregulatory properties of mouse limbal stem cells. J Immunol 184:2124–2129
218. Abumaree M, Al Jumah M, Pace RA, Kalionis B (2012) Immunosuppressive properties of mesenchymal stem cells. Stem Cell Rev 8:375–392
219. Ang LPK, Tanioka H, Kawasaki S, Ang LPS, Yamasaki K, Do TP et al (2010) Cultivated human conjunctival epithelial transplantation for total limbal stem cell deficiency. Invest Ophthalmol Vis Sci 51:758–764
220. Ricardo JR, Cristovam PC, Filho PA et al (2013) Transplantation of conjunctival epithelial cells cultivated ex vivo in patients with total limbal stem cell deficiency. Cornea 32(3):221–228
221. Nishida K, Yamato M, Hayashida Y et al (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 351:1187–1196
222. Chen H-CJ, Chen H-L, Lai J-Y et al (2009) Persistence of transplanted oral mucosal epithelial cells in human corneas. Invest Ophthalmol Vis Sci 50:4660–4668
223. Eslani M, Baradaran-Rafii A, Ahmad S (2012) Cultivated limbal and oral mucosal epithelial transplantation. Semin Ophthalmol 27:80–93
224. Burillon C, Huot L, Justin V et al (2012) Cultured autologous oral mucosal epithelial sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. Invest Ophthalmol Vis Sci 53:1325–1332
225. Chen H-CJ, Yeh L-K, Tsai YJ et al (2012) Expression of angiogenesis-related factors in human corneas after cultivated oral mucosal epithelial transplantation. Invest Ophthalmol Vis Sci 53(9):5615–5623
226. Kim JH, Chun YS, Lee SH et al (2010) Ocular surface reconstruction with autologous nasal mucosa in cicatricial ocular surface disease. Am J Ophthalmol 149:45–53
227. Reza HM, Ng BY, Gimeno FL, Phan TT, Ang LP (2011) Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. Stem Cell Rev 7:935–947
228. Jiang T-S, Cai L, Ji W-Y et al (2010) Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. Mol Vis 16:1304–1316
229. Reinshagen H, Auw-Haedrich C, Sorg RV et al (2011) Corneal surface reconstruction using mesenchymal stem cells in experimental limbal stem cells deficiency in rabbits. Acta Ophthalmol 89:741–748
230. Homma R, Yoshikawa H, Takeno M et al (2004) Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest Ophthalmol Vis Sci 45:4320–4326
231. Notara M, Hernandez D, Mason C, Daniels JT (2012) Characterization of the phenotype and functionality of corneal epithelial cells derived from mouse embryonic stem cells. Regen Med 7:167–178
232. Hanson C, Hardarson T, Ellerstrom C et al (2013) Transplantation of human embryonic stem cells into a partially wounded human cornea in vitro. Acta Ophthalmol 91(2):127–130
233. Hayashi R, Ishikawa Y, Ito M et al (2012) Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblasts and corneal epithelium. PLoS One 7:e45435
234. Daniels JT, Secker GA, Shortt AJ et al (2006) Stem cell therapy delivery: treading the regulatory tightrope. Regen Med 1:715–719

Adult Stem Cells in Teeth

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Abstract Stem cells hold enormous interest for basic and translational scientists because of their ability to self-renew and differentiate into multiple cell types. These properties have led stem cell biology to emerge as an important field of biomedical research over the past few decades, and a large effort to identify the locations of adult stem cells has been under way. Teeth and tooth-supporting structures house important adult stem cell niches in the body. In this chapter, we describe the known populations of adult dental stem cells. We focus on the location, stemness, and differentiation potential of these cells. In addition, we discuss recent advances in the applications of adult stem cells to the field of regenerative medicine.

Keywords Tooth • Dental • Regeneration • Pluripotent • Pulp

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Abbreviations

AP	Apical papilla
ChIP-seq	Chromatin immunoprecipitation sequencing
DFSCs	Dental follicle stem cells
DPPSCs	Dental pulp pluripotent stem cells
DPSCs	Dental pulp stem cells
EK	Enamel knot
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
HA	Hydroxyapatite
HERS	Hertwig's epithelial root sheath
IEE	Inner enamel epithelium
iPSCs	Induced pluripotent stem cells
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells
SCAP	Stem cells of apical papilla
SHED	Stem cells from human exfoliated deciduous teeth

1 Introduction

Stem cells are progenitors that are capable of both self-renewal and differentiation into multiple cell types. Because of the enormous therapeutic potential of stem cell research, it has emerged as an important field of biomedical research, and it encompasses such diverse applications as tissue engineering and repair, treatment of autoimmune disorders, and in vitro organ generation.

Stem cells can be divided into several major types, including embryonic stem cells (ESCs), adult (somatic) stem cells, and induced pluripotent stem cells (iPSCs). ESCs were first isolated from mouse blastocysts in the early 1980s and from human embryos almost 2 decades later [1–3]. More recently, the technique of converting virtually any cell type, including differentiated somatic cells, into iPSCs was developed, promising an almost unlimited source of easily obtained pluripotent stem cells [4, 5]. Production of iPSCs originally involved the introduction of a cocktail of four transcription factors—OCT3/4, SOX2, KLF4, and c-MYC—into adult cells [6]. However, further studies revealed that specific reprogramming factors differ among tissues [7, 8]. The resulting reprogrammed cells can then be redifferentiated into cells that represent derivatives of all three germ layers [5, 6]. This allows for the potential use of autologous cells in clinical applications and may help minimize recipient rejection.

In addition to ESCs and iPSCs, stem cells can be isolated from adult tissues. In some cases, multiple adult stem cell types of varying differentiation potential can be isolated from the same tissue. The notion that almost all tissues in the body contain somatic stem cells is now established. Examples of tissues with well-characterized

somatic stem cells include hair follicles, intestinal crypts, and skin [9–11]. In this chapter, we discuss another important organ that houses several distinct populations of adult stem cells: the tooth.

1.1 Strategies for Isolation of Dental Stem Cells

To accurately study the properties of adult stem cells, it is vital to correctly identify and isolate them from millions of somatic cells within the same tissue, a feat seemingly akin finding a needle in a haystack. One of the most common strategies for isolation of dental stem cells involves separation based on markers. However, the identification of specific stem cell markers is challenging. Historically, marker candidates were chosen based on several criteria, such as being targets of major signaling pathways that are active within the putative stem cell niche [12]. Definitive identification of stem cells requires linkage between them and their progeny through either transplantation or lineage-tracing experiments [13, 14]. Further, some stem cells are quiescent and can be localized using label-retention experiments. This technique relies on the idea that, over time, fast-cycling somatic cells dilute the label, whereas slow-cycling stem cells retain much of the original signal [15].

Once a stem cell marker is known, populations of cells can be isolated via fluorescence-activated cell sorting (FACS) or by antibody-conjugated microbead isolation. Other methods of prospective stem cell isolation exist, although these are less robust. One method, high efflux of a fluorescent nuclear stain, isolates cells capable of excluding a fluorescent DNA-binding dye. Studies show that cells capable of differentiating into adipocytes, chondrocytes, and osteoblasts can exclude the dye by utilizing cell-specific membrane efflux pumps [16, 17]. Additionally, the ability of some adult dental stem cells to exhibit a high proliferation capacity in vitro has been used as a method of isolation by focusing on cell growth. This requires frequent passaging at low densities to prevent the stem cells from undergoing differentiation [18, 19].

1.2 Common Markers of Stem Cells

Identification of markers specific to stem cells can be used to separate them from a heterogeneous cell population. Often, these markers are regulatory genes involved in development and maintenance of stem cells. In this section, we describe common stem cell markers used to identify adult stem cells in teeth.

OCT3/4, a member of the Pit-Oct-Unc family of transcription factors, was initially thought to be expressed almost exclusively in ESCs [20, 21]. Later studies identified OCT3/4^{+} embryonic and adult stem cells as well as the requirement for *Oct3/4* expression in iPSCs [6, 22, 23]. The key to remaining undifferentiated seems to be maintenance of a narrow range of *Oct3/4* expression, as both positive and

negative fluctuations in expression cause cell differentiation [24]. Genome-wide interaction studies, including chromatin immunoprecipitation sequencing (ChIP-Seq), suggest that OCT3/4 controls cell pluripotency by modulating major developmental signaling pathways, such as the Fgf and Wnt/β-catenin pathways [24]. One important OCT3/4 target is *Nanog*, which plays a key role in the transcription factor signaling network in mammalian pluripotent cells and developing germ cells [25, 26]. Disruption of Nanog signaling leads to early embryonic lethality, whereas constitutive expression promotes self-renewal of ESCs. Finally, nestin is an intermediate filament protein originally identified in neuroepithelial stem cells that is also expressed in non-neuronal tissues, such as pancreatic islets, hematopoietic cells, and teeth [27–30].

A number of common surface markers has also been used for identification and isolation of stem cell populations. Examples of these include CD29, CD44, CD73, CD90, CD105, CD146, and CD166 [31, 32]. Integrin β-1 (CD29) was first identified in lymphocytes and plays a role in matrix adhesion [33, 34]. The cell surface glycoprotein CD44 was first identified in hematopoietic stem cells and plays a role in adhesion and cell–cell interactions [35]. Ecto-5′-nucleotidase (CD73) and CD90 are mesenchymal stem cell markers involved in interactions between hematopoietic and endothelial cells [36, 37]. Another commonly used marker is Endoglin (CD105), which is a member of the TGF-β1 receptor complex and was originally identified in endothelial cells of the cardiovascular system [38]. Finally, CD146 and CD166 are common markers of mesenchymal stem cells used for identification of dental stem cells, with both playing a role in cell–cell adhesion [39, 40]. In the last 5 years, a number of articles have presented additional markers of adult dental stem cells, such as CD34, CD117, and CD271 [31].

2 Tooth Development and Morphogenesis

2.1 The Adult Tooth

The three fundamental parts of the tooth are the crown, roots, and supporting structures (Fig. 1). Teeth are anchored in the alveolar processes of the maxillary and mandibular bones by the periodontal ligament (PDL). These ligaments extend from the bone in tooth sockets and insert into the cementum layer, which is the outermost layer of the tooth root [41]. The crown is composed of enamel and is exposed to the oral cavity, providing masticatory (chewing) function for the tooth. The middle layer of the tooth in both roots and the crown is dentin. The dentin encases the dental pulp, the innermost part of the tooth in both crown and roots. The pulp tissue comprises the neurovascular bundle of the tooth and its supporting cells as well as a population of dentin-secreting odontoblasts [42].

Humans possess 20 primary teeth and 32 adult teeth: 8 incisors, 4 canines, 8 premolars, and 12 molars. The primary teeth appear at around 6 months of age and

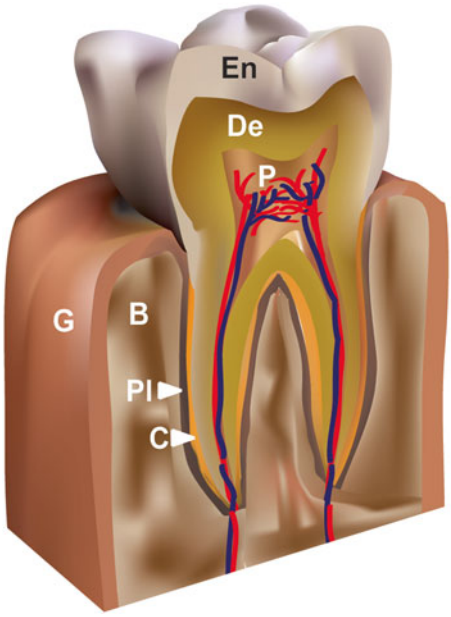

Fig. 1 Cartoon depiction of a human lower molar tooth. The crown is the part of the tooth covered by enamel (En) and the root is the part of the tooth covered by cementum (C). Underlying both enamel and cementum is dentin (De). The tooth is attached to bone (B) via periodontal ligaments (Pl). The neurovascular bundle of the tooth is located in the pulp (P). The supporting bone is covered by the gingiva (G)

are fully shed by the early teens. Once the tooth erupts into the oral cavity, the dental epithelial tissue is lost, such that adult human teeth lose the potential to regenerate enamel, and the remaining mesenchymal tissues have only a limited capacity to regenerate dentin, cementum, and pulp. In contrast, mice, which are an important model for investigation of tooth development, exhibit a highly specialized dentition [43]. They possess 4 incisors and 12 molars, which are separated by a toothless area called the diastema. Rodent incisors are unusual in their ability to grow throughout the lifetime of the animal through a continuous wear and formation of all tooth tissues including enamel, dentin, pulp, and cementum. This property is made possible by the presence of active adult stem cells in a niche called the cervical loop [44]. In addition, several species of rodents (and other mammals) exhibit ever-growing molars, called hypselodont molars. These molars retain adult epithelial stem cells in compartments known as intercuspal cervical loops, which are surrounded by stem cells of the mesenchyme [45]. In this chapter, we describe the adult stem cells of human teeth, which are all rooted.

2.2 Tooth Development

Mammalian tooth development requires tissues of both epithelial and mesenchymal origin (Fig. 2) [46, 47]. Whereas the epithelium originates from oral ectoderm, the mesenchyme is derived from cranial neural crest. The latter arises from the margins

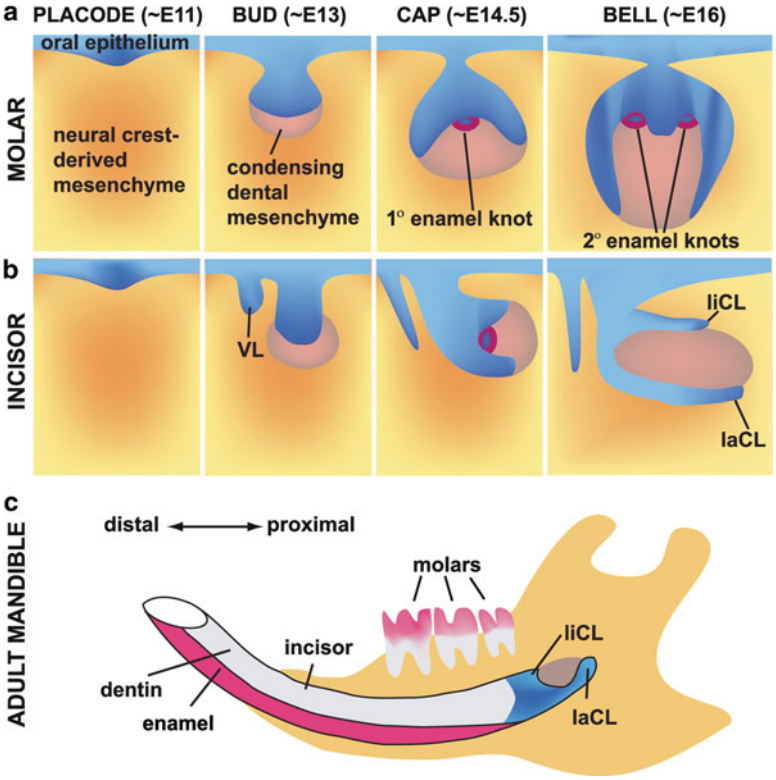

Fig. 2 Cartoon depiction of various stages of mouse molar (**a**) and incisor (**b**) tooth development and adult mouse mandible (**c**) in sagittal view. Tooth development begins with thickening and invagination of the oral epithelium into the underlying mesenchyme at ~E11. In the bud stage (~E13), the mesenchyme condenses. At the cap stage (~E14.5), the enamel knot, a central signaling center, appears. At the bell stage (~E16), the secondary enamel knots form, corresponding to the future location of cusps. In addition, the extracellular matrices of enamel and dentin are excreted by the differentiating ameloblasts and odontoblasts, respectively. Tooth development is similar in incisor and molar with a few key differences being a 90° turn of the incisor Bell, as well as the presence of the vestibular lamina (VL), the labial and lingual cervical loops (laCL and liCL, respectively), and the absence of 2° enamel knots in the incisor

of neuroepithelium and undergoes a ventro-lateral migration, giving rise to facial structures, facial sensory organs, and pharyngeal arches [48]. Neural crest-derived mesenchyme forms many dental tissues, such as dentin, pulp, and periodontal ligaments, whereas the ectodermal-derived epithelium forms enamel [49].

Much of the knowledge about tooth development (odontogenesis) stems from decades of investigations utilizing mouse models. In mice and the majority of mouse-like rodents (i.e., rats and voles), tooth development begins between embryonic day (E) 8.5 and 10. At that time, the first signaling molecules involved in development become apparent. The formation of signaling centers is followed by the thickening of the epithelium into the underlying mesenchyme at E11.

Subsequently, a series of developmental stages named bud, cap, and bell occur. At the bud stage (E12.5–13.5), the epithelium continues to proliferate and invaginate into the mesenchyme, which responds by condensing under the epithelial bud to form a structure known as the dental papilla. The enamel knot (EK), which acts as a transient signaling center and is characterized by expression of several secreted factors, forms during the bud to cap stage transition. The cap stage (E14–15) is characterized by further epithelial and mesenchymal proliferation. The dental epithelium proliferates and surrounds the dental papilla, whereas mesenchyme proliferates and forms the dental follicle. The late cap stage is also the time when significant differences emerge between the mouse incisor and molar, as the developing incisor begins to grow parallel to the long axis of the jaw. During the bell stage, the outline of the final tooth shape becomes apparent. The primary EKs are replaced by secondary EKs in the molars, which correspond to the places of future tooth cusps [50]. Thus, incisors and canines exhibit only one EK, whereas premolars and molars exhibit several. Finally, specific dental cell types become apparent at the bell stage.

The enamel-producing ameloblasts form from cells adjacent to the dental papilla, in a niche known as the inner enamel epithelium (IEE). These cells proliferate and begin to secrete enamel matrix. The dentin-secreting odontoblasts arise from the cells located on the outermost layer of the dental papilla. Together, the niches containing ameloblast and odontoblast progenitor cells make up the cervical loops. As the teeth begin to erupt, following the completion of crown formation, the cervical loop epithelium forms a bilayer structure called Hertwig's epithelial root sheath (HERS) to guide the development of tooth roots [51]. As HERS migrates apically together with the dental papilla (inner) and dental follicle (outer) mesenchymal tissues, it breaks up into epithelial rests and cords, allowing the dental follicle mesenchyme to migrate onto the root dentin matrix to form cementoblasts (cementum-secreting cells) and the periodontal ligament.

Since the mid-1990s, a significant effort to identify and characterize adult stem cells within teeth has been under way. Almost two decades later, such cells have been identified in virtually all tissues of immature and mature primary and permanent teeth as well as exfoliating primary teeth. These stem cells vary in location, origin, and differentiation potentials.

3 Adult Tooth Stem Cells

Primary human teeth begin to erupt at around 6 months of age and complete eruption by 5–6 years. Permanent dentition begins eruption at 6 years of age and can continue into the mid-twenties (eruption of third molars ranges from 17 to 25 years). The period between the eruption of first permanent tooth (~6 years) and loss of the last primary tooth (~12 years) is known as the mixed dentition stage (a time when both primary and permanent teeth are present in the mouth). At this time, stem cells of developing, primary, and permanent teeth are present (Fig. 3). In this section, we describe stem cells found in fully formed primary and permanent (mature) dentition and in developing teeth.

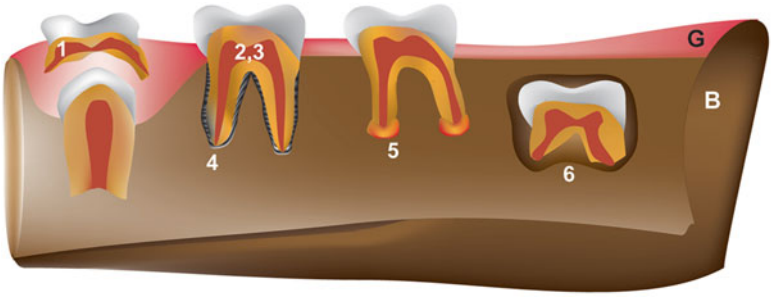

Fig. 3 Locations of adult dental stem cells in humans. Stem cells from (*1*) human exfoliated deciduous teeth (SHED); (*2*, *3*) dental pulp stem cells (DPSCs) and dental pulp pluripotent stem cells (DPPSCs); (*4*) periodontal ligament stem cells (PDLSCs); (*5*) stem cells of apical papilla (SCAP); (*6*) dental follicle stem cells (DFSC). *G* gingiva, *B* alveolar bone

3.1 Stem Cells of Mature Teeth

3.1.1 Dental Pulp Stem Cells

The dental pulp contains a neurovascular bundle as well as dentin-producing odontoblasts and supporting fibroblasts. The neural crest-derived dental pulp stem cells (DPSCs) reside in the perivascular and periodontoblastic compartments within the pulp and were originally thought of as primarily odontoblast precursors with a fibroblast-like phenotype and limited differentiation capacity [52, 53]. In vitro, DPSCs display a high growth rate and fibroblast-like ability to adhere to plastics [18, 53]. In vivo, the cells' morphology resembles that of smooth muscle actin-expressing pericytes [54]. Overall, the cells make up about 0.5–0.7 % of the total cell mass of the pulp, which ranges from 500,000 to 1,000,000 cells [52].

Expression of stem cell markers in the DPSCs varies, making it difficult to establish a universal marker for all DPSCs. In the pulp, STRO-1$^+$ DPSCs express pericyte-specific markers, which led to the proposal that DPSCs may be of pericyte origin [54]. Further, the inflammatory response of the pulp to enhance tertiary dentin production by odontoblasts is reported to exhibit increased *STRO-1* expression, suggesting increased proliferation of DPSCs and their differentiation into odontoblasts [55]. While no single universal marker is available due to the heterogeneity of the DPSCs, the most commonly reported markers are CD29, CD44, CD73, CD90, CD105, CD146, and CD166. In addition, DPSCs express several factors common to stem cells, such as *OCT3/4*, Nestin, Nanog, and Vimentin [56–58].

DPSCs exhibit a moderate potential for differentiation into dental tissues. DPSCs transplanted under rat kidney capsules formed dentin–pulp complexes and thus demonstrated the ability to differentiate into odontoblasts, expressing the odontocyte marker dentin sialophosphoprotein (DSPP) [59]. In addition, in vitro

stimulation of DPSCs by various techniques, such as exposure to dentinal tubules, also stimulated the stem cells to differentiate into odontoblasts [60].

DPSCs demonstrate a wide capability of differentiation into non-dental tissues. When placed into osteogenic media, the STRO-1$^+$ cells demonstrated osteogenic potential. In those experiments, DPSCs were initially capable of differentiating into both chondrogenic and osteogenic cells, with only osteogenic cells remaining in late passage populations [61]. Studies conducted in rat models have demonstrated that DPSCs transplanted in vivo to the sites of large cranial bone defects in addition to collagen matrices yielded new bone formation [62]. Moreover, DPSC transplantation into transected rat spinal cord resulted in recovery of locomotor functions [63]. As previously mentioned, subpopulations of DPSCs express α-smooth muscle actin, which is a common myogenic marker. Studies investigating the myogenic potential of pulpal stem cells revealed in vitro myocyte differentiation when treated with 5-Aza-2-Deoxycytidine. In addition, applications of DPSCs in animal models of muscular dystrophy and myocardial infarction resulted in increased muscle mass [64, 65]. Finally, DPSCs can differentiate into a number of neural crest-derived tissues, such as cornea and melanocytes, as well as neural cells and hair follicles [66–68]. Implantation of these cells into damaged rabbit cornea resulted in epithelial repairs, whereas implantation into inactive hair follicles resulted in resumed hair production [69, 70].

3.1.2 Dental Pulp Pluripotent Stem Cells

Recently, a subpopulation of DPSCs called dental pulp pluripotent stem cells (DPPSCs) has been isolated from pulp tissue of human third molars [71, 72]. These cells are smaller than DPSCs, with a high nucleus/cytoplasm ratio, and a fibroblast-like flattened and elongated morphology.

Like DPSCs, DPPSCs express *OCT3/4*, Nanog, and Nestin. In addition, DPPSCs share several markers with DPSCs, namely, CD29 and CD90. Unlike DPSCs, however, these cells are CD105$^-$, CD73$^-$, and CD146$^-$, in addition to being Sox2$^+$ [71]. Interestingly, both OCT3/4 and SOX2 are needed for conversion of somatic cells to iPSCs. Reprogramming of adult stem cells into iPSCs often yields better results than the conversion of somatic cells, perhaps due to the use of less reprogramming factors.

In the early days of stem cell research, ESC pluripotency was established by in vivo formation of teratomas, which are heterogeneous tumors containing tissues from all three embryonic layers (ectoderm, mesoderm, and endoderm) [2]. Similar teratomas form when DPPSCs are injected into mice, indicating that these cells have pluripotent potential similar to that of ESCs and iPSCs [72]. While the current effort of determining in vivo differentiation potential of DPPSCs has been primarily focused around the oral cavity, much remains to be explored about DPPSCs' differentiation potential into other tissue types.

3.1.3 Stem Cells from Human Exfoliated Deciduous Teeth

One of the hallmarks of mammalian dentition is the replacement of primary (milk) teeth with permanent adult ones. In humans, the process occurs from age 6 into the early teens. During replacement, the primary tooth roots become resorbed and the crown exfoliates. Despite this partial resorption, primary teeth still contain pulpal tissue at the time of exfoliation. A population of cells called stem cells from human exfoliated deciduous teeth (SHED) can be isolated from the coronal perivascular and periodontoblastic compartments of the pulp and sometimes the radicular (root) pulp of incompletely resorbed roots of deciduous teeth [73]. Despite the reduced number of pulp cells due to root resorption, SHED are an appealing source of adult stem cells due to the natural exfoliation process of the teeth.

The cells are similar to DPSCs in their morphology and some of their defining markers, but they also differ in a number of ways. First, SHED exhibit a much higher in vitro proliferation rate than DPSCs [74, 75]. Second, SHED constitute a higher proportion of cells within the pulp, comprising about 1.0–1.2 % of the total cell mass [75]. Third, SHED have an innate ability to induce formation of new bone-like matrix within lamellar bone [74, 76]. This osteoinductive property is consistent with the observation that primary tooth root resorption is often accompanied by new bone formation [77].

In addition to common stem cell markers shared with DPSCs, such as CD44, CD73, CD90, CD105, and CD146, SHED also express CD13. Further, SHED express *STRO-1*, *OCT3/4*, Nanog, and Nestin [73, 78]. In addition, SHED express the pluripotency markers stage-specific embryonic antigens 3 and 4 (*SSEA-3* and *SSEA-4*) [78].

Like DPSCs, SHED show an almost universal differentiation potential in vitro, as they are capable of forming odontoblasts, osteoblasts, adipocytes, myoblasts, endothelial cells, and neuronal cells [79]. In vivo SHED transplantation experiments revealed the cells' ability to differentiate into osteoblasts and odontoblasts. Finally, when placed in mouse brain, the cells were able to survive and express neural markers [80].

3.1.4 Periodontal Ligament Stem Cells

In addition to the somatic stem cells that can be obtained from adult tooth pulp, the structures supporting the teeth also present an important niche for stem cells. The teeth are anchored in the jaw bone by the periodontal ligament (PDL). The PDL consists of both epithelial cells (remnants of enamel epithelium) and cells derived from the neural crest mesenchyme. The majority of PDL mass consists of extracellular collagen, organized into fibers extending from alveolar bone to root cementum. Studies investigating extracted human teeth have identified PDL stem cells (PDLSCs), which can make up as much as 3 % of total PDL cell mass. The location of these cells impacts their properties, as PDLSCs collected closer to alveolar bone exhibit superior bone regeneration properties compared to PDLSCs collected in

proximity to cementum, despite the average width of the PDL space being only 350 μm [81].

As expected, PDLSCs share many surface markers with stem cells from the pulp, such as CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, and CD166. In addition, these cells express STRO-1 and Scleraxis. The latter is a helix-loop-helix transcription factor expressed in precursor cells of tendons and ligaments [58, 81, 82].

When transplanted in vivo in conjunction with hydroxyapatite (HA) scaffolds, PDLSCs are capable of differentiating into cells forming different parts of the PDL, including Sharpey's fibers (primary fibers of the PDL), cementum, and even alveolar bone [73, 82]. When grown in vitro in adipogenic, chondrogenic, and osteogenic conditions, PDLSCs differentiated into adipocyte-, chondroblast-, and osteoblast-like cells, respectively [77, 83].

3.2 Stem Cells from Developing Teeth

In humans, tooth development begins in utero and ends with the completion of root formation of the maxillary third molars at 17–25 years. In addition to adult dental tissues, developing teeth also present an important and readily available source of stem cells.

3.2.1 Stem Cells of Apical Papilla

The apical papilla (AP) tissue is found at the apices of the developing roots and contributes to root formation, eventually becoming a part of the radicular pulp. However, studies have shown a distinct histological difference between the pulp and the AP. Separated from the pulp by a cell-rich junction, the apical papilla is less vascular but more cellular. It has been hypothesized that the AP is the source of primary odontoblasts, which migrate through the cell-rich border into the pulp tissue and lay down dentin of the developing roots [84]. Recently, a population of stem cells has been found in apical papillae from extracted immature permanent teeth, termed stem cells of apical papilla (SCAP).

SCAP exhibit threefold greater in vitro proliferation ability than stem cells of pulpal origin. SCAP express a number of stem cell markers, such as CD49, CD51, CD73, CD90, CD105, CD106, CD146, and CD166. In addition, they express *STRO-1* and Nestin [84]. While SCAP in vitro differentiation is limited to odontoblasts, adipocytes, and neuronal cells, they possess greater capacity to differentiate in vivo into both odontoblasts and pulpal fibroblasts in comparison to stem cells of pulpal or periodontal origin [85]. SCAP are especially useful in root regeneration. Studies utilizing hydroxyapatite and decellularized dentin scaffold matrix carriers and SCAP were successful in obtaining root regeneration in animal models [86]. Thus, SCAP may provide a better source of cells for dentin reparative therapies than the DPSCs from fully developed teeth.

3.2.2 Dental Follicle Stem Cells

The developing dental tissues are surrounded by the dental follicle, which is necessary for tooth eruption and plays a role in alveolar bone remodeling during eruption. Subsequently, the dental follicle differentiates into the periodontal ligament. In addition, it is thought that cells of the dental follicle differentiate into cementoblasts and osteoblasts [87]. Efforts to identify the origin of these cells have resulted in the discovery of a population of dental follicle stem cells (DFSCs).

The DFSCs are small compared to the surrounding supporting fibroblasts of the dental follicle. When cocultured, DFSCs quickly encircle DPSCs, mimicking the in vivo developmental process. The stem cells of the dental follicle express the expected set of markers: CD13, CD29, CD44, CD90, CD105, CD105, and *STRO-1* [31].

Currently, the differentiation potential of DFSCs is thought to be rather limited. Under in vitro induction, these cells are capable of expressing adipocyte, chondrocyte, and osteocyte markers [87, 88]. In addition, when transplanted into areas of jawbone defects in mice, DFSCs differentiated and secreted dentin, cementum, and bone matrix. In addition, the cells were also capable of forming periodontal ligament fibers that inserted into the secreted cementum matrix [89]. Thus, like SCAP, DFSCs are good candidates for therapies involving the regeneration and repair of the periodontal ligament complex.

4 Applications of Tooth Stem Cells

4.1 Sources of Stem Cells

Stem cells can be obtained through minimally invasive routine dental procedures, such as root canal therapy (stem cells of the pulp), periodontal therapy (PDLSCs), and tooth extraction. In addition, naturally exfoliating primary teeth can be collected for the isolation of SHED by dentists. Perhaps the most widespread source of dental adult stem cells is the third molar tooth [57, 71, 72]. Human third molars often present clinically unfavorable eruption patterns, such as erupting at angles off to the main occlusal plane, erupting partially, or not erupting at all. Due to their location in the back of the oral cavity, these teeth are difficult to maintain and often present with pathologies of dental and supporting tissues, such as caries and periodontal disease. For those reasons, third molars are often extracted prophylactically when the patients are in their mid to late teens. These teeth are extracted at different stages of their development, with routine cases being done when the roots are still developing or even when the teeth are still in the bell stage. Thus, third molars are a good source for both DFSCs and SCAP in addition to the somatic dental stem cells of the pulp and the PDL.

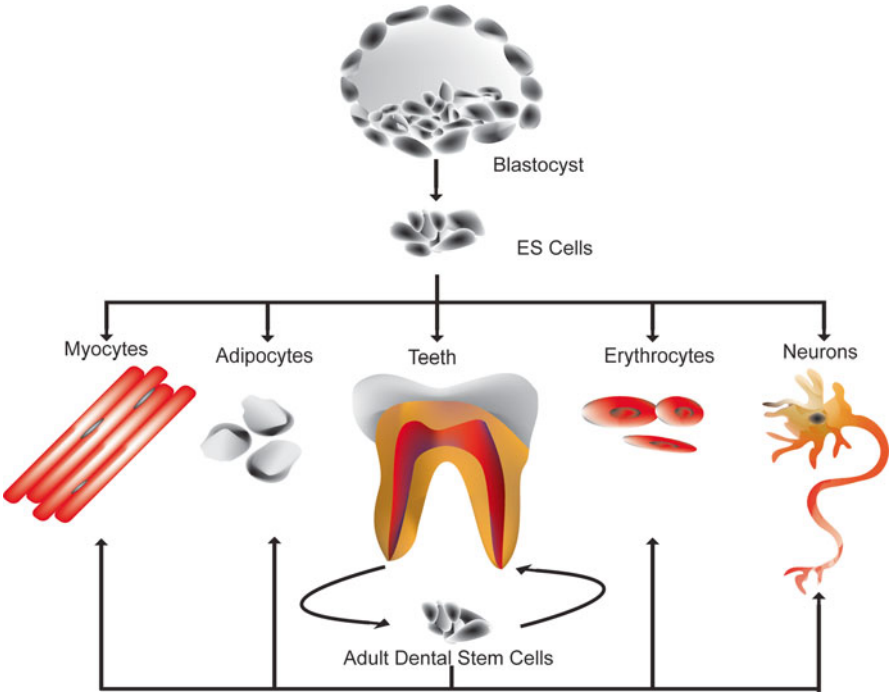

Fig. 4 Differentiation potentials of adult dental stem cells. Embryonic stem cells from the inner cell mass of the blastocyst have the potential to give rise to all tissues in the body. In turn, many adult tissues retain populations of adult stem cells with varying degrees of differentiation and transdifferentiation potentials. Teeth contain several populations of adult stem cells that hold the potential to not only regenerate tooth structure in a limited capacity, but also regenerate teeth entirely as well as transdifferentiate into other tissue types

4.2 Clinical Applications

The ultimate desired outcome of dental stem cell therapy is the complete generation of a functional tooth, PDL, and surrounding alveolar bone. In the past few years, advances have been made towards achieving this goal, such as combination of SCAP and PDLSCs to construct a functional root and PDL in vivo [90]. Such a root can later be restored with an artificial crown and serve as a functioning tooth. In addition, placement of PDLSCs with mineral scaffolds into alveolar bone defects has been successfully used to regenerate new bone [73, 82]. Further, teeth that have undergone a root canal procedure (complete or partial removal of the pulp) may soon be restored by placement of DPSCs. The potential of transdifferentiation of adult dental stem cells into other tissues suggests that one day these cells could be modified to achieve the same level of differentiation potential as ESCs (Fig. 4).

5 Conclusion

The tooth is an exciting model for investigation of adult stem cells. In this chapter, we have described the recent advances in understanding the locations, properties, and application potential of adult dental stem cells. However, a plethora of important basic and applied questions, ranging from determination of the origin of cells that give rise to adult stem cell niches to therapeutic application of dental stem cells in other organ systems, requires further investigation.

References

1. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
2. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
4. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
5. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
6. Stadtfeld M, Hochedlinger K (2010) Induced pluripotency: history, mechanisms, and applications. Genes Dev 24:2239–2263
7. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26:101–106
8. Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1:39–49
9. Simons BD, Clevers H (2011) Stem cell self-renewal in intestinal crypt. Exp Cell Res 317:2719–2724
10. Fuchs E (2009) Finding one's niche in the skin. Cell Stem Cell 4:499–502
11. Beck B, Blanpain C (2012) Mechanisms regulating epidermal stem cells. EMBO J 31:2067–2075
12. van der Flier LG, Clevers H (2009) Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 71:241–260
13. Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245
14. Joyner AL, Zervas M (2006) Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. Dev Dyn 235:2376–2385
15. Hsu YC, Fuchs E (2012) A family business: stem cell progeny join the niche to regulate homeostasis. Nat Rev Mol Cell Biol 13:103–114
16. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806
17. Iohara K, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M (2006) Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. Stem Cells 24:2493–2503
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97:13625–13630

19. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20:530–541
20. Scholer HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P (1990) New type of POU domain in germ line-specific protein Oct-4. Nature 344:435–439
21. Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M, Hamada H (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 60:461–472
22. Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH (2006) A protein interaction network for pluripotency of embryonic stem cells. Nature 444:364–368
23. Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hubner K, Scholer HR (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122:881–894
24. Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S (2011) Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145:875–889
25. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642
26. Pan G, Thomson JA (2007) Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res 17:42–49
27. Michalczyk K, Ziman M (2005) Nestin structure and predicted function in cellular cytoskeletal organisation. Histol Histopathol 20:665–671
28. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829–834
29. Li LRL, Qi H, Li F (2008) Multipotential character of nestin-positive cells differentiated from adult mouse pancreatic islets. Cell Res 18:156–162
30. About I, Laurent-Maquin D, Lendahl U, Mitsiadis TA (2000) Nestin expression in embryonic and adult human teeth under normal and pathological conditions. Am J Pathol 157:287–295
31. Kawashima N (2012) Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch Oral Biol 57:1439–1458
32. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736
33. Goodfellow PJ, Nevanlinna HA, Gorman P, Sheer D, Lam G, Goodfellow PN (1989) Assignment of the gene encoding the beta-subunit of the human fibronectin receptor (beta-FNR) to chromosome 10p11.2. Ann Hum Genet 53:15–22
34. Ruoslahti E, Pierschbacher MD (1987) New perspectives in cell adhesion: RGD and integrins. Science 238:491–497
35. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell 61:1303–1313
36. Airas L, Hellman J, Salmi M, Bono P, Puurunen T, Smith DJ, Jalkanen S (1995) CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. J Exp Med 182:1603–1608
37. Rege TA, Hagood JS (2006) Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. FASEB J 20:1045–1054
38. Rius C, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, Vary CP, Bernabeu C (1998) Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood 92:4677–4690
39. Alongi DJ, Yamaza T, Song Y, Fouad AF, Romberg EE, Shi S, Tuan RS, Huang GT (2010) Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. Regen Med 5:617–631
40. Guezguez B, Vigneron P, Lamerant N, Kieda C, Jaffredo T, Dunon D (2007) Dual role of melanoma cell adhesion molecule (MCAM)/CD146 in lymphocyte endothelium interaction:

MCAM/CD146 promotes rolling via microvilli induction in lymphocyte and is an endothelial adhesion receptor. J Immunol 179:6673–6685
41. Ten Cate AR, Mills C (1972) The development of the periodontium: the origin of alveolar bone. Anat Rec 173:69–77
42. Nanci A, Ten Cate A (2002) Ten Cate's oral histology: development, structure, and function, vol 1. Elsevier, St. Louis
43. Line SR (2003) Variation of tooth number in mammalian dentition: connecting genetics, development, and evolution. Evol Dev 5:295–304
44. Smith CE, Warshawsky H (1975) Cellular renewal in the enamel organ and the odontoblast layer of the rat incisor as followed by radioautography using 3H-thymidine. Anat Rec 183:523–561
45. Keranen SV, Aberg T, Kettunen P, Thesleff I, Jernvall J (1998) Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents. Dev Genes Evol 208:477–486
46. Ohazama A, Haworth KE, Ota MS, Khonsari RH, Sharpe PT (2010) Ectoderm, endoderm, and the evolution of heterodont dentitions. Genesis 48:382–389
47. Jheon A, Seidel K, Biehs B, Klein OD (2013) From molecules to mastication: the development and evolution of teeth. Wiley Interdiscip Rev Dev Biol 2(2):165–182
48. Noden DM, Schneider RA (2006) Neural crest cells and the community of plan for craniofacial development: historical debates and current perspectives. Adv Exp Med Biol 589:1–23
49. Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127:1671–1679
50. Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I (1994) Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating Fgf-4 gene. Int J Dev Biol 38:463–469
51. Avery J (2002) Development of teeth: crown formation, 3rd edn. Thieme, Stuggart
52. Janebodin K, Horst OV, Ieronimakis N, Balasundaram G, Reesukumal K, Pratumvinit B, Reyes M (2011) Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PLoS One 6:e27526
53. Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88:792–806
54. Zhao X, Gong P, Lin Y, Wang J, Yang X, Cai X (2012) Characterization of alpha-smooth muscle actin positive cells during multilineage differentiation of dental pulp stem cells. Cell Prolif 45:259–265
55. Pereira LO, Rubini MR, Silva JR, Oliveira DM, Silva IC, Pocas-Fonseca MJ, Azevedo RB (2012) Comparison of stem cell properties of cells isolated from normal and inflamed dental pulps. Int Endod J 45:1080–1090
56. Shi S, Robey PG, Gronthos S (2001) Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. Bone 29:532–539
57. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531–535
58. Lindroos B, Maenpaa K, Ylikomi T, Oja H, Suuronen R, Miettinen S (2008) Characterisation of human dental stem cells and buccal mucosa fibroblasts. Biochem Biophys Res Commun 368:329–335
59. Yu J, Jin F, Deng Z, Li Y, Tang L, Shi J, Jin Y (2008) Epithelial-mesenchymal cell ratios can determine the crown morphogenesis of dental pulp stem cells. Stem Cells Dev 17:475–482
60. Ferro F, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, Beltrami AP, Cesselli D, Falini G, Beltrami CA, Curcio F (2012) Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. PLoS One 7:e41774

61. Zhang W, Walboomers XF, Van Kuppevelt TH, Daamen WF, Van Damme PA, Bian Z, Jansen JA (2008) In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. J Tissue Eng Regen Med 2:117–125
62. de Mendonca Costa A, Bueno DF, Martins MT, Kerkis I, Kerkis A, Fanganiello RD, Cerruti H, Alonso N, Passos-Bueno MR (2008) Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. J Craniofac Surg 19:204–210
63. Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J Clin Invest 122:80–90
64. Nakatsuka R, Nozaki T, Uemura Y, Matsuoka Y, Sasaki Y, Shinohara M, Ohura K, Sonoda Y (2010) 5-Aza-2′-deoxycytidine treatment induces skeletal myogenic differentiation of mouse dental pulp stem cells. Arch Oral Biol 55:350–357
65. Gandia C, Arminan A, Garcia-Verdugo JM, Lledo E, Ruiz A, Minana MD, Sanchez-Torrijos J, Paya R, Mirabet V, Carbonell-Uberos F, Llop M, Montero JA, Sepulveda P (2008) Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 26:638–645
66. Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, Formstecher P, Bailliez Y, Polakowska RR (2008) Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 17:1175–1184
67. Mikami Y, Ishii Y, Watanabe N, Shirakawa T, Suzuki S, Irie S, Isokawa K, Honda MJ (2011) CD271/p75(NTR) inhibits the differentiation of mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages. Stem Cells Dev 20:901–913
68. Nakashima M, Iohara K, Sugiyama M (2009) Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration. Cytokine Growth Factor Rev 20:435–440
69. Gomes JA, Geraldes Monteiro B, Melo GB, Smith RL, Cavenaghi Pereira da Silva, Lizier NF, Kerkis A, Cerruti H, Kerkis I (2010) Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. Invest Ophthalmol Vis Sci 51:1408–1414
70. Reynolds AJ, Jahoda CA (2004) Cultured human and rat tooth papilla cells induce hair follicle regeneration and fiber growth. Differentiation 72:566–575
71. Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, Giner L, Casals N (2011) Isolation of pluripotent stem cells from human third molar dental pulp. Histol Histopathol 26:1057–1070
72. Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, Ferres-Padro E, Giner L (2012) Dental pulp of the third molar: a new source of pluripotent-like stem cells. J Cell Sci 125:3343–3356
73. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807–5812
74. Nakamura S, Yamada Y, Katagiri W, Sugito T, Ito K, Ueda M (2009) Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J Endod 35:1536–1542
75. Hara K, Yamada Y, Nakamura S, Umemura E, Ito K, Ueda M (2011) Potential characteristics of stem cells from human exfoliated deciduous teeth compared with bone marrow-derived mesenchymal stem cells for mineralized tissue-forming cell biology. J Endod 37:1647–1652
76. Otaki S, Ueshima S, Shiraishi K, Sugiyama K, Hamada S, Yorimoto M, Matsuo O (2007) Mesenchymal progenitor cells in adult human dental pulp and their ability to form bone when transplanted into immunocompromised mice. Cell Biol Int 31:1191–1197
77. Seo BM, Sonoyama W, Yamaza T, Coppe C, Kikuiri T, Akiyama K, Lee JS, Shi S (2008) SHED repair critical-size calvarial defects in mice. Oral Dis 14:428–434

78. Egusa H, Sonoyama W, Nishimura M, Atsuta I, Akiyama K (2012) Stem cells in dentistry – part I: stem cell sources. J Prosthodont Res 56:151–165
79. Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, Smith AJ, Nor JE (2008) Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod 34:962–969
80. Nourbakhsh N, Soleimani M, Taghipour Z, Karbalaie K, Mousavi SB, Talebi A, Nadali F, Tanhaei S, Kiyani GA, Nematollahi M, Rabiei F, Mardani M, Bahramiyan H, Torabinejad M, Nasr-Esfahani MH, Baharvand H (2011) Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. Int J Dev Biol 55:189–195
81. Gay IC, Chen S, MacDougall M (2007) Isolation and characterization of multipotent human periodontal ligament stem cells. Orthod Craniofac Res 10:149–160
82. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364:149–155
83. Seo BM, Miura M, Sonoyama W, Coppe C, Stanyon R, Shi S (2005) Recovery of stem cells from cryopreserved periodontal ligament. J Dent Res 84:907–912
84. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT (2008) Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. J Endod 34:166–171
85. Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, Palotas A, Kose GT (2010) Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo- and neurogenesis. Pharmacogenomics J 10:105–113
86. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S (2006) Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One 1:e79
87. Yao S, Pan F, Prpic V, Wise GE (2008) Differentiation of stem cells in the dental follicle. J Dent Res 87:767–771
88. Vollner F, Ernst W, Driemel O, Morsczeck C (2009) A two-step strategy for neuronal differentiation in vitro of human dental follicle cells. Differentiation 77:433–441
89. Kemoun P, Laurencin-Dalicieux S, Rue J, Farges JC, Gennero I, Conte-Auriol F, Briand-Mesange F, Gadelorge M, Arzate H, Narayanan AS, Brunel G, Salles JP (2007) Human dental follicle cells acquire cementoblast features under stimulation by BMP-2/-7 and enamel matrix derivatives (EMD) in vitro. Cell Tissue Res 329:283–294
90. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S (2008) The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. J Endod 34:645–651

Adult Mammary Stem Cells: Identity, Location, and Functional Assays

Pirashaanthy Tharmapalan and Rama Khokha

Abstract The mammary gland is an apocrine organ that undergoes multiple periods of robust change marked with proliferation, differentiation and apoptosis. The profound regenerative potential observed in the mammary gland implies the presence of a population of mammary stem cells (MaSCs) with the capacity to both self-renew and give rise to all mammary lineages. Furthermore, a single mammary epithelial cell enriched for specific cell surface markers has been shown to reconstitute an entire, functional mammary gland in vivo, thereby demonstrating multipotent stem cell potential. The purpose of this chapter is to briefly outline the current state of knowledge on the identity and location of the MaSC, as well as provide a critical overview of the assays utilized to examine MaSC potential.

Keywords Mammary stem cell • Mammary progenitor cell • Breast • Mammary remodeling • Clonogenic assays

Abbreviations

FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GFP	Green fluorescent protein
LRC	Label-retaining cells
MaSC	Mammary stem cell
MRU	Mammary repopulating unit
PI-MEC	Parity-induced mammary epithelial cells

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SP	Side population
TDLU	Terminal ductal lobular units
TEB	Terminal end buds
YFP	Yellow fluorescent protein

1 Introduction

The mammary gland is composed of an organized bi-layered epithelial ductal network, embedded within mesenchymal components, and serves to effectively deliver milk containing vital nutrients and immune factors to offspring. In humans, the epithelial ductal network arises as a bundle of 5–10 lactiferous ducts extending from the nipples into the mammary fat pads. Bifurcating radially, each lactiferous duct branches off into segmental ducts that end in discrete pyramidal lobules. These lobular structures, called terminal ductal lobulo-alveolar units (TDLUs), are the main functional secretory units of the gland and include an intralobular duct that diverges into terminal ducts (Fig. 1a) [1–3]. These terminal ducts contain clusters of smaller blind-ended ductules that differentiate into milk-secreting acini during lactation. At the cellular level, both the TDLUs and the subtending mammary ducts are bi-layered in nature, with an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. These latter cells, also referred to as basal cells, are in direct contact with the basement membrane and contract to aid in milk ejection during lactation while luminal cells differentiate into milk-secreting cells during pregnancy and lactation [4, 5]. Mesenchymal components of the mammary fat pad consist of fibroblasts and adipocytes that are interspersed with a variety of immune cells and blood vessels [6]. Connective tissue proteins such as collagen, laminin, fibronectin, tenascin, and others lend structural support to the intricate epithelial ductal tree to build the breast tissue [7, 8].

The murine mammary gland often serves as an instructive model and has proven to be an insightful tool for investigating mammary stem cell dynamics. There are five pairs of mammary glands in mice, with each gland bearing a single lactiferous duct that bifurcates linearly into 5–10 secondary ducts, with multiple side branches [1]. Analogous in function to TDLUs, lobuloalveoli are the main secretory unit in the murine gland [2, 3]. However, unlike TDLUs, lobuloalveoli have the propensity to develop along both a duct and at the end of a terminal duct (Fig. 1b) [1]. The mesenchyme surrounding the mouse mammary epithelial network is less fibrous and has higher adipocyte content compared to the human breast. Unlike the human breast that has loose intralobular connective tissue and dense interlobular connective tissue forming a slightly exclusive collagenous compartment around the epithelial network, murine epithelial cells are encased in a periductal stroma which is in turn imbedded in fat tissue [9, 10].

Despite these structural differences, similarities in the development and function of the mouse mammary gland inform human mammary biology. The developmental progression of the murine mammary gland observed over a female's reproductive

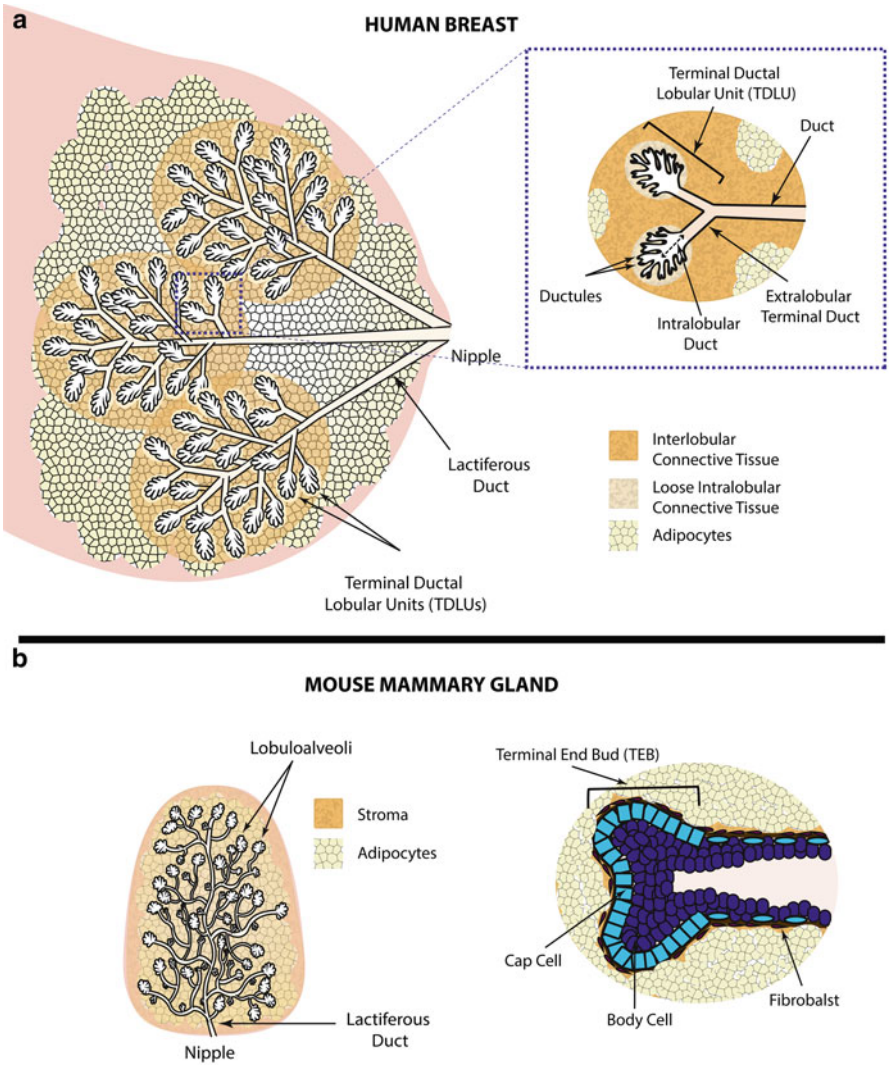

Fig. 1 The mammary gland structure. (**a**) Schematic of the human ductal system. Arising as 5–10 lactiferous ducts from the nipple, the mammary ductal tree bifurcates in a radial manner with terminal ductal lobular units (TDLUs) as the functional unit. TDLUs form at the end of terminal ducts and consist of an intralobular terminal duct and smaller blind-ended tubules lined with secretory cells. (**b**) Schematic of the murine mammary gland. The murine mammary gland consists of a single lactiferous duct that bifurcates into 5–10 secondary ducts linearly. The functional units of the murine gland are lobuloalveoli. During puberty, growth primarily occurs at the club-like structures at the distal tip of ducts called terminal end buds. Terminal end buds contain of an inner layer of body cells that align with luminal cells and an outer layer of cap cells that align with basal cells of the subtending duct

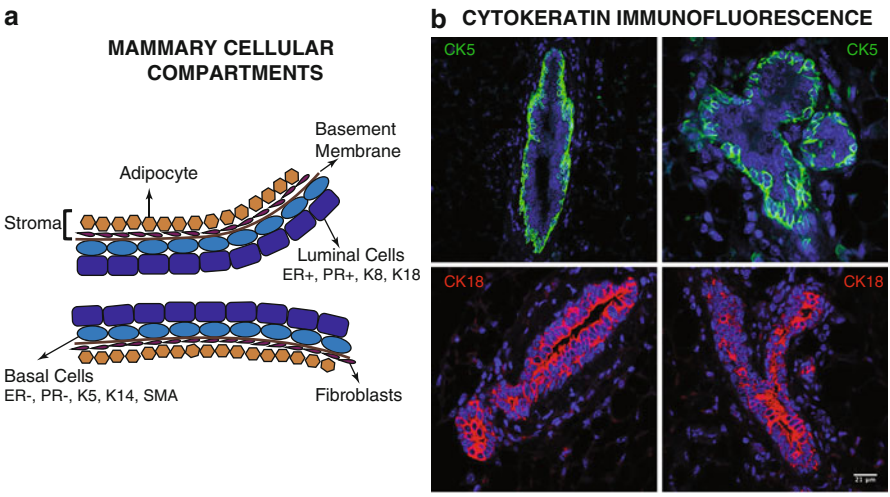

Fig. 2 Mammary cell compartments. (**a**) The mammary ducts are bi-layered in structure that consist of an inner layer of luminal cells lining the lumen of the duct and an outer layer of basal cells which is in contact with the basement membrane. (**b**) Cross section of the bi-layered mammary ducts stained with antibodies for cytokeratin 5 (*green*) and cytokeratin 18 (*red*), labeling basal and luminal cell layers, respectively

lifetime, from embryonic development to pregnancy and lactation, recapitulates critical aspects of the human breast. Similarly, the cellular organization of the murine ductal system mirrors the human bi-layered epithelial network consisting of luminal and basal cells (Fig. 2). Comparative transcriptome analyses of normal mouse mammary epithelial populations and human counterparts revealed many conserved gene signatures and pathways with the MaSC-enriched subpopulation showing the highest rate of conservation [11]. Thus, similarities between the two species allow emerging knowledge on murine MaSCs to guide the study of human mammary stem cells.

2 Mammary Gland Development and MaSCs

The mammary ductal network primarily develops postnatally and undergoes episodes of distinct but highly regulated morphological changes before maturing into a functional organ. The striking growth and structural remodeling which occur repeatedly over the reproductive life span of a female have been well characterized in both the human breast and murine mammary tissue. Epidemiological studies link these developmental phases to an altered predisposition for breast cancer and experimental evidence from murine models supports a role for MaSCs in driving these morphological developments.

2.1 Prepubertal Mammary Gland and MaSCs

Mammary tissue formation first begins embryonically around day E10–11 as a mammary streak from the anterior to the posterior limb bud, forming a bulbous mammary rudiment by day E12.5 with ducts arising by day E16 in the mouse [12]. Comparably, human breast development begins as the mammary epithelium forms between week 7 and 8 of gestation (typically when the embryo is 5.5 mm in size) and subsequently invades the stroma whilst continuing through various stages of development [13, 14]. Chimera studies using fused blastomeres initially indicate the presence of at least two stem cells embryonically, but the frequency of fetal mammary stem cells has since been characterized [15, 16].

At birth, the gland in both species consists of a primitive rudimentary ductal tree. The gland undergoes isometric growth postnatally until the onset of puberty, when hormones from the hypothalamic–pituitary–ovarian axis trigger the development of the intricate ductal network. Specifically, the ovarian hormone estrogen elicits ductal elongation and expansion while the ovarian hormone progesterone stimulates tertiary branching and lobuloalveologenesis. In mice, growth of the mammary duct during puberty primarily occurs at the distal tips to form enlarged bulbous structures called terminal end buds (TEBs) [17]. Consisting of inner body cells that align with luminal cells of the subtending duct and a single outer layer of cap cells that is continuous with the basal layer, TEBs are the site of active proliferation as the ductal system is generated. During this process, some of the cap cells from the TEBs have been shown to reposition themselves along the extended duct as basal cells. It has been postulated that these cells are a stem cell population in rodents. In humans, although TEB-like structures are found and are the sites of active epithelial proliferation, the corresponding cap cell population is somewhat indiscernible. As a result, the precise nature of the population driving human pubertal mammary changes remains unknown. The epithelial ductal network continues to invade the surrounding stroma until the boundaries of the mammary fat pad are reached, giving rise to the virgin mammary gland by the end of puberty.

The occurrence of label-retaining epithelial cells in the mammary pubertal gland has been examined through the long-term maintenance of bromodeoxyuridine during DNA replication [18–20]. Label retention is thought to be a characteristic of stem cells through asymmetric cell division and label-retaining cells (LRCs) have been specifically detected in the basal fraction during puberty [21]. Identified as a stem cell marker in the hematopoietic system, src homology 2 domain-containing 5′-inositol phosphatase (s-SHIP) is another proposed marker for activated MaSCs. Green fluorescent protein (GFP) expression driven by s-SHIP promoter was found in a subpopulation of cap cells at puberty. This supports the presence of an activated stem cell pool within the cap cell population [22].

2.2 MaSCs and the Adult Mammary Gland

In the adult female, an expansion and regression of the mammary epithelium is observed during each reproductive cycle. The human reproductive cycle, known as the menstrual cycle, is 28 days long on average whereas the rodent estrous cycle generally lasts 4–6 days. Cyclical hormonal changes in the hypothalamic–pituitary–ovarian axis lead to potent fluctuations in ovarian estrogen and progesterone, evoking transient but repeated morphological alterations in the mammary gland. These cellular changes in the gland are often overlooked due to the fact that they are less extensive than the growth observed at puberty or pregnancy. However, the peak of progesterone during the murine diestrus stage, corresponding to the human luteal phase, results in significant side branching and lobuloalveologenesis [23, 24]. Recent studies have demonstrated that mammary epithelium and MaSC frequency undergo notable alterations during each reproductive cycle with MaSCs being defined as cells that have the ability to reconstitute all lineages of a mammary gland in vivo [23]. Specifically, increased progesterone during diestrus drives an up to sixfold expansion in the basal populations and a threefold expansion in the luminal population relative to the estrous stage [23]. These alterations are accompanied by a 7.6-fold increase in functional MaSC activity when comparing diestrus- and estrous-staged mammary cell transplants [23]. This expansion accompanies increased cell proliferation that is tightly regulated and counteracted by increased cell death, thereby preventing an accumulation of mammary epithelium [23]. Overall, the dynamic nature of the gland can be appreciated even in the adult premenopausal female regardless of parity.

2.3 MaSCs During Pregnancy, Lactation, and Involution

The gland undergoes a period of copious proliferation and differentiation during pregnancy and lactation. A prominent formation of alveolar buds takes place under the influence of placental progesterone and prolactin during murine gestation, with these buds differentiating into the milk-secreting alveoli by the end of pregnancy [25]. During human gestation, TDLUs transition from lobule type (Lob)-1 that resemble TEBs to Lob-3 which is the most differentiated lobule type [13, 26, 27]. By the end of pregnancy, not only does the number of cells per TDLU increase dramatically due to proliferation, but the size of each cell also increases due to cytoplasmic enlargement [13, 26, 27].

Post-lactation involution depends on extensive apoptosis peaking at 3–4 days after weaning and results in mammary gland remodeling back to a non-parous-like state by 8 days in the mouse. Although these events have been more intensely studied in rodents, the process occurs in a similar manner in humans. It is characterized by cellular autolysis leading to the collapse of acinar lobules and narrowing tubules, infiltration of phagocytes and round cells, and connective tissue regeneration

surrounding the ducts and lobules. The post-involutional breast tissue does not completely return to the virgin state. Instead, the human parous gland contains slightly more glandular tissue and Lob-3 type lobules, reflecting a more differentiated state [13, 26, 27]. These changes in the mammary gland occur again upon subsequent pregnancies.

A unique population of cells, termed parity-induced mammary epithelial cells (PI-MECs), were found to expand within alveolar units during pregnancy, survive involution to persist in the nonpregnant parous female, and serve as progenitors in subsequent pregnancies [28]. Transplantation into cleared mammary fat pads demonstrated that PI-MECs could contribute to both ductal and alveolar development, further implicating self-renewing and multipotent capabilities. In the mouse, s-SHIP expressing cells are also found restricted to the distal tips of alveolar buds during early-mid gestation, before the formation of differentiated alveoli suggesting the alveolar unit to be the putative niche for activated MaSCs during pregnancy [22].

Overall, the mammary gland endures many cycles of remodeling throughout the female life span where it undergoes significant changes in size and function. Pubertal development of the mammary gland alludes to the existence of cells that have the ability to give rise to the full spectrum of mammary epithelial cell types. The successive cycles of epithelial cell turnover that occur as a function of the reproductive cycle or pregnancy further indicate the presence of activated stem or progenitor cell pool(s) in the mature gland which have an inherent ability to self-renew. What is not clear is whether a subpopulation of MaSCs drives the morphological changes observed over the female reproductive life span in vivo or if concerted progenitor activity also contributes to these changes.

3 Mammary Epithelial Stem and Progenitor cells

3.1 Murine MaSCs

Early transplantation studies first introduced the concept of a self-renewing multipotent mammary cell in murine models. In these experiments, mammary epithelial fragments as small as 0.5 mm could regenerate a functional mammary gland when transplanted into the mammary fat pad of a syngeneic host cleared of all endogenous mammary epithelium [29, 30]. The regenerated glands retained hormone responses and successfully lactated, demonstrating the regenerative capacity of select mammary cells to repopulate a mammary fat pad with the appropriate mammary epithelial differentiation program [29–31]. Furthermore, the regenerated gland possessed a finite ability to serially transplant for five to eight generations, unlike neoplastic tissues that have unlimited outgrowth potential [32]. Concurrently, early attempts to identify a putative stem cell population were also based on electron microscopy analyses. Specifically, based on in vitro differentiation potential, small light cells were the candidate MaSC population, characterized with unique ultrastructural features, mitotic figures, and situated between the luminal and basal layers of the gland [33, 34].

Building on the above pioneering work by the Deome laboratory subsequent studies demonstrated that regenerative potential was scattered throughout the epithelial network and persists throughout the life span of a mouse, irrespective of parity [34]. For instance, the mammary epithelial cells from both 26-month-old virgin mice and 3-week-old prepubertal mice were successfully propagated and transplanted for up to five generations [34, 35]. In the same manner, the reconstitution potential of cells from virgin glands, nulliparous, uniparous, and multiparous mice were also found to have little variation [34, 35]. Thus, the consistent presence of select multipotent cells within the mammary gland was proposed since outgrowth potential was affected neither by the age of transplanted mammary tissue nor the developmental stage.

However, the existence of a multipotent MaSC in the gland was most convincingly supported by the formation of a functional mammary gland from a single cell. This phenomenon was established using a retroviral-tagged cell that clonally expanded to produce an extensive ductal tree in an epithelium-divested mammary fat pad [36]. The resulting gland contained both luminal and basal epithelial components and retained the ability to serially transplant [36].

With the foundation built by these studies, the primary goal within the mammary stem cell field subsequently transitioned towards purifying a highly select MaSC population. Initially, based on approaches used to identify stem cells in other systems, a number of candidate populations were examined for putative MaSC activity. Drawing from the hematopoietic system, the dye hoechst33342 was used to isolate a subpopulation of putative stem cells, referred to as the side population (SP) [37]. Stem cells generally have the ability to efflux dye more effectively due to the presence of ABC transporters. The ability to effectively efflux Hoechst dye more rapidly in MaSCs than differentiated cells may arise from the presence of ABCG2, a breast cancer resistance protein belonging to the ABC transporter super family [38, 39]. Originally, the SP population in the mammary gland was thought to enrich for MaSCs since this population was able to generate ductal and alveolar structures in epithelium-divested fat pads [20, 40]. However, current evidence suggests this population enriches for a luminal progenitor. Similarly, cells expressing the marker stem cell antigen-1 (Sca-1) were found to regenerate limited structures upon transplantation, but it too is now proposed to enrich for a luminal hormone receptor positive population [21, 22]. At present, dissociated mammary epithelial cells continue to be separated into distinct subpopulations on the basis of various cell surface markers and assayed for repopulating potential. Notably, studies by Shackleton et al. [21] and Stingl et al. [41] have provided a cell surface marker profile that distinctly isolates the luminal, basal, and stromal cellular subpopulations. Furthermore, a single basal cell has been shown to be capable of reconstituting an entire, functional mammary gland when transplanted into an epithelium-divested fat pad in vivo [21, 41]. Despite the significant progress in characterizing various mammary epithelial subpopulations and in the enrichment of MaSCs through the use of combinations of various cell surface markers, a cell surface signature exclusive to MaSCs remains to be defined.

In addition to evidence from transplantation assays, other approaches utilizing unique transgenic reporter mice have been insightful in understanding MaSC dynamics. A lineage-tracing study has been done to examine stem cell dynamics from embryogenesis to just after birth, through puberty, and following multiple pregnancies [42]. Using yellow fluorescent protein (YFP) expression under tamoxifen or doxycycline inducible lineage-specific cytokeratin promoters, stem cell and progenitor activity was monitored. In this manner, multipotent MaSC were identified embryonically, giving rise to both luminal and basal cell. When YFP expression was induced during puberty, candidate unipotent progenitors were instead identified that solely gave rise to either luminal cell or basal cell, but not both. Moreover, the study proposed that a unipotent basal progenitor reverts to a multipotent progenitor in order to reconstitute a cleared mammary fat pad following transplantation and that this cell does not assume such a function physiologically [42]. Contrastingly, however, more recent lineage-tracing experiments have suggested that Axin-2-positive cells, which are largely restricted to the basal population in adult virgin females, are able to contribute to both the luminal alveolar and basal lineages during pregnancy [43]. To date, the field remains divided as to whether or not MaSCs and/or bipotent progenitors do in fact contribute to mammary gland remodeling, or if physiologically these events are mediated solely by a combined action of basal and luminal unipotent progenitor cells.

3.2 Human MsSC

Studies of microdissected human breast tissues showed conserved X inactivation patterns in contiguous regions of breast epithelium implying that the cells originated from the same progenitor [44]. Similarly, entire ducts or lobules with identical patterns of loss of heterozygosity were also observed, again implicating a common progenitor [45]. Even luminal and basal cells in the same region were found to possess identical chromosomal alterations insinuating a shared ancestor [46]. However, until recently, evidence for human MaSCs was mainly observational due to limitations in our technical ability to test human stem cell potential in vivo. Lack of appropriate in vitro and in vivo assays initially delayed the characterization of human mammary epithelial cells for stem cell potential. In vivo transplantation assays of human mammary cells were compromised by differences in the mouse host stroma in comparison to the human stroma resulting in unsuccessful transplants. Primary mammary epithelial cells also have restricted colony-forming ability in vitro due to limited replication and differentiation capacity in solid matrix culturing systems. Thus advances in establishing in vivo and in vitro measures for human stem cell potential have been paramount in strengthening evidence for human MaSCs.

Progressive improvements in the dissociation of mammary tissue, the use of feeder layers, and the development of special culturing media have enabled human epithelial cells to be successfully cultured in vitro [47–50]. The technique of culturing mammospheres has even provided the first evidence of human mammary

epithelial differentiation ex vivo through the formation of mixed and basal staining colonies from a single clonal monolayer under differentiating stimuli [49]. Additionally, improvements have also been made towards measuring stem cell potential in vivo by "humanizing" the murine mammary fat pad. Cleared murine fat pads colonized with immortalized human fibroblasts have helped render the murine fat pad a suitable environment for supporting human mammary outgrowths [51, 52]. Transplanting mammary tissue under the renal capsule of CD-1 nude mice maintained viable mammary epithelium that expressed appropriate luminal and basal markers and hormone receptors and even produced beta-casein and milk fat globule membrane proteins when the hosts became pregnant [53]. A method for quantifying human MaSC frequency has been established by combining human breast epithelium with immortalized human breast fibroblasts and co-inoculating this mix into either the mammary fat pad or under the renal kidney capsule of an immunocompromised mouse which is followed by in vitro assays [54]. This technique has been successful in generating outgrowths from select subpopulations of mammary epithelial cells, paralleling what is often observed in murine mammary epithelial transplant studies [51, 54, 55]. Using these in vivo and in vitro techniques in conjunction has also proved fruitful as staining mammospheres with PKH26, a lipophilic dye that is retained in slowly dividing cells, has shown to further enrich for cells with MaSC activity. This activity was tested by transplantation into a humanized mouse mammary gland [56]. In this manner, many new avenues of characterizing human MaSCs are now possible and will undoubtedly broaden the field of knowledge.

3.3 Progenitors

In the attempts to uncover the multipotent mammary stem cell, distinct progenitors with a parent–progeny relationship to the multipotent stem cell have also been identified. For instance, early limiting dilution transplantations lead to the identification of three distinct progenitor populations: a progenitor that forms both ducts and alveoli, a progenitor that gives rise to ducts alone, and another that solely forms alveoli [57]. As a result, the notion of a mammary epithelial hierarchy was established. However, over the years, due to the use of different experimental approaches, the above hierarchy has been questioned. Other studies have suggested the presence of progenitors that form colonies with only luminal epithelial cells, only myoepithelial cells, or colonies with a mixture of both luminal and myoepthelial cells, indicating the presence of a bipotent progenitor [47, 48, 58]. As a result, it remains to be determined whether the lineage-restricting step for ductal vs. alveolar progenitor commitment or basal vs. luminal progenitor commitment occurs first.

Overall, there are two proposed mammary epithelial hierarchies supported by different experimental approaches, but it still remains unclear as to which is the more biologically relevant hierarchy. The first model proposes that a bipotent progenitor downstream of the mammary stem cell gives rise to a luminal progenitor and a myoepithelial progenitor. The luminal progenitor is postulated to then give rise to

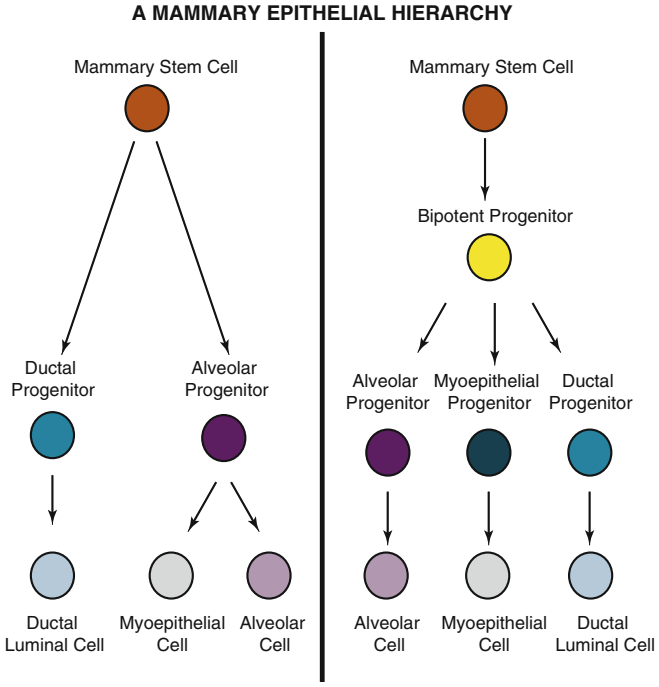

Fig. 3 Mammary epithelial cell hierarchy models. Based on different experimental approaches, there are currently two main models for the mammary epithelial hierarchy. The first model (*left*) contains a ductal progenitor and an alveolar progenitor with the latter giving rise to both basal cells and alveolar cells. The second model (*right*) contains a bipotent progenitor which gives rise to basal, alveolar, and luminal progenitors

ductal progenitors and alveolar progenitors, which in turn form ductal cells and alveolar cells respectively. The myoepithelial progenitor is thought to form basal cells. In the second model, the multipotent stem cell gives rise to a ductal progenitor and an alveolar progenitor. The ductal progenitor then goes on to form ductal cells or basal cells while the alveolar progenitor goes on to form alveolar cells or basal cells (Fig. 3).

4 Characterizing Mammary Stem Cells

Considerable effort has been focused on isolating a pure MaSC population. Using purification strategies from other systems, such as the hematopoietic and digestive system where the stem cell hierarchy is better established, various putative markers have been tested. Although current methods have enabled for the enrichment of this small cell fraction, a signature strictly unique to MaSC remains to be elucidated.

4.1 Murine and Human MaSC Markers

Based on the expression of heat stable antigen (CD24) in conjunction with either α6 integrin (CD49f) or β1 integrin (CD29), the mammary gland can be resolved into three distinct isolated subpopulations [21, 41, 59]. Luminal epithelial cells are characterized as Lin^- $CD24^{med/+}$ $CD49f^{lo}$ or Lin^- $CD24^{med/+}$ $CD29^{lo}$, basal cells as Lin^- $CD24^{med/+}$ $CD49f^{hi}$ or Lin^- $CD24^{med/+}$ $CD29^{hi}$, and stromal cells as Lin^- $CD24^{lo/-}$ $CD49f^{lo}$ or Lin^- $CD24^{lo/-}$ $CD29^{lo}$.

MaSCs are enriched specifically within the basal compartment, with transplantation of FACS-purified basal cells, but not luminal or basal cells, yielding functional mammary outgrowths. Containing both ductal and alveolar structures, glands generated from basal cells possessed the full spectrum of epithelial cells and exhibited the ability to serially transplant. The generation of an entire mammary gland from a single cell from the basal population further solidified the location of the MaSC within the basal compartment [21, 41]. It has also been suggested that the tip of the basal population highest in expression for CD49 and CD24 may further enrich for MaSCs, also referred to as the mammary repopulating unit (MRU) population [41]. The marker CD29 has even been suggested to play a functional role in mammary stem cell biology as deletion of the β1 integrin from the basal compartment resulted in a lower reconstitution frequency in secondary transplants [60]. LRG5 and Axin2 are two other markers shown to further enrich for MaSCs within the basal compartment [61, 62]. The multipotent MaSCs enriched in the basal population have also been further characterized as hormone receptor negative and were not observed to express the estrogen receptor (ERα), the progesterone receptor, or the ErbB2 receptor [63]. Transplantations of sorted murine basal cells at limiting dilution have led to the estimate that a mammary stem cell is situated at a frequency of about 1 in a few hundred cells within the basal population, although estimates range from 1 in 100 to 1 in 2,500 basal cells [23, 41].

Using hormone-treated immunodeficient mice, human breast epithelial cells incorporated with human fibroblasts and collagen injected at a non-orthotopic site under the kidney capsule have resulted in the regeneration of a mammary gland [54]. The gland was only regenerated from the $CD49f^{hi}$ $EpCAM^{-/lo}$ basal cell population but not the luminal fraction and the regenerated glands were also able to form clonogenic progenitors in vitro [54]. Implantation of the $CD49f^{hi}$ $EpCAM^{-/lo}$ basal cell population was again shown to regenerate a functional mammary gland when combined with immortalized human breast fibroblasts in an immunodeficient mouse mammary fat pad [55]. At this orthotopic site, the resulting regenerated gland contained lobular regions similar to TDLUs that were capable of fully differentiating into terminal alveolar structures. Although a suboptimal regenerative ability was observed in serial transplants, likely resulting from nonoptimal growth conditions, the $CD49f^{hi}$ $EpCAM^{-/lo}$ basal human breast epithelial cell population is thought to contain human MaSCs. These developments in quantifying MaSC frequency in humans have led to estimates of between 1/1,000 and 1/10,000 MaSc in the human breast [54].

4.2 Murine Progenitor Markers

Various candidate luminal progenitors pools have been identified in the mouse mammary gland within the Lin$^-$ CD24$^{med/+}$ CD49f^{lo}/CD29^{lo} population. Based on colony forming assays, the luminal fraction seems to contain luminal progenitor cells that form discrete colonies in vitro when placed in low cell-density adherent cultures. Cells derived from MRU outgrowths also result in these types of colonies and are therefore believed to be the parent population to the luminal progenitors. Different populations of luminal progenitors have been further resolved from the Lin$^-$ CD24$^{med/+}$ CD49f^{lo}/CD29^{lo} population using additional markers. Notably, enrichment for a luminal progenitor from a more differentiated luminal cell has been shown using the β3 integrin marker (CD61$^+$) or the lack of CD133 prominin1 or Sca-1 [64, 65]. The exact degree to which these two populations overlap is still unclear; however, the CD61$^+$ luminal progenitor is the first cell in the mammary epithelial hierarchy believed to express estrogen receptor (ER α), while the Sca-1$^-$ luminal progenitor population is generally thought to be a hormone receptor negative progenitor population based on high colony-forming ability [66]. Sca-1$^-$ cells are also candidate alveolar progenitors since they have been shown to express more milk protein genes while Sca-1$^+$ populations are thought to be hormone receptor positive. Moreover, the expression of c-Kit in conjunction with Sca-1 expression was shown to enrich for estrogen receptor positive luminal progenitors (c-Kit$^+$ Sca-1$^+$) and estrogen receptor negative luminal progenitors (c-Kit$^+$ Sca-1$^-$) that are also believed to be the alveolar progenitors [67]. Recently the expression of the α2 integrin (CD49b) has been found to better resolve these luminal progenitors into the estrogen receptor positive (CD49b$^+$ Sca-1$^+$) and estrogen receptor negative luminal progenitor populations (CD49b$^+$ Sca-1$^-$) [68].

4.3 Human Progenitor Markers

Although the ability to test stem potential in vivo has been limited until recently, bipotent mammary epithelial progenitors that form either luminal or basal colonies as well as mixed luminal plus basal colonies have been detected previously based on in vitro cultures. These bipotent progenitors have been enriched using flow cytometry and immunomagnetic sorting strategies based on the expression of a cohort of markers including MUC1 [69], CD10/CALLA [70], ESA/EpCAM [58, 71], CD49f [72], CD24, CD133, and Thy1 [47, 54, 55, 73]. EpCAM$^+$ MUC1$^+$ cells have been shown to enrich for progenitors that form luminal colonies while CD10$^+$ progenitors form basal colonies [47, 54, 55, 73, 74]. Cells that are EpCAM$^-$MUC$^{-/weak}$CD10$^{+/weak}$ have been shown to make mixed colonies and were also found to express high levels of α6 integrin (CD49f), indicating a basal position in vivo. This data insinuates that the bipotent progenitor in humans is EpCAM$^-$MUC$^{-/weak}$CD10$^{+/weak}$ and immortalized EpCAM$^-$ MUC1$^-$ further support this idea since they are able to self-renew and

generate luminal as well as basal cells while immortalized EpCAM$^+$ MUC1$^+$ cells are restricted to the luminal lineage [47]. EpCAM$^+$ MUC1$^-$ also expressed high level of keratin 19, a feature of TDLUs in vivo and EpCAM$^+$ MUC1$^-$ cells formed branching structures similar to uncultured TDLUs in 3D cultures as well as in vivo transplantations, suggesting that they may be a TDLU precursor in the breast. The markers EpCAM and CD49f in conjunction with the marker ALDH are also proposed to separate nonclonogenic luminal cells from relatively differentiated luminal progenitors (EpCAM$^+$ CD49f$^+$ ALDH$^-$) and undifferentiated luminal progenitors (EpCAM$^+$ CD49f$^+$ ALDH$^+$). Possessing a gene signature related to alveolar differentiation, ALDH luminal progenitors are proposed to be analogous to CD49b$^+$ Sca-1$^-$ luminal progenitors in the mouse [68]. Recent evidence also suggests GATA3 as a luminal marker and ErbB2 as an estrogen receptor positive luminal progenitor marker [52, 64]. Other basal cell markers in humans include p63 and SMA [75].

5 Assessing Stem Cell Potential

Until quite recently, a select number of assays have been utilized as standard techniques to measure the stem cell potential of cells in the mammary gland. However, recent advances in the way stem cell potential is examined have led to a number of unique insights regarding MaSC dynamics. Although all of these tools have been informative, there is still an array of underlying limitations and caveats that must not be overlooked.

5.1 FACS-Based Analyses

The application of fluorescence-activated cell sorting (FACS) has greatly advanced mammary epithelial characterization. Using total dissociated mammary cells, a cocktail of antibodies, and a series of gates that deplete for doublets, immune cells, and dead cells while marking cells of interest, the mammary epithelial subpopulations can specifically be isolated and purified from the total gland (Fig. 4).

Although FACS-based transplantation assays have yielded an inscrutable amount of useful data, there are a number of technical issues that should be taken into consideration. To begin with, using freshly dissociated mammary cells is imperative for gaining biologically relevant data. Although this is often difficult for human breast epithelium, it is important to avoid culturing cells before analysis as the inherent biology of the cells becomes altered. For instance, Sca-1, a progenitor cell surface marker, becomes induced in mammary epithelial cells after culturing, which can confound results. The dissociation protocol itself can significantly affect results and has the potential to alter the types of cells that are successfully dissociated. A number of different techniques have been developed to circumvent some of the concerns associated with dissociating the mammary gland into single cells, although each

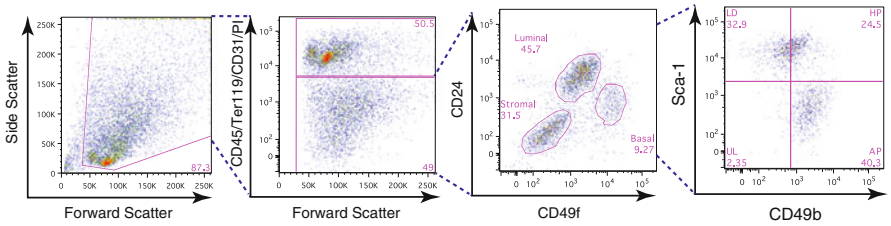

Fig. 4 Cell surface characterization of mammary cell subpopulations by flow cytometry. FACS plots showing the gating strategy used to isolate mammary epithelial subpopulations. Excluding for debris, dead cells (PI+), and lineage-positive cells (CD45+, Ter 119+, CD31+), mammary cells can be separated into the luminal, basal, and stromal compartment using the markers CD24 and CD49f. Further gating on just the luminal subpopulation, the markers Sca-1 and CD49b can further segregate the population into distinct progenitor and differentiated luminal cell fractions

still has its own caveats [76]. Other factors that should be taken into account include the antibodies themselves since some antibodies, such as those for CD24, have been shown to bind with variable efficacy when doing flow cytometry and alter the cellular profiles obtained [76].

When using mouse models, the stage of the mouse during the reproductive cycle is often overlooked which can have profound effects, particularly when conducting transplantations assays or FACS-based analyses. The profound influence of hormones on the mammary gland has always been accepted, but the specific mitogenic effects of progesterone during the reproductive cycle on the mammary gland and MaSCs in particular have been clearly reported [23]. As a result, it is important to take into account the reproductive stage of a mouse when conducting these analyses since MaSC numbers can be greatly confounded by the hormone status of the animal.

5.2 *In Vitro Colony-Forming Assays*

Since transplantation studies were not possible with human breast epithelium until recently, the colony-forming assays became an imperative tool for exploring multipotent ability of human mammary cells. There are two major methods of conducting colony-forming assays, the first is a culture that utilizes a feeder layer of NIH3T3 cells and the second is a 3D culture in Matrigel [21, 41, 64, 65] (Fig. 5).

The colony-forming assay using the feeder layer requires the initial irradiation of NIH3T3 cells. The irradiated cells are then mixed with mammary epithelial cells and plated on a dish. To generate luminal type colonies, the plates are cultured for 7 days at 37 °C at 20 % oxygen levels while basal colonies form when cultured for 7 days at 5 % oxygen levels at 37 °C. This method is primarily used to quantify progenitor frequencies and colony-forming capacity within a population.

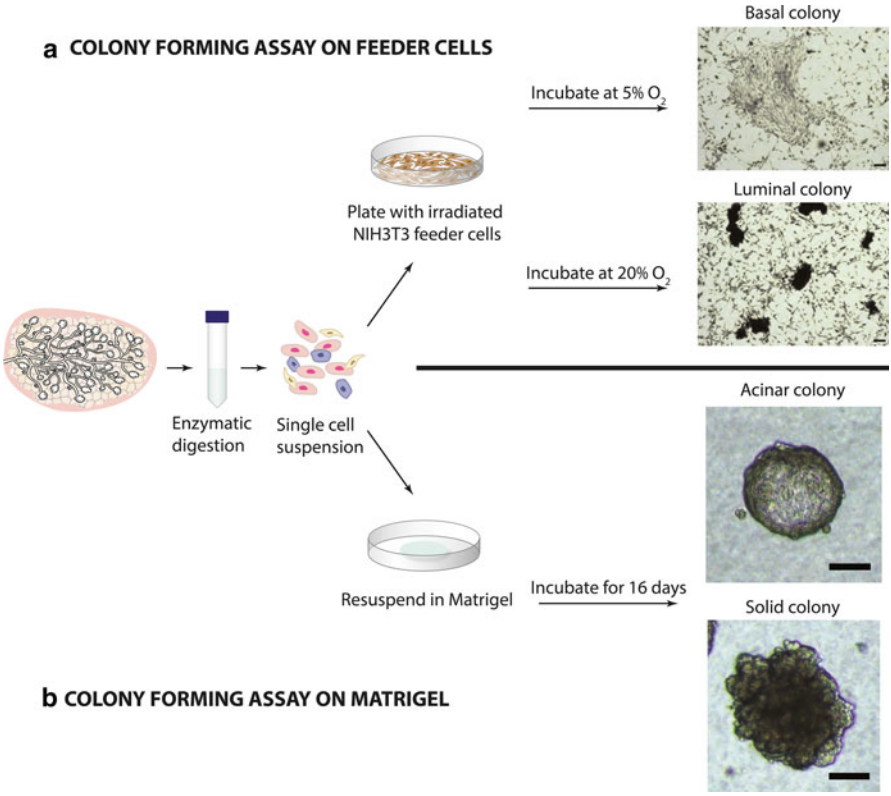

Fig. 5 An outline of colony-forming assays. Mammary tissue is dissociated using collagenase treatment to generate a single cell preparation. CFC assays can be performed on total mammary cells or on FACS-purified cell populations. The cells are plated onto a layer of irradiated feeder cells and incubated in 20 % O_2 if assessing luminal colony-forming potential and in 5 % O_2 if assessing basal stem cell potential, followed by scoring for number of colonies generated. Cells can alternatively be resuspended in matrigel and grown for 16 days to develop 3D colonies that are either acinar or solid in nature. These 3D colonies can be subsequently fixed, sectioned, and stained for luminal and basal markers

Matrigel cultures are done by resuspending mammary epithelial cells in 50 µl of Matrigel covered with 4 ml of Epicult B medium containing 5 % FCS. After 16 days of culture, the 50 µl Matrigel culture is fixed in 4 % paraformaldehyde, embedded in 1 % agarose, and then fixed again in 4 % paraformaldehyde. Finally the colonies can be sectioned and stained using hematoxylin and eosin. Mainly used to stain colonies, this method is not typically used for calculating progenitor frequencies due to concerns about obtaining accurate colony counts across the various planes of the plate.

A caveat with culturing cells in vitro is that it likely alters the inherent biological nature of the cells being examined. In a dynamic system such as the mammary

gland where there is a complex interplay of signaling factors from not only the microenvironment but also systemically in the case of hormones, it is difficult to recapitulate the biologically relevant signaling milieu. As a result, capturing a response in vitro may not always be possible. Furthermore, mammary epithelial cells have already been shown to alter their expression of certain cell surface markers upon culture. Therefore, in vitro studies provide a readout for cellular potency, although they are not the ideal assays.

5.3 In Vivo Transplantation Assays

Transplantation assays have become the gold standard for assessing stem cell potential and have been widely used in the field for some time. Using prepubertal mice between the ages of day 19 and 21, mice are cleared of endogenous mammary epithelium in their fourth inguinal glands and cells for transplantation are injected into the mammary fat pad. Serial transplantations done in this manner thus assess stem cell potential since both the ability to self-renew and the multipotent ability to generate the full epithelial hierarchy can be examined. Transplanting cells at limiting numbers also allows for the quantification of MaSC frequency. In conjunction with recent advances in flow cytometry, the ability to sort specific subpopulations and transplant cells to evaluate stem cell function has been invaluable.

The ability to do transplantation assays in humans was originally limited due to differences in the mouse stroma in comparison to the human breast leading to unsuccessful transplants. However, advances in "humanizing" the murine mammary fat pad with immortalized human breast fibroblasts in immunocompromised mice by simulating human stroma have resulted in the successful transplantation of human breast epithelium. Outgrowths in the orthotopic site of a mouse mammary gland as well as the non-orthotopic site under the kidney capsule have been successfully generated when a combination of immortalized human breast fibroblasts and human breast epithelium were injected in a collagen gel [51, 54, 55].

Although transplantations allow for the assessment of multipotency through the regeneration of a complete functional mammary gland and self-renewal through serial transplantations, lineage-tracing studies suggest that this does not accurately reflect the way basal cells behave physiologically. Instead it is proposed that basal unipotent progenitors revert to a bipotent state during transplantation assays responding to meet the homeostatic maintenance demands of a system which perhaps simulates injury [42]. Thus although single cells have been shown to generate an entire mammary gland, it is important not to overlook the potential impact of placing cells in a environment that may extraneously stimulate MaSC activity which would not occur in the intact physiological state. It is important to consider the critical role the stem cell niche plays in directing MaSC activity and how transplantation assays may not necessarily reflect this crucial component [77].

6 Conclusions

There have been significant advances in providing evidence for a mammary stem cell but much remains to be uncovered about the true identity as well as the precise location of this stem cell population. The mammary epithelial hierarchy is constantly beginning redefined. Specifically, the identification of a highly defined cell surface marker signature for MaSCs, apart from mature basal cells, awaits identification. Various mammary progenitor pools are also being identified, refined, and characterized. Moreover, lineage-tracing studies are beginning to raise new questions and suggest that the gold standard transplantation assay may not accurately reflect the physiological behavior of mammary epithelial cells. Further murine studies coupled with advances in the ability to assess human MaSC/progenitor activity will undoubtedly lead to a better understanding of the human breast.

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References

1. Cardiff RD, Wellings SR (1999) The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia 4(1):105–122
2. Cardiff RD (1998) Are the TDLU of the human the same as the LA of mice? J Mammary Gland Biol Neoplasia 3(1):3–5
3. Russo J et al (1990) Comparative study of human and rat mammary tumorigenesis. Lab Invest 62(3):244–278
4. Murrell TG (1995) The potential for oxytocin (OT) to prevent breast cancer: a hypothesis. Breast Cancer Res Treat 35(2):225–229
5. Ronnov-Jessen L, Petersen OW, Bissell MJ (1996) Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 76(1):69–125
6. Wiseman BS, Werb Z (2002) Stromal effects on mammary gland development and breast cancer. Science 296(5570):1046–1049
7. Schedin P et al (2004) Mammary ECM composition and function are altered by reproductive state. Mol Carcinog 41(4):207–220
8. Muschler J, Streuli CH (2010) Cell-matrix interactions in mammary gland development and breast cancer. Cold Spring Harb Perspect Biol 2(10):a003202
9. Hovey RC, McFadden TB, Akers RM (1999) Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. J Mammary Gland Biol Neoplasia 4(1):53–68
10. Weaver VM et al (1996) The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. Biochem Cell Biol 74(6):833–851
11. Lim E et al (2010) Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 12(2):R21

12. Veltmaat JM et al (2003) Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation. Differentiation 71(1):1–17
13. Russo J, Russo IH (2004) Development of the human breast. Maturitas 49(1):2–15
14. Hovey RC, Trott JF, Vonderhaar BK (2002) Establishing a framework for the functional mammary gland: from endocrinology to morphology. J Mammary Gland Biol Neoplasia 7(1):17–38
15. Mintz B, Slemmer G (1969) Gene control of neoplasia. I. Genotypic mosaicism in normal and preneoplastic mammary glands of allophenic mice. J Natl Cancer Inst 43(1):87–109
16. Spike BT et al (2012) A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer. Cell Stem Cell 10(2):183–197
17. Williams JM, Daniel CW (1983) Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol 97(2):274–290
18. Smith GH (2005) Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. Development 132(4):681–687
19. Zeps N et al (1998) Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. Differentiation 62(5):221–226
20. Welm BE et al (2002) Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 245(1):42–56
21. Shackleton M et al (2006) Generation of a functional mammary gland from a single stem cell. Nature 439(7072):84–88
22. Bai L, Rohrschneider LR (2010) s-SHIP promoter expression marks activated stem cells in developing mouse mammary tissue. Genes Dev 24(17):1882–1892
23. Joshi PA et al (2010) Progesterone induces adult mammary stem cell expansion. Nature 465(7299):803–807
24. Andres AC, Strange R (1999) Apoptosis in the estrous and menstrual cycles. J Mammary Gland Biol Neoplasia 4(2):221–228
25. Richert MM et al (2000) An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia 5(2):227–241
26. Russo J et al (2005) The protective role of pregnancy in breast cancer. Breast Cancer Res 7(3):131–142
27. Russo J, Rivera R, Russo IH (1992) Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23(3):211–218
28. Matulka LA, Triplett AA, Wagner KU (2007) Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells. Dev Biol 303(1):29–44
29. Deome KB et al (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 19(5):515–520
30. Faulkin LJ Jr, Deome KB (1960) Regulation of growth and spacing of gland elements in the mammary fat pad of the C3H mouse. J Natl Cancer Inst 24:953–969
31. Daniel CW, Deome KB (1965) Growth of mouse mammary glands in vivo after monolayer culture. Science 149(3684):634–636
32. Daniel CW et al (1975) Unlimited division potential of precancerous mouse mammary cells after spontaneous or carcinogen-induced transformation. Fed Proc 34(1):64–67
33. Chepko G, Smith GH (1997) Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. Tissue Cell 29(2):239–253
34. Smith GH, Medina D (1988) A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci 90(Pt 1):173–183
35. Young LJ et al (1971) The influence of host and tissue age on life span and growth rate of serially transplanted mouse mammary gland. Exp Gerontol 6(1):49–56
36. Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125(10):1921–1930
37. Goodell MA et al (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183(4):1797–1806

38. Zhou S et al (2002) Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A 99(19):12339–12344
39. Jonker JW et al (2005) Contribution of the ABC transporters Bcrp1 and Mdr1a/1b to the side population phenotype in mammary gland and bone marrow of mice. Stem Cells 23(8):1059–1065
40. Alvi AJ et al (2003) Functional and molecular characterisation of mammary side population cells. Breast Cancer Res 5(1):R1–R8
41. Stingl J et al (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439(7079):993–997
42. Van Keymeulen A et al (2011) Distinct stem cells contribute to mammary gland development and maintenance. Nature 479(7372):189–193
43. van Amerongen R, Bowman AN, Nusse R (2012) Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell 11(3):387–400
44. Tsai YC et al (1996) Contiguous patches of normal human mammary epithelium derived from a single stem cell: implications for breast carcinogenesis. Cancer Res 56(2):402–404
45. Lakhani SR et al (1999) Genetic alterations in 'normal' luminal and myoepithelial cells of the breast. J Pathol 189(4):496–503
46. Deng G et al (1996) Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 274(5295):2057–2059
47. Stingl J et al (2001) Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat 67(2):93–109
48. Gudjonsson T et al (2002) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes Dev 16(6):693–706
49. Dontu G et al (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17(10):1253–1270
50. Villadsen R et al (2007) Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 177(1):87–101
51. Kuperwasser C et al (2004) Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A 101(14):4966–4971
52. Ginestier C et al (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1(5):555–567
53. Parmar H et al (2002) A novel method for growing human breast epithelium in vivo using mouse and human mammary fibroblasts. Endocrinology 143(12):4886–4896
54. Eirew P et al (2008) A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. Nat Med 14(12):1384–1389
55. Lim E et al (2009) Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 15(8):907–913
56. Pece S et al (2010) Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell 140(1):62–73
57. Smith GH (1996) Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. Breast Cancer Res Treat 39(1):21–31
58. Stingl J et al (1998) Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast. Differentiation 63(4):201–213
59. Sleeman KE et al (2006) CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 8(1):R7
60. Taddei I et al (2008) Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. Nat Cell Biol 10(6):716–722
61. Zeng YA, Nusse R (2010) Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell 6(6):568–577
62. Plaks V et al (2013) Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell Rep 3(1):70–78

63. Asselin-Labat ML et al (2006) Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 98(14):1011–1014
64. Asselin-Labat ML et al (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nat Cell Biol 9(2):201–209
65. Sleeman KE et al (2007) Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. J Cell Biol 176(1):19–26
66. Kendrick H et al (2008) Transcriptome analysis of mammary epithelial subpopulations identifies novel determinants of lineage commitment and cell fate. BMC Genomics 9:591
67. Regan JL et al (2012) c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer. Oncogene 31(7):869–883
68. Shehata M et al (2012) Phenotypic and functional characterization of the luminal cell hierarchy of the mammary gland. Breast Cancer Res 14(5):R134
69. Taylor-Papadimitriou J et al (2002) MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 7(2):209–221
70. Gusterson BA et al (1986) Identification of myoepithelial cells in human and rat breasts by anti-common acute lymphoblastic leukemia antigen antibody A12. J Natl Cancer Inst 77(2):343–349
71. Latza U et al (1990) Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelial. J Clin Pathol 43(3):213–219
72. Koukoulis GK et al (1991) Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Correlations with their functions as receptors and cell adhesion molecules. Am J Pathol 139(4):787–799
73. Raouf A et al (2008) Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell 3(1):109–118
74. Clayton H, Titley I, Vivanco M (2004) Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 297(2):444–460
75. Skalli O et al (1986) A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. J Cell Biol 103(6 Pt 2):2787–2796
76. Smalley MJ et al (2012) Isolation of mouse mammary epithelial subpopulations: a comparison of leading methods. J Mammary Gland Biol Neoplasia 17(2):91–97
77. Joshi PA, Di Grappa MA, Khokha R (2012) Active allies: hormones, stem cells and the niche in adult mammopoiesis. Trends Endocrinol Metab 23(6):299–309

Adult Ovary Stem Cells

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Abstract Adult ovarian stem cells represent one of the most intriguing subjects in the field of reproductive medicine and biology, even as we continue to pose the question whether they really exist. Currently there is increasing knowledge on ovarian physiology and function. This presents some new findings on ovarian stem cells which have become the center of interest for different clinical and scientific disciplines, such as reproductive medicine and biology, regenerative medicine, and oncology. The aim of this review is to summarize the quickly evolving knowledge on stem cells in adult human and other mammalian ovaries.

Keywords Ovary • Adult stem cells • Surface epithelia • Cancer stem cells • Regeneration

Abbreviations

AD cells	Adherent cells
CA125	Cancer antigen 125
CK	Cytokeratin
CSCs	Cancer stem cells
DXR	Doxorubicin

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EpCAM	Epithelial cell adhesion molecule
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
GSCs	Germline (germinal) stem cells
hESCs	Human embryonic stem cells
LIF	Leukemia-inhibitory factor
mGSCs	Mouse germline (germinal) stem cells
mOSE	Mouse ovarian surface epithelium
NAD cells	Non-adherent cells
OSE	Ovarian surface epithelium
PGCs	Primordial germ cells
SCID	Severe combined immunodeficiency
SCP3	Synaptonemal complex protein 3
SP	Side population
SSEA-4	Stage-specific embryonic antigen-4
TA	Tunica albuginea
TGFB1	Transforming growth factor beta 1
VSELs	Very small embryonic-like stem cells
ZP	Zona pellucida

1 Introduction

The human ovary is a very complex organ with several functions, including folliculogenesis to produce oocytes for fertilization and creation of new human beings. The cyclic nature of the reproductive process in females depends on the ability of the ovary to change in both structure and function. This is reflected at the molecular level by combining many different mechanisms and pathways leading to successful human reproduction, if healthy. The complex instrumentation in the ovary that allows follicular growth is regulated by gonadotropins, steroid hormones, and several factors produced within the ovary. In all these processes different types of cells are involved, including stem cells, which interact with other types of cells to produce mature oocytes and cells supporting them.

2 Structure of the Adult Human Ovary

The ovary consists of three different regions: (1) an outer cortex, which contains the ovarian follicles, (2) a central medulla consisting of ovarian stroma, and (3) an inner hilum with arboring blood vessels surrounding the area of attachment of the ovary to the mesovarian region [1]. The surface of the ovary is covered with ovarian surface epithelium, a simple epithelium, which changes from squamous to cuboidal

morphology with age. During embryonic development the epithelial cells are derived from the mesoderm and are closely related to the mesothelium of the peritoneum. Immediately beneath the surface epithelium lies a dense connective tissue sheath, the tunica albuginea ovarii.

3 Ovarian Surface Epithelium

The ovarian surface epithelium is a low-differentiated mesothelial layer that surrounds the ovary and undergoes many repeated injury and repair cycles after ovulation-associated inflammation. In general, little is known about the changes that occur in the ovarian surface epithelium before or during ovulation and even less about the regenerative processes that occur after the surface is ruptured to release a mature oocyte.

The process of repeated ovulation-related disruption and repair is accompanied by complex remodeling, which include somatic stem/progenitor cell-mediated processes. It was proposed that the ovarian surface epithelium exfoliated from the dome of ovulatory follicles is replenished by generative stem cell proliferation and migration from the wound edges [2]. Indeed the gene expression profiling of human adult ovarian surface epithelium brushings performed by Bowen et al. supported the hypothesis that human ovarian surface epithelia are multipotent, are capable of regeneration, and additionally serve as ovarian cancer initiating cells [3]. By comparative gene expression profiling they confirmed a high expression of many genes known to be involved in the canonical cell cycle pathways as well as signaling pathways previously related to development (i.e., the TGFB/BMP, Wnt, Notch, Hedgehog, and Retinoid pathways) and the maintenance of stem cells in a quiescent state (i.e., TGFB, cyclin-dependent kinase inhibitor 1B—*CDKN1B*). These data were consistent with the hypothesis that ovarian surface epithelial cells are arrested in the G0/G1 phase of the cell cycle and indicated that many, if not all, of the surface epithelial cells on the surface of adult ovaries are not terminally differentiated, but arrested in a quiescent state, characteristic of most adult stem cell populations [4]. Moreover, they also provided an alternative hypothesis that the adult ovarian surface epithelial cells are stem cell-like and maintain a degree of pluripotency sufficient to allow them to transform (e.g., into cancer cells). This might be supported by some of their observations: for example, the Wnt receptor FZD7 that has been shown to be expressed in human embryonic stem cells (hESCs) and to play a role in the self-renewal capacity of these cells [5] was highly expressed in the brushed ovarian surface epithelium. Furthermore, their results confirmed the expression of genes *LHX2* and *LHX9* and supported the idea that asymmetric cellular division occurs in the adult human ovarian surface epithelium [3]. It is known that the processes of stem cell self-renewal and differentiation are accomplished by a combination of symmetric and asymmetric cell divisions [6, 7]. Each symmetric division gives rise to two identical daughter stem cells. In contrast, each asymmetric division results in one stem cell and one progenitor cell with limited self-renewal potential.

In spite of all these findings, they did not provide any idea about a separate population of ovarian stem cells expressing some markers of pluripotency which might be present among surface epithelial cells. Recently, Amsterdam et al. found the expression of the pluripotency-related marker NANOG in the surface epithelium of healthy human ovaries [8]. All these findings may be related to the stemness of adult human ovaries.

4 Stem Cells in Ovarian Surface Epithelium

Several studies (discussed below) provide direct evidence for the presence of stem cells among epithelial cells in the ovarian surface epithelium of both the animal and human adult ovaries.

5 Stem Cells in Mouse Ovarian Surface Epithelium

Recently, a population of mouse ovarian surface epithelial (MOSE) cells that exhibit progenitor/stem cell characteristics was identified by Gamwell et al. [9]. They selected a population of MOSE cells with progenitor cell characteristics that expressed the stem cell marker lymphocyte antigen 6 complex, locus A (LY6A; also known as stem cell antigen-1 or SCA-1). The size of the LY6A-expressing (LY6A+) progenitor cell population was regulated by at least two ovulation-associated factors present in the follicular fluid: transforming growth factor beta 1 (TGFB1) and leukemia-inhibitory factor (LIF). They concluded that this population of LY6A+ MOSE progenitor cells on the ovarian surface may play a role in ovulatory wound healing [9]. But neither a genetic marker nor how these cells are regulated was determined in this study; this therefore remains to be resolved in the future.

Wnts are a family of secreted signaling molecules, which are involved in a number of developmental processes including the establishment of cell fate, polarity, and proliferation. Some recent studies also implicated Wnts in adult stem cell maintenance, renewal, and differentiation [10]. The role of Wnts is to transduce their signal through one of three signaling pathways. The best studied, the Wnt/β-catenin pathway, leads to an increase in intracellular β-catenin, which acts as a co-transcription factor with members of the TCF/LEF family. It was confirmed that a number of Wnts are expressed in the ovaries, specifically in the membrana granulosa and ovarian surface epithelium. Using responsive transgenic (TopGal) mice, Usongo and Farookhi investigated the spatiotemporal pattern of β-catenin/TCF expression in the ovarian surface epithelium [10]. By generating the β-galactosidase response (lacZ+) they identified the cell population that covered the medio-lateral surface of the indifferent gonad at embryonic day (E) 11.5 in a mouse. From E12.5 onwards lacZ expression disappeared in cells covering the testis, but interestingly, it still persisted in developing ovaries of female mice. They observed that lacZ+

ovarian surface epithelium cells were present throughout embryonic and postnatal ovarian development, but being age dependent they decreased to a small proportion when the animals were weaned and remained at this proportion with aging. By flow cytometric (FACS) and ovarian section analyses in postnatal (day 1) mice they showed that lacZ+ cells represented approximately 20 % of ovarian surface epithelium, falling to 8 % in 5-day-old animals and accounted for only 0.2 % of ovarian surface epithelium in prepubertal and adult mice [10]. The process of apoptosis was undetected in the ovarian surface epithelium of neonates, and β-catenin/TCF-signaling cells were proliferating in their ovaries, both of which indicate that neither cell death nor proliferation failure was responsible for the proportion of alteration in these cells. It appeared that lacZ+ cells gave rise to lacZ− cells, as confirmed in cell cultures. Furthermore, they used the DNA-binding dye DyeCycle Violet for a side population (SP) assay, which aimed to identify subpopulations of ovarian surface epithelium cells with chemoresistance phenotype associated with ABCG2 transporter activity. Before the end, the FACS analysis revealed that the lacZ+ cells exhibit cytoprotective mechanisms, indicated by enrichment within the SP. They concluded that their study raised the possibility that WNT/β-catenin-signaling cells represent a progenitor cell population in the mouse ovarian surface epithelium [10].

Similarly, Szotek et al. found that normal mouse ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics [11]. Using BrdU incorporation and doxycycline-inducible histone2B-green fluorescent protein pulse-chase techniques, they identified a label-retaining cell population in the surface epithelium of adult mouse ovaries and proposed these cells as candidate somatic stem/progenitor cells. They found that the newly identified cell population exhibited quiescence with asymmetric label retention, functional response to estrous cycling in vivo by proliferation, enhanced growth characteristics by in vitro colony formation, and cytoprotective mechanisms by enrichment for the side population. They concluded that these characteristics identify the label-retaining cell population as a candidate for putative somatic stem/progenitor cells of the coelomic epithelium of the mouse ovary.

6 Mouse Ovarian Stem Cell Lines

More studies confirmed that adult mouse ovarian surface epithelium might be an interesting source of stem cells. Moreover, these studies confirmed that stem cells present among the epithelial cells at the ovarian surface expressed some markers of pluripotency and can form cell colonies and embryoid body-like structures in vitro and teratoma in vivo when injected into immunodeficient mice.

Until recently the existence of female germline stem cells (GSCs) in postnatal mammalian ovaries remained a controversial issue among reproductive biologists and stem cell researchers [12, 13]. After some previous indirect and relatively weak evidence on the presence of stem cells in adult mouse ovaries [14, 15] an important breakthrough was made by Zou and his coworkers [16] who found the presence of

mouse *Vasa* homologue (*Mvh*)-positive cells in the ovarian surface epithelium of neonatal mouse ovaries. They isolated these cells by immunomagnetic isolation and established a neonatal mouse germ stem cell (mGSC) line persisting for more than 15 months. Similarly, GSCs from adult mouse ovaries were isolated and cultured for more than 6 months. It was confirmed that these cells retained high telomerase activity and a normal karyotype during prolonged culture in vitro. After infection with a green fluorescent protein (GFP) virus and transplantation into infertile mice, transplanted GSCs underwent oogenesis and the mice produced GFP transgene-marked offspring [16]. Moreover, germ stem cells were also found in aged mouse ovaries and were associated with folliculogenesis and neo-oogenesis. Niikura et al. found that aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment [17]. Among more experiments based on their previous finding that immature follicles are rapidly regenerated in young adult mouse ovaries after acute oocyte loss induced by doxorubicin (DXR) exposure [15], they established a strong positive correlation between ovarian *Stra8* expression and regeneration of follicles following DXR treatment in young adult female mice. The Stra8-immunopositive cells were localized to the ovarian surface epithelium after DXR exposure and were detected at a time coincident with oocyte regeneration [17]. In another study multipotent stem cell lines were isolated and established by Pacchiarotti et al. [18] from mouse postnatal and adult ovaries using a transgenic mouse model in which the GFP was expressed under a germ cell-specific *Oct4* promoter. Two distinct populations of GFP-O*ct4*-positive cells, based on their distribution and size, were found in the mouse ovaries: (1) a group of small cells with an average diameter of 10–15 μm located at the ovarian surface epithelium and (2) larger cells with an average diameter of 50–60 μm, resembling oocytes, which were located in the center of the follicular compartment. The flow cytometry analysis revealed that the percentage of GFP-*Oct4*-positive cells in the mouse ovaries significantly decreased with age; while 1–2 % positive cells were found in the neonatal mice ovaries, only 0.05 % was still present in the adult ovaries [18]. These established ovarian GSC lines maintained their stem cell characteristics, high telomerase activity, and normal karyotype after many passages for more than 1 year. Additionally, they formed embryoid body-like structures with some differentiation capacity into all three germ cell layers (endoderm, mesoderm, and ectoderm). It was important that the germline stem cells were distinct from the CD133-positive cells circulating in the bloodstream. Gong et al. established two additional lines of colony-forming cells isolated from adult mouse ovaries after enzymatic degradation of ovarian cortex tissue and culture on fibroblasts [19]. These cells expressed some markers of pluripotent embryonic stem cells and formed embryoid bodies and teratoma after injection into immunodeficient SCID mice. The embryoid bodies and teratoma were positive for markers of all three germ layers (mesoderm, endoderm, and ectoderm). Additionally, these cells were differentiated in vitro into neuronal cells. The discovered putative stem cells were alkaline phosphatase positive and expressed high telomerase activity and a normal female karyotype, but the methylation status was different than in ESCs. Despite that, they were supposed to be pluripotent.

All these studies confirmed that a population of stem cells exists among epithelial cells in the layer of mouse ovarian surface epithelium which expresses a degree of pluripotency, and they opened an important question regarding the similar population of cells that exists in adult human ovaries.

7 Stem Cells in Human Ovarian Surface Epithelium: Do They Really Exist?

Some experimental evidence for stem cells in human adult ovarian surface epithelium does exist. Human ovarian tissue is not an easily available material to research for the presence of stem cells. In the past, the biology of human ovarian surface epithelium was intensely researched in terms of ovulation and ovarian cancer manifestation, but to a much lesser extent for the presence of putative stem cells. Nevertheless, Bukovsky and his coworkers scraped the ovarian surface epithelium of postmenopausal women and cultured the scraped populations of cells in vitro [20]. They were the first to observe development of large oocyte-like cells in postmenopausal ovarian surface epithelium cell cultures in the presence of estrogenic stimuli (phenol red). These large oocyte-like cells with a diameter of 180 μm exhibited germinal vesicle breakdown, expulsion of the polar body, and a surface expression of zona pellucida proteins, as revealed by immunocytochemistry. The experimental confirmation of oocyte-like cells was weak, but this work provided a new approach to study the ovarian surface epithelium for the presence of stem cells in humans. Additionally, this was indirect evidence of putative stem cells in the ovarian surface epithelium of postmenopausal women with no naturally present follicles/oocytes. In the next step, they found the steroid-mediated differentiation of neural/neuronal cells from the epithelial ovarian precursors in vitro [21].

Some further steps were made by our group. We identified an unknown population of small putative stem cells with diameters of up to 4 μm in the adult ovarian sections in situ and in a population of cells scraped from the ovarian surface epithelium of women with no naturally present follicles/oocytes—postmenopausal women and women with premature ovarian failure [22]. These putative stem cells developed into oocyte-like cells when cultured in vitro. This was the first time such in vitro-developed oocyte-like cells were analyzed on their genetic status and were confirmed to express some transcription factors of pluripotent embryonic stem cells, such as OCT4, SOX2, and NANOG, and some oocyte-specific markers (i.e., ZP2 and VASA). Moreover, we found parthenogenetic blastocyst-like structures developed in the ovarian surface epithelium cell cultures of postmenopausal women with no naturally present follicles/oocytes [23, 24]. Our experimental data showed that ovarian surface epithelium in patients with severe ovarian infertility—premature ovarian failure—is a potential source of stem cells expressing some markers of pluripotent/multipotent stem cells such as alkaline phosphatase activity, SSEA-4 surface antigen, and SOX-2 nuclear marker, as revealed by immunocytochemistry [25]. We found small, round cells with diameters of up to 4 μm among epithelial cells in

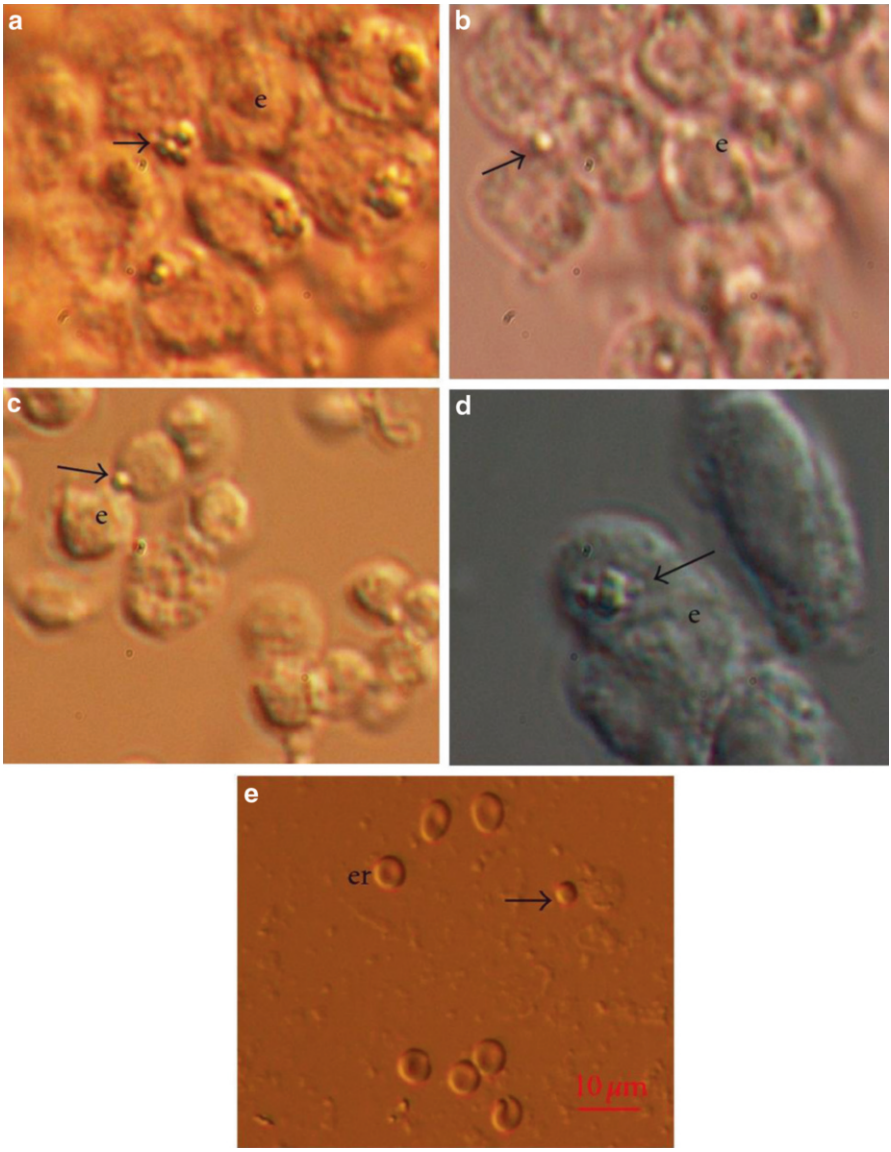

Fig. 1 Small, round cells (*arrows*)—putative stem cells in the ovarian surface epithelium. (**a**–**d**) Among the epithelial cells in the ovarian surface epithelium scrapings (magnification 6,000-times). (**e**) Among the erythrocytes in a scraped population of cells (magnification 200-times). (Inverted microscope, dic-Nomarski illumination, immersion objective for magnification 6,000-times, and Hoffman illumination for magnification 200-times.) *e* epithelial cells, *er* erythrocytes. Figure from [25]

the ovarian surface epithelium layer (Fig. 1). The single oocyte-like cells (Fig. 2) developed in vitro in the ovarian surface epithelium cultures of women with premature ovarian failure expressed the genes (*OCT4A*, *SOX-2*, *NANOG*, *NANOS*, *STELLA*, *CD9*, *LIN28*, *KLF4*, *GDF3*, and *MYC*) characteristic for pluripotent stem

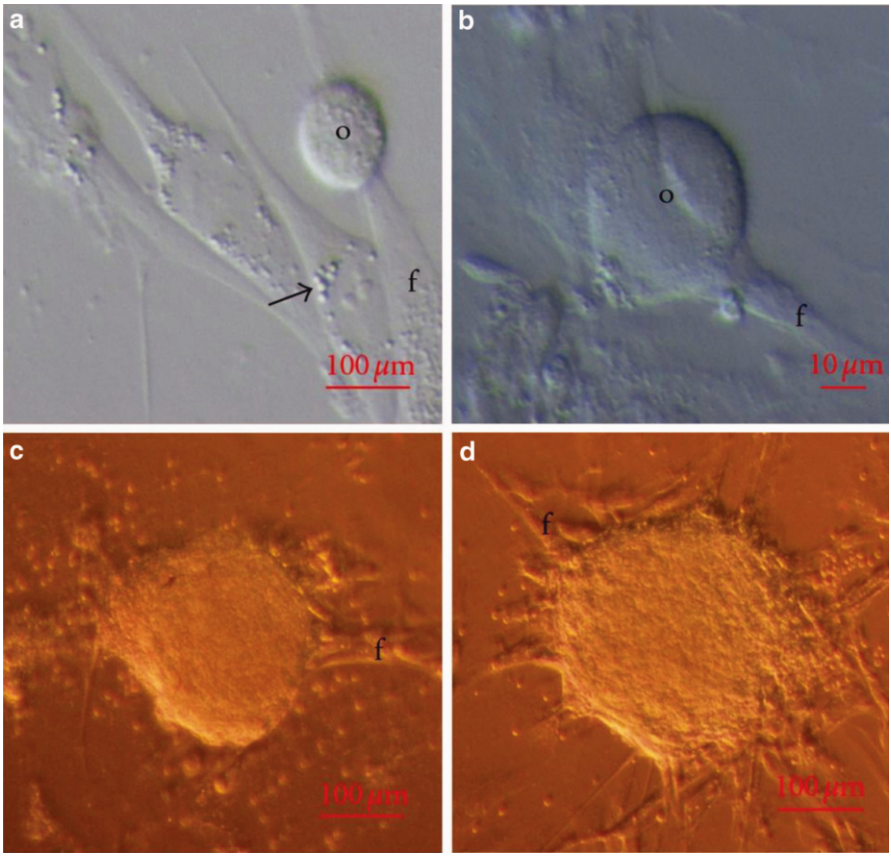

Fig. 2 *In vitro* culture of cells scraped from the ovarian surface epithelium. (**a**) Oocyte-like cell developed *in vitro* on autologous ovarian fibroblasts and small, round cells (*arrow*) with diameters of 2–4 μm proliferating in the surroundings as attached to fibroblasts on day 4 of the culture (magnification 100-times). (**b**) Oocyte-like cell developed *in vitro* on autologous ovarian fibroblasts on day 10 of the culture (magnification 200-times). (**c**, **d**) Cell clusters developed *in vitro* on autologous ovarian fibroblasts on day 4 of the culture (magnification 100-times). (Inverted microscope, Hoffman illumination.) *f* autologous ovarian fibroblast, *o* oocyte-like cell. Figure from [25]

cells, as revealed by single-cell RT-PCR. These results showed that even the ovaries of women with severe ovarian infertility might possess some regenerative potential, which needs to be further researched. In spite of promising results, the research of mouse embryonic stem cells revealed that oocyte-like cells developed in vitro failed to complete the process of meiosis [26, 27] and that transplanting these cells to develop them to maturity in vivo was proposed as a better option [27].

The above-mentioned findings were confirmed by another study by Parte et al. who observed the presence of small, putative stem cells in adult ovarian surface epithelium in humans but also in some other mammalian species such as sheep and marmoset monkey [28]. Moreover, they proposed these small, putative stem cells in the ovarian surface epithelium to be “very small embryonic-like” stem cells

regarding the similar population of small stem cells discovered in some other human adult tissues and organs. The group of Ratajczak first found a novel population of CXCR4+, SSEA-1+, Oct-4+, and CD45− cells in human adult bone marrow [29, 30] and CXCR4+, SSEA-4+, and Oct-4+ cells in umbilical blood [31]. They termed these cells "very small embryonic-like stem cells" (VSELs). The small, putative stem cells found in adult human ovaries indeed resemble the VSELs in terms of morphology and sizes. The molecular analysis of purified VSELs revealed the expression of several epiblast/primordial germ cell (PGC) markers, migrating PGC-like epigenetic reprogramming profiles of OCT4, NANOG, and STELLA loci, and a unique pattern of genomic imprinting [32]. It has also been demonstrated that VSELs show hypomethylation or erasure of imprints in paternally methylated and hypermethylation of imprints in maternally methylated genes [33] resulting in upregulation of genes *H19* and *P57* (*KIP2* or *CDKN1C*) and repression of *IGF2* and *RASGRF1*. This may explain the VSEL's quiescent status and lack of teratoma formation in human adult tissues and organs. These findings raised an important question: if small putative stem cells in human adult ovaries are really of ovarian origin or are deposited from elsewhere (i.e., bloodstream, bone marrow). Yet the work of our group confirmed that they are an integral part of the ovarian surface epithelium [25]. In three patients with premature ovarian failure we found small, round yellow-colored cells with diameters of 2–4 μm—putative stem cells; they were found having been captured among epithelial cells or were attached to them in ovarian surface epithelium brushings (Fig. 1). After May–Grünwald–Giemsa staining, usually used for blood cell staining, the small putative stem cells scraped from the ovarian surface epithelium did not stain or were weakly stained. On the other hand, all blood cells, including lymphocytes, nicely stained blue. Erythrocytes were the predominating blood cells in a population of cells scraped from the ovarian surface epithelium, and small putative stem cells were significantly lower than the erythrocytes. Other types of blood cells represented about 1 % of all scraped cells, as revealed by May–Grünwald–Giemsa staining. Small putative stem cells could be comparable to lymphocytes due to their round shape and high nuclear/cytoplasm ratio, but were significantly smaller. The lymphocytes were slightly bigger than the erythrocytes and strongly stained using May–Grünwald–Giemsa, while the small putative stem cells did not stain or only weakly stained. In any case, further research is needed to elucidate whether small putative ovarian stem cells are really VSELs or something else.

The embryonic stem cell-like cells in the adult human ovarian surface epithelium are proposed to be further researched because they may represent an alternative for establishing autologous stem cell lines from adults without any genetic manipulation.

8 Ovarian Surface Epithelium, Stem Cells, and Ovarian Cancer

The ovarian surface epithelium is involved in the manifestation of the majority of ovarian cancers, which are experienced as very aggressive and resistant to chemo- and radiotherapy, thus receiving great attention from researchers. Ovarian cancer is

the most lethal of all gynecological malignancies, and the identification of novel prognostic and therapeutic targets for ovarian cancer is very important. It is believed that stem cells are involved in the manifestation of ovarian cancer, but only a small proportion of them are really endowed with the stem cell properties responsible for tumor growth, metastatic progression, and recurrence. In recent years, much attention has been paid to the concept of cancer stem cells and pluripotency/self-renewal-related pathways in cancer biology.

9 Markers of Pluripotency in Ovarian Cancer

Many studies indirectly showed that the ovarian epithelial cancers might be related to stem cells [34]. It was confirmed that ovarian tumor tissues express some markers related to pluripotency and embryonic stem cells. The Hedgehog (Hh) and Notch signaling pathways are important in tissue pattern programming and cell fate determination during embryonic development [34]. The hyperactivation of these two pathways is frequently observed in gynecological malignancies, including ovarian cancer. In contrast, the expression profiles of pluripotency-regulating core transcriptional factors NANOG, OCT4, and SOX2 appear to be heterogeneous. Among these transcription factors, only overexpression of NANOG was found to have a prominent effect on gynecological tumorigenesis, while dysregulations of OCT4 and SOX2 may vary in a dependent manner [34].

The marker NANOG is one of the key transcription factors essential for maintaining self-renewal and pluripotency in stem cells. The study of Siu et al. showed overexpression of NANOG mRNA and protein in the nucleus of ovarian cancers compared with benign ovarian lesions [35]. In this study the increased nuclear *NANOG* expression was significantly associated with high-grade cancers, serous histological subtypes, reduced chemosensitivity, and poor overall and disease-free survival. Additionally, it was found that the stable knockdown of *NANOG* impeded ovarian cancer cell proliferation, migration, and invasion, which was accompanied by an increase in mRNA expression of *E-cadherin*, *caveolin-1*, *FOXO1*, *FOXO3a*, *FOXJ1*, and *FOXB1*. On the other hand, ectopic *NANOG* overexpression enhanced ovarian cancer cell migration and invasion together with decreased *E-cadherin*, *caveolin-1*, *FOXO1*, *FOXO3a*, *FOXJ1*, and *FOXB1* mRNA expression. The NANOG-mediated cancer cell migration and invasion involved its regulation of *E-cadherin* and *FOXJ1*. Similarly, Lee et al. found that the gene *NANOG* expression was positive in 21.6 % of 74 ovarian serous carcinoma tissues, but it was not expressed in the ovarian serous cystadenoma tissues [36]. The positive *NANOG* expression was associated with residual tumor size after surgery and with the overall poorer survival of patients with positive *NANOG* than in patients with negative *NANOG* expression. They concluded that the positive *NANOG* expression in ovarian tumor tissue is associated with a poor prognosis of ovarian serous carcinoma and that the marker NANOG has a potential to predict the survival of patients and may be involved in the mechanism of chemoresistance. Based on their experimental data, Amsterdam et al. further suggested that the gene *NANOG*, which very possibly plays an important role in

development of embryonic ovaries, may be partially silenced in the ovaries of fertile and post-menopausal women, but is reexpressed in ovarian cancer by epigenetic activation [8]. The expression of *NANOG* in normal ovaries and in borderline ovarian tumors may assist in the early detection and improved prognosis of ovarian cancer, and its targeting by inhibitory miRNA or other means may assist in treating this difficult disease. Similarly, also RUNX1 was confirmed to be a broader epithelial stem cell and ovarian cancer factor, which is an attractive potential target for both prevention and therapy of several ovarian epithelial cancers [37]. The RUNX1 stimulates STAT3 signaling via direct transcriptional repression of *SOCS3* and *SOCS4*; this is essential for ovarian cancer cell growth. In spite of potential clinical importance, these studies provided only indirect evidence of ovarian cancer stem cells.

10 Ovarian Cancer Stem Cells

The cancer stem cells (CSCs) are defined as cells within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. Current studies suggested that the tumor is initiated and maintained by an unique population of cells with stem-like properties [38]. There were more studies which provided some direct confirmation and characterization of putative ovarian cancer stem cells, but the discovered populations of cells were heterogeneous so we cannot talk about one uniform population of ovarian cancer stem cells. One of the reasons is that no universal single marker or marker combination has been found to successfully isolate (ovarian) CSCs until now. The isolation of different populations of putative CSCs illustrates a hierarchical model of ovarian tumor heterogeneity in which only a small proportion of cells among biologically distinct populations can initiate tumor growth [39]. Reactivation of pluripotent transcription factors NANOG, OCT4, and/or SOX2 in association with distinct tumorigenic properties could be found in cell clones isolated from ovarian tumors by various approaches.

Bapat et al. presented direct evidence that the aggressiveness of human ovarian cancer might be a result of transformation and dysfunction of stem cells in the adult human ovary [40]. They reported that a single tumorigenic cell clone was isolated among a mixed population of cells derived from the ascites of a patient with advanced ovarian cancer. At the same time, another cell clone underwent spontaneous transformation in a cell culture, thus providing a model of ovarian cancer progression. Both cancer-transformed clones possessed stem cell-like characteristics and grew as spheroids in vitro, although further maturation and tissue-specific differentiation did not occur. The tumors grown from these two cell clones after the transplantation into the animal models were similar to those in the human disease in their histopathology. Ovarian CSCs are considered to be associated with chemo-resistance and radio-resistance that lead to the failure of traditional therapies [41]. Most therapies are directed at the fast-growing ovarian tumor mass, but not the slow-dividing cancer stem cells. It is proposed that eradicating cancer stem cells, the root of the cancer's origin and recurrence, represents a promising approach to improve ovarian cancer survival or even to cure ovarian cancer patients. It is believed that CSCs are able to survive

conventional chemotherapies, which usually target fast-dividing cells, and give rise to recurrent tumors that are more resistant and more aggressive [42]. Recently, chemoresistance of ovarian cancers has also been related to the acquisition of epithelial to mesenchymal transition in cancer cells that might be linked with the activation of the Notch signaling pathway [43]. Ahmed et al. found that the epithelial to mesenchymal transition and cancer stem cell-like phenotypes facilitate chemoresistance in recurrent ovarian cancer [44]. The process of epithelial to mesenchymal transition in ovarian cancer enables the otherwise stationary epithelial cells to become motile and invasive and to spread and recolonize into surrounding tissues [45]. It has already been reported that the epithelial–mesenchymal transition generated cells with properties of stem cells. Indeed, these features of epithelial to mesenchymal transition in ovarian cancer have been shown to correlate with a cancer stem cell-like phenotype [46].

One of the biggest problems related to ovarian cancer is a lack of appropriate markers to enable early detection of this difficult disease. But recently it was found that the markers CD44, CD133, cytokeratin 18 (CK18), and aldehyde dehydrogenase isoform 1 (ALDH1) are highly expressed in ovarian CSCs and function as tools for their identification and characterization. The expression of the CD133 antigen in primary ovarian cancer cell lines is regulated by epigenetics [47]. Steffensen et al. reported on epithelial ovarian CSCs which were CD44, CK18, and ALDH1 positive and located as clusters close to the stroma, forming the cancer stem cell "niche" [48]. They found that 17.1 % of the ovarian tumor samples revealed a high number of CD44+ stem cells with more than 20 % positive cells per sample. The number of CD44+ stem cells was significantly higher in patients with early-stage ovarian cancer and was associated with shorter progression-free survival. This study suggested that evaluation of the number of epithelial ovarian CSCs in the ovarian tumor can be used as a predictor of disease and could be applied for treatment selection in early-stage ovarian cancer. Some other groups also published the experimental evidence for ovarian cancer stem cells [49–51]. Empirically, tumor cells in ascites are a major source of disease recurrence in ovarian cancer patients. Latifi et al. developed a novel method to separate adherent (AD) and non-adherent (NAD) cells in ascites cell culture [49]. The AD cells from both chemonaive and chemoresistant ovarian cancer patients exhibited a mesenchymal morphology with an antigen profile of mesenchymal stem cells and fibroblasts, while NAD cells had an epithelial morphology with enhanced expression of cancer antigen 125 (CA125), epithelial cell adhesion molecule (EpCAM), and cytokeratin 7 (CK7). They found that NAD cells developed infiltrating tumors and ascites in 12–14 weeks after intraperitoneal injections into nude mice, while AD cells remained non-tumorigenic for up to 20 weeks. In CR patients there was a tendency to enhance mRNA expression of E-cadherin, EpCAM, STAT3, and OCT4 in the NAD population of ascites cells. Ricci et al. found that solid ovarian tumors contained a subpopulation of cells (tumor-initiating cells, TICs) that drove and sustained tumor growth and were possibly responsible for recurrence [50]. After enzymatic digestion of primary ovarian carcinoma samples, they isolated a subpopulation of cells as non-adherent (NAD) spheres in a medium suitable for tumor stem cells. These cells were able to self-renew in vitro, were tumorigenic, and expressed an epithelial-like morphology when grown in FBS-supplemented

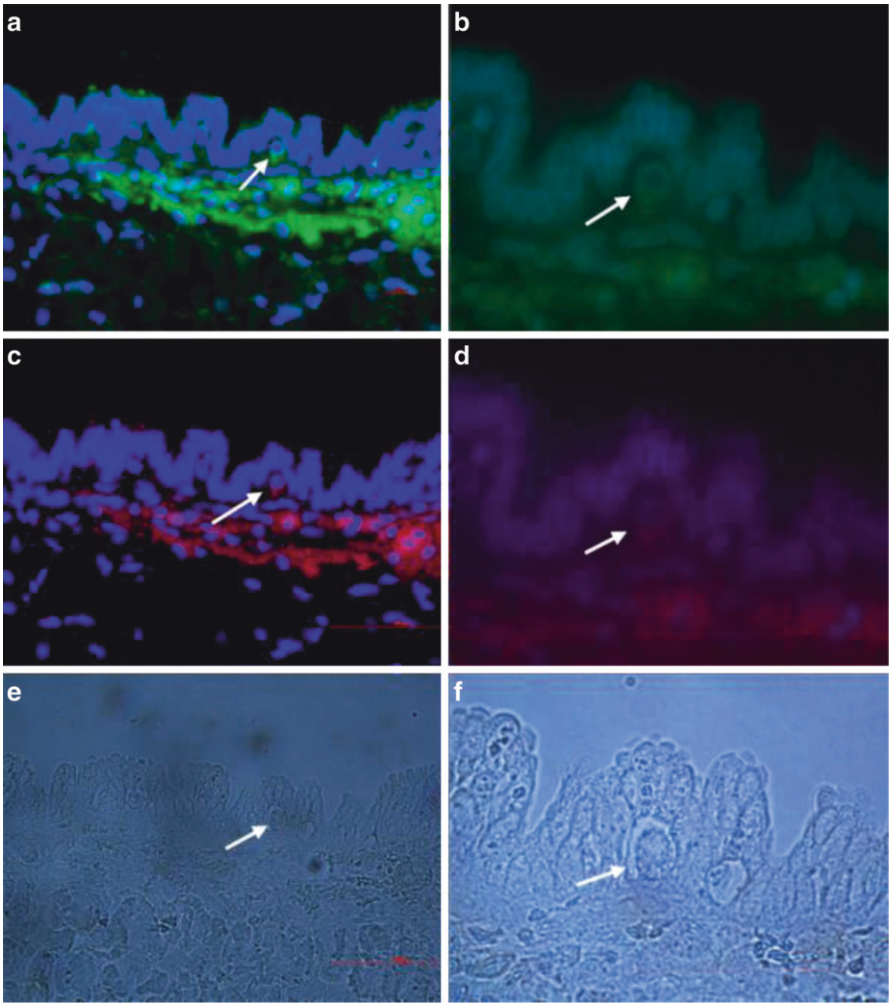

Fig. 3 Primitive oocyte-like cells (*arrows*) in the ovarian surface epithelium above the autofluorescent necrotic malignant tissue of a patient with serous papillary adenocarcinoma. (**a**, **b**) SSEA-4-positive cells (*green*). (**c**, **d**) SOX-2-positive cells (*red*). (**e**, **f**) Non-stained cells (**a**, **c**, **e**: fluorescent and light microscopy, magnifications ×400; **b**, **d**, **f**: fluorescence and light microscopy, magnifications ×1,000). Figure from [51]

medium, losing their tumorigenic potential. They showed higher expression of genes involved in stemness than differentiated cells derived from them and were more resistant to the cytotoxic effects of some drugs. Additionally, these cells expressed some mesenchymal markers, and epithelial transition was induced when cultured in differentiating conditions, combined with a loss of invasive potential. Based on their experimental data, they concluded that ovarian cancer is a stem cell disease [50]. Additionally, our group has presented a clinical case on primitive oocyte-like cells in the ovarian surface epithelium of a 67-year-old woman (Fig. 3)

which probably persisted from the fetal period of life or developed from putative stem cells and expressed pluripotency-related markers SSEA-4, SOX-2, and germinal marker VASA. These cells represented a pathological condition, which was not observed in control healthy ovaries and might be related to serous papillary adenocarcinoma manifestation and malignant tissue in this woman [51]. Moreover, recently we discovered a population of small, round cells with diameters of up to 4 μm expressing some markers of pluripotency in the ovarian cell cultures set up after enzymatic degradation of cortex tissue in women with borderline ovarian cancer. These cells were also found in healthy human adult ovarian surface epithelium, as already discussed above [22–26]. But in women with borderline ovarian cancer, comparable small cells were hyperproliferating and formed cell colonies and tumor-like structures in vitro (Fig. 4) which were positively stained on some markers of pluripotency (OCT4, NANOG), as can be seen in Fig. 5; they also expressed some genes related to pluripotency and germinal cells, as revealed by RT-PCR and microarrays. These data may indicate the possible relation of putative ovarian CSCs to pluripotency and germinal lineage. We suggest that there is a population of small putative stem cells in adult human ovaries which might be involved in human

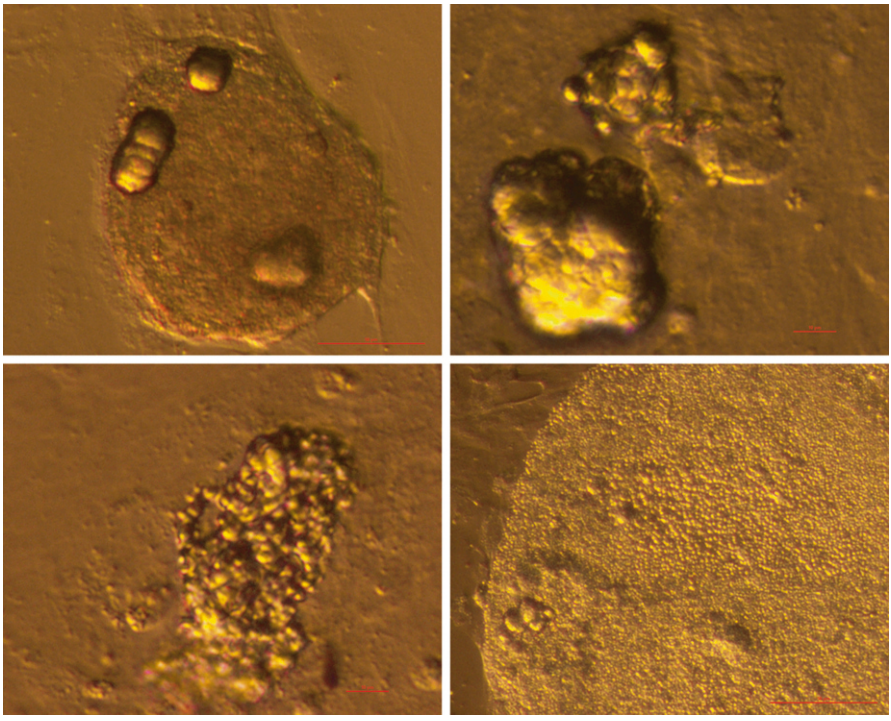

Fig. 4 Small putative stem cells with diameters of up to 4 μm, hyperproliferating and forming cell colonies, and tumor-like structures in the ovarian cell culture set up after enzymatic degradation of ovarian cortex tissue in women with borderline ovarian cancer

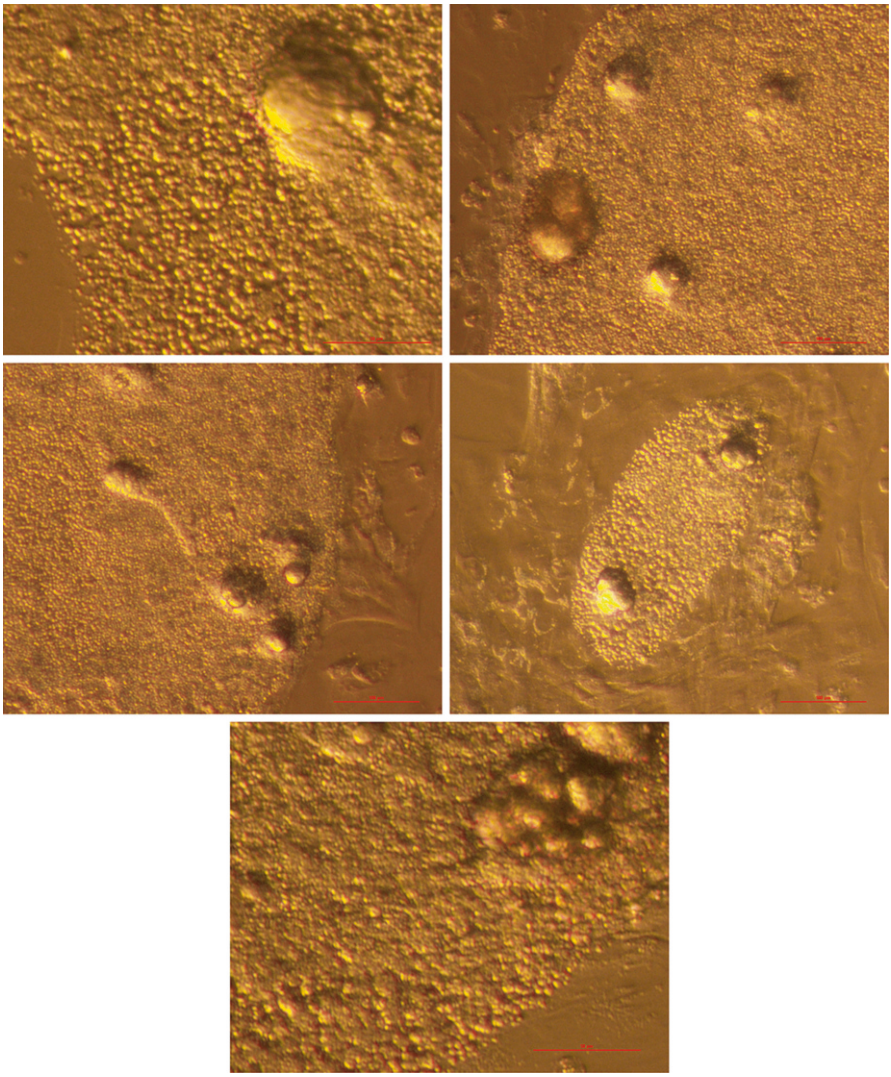

Fig. 4 (continued)

reproduction and tissue regeneration, but upon an inappropriate condition in the body they start to develop in the direction of aggressive ovarian cancer. Further research is needed to make a real conclusion in the future.

Mezencev et al. were trying to identify inhibitors of ovarian cancer stem-like cells by high-throughput screening [52]. From 793 analyzed compounds with evaluable data, 158 were found to have significant inhibitory effects on ovarian cancer stem cells. Further computational analysis indicated that the majority of these compounds were associated with mitotic cellular responses.

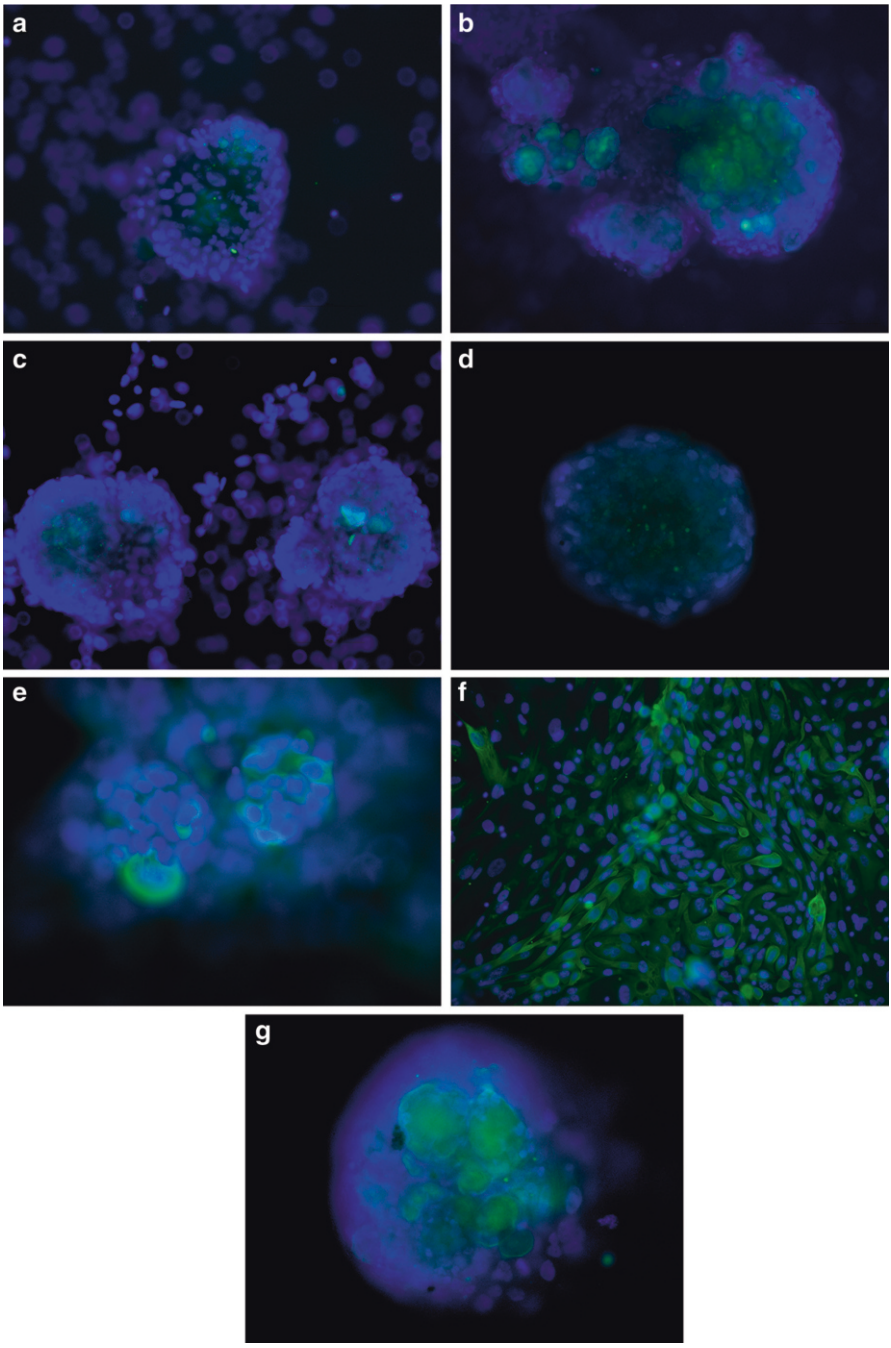

Fig. 5 Ovarian cell culture with forming tumor-like structures which were positively stained for: (**a**, **c**) OCT4, (**b**, **d**) NANOG, (**e**, **f**) CYTOKERATIN, and (**g**) negative control

11 Ovarian Cortex

Beneath the ovarian surface epithelium is a dense connective tissue sheath, the tunica albuginea ovarii. The layer of the ovarian stroma lies below the tunica albuginea, composed of connective tissue cells and fibers, among which primary and secondary follicles in various stages of development are scattered [1].

12 Ovarian Cortex: Place of Folliculogenesis/Oogenesis De Novo?

There is an important scientific question about the potential folliculogenesis and oogenesis de novo in adult human ovaries which remains unresolved. A main reason is that it is very difficult to approach this scientific problem due to the lack of the human ovarian tissue available for research and the impossibility of performing the study in vivo. Most studies based on the principles of follicle counting and immunostaining of ovarian sections excluded the dynamics of processes such as oogenesis and folliculogenesis. For a long time it has been generally accepted that the total number of follicles is set at birth. This statement was supported by several studies including some recent ones. In one of the last studies Byskov et al. found that there is no evidence for the presence of oogonia in the human ovary after their final clearance during the first 2 years of life [53]. They tried to elucidate whether oogonia detected by immunohistochemical methods in the human ovary during the first trimester were also present in peri- and postnatal ovaries. Almost all oogonia in the fetal ovaries were positively stained for SSEA4, NANOG, OCT4, and C-KIT, whereas only a small proportion of oogonia were positively stained for MAGE-A4. At birth only a few oogonia were positively stained for these markers and even these disappeared before 2 years of age, leaving only some diplotene oocytes stained for C-KIT. Nevertheless, they found that up to 2 years of age the medulla contained conglomerates of healthy and degenerating oogonia and small follicles, “waste baskets”, and oogonia enclosed in growing follicles. Also, medulla of older ovaries contained groups of healthy primordial follicles. They concluded that they did not find any evidence for the presence of oogonia in the human ovary during the first 2 years of life and suggested that perinatal medullary “waste baskets” and oogonia enclosed in the growing follicles gave rise to the groups of small, healthy follicles in the medulla. Similarly this was reconfirmed by another “genetic” approach. Liu et al. found that no early meiotic-specific or oogenesis-associated mRNAs for *SPO11*, *PRDM9*, *SCP1*, *TERT*, and *NOBOX* were detectable in adult human ovaries using RT-PCR, compared to fetal ovaries and adult testis controls [54]. It should be stressed that the folliculogenesis/oogenesis is usually estimated only in terms of the presence of oogonia and expression of oogonia/oocyte-specific markers (transcription factors) in ovarian tissue. Still, the follicles/oocytes could also develop from some earlier stages—putative stem cells—in different compartments of the ovary during the adult period of life. Several studies confirmed the development of oocyte/

follicle-like structures in vitro from putative ovarian stem cells [22–26], embryonic stem cells or induced pluripotent stem cells [55–57], and stem cells from amniotic fluid in humans [58].

There were some studies both in the animal models and in humans which showed that the possibility of neo-folliculogenesis/oogenesis in adult ovaries is not excluded. Johnson et al. treated the prepubertal female mice with the mitotic germ cell toxicant busulfan, which eliminated the primordial follicle reserve by early adulthood without inducing atresia. They demonstrated the cells expressing the meiotic entry marker synaptonemal complex protein 3 (SCP3) in juvenile and adult ovaries of the same mice. Additionally, wild-type ovaries grafted into the transgenic female mice with the expression of green fluorescent protein (GFP) became infiltrated with GFP-positive germ cells that form new follicles. They suggested the existence of proliferative germ cells—putative stem cells—that sustain oocyte and follicle production in the postnatal mammalian ovary [14]. Moreover, they found that bone marrow transplantation restored oocyte production in wild-type mice sterilized by chemotherapy and in ataxia telangiectasia-mutated gene-deficient mice, which are incapable of making oocytes. Their results confirmed bone marrow as a potential source of stem cell-derived germ cells that could additionally sustain the oocyte production in adult mouse ovaries [15]. Some other research groups also confirmed this finding [59, 60].

Based on some experimental evidence, Bukovsky et al. hypothesized that in adult human ovaries, mesenchymal cells in the *tunica albuginea* (TA) are bipotent progenitors with a commitment for both primitive granulosa and germ cells [61]. Using immunocytochemistry they showed that cytokeratin (CK)+ mesenchymal cells in ovarian TA can differentiate into surface epithelium (SE) cells by a mesenchymal–epithelial transition. Some segments of SE directly associated with ovarian cortex were overgrown by TA and formed solid epithelial cords; these cords fragmented into small epithelial nests descending into the lower ovarian cortex and then assembled with zona pellucida (ZP)+ oocytes. They observed that germ cells could originate from SE cells which covered the TA by asymmetric division. During vascular transport, the putative germ cells grew to the oocyte size and were picked up by epithelial nests associated with the vessels. It was further observed that some extensions of granulosa cells entered the oocyte cytoplasm during follicle formation. In the ovarian medulla some occasional vessels showed an accumulation of ZP+ oocytes with diameters of 25–30 μm or particles of degenerating oocytes. Their results indicated that the pool of primary follicles in adult human ovaries may not represent a static but a dynamic population of differentiating and regressing structures. De Felici proposed that a small number of primordial germ cells (PGCs)/oogonia or of PGC-derived undifferentiated cells with some stem cell characteristics could persist in the postnatal ovary and under certain conditions resume mitosis, enter meiosis, and give rise to oocytes [62]. Recently, White et al. found rare mitotically active cells purified from frozen-thawed ovarian cortical tissue of reproductive age women—germline stem cells—that showed a gene expression profile consistent with primitive germ cells [63]. These cells were successfully expanded in vitro for months and spontaneously generated 35–50 μm oocytes, confirmed by their morphology, gene expression, and haploid chromosomal status. Injection of the GFP-labeled human germline stem cells into human ovarian cortical biopsies led to the

formation of follicles containing GFP-positive oocytes 1–2 weeks after xenotransplantation into immunodeficient female mice. Additionally, different studies confirmed that telomerase, an enzyme complex that binds to the chromosome ends—telomeres—and maintains telomere length and integrity, is expressed in germ cells, proliferative granulosa cells, germline stem cells, and neoplastic cells in the adult ovary, but it is absent in differentiated or aged cells [64].

Some other studies indicated the stemness of follicular cells. The multipotent subpopulation of luteinizing granulosa cells was isolated from the follicular cells in the follicular fluid of infertile women retrieved by ultrasound-guided oocyte aspiration in the in vitro fertilization program [65]. These cells were successfully cultured over a prolonged period of time in the presence of leukemia-inhibitory factor (LIF). They expressed mesenchymal lineage markers (i.e., CD29, CD44, CD90, CD105, CD117, and CD166) and were differentiated in vitro into different cell types, such as neurons, chondrocytes, and osteoblasts. After their transplantation into immunodeficient (SCID) mice, these cells survived and generated in vivo tissues of mesenchymal origin. Furthermore, Honda et al. found that thecal stem cells appear to be present in neonatal mouse ovaries and can be isolated, purified, and induced to differentiate in vitro [66]. Until now thecal stem cells had not been confirmed in humans.

The principle of folliculogenesis/oogenesis de novo is still strongly criticized [67]. It can be generally summarized from the literature that more evidence is needed to sustain the notion of new formation of oocytes in the normal adult mammalian ovary [62, 68–73]. In any case, further research is needed to answer a difficult scientific question about potential folliculogenesis/oogenesis and to make a real conclusion in the future.

13 Ovarian Cortex Tissue, Stem Cells, and Regenerative Medicine

The work of our group shows that it is possible to establish long-term ovarian cell cultures after enzymatic degradation of ovarian tissue in women of different ages. These cell cultures constitute a relatively low proportion of cells expressing some markers of pluripotency/multipotency, the small, round cells with diameters of up to 4 μm and bigger mesenchymal-like cells, and express a degree of plasticity. We were able to differentiate these cells in vitro into somatic cells of all three germ layers: adipogenic and osteogenic cells (mesoderm), neural-like (ectoderm), and pancreatic-like cells (endoderm) releasing insulin when exposed to glucose (see Fig. 6). Further research is needed to better characterize the putative stem cells from

Fig. 6 (continued) (**d**) Nestin-positive neuronal-like cells. (**e**) β-tubulin III-positive neuronal-like cells. (**f**) S-100-positive neuronal-like cells. (**g**) Pancreatic-like cells (endoderm) positively stained on the expression of insulin. (**h**) Alkaline phosphatase-positive cluster of ovarian stem cells. *Scale bar* = 50 μm, except (**b**, **g**, **h**) 100 μm

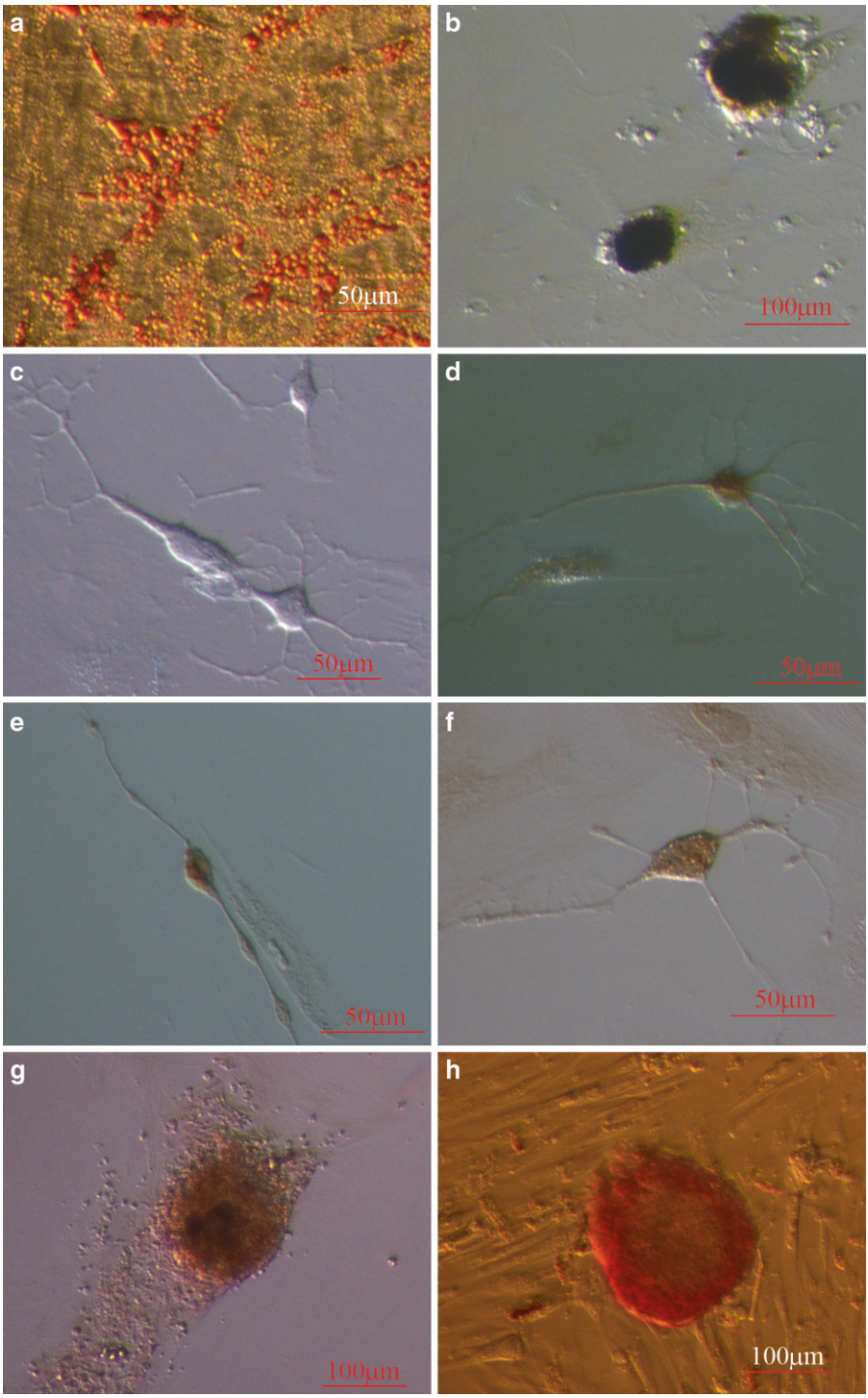

Fig. 6 *In vitro* differentiation of ovarian stem cells into cell types of all three germ layers. (**a**) Adipogenic cells, which released lipid droplets, stained with Oil Red O (mesoderm). (**b**) Osteogenic cells, confirmed by von Kossa staining (mesoderm). (**c**) Net of neuronal-like cells (ectoderm).

the ovarian cortex and the functionality of in vitro differentiated somatic cells. In spite of promising results, the human ovarian tissue represents a difficult available source of stem cells and might be used for a purpose of regenerative medicine in rare cases such as the removal of ovaries to prevent breast cancer (ovariectomy).

14 Conclusion

From all mentioned findings and quickly evolving new knowledge we can conclude that ovarian stem cells are very interesting from different aspects of human life: from the reproduction and manifestation of ovarian cancer to the potential use in regenerative medicine to autologously treat degenerative disease. Yet further research is needed to safely apply them toward creating new strategies of diagnostics and treatment in human medicine. Adult ovary stem cells demand the greatest attention and effort in further research.

References

1. Carr BR, Blackwell RE, Azziz R (eds) (2005) Essential reproductive medicine, 1st edn. McGraw-Hill, New York, p 778
2. Murdoch WJ, McDonnel AC (2002) Roles of the ovarian surface epithelium in ovulation and carcinogenesis. Reproduction 123:743–750
3. Bowen NJ, Walker LD, Matyunina LV, Logani S, Totten KA, Benigno BB, McDonald JF (2009) Gene expression profiling supports the hypothesis that human ovarian surface epithelia are multipotent and capable of serving as ovarian cancer initiating cells. BMC Med Genomics 2:71
4. Orford KW, Scadden DT (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet 9:115–128
5. Melchior K, Weiss J, Zaehres H, Kim YM, Lutzko C, Roosta N, Hescheler J, Muschen M (2008) The WNT receptor FZD7 contributes to self-renewal signaling of human embryonic stem cells. Biol Chem 389:897–903
6. Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441:1068–1074
7. Knoblich JA (2008) Mechanisms of asymmetric stem cell division. Cell 132:583–597
8. Amsterdam A, Raanan C, Schreiber L, Freyhan O, Schechtman L, Givol D (2013) Localization of the stem cells markers LGR5 and Nanog in the normal and the cancerous human ovary and their inter-relationship. Acta Histochem 115(4):330–338
9. Gamwell LF, Collins O, Vanderhyden BC (2012) The mouse ovarian surface epithelium contains a population of LY6A (SCA-1) expressing progenitor cells that are regulated by ovulation-associated factors. Biol Reprod 87:80
10. Usongo M, Farookhi R (2012) β-catenin/Tcf-signaling appears to establish the murine ovarian surface epithelium (OSE) and remains active in selected postnatal OSE cells. BMC Dev Biol 12:17
11. Szotek PP, Chang HL, Brennand K, Fujino A, Pieretti-Vanmarcke R, Lo Celso C, Dombkowski D, Preffer F, Cohen KS, Teixeira J, Donahoe PK (2008) Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics. Proc Natl Acad Sci U S A 105:12469–12473

12. Skaznik-Wikiel M, Tilly JC, Lee HJ, Niikura Y, Kaneko-Tarui T, Johnson J, Tilly JL (2007) Serious doubts over "Eggs forever?". Differentiation 75:93–99
13. Tilly JL, Telfer EE (2009) Purification of germline stem cells from adult mammalian ovaries: a step closer towards the control of the female biological clock? Mol Hum Reprod 15:393–398
14. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL (2004) Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428:145–150
15. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122:303–315
16. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J (2009) Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 11:631–636
17. Niikura Y, Niikura T, Tilly JL (2009) Aged mouse ovaries possess rare premeiotic germ cells that can generate oocytes following transplantation into a young host environment. Aging (Albany NY) 1:971–978
18. Pacchiarotti J, Maki C, Ramos T, Marh J, Howerton K, Wong J, Pham J, Anorve S, Chow YC, Izadyar F (2010) Differentiation potential of germ line stem cells derived from the postnatal mouse ovary. Differentiation 79:159–170
19. Gong SP, Lee ST, Lee EJ, Kim DY, Lee G, Chi SG, Ryu BK, Lee CH, YumKE LHJ, Han JY, Tilly JL, Lim JM (2010) Embryonic stem cell-like cells established by the culture of adult ovarian cells in mice. Fertil Steril 93:2594–2601
20. Bukovsky A, Svetlikova M, Caudle MR (2005) Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol 3:17
21. Bukovsky A, Caudle MR, Svetlikova M (2008) Steroid-mediated differentiation of neural/neuronal cells from epithelial ovarian precursors in vitro. Cell Cycle 7:3577–3583
22. Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P, Malicev E, Meden-Vrtovec H (2008) Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. Differentiation 76:843–856
23. Virant-Klun I, Rozman P, Cvjeticanin B, Vrtacnik-Bokal E, Novakovic S, Rülicke T, Dovc P, Meden-Vrtovec H (2009) Parthenogenetic embryo-like structures in the human ovarian surface epithelium cell culture in postmenopausal women with no naturally present follicles and oocytes. Stem Cells Dev 18:137–149
24. Virant-Klun I, Skutella T (2010) Stem cells in aged mammalian ovaries. Aging (Albany NY) 2:3–6
25. Virant-Klun I, Skutella T, Stimpfel M, Sinkovec J (2011) Ovarian surface epithelium in patients with severe ovarian infertility: a potential source of cells expressing markers of pluripotent/multipotent stem cells. J Biomed Biotechnol 2011:381928
26. Novak I, Lightfoot DA, Wang H, Eriksson A, Mahdy E, Höög C (2006) Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. Stem Cells 24:1931–1936
27. Nicholas CR, Haston KM, Grewall AK, Longacre TA, Reijo Pera RA (2009) Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility. Hum Mol Genet 18:4376–4389
28. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, Hinduja I (2011) Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. Stem Cells Dev 20:1451–1464
29. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869
30. Kucia M, Zuba-Surma E, Wysoczynski M, Dobrowolska H, Reca R, Ratajczak J, Ratajczak MZ (2006) Physiological and pathological consequences of identification of very small embryonic like (VSEL) stem cells in adult bone marrow. J Physiol Pharmacol 57(Suppl 5):5–18

31. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E, Czajka R, Wojakowski W, Machalinski B, Ratajczak MZ (2007) Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia 21:297–303
32. Shin DM, Liu R, Klich I, Wu W, Ratajczak J, Kucia M, Ratajczak MZ (2010) Molecular signature of adult bone marrow-purified very small embryonic-like stem cells supports their developmental epiblast/germ line origin. Leukemia 24:1450–1461
33. Shin DM, Zuba-Surma EK, Wu W, Ratajczak J, Wysoczynski M, Ratajczak MZ, Kucia M (2009) Novel epigenetic mechanisms that control pluripotency and quiescence of adult bone marrow-derived Oct4(+) very small embryonic-like stem cells. Leukemia 23:2042–2051
34. Mak VC, Siu MK, Wong OG, Chan KK, Ngan HY, Cheung AN (2012) Dysregulated stemness-related genes in gynecological malignancies. Histol Histopathol 27:1121–1130
35. Siu MK, Wong ES, Kong DS, Chan HY, Jiang L, Wong OG, Lam EW, Chan KK, Ngan HY, Le XF, Cheung AN (2013) Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers. Oncogene 32(30):3500–3509. doi:10.1038/onc.2012.363 [Epub ahead of print]
36. Lee M, Nam EJ, Kim SW, Kim S, Kim JH, Kim YT (2012) Prognostic impact of the cancer stem cell-related marker NANOG in ovarian serous carcinoma. Int J Gynecol Cancer 22:1489–1496
37. Scheitz CJ, Lee TS, McDermitt DJ, Tumbar T (2012) Defining a tissue stem cell-driven Runx1/Stat3 signalling axis in epithelial cancer. EMBO J 31(21):4124–4139
38. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
39. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 367:645–648
40. Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65:3025–3029
41. Hu Y, Fu L (2012) Targeting cancer stem cells: a new therapy to cure cancer patients. Am J Cancer Res 2:340–356
42. Morrison R, Schleicher SM, Sun Y, Niermann KJ, Kim S, Spratt DE, Chung CH, Lu B (2011) Targeting the mechanisms of resistance to chemotherapy and radiotherapy with the cancer stem cell hypothesis. J Oncol 2011:941876, Article ID 941876
43. Wang Z, Li Y, Kong D, Banerjee S, Ahmad A, Azmi AS, Ali S, Abbruzzese JL, Gallick GE, Sarkar FH (2009) Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. Cancer Res 69:2400–2407
44. Ahmed N, Abubaker K, Findlay J, Quinn M (2010) Epithelial mesenchymal transition and cancer stem cell-like phenotypes facilitate chemoresistance in recurrent ovarian cancer. Curr Cancer Drug Targets 10:268–278
45. Ahmed N, Thompson EW, Quinn MA (2007) Epithelial-mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. J Cell Physiol 213:581–588
46. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704–715
47. Min KJ, So KA, Ouh YT, Hong JH, Lee JK (2012) The effects of DNA methylation and epigenetic factors on the expression of CD133 in ovarian cancers. J Ovarian Res 5:28
48. Steffensen KD, Alvero AB, Yang Y, Waldstrøm M, Hui P, Holmberg JC, Silasi DA, Jakobsen A, Rutherford T, Mor G (2011) Prevalence of epithelial ovarian cancer stem cells correlates with recurrence in early-stage ovarian cancer. J Oncol 2011:620523

49. Latifi A, Luwor RB, Bilandzic M, Nazaretian S, Stenvers K, Pyman J, Zhu H, Thompson EW, Quinn MA, Findlay JK, Ahmed N (2012) Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One 7:e46858
50. Ricci F, Bernasconi S, Perego P, Ganzinelli M, Russo G, Bono F, Mangioni C, Fruscio R, Signorelli M, Broggini M, Damia G (2012) Ovarian carcinoma tumor-initiating cells have a mesenchymal phenotype. Cell Cycle 11:1966–1976
51. Virant-Klun I, Skutella T, Cvjeticanin B, Stimpfel M, Sinkovec J (2011) Serous papillary adenocarcinoma possibly related to the presence of primitive oocyte-like cells in the adult ovarian surface epithelium: a case report. J Ovarian Res 4:13
52. Mezencev R, Wang L, McDonald JF (2012) Identification of inhibitors of ovarian cancer stem-like cells by high-throughput screening. J Ovarian Res 5:30
53. Byskov AG, Høyer PE, Yding Andersen C, Kristensen SG, Jespersen A, Møllgård K (2011) No evidence for the presence of oogonia in the human ovary after their final clearance during the first two years of life. Hum Reprod 26:2129–2139
54. Liu Y, Wu C, Lyu Q, Yang D, Albertini DF, Keefe DL, Liu L (2007) Germline stem cells and neo-oogenesis in the adult human ovary. Dev Biol 306:112–120
55. Richards M, Fong CY, Bongso A (2010) Comparative evaluation of different in vitro systems that stimulate germ cell differentiation in human embryonic stem cells. Fertil Steril 93:986–994
56. West FD, Roche-Rios MI, Abraham S, Rao RR, Natrajan MS, Bacanamwo M, Stice SL (2010) KIT ligand and bone morphogenetic protein signaling enhances human embryonic stem cell to germ-like cell differentiation. Hum Reprod 25:168–178
57. Medrano JV, Ramathal C, Nguyen HN, Simon C, Reijo Pera RA (2012) Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. Stem Cells 30:441–451
58. Cheng X, Chen S, Yu X, Zheng P, Wang H (2012) BMP15 gene is activated during human amniotic fluid stem cell differentiation into oocyte-like cells. DNA Cell Biol 31:1198–1204
59. Eggan K, Jurga S, Gosden R, Min IM, Wagers AJ (2006) Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature 441:1109–1114
60. Lee HJ, Selesniemi K, Niikura Y, Niikura T, Klein R, Dombkowski DM, Tilly JL (2007) Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a pre-clinical mouse model of chemotherapy-induced premature ovarian failure 1. J Clin Oncol 25:3198–3204
61. Bukovsky A, Caudle MR, Svetlikova M, Upadhyaya NB (2004) Origin of germ cells and formation of new primary follicles in adult human ovaries. Reprod Biol Endocrinol 2:20
62. De Felici M (2010) Germ stem cells in the mammalian adult ovary: considerations by a fan of the primordial germ cells. Mol Hum Reprod 16:632–636
63. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL (2012) Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med 18:413–421
64. Liu JP, Li H (2010) Telomerase in the ovary. Reproduction 140:215–222
65. Kossowska-Tomaszczuk K, De Geyter C, De Geyter M, Martin I, Holzgreve W, Scherberich A, Zhang H (2009) The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. Stem Cells 27:210–219
66. Honda A, Hirose M, Hara K, Matoba S, Inoue K, Miki H, Hiura H, Kanatsu-Shinohara M, Kanai Y, Kono T, Shinohara T, Ogura A (2007) Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells. Proc Natl Acad Sci U S A 104:12389–12394
67. Notarianni E (2011) Reinterpretation of evidence advanced for neo-oogenesis in mammals, in terms of a finite oocyte reserve. J Ovarian Res 4:1–20
68. Gosden RG (2004) Germline stem cells in the postnatal ovary: is the ovary more like a testis? Hum Reprod Update 10:193–195

69. Greenfeld C, Flaws JA (2004) Renewed debate over postnatal oogenesis in the mammalian ovary. Bioessays 26:829–832
70. Telfer EE (2004) Germline stem cells in the postnatal mammalian ovary: a phenomenon of prosimian primates and mice? Reprod Biol Endocrinol 2:24
71. Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S, Tsafriri A (2005) On regenerating the ovary and generating controversy. Cell 122:821–822
72. Byskov AG, Faddy MJ, Lemmen JG, Andersen CY (2005) Eggs forever? Differentiation 73:438–446
73. Sottile V (2007) Bone marrow as a source of stem cells and germ cells? Perspectives for transplantation. Cell Tissue Res 328:1–5

Adult Prostate Stem Cells

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Abstract The susceptibility of the prostate to benign prostatic hyperplasia and prostate cancer has prompted research into normal prostate stem cells in order to reveal the underlying mechanisms of these diseases. Yet, it can be challenging to study prostate stem cells because the prostate is a slow cycling tissue. In this chapter, we compare the assays used to study prostate stem cells including castration, lineage tracing and tissue recombination and discuss how they may influence the phenotype of stem cells. We also review the location and characteristics of prostate stem cells. In particular, we focus on the evidence for basal and luminal stem cells, the role of intermediate cells as transit amplifying cells and the relationship of neuroendocrine cells to the other epithelial cell types.

Keywords Prostate • Stem cell • Castration • Lineage tracing • Tissue recombination

Abbreviations

2D, 3D	Two dimensional, three dimensional
ALDH	Aldehyde dehydrogenase
AR	Androgen receptor
BCL2	B-cell CLL/lymphoma 2
BMI1	BMI1 polycomb ring finger oncogene

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BrdU	Bromodeoxyuridine (5-bromo-2′-deoxyuridine)
CARN	Castrate-resistant NKX3.1-expressing cell
CD44, etc.	Cluster of differentiation 44, etc.
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27 Kip1)
CGA	Chromogranin A
COX	Cytochrome c oxidase
ERG	v-ets erythroblastosis virus E26 oncogene homolog
FGF10	Fibroblast growth factor 10
FOXA2	Forkhead box a2
GSTP1	Glutathione S-transferase pi 1
HMWK	High-molecular weight keratin
HMGA2	High-mobility group AT-hook 2
K8, etc.	Keratin 8, etc.
Ki67	Antigen identified by monoclonal antibody Ki-67
Lin	Lineage expression of haematopoietic markers (CD31, CD45, Ter119)
LSC	$Lin^{-}Sca\text{-}1^{+}CD49f^{+}$
LSCT	$Lin^{-}Sca\text{-}1^{+}CD49f^{hi}Trop2^{hi}$
NKX3.1	NK3 homeobox 1
p63	Tumour protein p63 TP63
PCNA	Proliferating cell nuclear antigen
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
Sca-1	Stem cell antigen-1
UGE	Urogenital epithelium
UGM	Urogenital mesenchyme
UGS	Urogenital sinus

1 Introduction

Stem cells are a dynamic and exciting area of prostate research and there has been rapid progress in determining their properties and identity. Changes in the notion of what defines tissue stem cells are unifying what once seemed to be conflicting results, although there is still no complete consensus. It was previously assumed that prostate stem cells would be a single, constant and immutable pool of cells. This has evolved to a more nuanced view where there are multiple populations of prostate stem/progenitor cells, the fate of which is determined by context, including the developmental stage and type of tissue regeneration. This shift in thinking has been driven by the increasingly sophisticated methods for studying prostate stem cells. Early studies relied on morphological and immunohistochemical analyses, but it is now possible to isolate, label and grow putative stem cells to study more specific populations of cells in much greater detail.

Two ongoing challenges for the field are to define the identity of prostate stem cells and to determine their properties in different contexts. This chapter will examine

the identity of different subsets of prostate stem/progenitor cells by separately focussing on basal, luminal, intermediate and neuroendocrine cells. The functional assays for studying prostate stem cells will also be compared since these models determine the context and, potentially, the characteristics of prostate stem cells.

2 Background: Cellular Organisation of the Prostate

The adult prostate is a system of branching epithelial ducts and glands surrounded by fibromuscular stroma. It produces prostatic fluids that are secreted into the urethra to become a major component of seminal fluid, which aids the survival of sperm [1]. The gross morphology of the human prostate has some key differences from the mouse and rat prostates, which are commonly used to study stem cell biology. The human prostate has three distinct zones, which develop from different segments of the prostatic urethra and are positioned in different anatomical planes of the adult organ [2]. The peripheral zone contains the majority (70 %) of the glandular epithelium, while the central zone extends anterior to the prostatic urethra to surround the ejaculatory ducts and the smallest component, the transition zone [2]. In contrast, the mouse and rat prostates contain four pairs of lobes; the anterior, dorsal, lateral and ventral prostate, named after their anatomical positions in the reproductive tract [3, 4]. The dorsal–lateral lobes are believed to most closely resemble the main peripheral zone of the human prostate, but there are still inevitable differences [3]. Nonetheless, the specific cell types comprising the epithelial compartment of the rodent and human prostate share close homology and will be the main focus in terms of the identity of prostatic stem cells.

Apart from the stroma, the adult prostate consists of four epithelial cell types: luminal, basal, intermediate and neuroendocrine cells. The arrangement of each cell type is shown in Fig. 1. Ducts and glands are lined by a layer of tall columnar luminal cells that secrete kallikrein proteases, including prostate-specific antigen (PSA) and prostatic acid phosphatase into seminal plasma [5]. Luminal cells are separated from the basement membrane by basal cells with cuboid or flattened morphology that form a continuous layer in humans, but a discontinuous layer in rodents [6]. Apart from their morphology, luminal and basal cells can be distinguished by the expression of several markers. Luminal cells typically express K8, K18, high levels of AR and, in humans, PSA [7–9]. Basal cells are marked by high-molecular weight keratins, K5 and K14, p63, CD44 and low levels of AR and K18 [7–11]. Intermediate cells are a third, rare cell type present in either the luminal or basal layer. They are identified based on their keratin profile rather than morphology, expressing K5 but not K14, or a combination of both luminal and basal keratins ($K8^+$, $K18^+$, $K5^+$ $K14^{+/-}$) [7–9, 12]. K19 has also been proposed as a marker of intermediate cells [9]. A fourth cell type, neuroendocrine cells, are scattered as rare, single cells extending from the basal layer. They have either “closed” morphology, with processes that only extend to surrounding cells, or “open” morphology, with dendrites that also project towards the glandular lumen [13]. In addition to their morphological

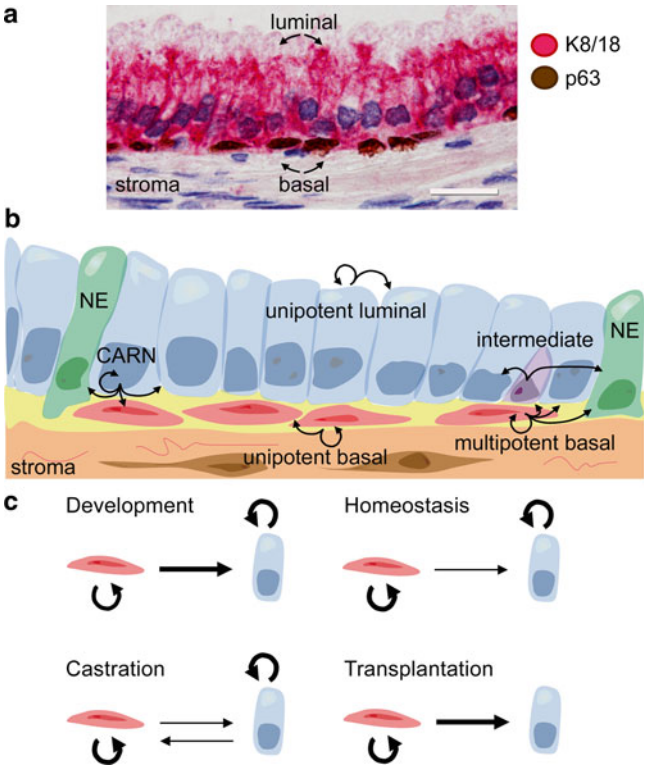

Fig. 1 Epithelial stem cells in prostate tissue. (**a**) Adult human prostate tissue with luminal cells stained with keratin 8/18 (K8/18; *red*) and basal cells stained with p63 (*brown*). *Scale bar* equals 20 μm. (**b**) Schematic representation of adult prostate tissue showing the ability of luminal and basal progenitors to differentiate into each cell type. Luminal cells are shown in *blue*, basal cells in *red*, intermediate cells in *purple* and neuroendocrine (NE) cells in *green*. CARN denotes castrate-resistant NKX3.1-expressing cells. (**c**) Schematic representation of the ability of basal and luminal progenitors to differentiate under different conditions. *Thick arrows* represent common and *thin arrows* rare events, respectively. Note that except for CARNs, the ability of luminal cells to differentiate in transplantation assays is unknown

heterogeneity, prostatic neuroendocrine cells secrete different combinations of neuropeptides, although they usually express chromogranin A (CGA), neuron-specific enolase and serotonin [13, 14].

3 From Prostate Development to Differentiation

Development of the prostate from small, homogenous clusters of epithelial cells into a complex tree-like network of stratified ducts and glands involves a process of epithelial cell lineage differentiation and may, therefore, provide insight into the possible hierarchical relationships between each prostate cell type in the mature

organ. In the tenth week of human gestation, or embryonic day 17.5 in the mouse, prostate development is initiated by a pulse of testicular androgens acting on the mesodermal urogenital mesenchyme (UGM) [15, 16]. This stimulates the endodermal urogenital epithelial (UGE) to form solid buds that elongate into the surrounding UGM and express markers of the prostate lineage, NKX3.1 and FOXA2 [17, 18]. Inductive cues from the UGM then promote the UGE to undergo branching morphogenesis around the third trimester in humans, but predominantly postnatally in mice [19, 20]. This is accompanied by canalisation of the solid cords of cells into pseudo-stratified epithelium, beginning in the proximal region near the urethra and extending to the branching distal tips. The prostate then remains dormant until the sharp increase in androgens at puberty induces proliferation and maturation of the tracts of epithelial cells into functional ducts and luminal glands.

The increasingly complex architecture of the developing prostate is matched by progressive changes in the cell types that comprise the epithelium. Prior to the formation of prostatic buds, the UGE has an intermediate phenotype, co-expressing luminal (K8, K18), basal (HMWK, p63, GSTP1) and intermediate (K19) markers [12]. As the epithelium buds and elongates, the solid cords of cells have uniform undifferentiated morphology. The cells all maintain an intermediate phenotype, based on the expression of luminal markers and reactivity with the pan-HMWK antibody, 34βE12, which detects K1, 5, 10 and 14 and labels all adult basal cells [12, 21, 22]. Staining with specific K5 and K14 antibodies, however, reveals a more sporadic expression pattern than with the pan-keratin antibody, suggesting that primitive prostate epithelium is more heterogeneous than first appreciated [21]. The discrepancy between staining patterns might also be due to developmental expression of K1 and K10, which are expressed in basal metaplasia in adult tissue [23, 24]. Nevertheless, as the epithelium canalises, more distinct cell types become evident [12, 25–27]. Luminally located cells lining acini and an intermediate layer of cells below them begin to lose basal cell markers (p63, HMWK), but still express high levels of luminal keratins [12, 21, 26, 28]. The underlying basal cells maintain basal markers and decreasing levels of luminal keratins [12, 26]. Yet, most cells still express K19, and the basal marker, GSTP1, indicating that basal and luminal differentiation are not completed until puberty [12, 21, 26].

Different conclusions have been drawn about the identity of adult prostate stem cells based on the phenotype of developing epithelium. It has been suggested that the multitude of intermediate cells in development represent transit amplifying cells derived from basal stem cells and poised to differentiate into luminal cells [27]. Alternatively, it has been argued that the prevalence of intermediate cells in development implies that the rare intermediate cells in adult tissue are multipotent stem cells that persist from early development [12]. Finally, based on heterogeneous expression of K5 and K14, but consistent K8 expression, it was proposed that prostate stem cells have a simple phenotype consisting of the luminal marker K8 alone [21]. These three different hypotheses typify the debate about the identity of prostate stem cells. Similar datasets can be interpreted in different ways to suggest that prostate stem cells have either a basal, intermediate or luminal phenotype. Indeed, it is also possible that embryonic and adult prostate stem cells have quite different properties. With growing understanding that the characteristics of stem

cells can vary depending on the context, be it developmental stage or stem cell assay, and ever more sophisticated methods to study stem cells, previous data are being reconsidered and retested. In the following sections, we summarise the methods used to characterise prostate stem cells. We also focus on each cell type within the prostate and examine whether, and under what circumstances, they function as prostate stem cells.

4 Stem Cells in Context: Functional Prostate Stem Cell Assays

4.1 Tracking Cells During Homeostasis

A range of assays has been used to study prostate stem cells; each has advantages and disadvantages and it is possible that the characteristics of prostate stem cells are influenced by the experimental conditions. It is difficult to study prostate stem cell activity in normal homeostasis because the adult prostate is growth quiescent; the average lifespan of adult human epithelial cells is estimated to be more than 2 years [29]. Early studies used immunohistochemistry to examine the expression of markers, like keratins, in fixed tissue specimens. Hierarchical relationships between cell types were inferred based on the co-expression of multiple lineage markers. More recently, groups of clonally related cells sharing mutations in the mitochondrial cytochrome c oxidase (COX) gene have been identified in human tissues based on the loss of COX enzyme activity [30, 31]. These groups of cells are thought to arise from a single mutated progenitor that has slowly divided over time. Therefore, by examining the phenotype of the clones, it is possible to retrospectively trace the long-term characteristics of the progenitor in normal homeostasis and deduce whether it was unipotent or multipotent.

Lineage tracing in transgenic mice has been used as a more dynamic way to follow the fate of cells without risking changes to their normal cellular hierarchy due to disruption of the tissue. This technique indelibly labels cells and their progeny when a CRE recombinase expressed under the control of a cell lineage-specific promoter is used to activate a reporter gene that is otherwise repressed by a stop cassette [32]. Keratins are often used for lineage-specific promoters of CRE recombinase. An important advantage of transgenic lineage tracing over clonal COX mutation analysis is that the phenotype of the original labelled cell is known. By comparing the number of labelled cells over time, it is also possible to infer whether or not a subset of cells has the ability to self-renew. A constant ratio of labelled to unlabelled cells implies that self-renewing cells sustain the lineage-marked population. However, unlike rapidly proliferating organs such as the digestive tract and skin, only a limited number of studies have conducted lineage tracing in the prostate under normal physiological conditions [28, 33]. Instead, it has usually been combined with castration–regeneration assays where there is greater turnover of cells.

4.2 Castration–Regeneration Assay

The castration–regeneration assay overcomes the problems associated with the slowly cycling prostate. Normal prostate homeostasis relies on systemic levels of testosterone. When male rodents are castrated, the prostate quickly regresses and the bulk of epithelial cells undergo apoptosis. The remaining growth-quiescent cells survive indefinitely in the castrate milieu and can rapidly proliferate to restore the normal prostate if testosterone is re-administered [34–36]. Remarkably, this regenerative capacity is retained for multiple cycles of castration and testosterone replacement [37, 38]. This technique provided some of the first evidence for the existence of stem cells in the adult prostate. It is now commonly used to enrich putative stem cells, since castration eliminates the bulk of the epithelium. The assay is also used to accelerate the turnover of cells and to study a phase of rapid epithelial regeneration. Multiple cycles of castration and regeneration can also be used to test for self-renewal [39].

4.3 Cell Culture and Transplantation Assays

Another common way to study prostatic stem cells is to isolate subsets of epithelial cells based on cell surface markers and then test their ability to proliferate and differentiate in vitro or in vivo. In vitro methods include colony formation assays under standard cell culture conditions, with or without a feeder layer of fibroblasts [40–42], and three-dimensional spheroid assays where progenitors form clonal colonies in Matrigel [43]. These colonies can be disaggregated and regrown over multiple cycles to assess the ability of cells to undergo self-renewal [43, 44]. Further evidence of stem cell properties can be obtained from in vivo assays, where cells are transplanted into mice to test their ability to regenerate prostatic glands with the full repertoire of cell types. However, this in vivo approach has proved troublesome in the prostate field, since isolated prostatic epithelial cells exhibit limited survival when grafted alone into mice. This hurdle has been overcome by combining the stem cell regeneration assay with tissue recombination. This method, which was pioneered in the prostate field by the Cunha laboratory, involves the isolation and recombination of different stromal and epithelial components to study their interactions when grafted together in vivo [45]. Putative prostate stem cells are typically recombined with UGM rather than adult prostatic stroma, because it provides a more supportive niche for the survival, proliferation and differentiation of prostate epithelial cells [11, 39, 42, 46–51]. However, whether inductive mesenchyme stimulates regeneration that accurately replicates normal adult homeostasis has been questioned [28, 33, 52].

5 Localisation of Prostate Stem Cells

The localisation of adult prostate stem cells mirrors the proximal to distal pattern of prostate development with slow cycling stem cells in the proximal region and actively proliferating, possibly transit amplifying, cells towards the distal tips [38]. However, clonal analysis of COX mutations in human prostate tissue identified evidence of multipotent cells throughout the gland, suggesting that stem cells may be concentrated, but not exclusively located in the proximal region [31]. In rodents, the proximal region close to the urethra has the greatest telomerase activity and the highest expression of stem cell markers, including Sca-1, CD49f, BCL2, CD166 and BMI1 [42, 44, 53–55]. Functionally, the proximal region also has the greatest regenerative activity in colony forming assays in vitro and in tissue recombination assays in vivo [38, 56]. Most label-retaining cells in serial castration–regeneration experiments are also in the proximal region, demonstrating that it harbours a population of castrate-resistant repopulating cells. In intact proximal prostate tissue, putative stem cells marked by BMI1 or $Lin^{-}/CD49f^{+}/Sca1^{+}$ (LSC) are predominantly $K5^{+}$ basal cells [42, 44]. Yet, there are both basal and luminal label-retaining cells after castration [38]. This suggests that the proximal region, where the majority of prostate stem cells are localised, may have different populations of stem cells.

6 Prostate Stem Cell Identities

6.1 Basal Cells

6.1.1 Basal Cells in Homeostasis

It has long been suspected that basal cells contain a subpopulation of stem cells; indeed, they were once referred to as reserve, generative or precursor cells [35]. However, there are differing views about whether adult basal cells are a self-sustaining population of unipotent cells or are multipotent progenitors that can differentiate into luminal and neuroendocrine cells (Fig. 1b). Nevertheless, there is evidence that basal cells have an important role in the turnover of cells in normal homeostasis. Immunohistochemistry for Ki-67 and PCNA shows that most, although not all, proliferative cells in adult human prostate tissue are basal [9, 57, 58]. These proliferative cells may be amplifying cells arising from a subset of quiescent basal cells. This is reinforced by the differential expression of CDKN1B ($p27^{Kip1}$), a cell cycle inhibitor, amongst basal cells; there are proliferative CDKN1B negative, and quiescent, CDKN1B positive basal cells [59]. The long-term survival of basal stem cells may be supported by the anti-apoptotic protein, BCL2, and telomerase, both of which are only expressed in basal cells in benign prostate epithelium [60–62]. Immunohistochemistry studies have not only suggested that basal cells have high proliferative potential but also that they may be capable of undergoing multilineage

differentiation. Rare cells that co-express basal and luminal ($HMWK^+$, PSA^+) or basal and neuroendocrine ($HMWK^+$, CGA^+) markers have been regarded as intermediate or transitional cells arising from basal progenitors and undergoing luminal or neuroendocrine differentiation [8, 63]. The benefit of these studies is that they do not involve manipulation of the tissue and preserve normal cellular features and relationships; however, the results are mostly correlative and fail to provide functional information about the ability of cells to differentiate.

The development of methods for clonal analyses and lineage tracing has enabled more rigorous examination of the relationships between different cell types in normal, unmanipulated tissues. Small groups of cells with COX mutations have been identified in adult human prostate tissue [30, 31]. Some clusters only contain basal cells, suggesting that they arose from unipotent basal progenitors. Other clusters contain basal, luminal and neuroendocrine cells, supporting the existence of multipotent prostate progenitors, as inferred from earlier studies that detected transitional cells with immunohistochemistry.

Lineage tracing of basal cells in mice produced similar results to clonal analyses of human tissue [28, 33]. $K5^+$ and $K14^+$ basal cells tagged in newborn mice, when branching morphogenesis is still underway, expanded and underwent multilineage differentiation into basal, luminal, intermediate and neuroendocrine cells as the prostate matured [28]. Clonal analysis of small clusters of labelled cells revealed different types of basal progenitors in the developing prostate. Luminal-committed progenitors only produced luminal cells, while bipotent progenitors generated clones with luminal, basal and occasional intermediate cells. Clusters that only contained basal cells may have arisen from unipotent basal progenitors or bipotent progenitors that, by chance, only underwent symmetric cell division to produce more basal cells and not asymmetric cell division to produce luminal cells. Compared to the developing prostate, basal to luminal differentiation is less common in the adult prostate according to K5 lineage tracing experiments [33]. Over 10 months, $K5^+$ basal cells produced mostly basal cells and a small, but increasing, subpopulation of luminal cells. This suggests that either the adult prostate has rare multipotent basal progenitors and more common unipotent basal cells or that basal cells have the stochastic ability to occasionally differentiate into luminal cells if required. Collectively, these studies have refined previous theories about the role of basal stem cells in homeostasis, indicating that there are subpopulations of unipotent and multipotent basal progenitors (Fig. 1b).

6.1.2 Basal Cells in Castration

Experiments that accelerate the turnover of cells by castration and testosterone replacement provide further evidence for basal stem cells. Castration induces apoptosis in the majority of luminal cells, but not basal cells, leading to an increase in the ratio of basal to luminal cells [35]. Given that castration and testosterone replacement can be repeated over multiple cycles, it has been suggested that basal cells contain a subpopulation of castrate-resistant multipotent stem cells that are capable

of regenerating the entire epithelium. Indeed, after re-administration of testosterone, a greater percentage of basal compared to luminal cells are actively proliferating [35]. An important caveat however, is that luminal cells outnumber basal cells, even after castration, so a greater number proliferating cells are actually luminal cells [35]. Furthermore, basal and luminal cells simultaneously begin to proliferate after testosterone re-administration. These observations led to the alternative hypothesis that basal and luminal cells are separate self-replicating populations, rather than basal cells being solely responsible for regenerating the epithelium. Lineage tracing has recently been used to help resolve this uncertainty [33, 64]. When $K14^+$ basal cells were followed for two cycles of castration and testosterone replacement, only basal progeny were observed, confirming that basal cells are indeed predominantly unipotent in this context [64]. However, when $K5^+$ basal cells were followed for five cycles, a small but increasing number of labelled luminal cells were detected [33]. These studies demonstrate that there is a population of regenerative, castrate-resistant basal cells in the adult prostate, but that multilineage differentiation is rare as is the case for tissue homeostasis.

6.1.3 Basal Cells in Culture and Transplantation

Basal cells have remarkable ability to proliferate and differentiate after isolation from prostate tissue. Most epithelial cells fail to grow when digested prostate tissue is transferred to in vitro culture. However, a fraction of basal cells do attach and establish primary cultures before rapidly differentiating into intermediate cells [7, 40, 65–68]. They can further differentiate towards a luminal phenotype with extended culture [7, 22]. Primary basal cells also form spheroids in Matrigel [33, 42, 43, 48]. The spheroids are clonal and can be digested and regrown for several generations, suggesting that they are sustained by a subpopulation of cells with the ability self-renew. When primary cells or spheroids are recombined with UGM and grown in vivo, they form glandular structures with basal and luminal cells [43, 69–71]. This demonstrates that primary cell culture selects a subset of multipotent basal cells with repopulating activity.

Cell sorting for different combinations of cell surface markers is a more direct approach to selectively enrich putative basal progenitors from freshly dissociated prostate tissue. One method is to select $\alpha_2\beta_1$ $integrin^{hi}$ cells based on rapid attachment to collagen I, followed by further enrichment using the AC133 antibody against glycosylated CD133 [41]. Without prior enrichment of $\alpha_2\beta_1$ $integrin^{hi}$ basal cells, CD133 sorting is unsuccessful, possibly because $AC133^+$ cells are extremely rare and even sometimes undetectable [41, 72, 73]. A second method to enrich basal progenitors from dissociated prostate tissue is to deplete haematopoietic cells (Lin^+; $CD31^+CD45^+Ter119^+$) and then select the subset of $CD49f^+Trop2^{hi}$ basal cells [48]. Sca-1 can be used to further enrich mouse basal progenitors as part of the $Lin^-Sca\text{-}1^+CD49f^{hi}Trop2^{hi}$ (LSCT) combination of markers [42, 48]. There is no human orthologue of Sca-1; however, CD166 can be used to further select repopulating basal cells from both the human $CD49f^+Trop2^{hi}$ and mouse LSCT populations [55].

A third method for sorting repopulating cells is to select Lin$^-$Sca-1$^+$CD133$^+$CD44$^+$CD117$^+$ cells [47]. Notably, CD117 is expressed by both basal and luminal cells in mice, but only by rare p63+ basal cells in humans [47]. Other protocols that purify potential stem cells based on functional characteristics, rather than cell surface antigens, including assays for Hoechst efflux and high ALDH activity, also enrich prostate basal cells [74, 75]. Therefore, selecting basal cells with cell sorting is a useful strategy to enrich regenerative cells from fresh prostate tissue.

It is unclear whether the different methods of cell sorting enrich distinct or overlapping populations of basal progenitors. Nevertheless, all methods enhance in vitro or in vivo repopulating activity. For example, human $\alpha_2\beta_1{}^{hi}$CD133$^+$ cells form more colonies than unselected basal cells in 2D assays and differentiate into basal and luminal cells in vivo when they are xenografted with cultured stroma [41]. Human CD49f^{hi}Trop2^{hi} and mouse LSCT cells also have greater ability to form 2D colonies and 3D spheroids than unsorted cells, which is further increased by sorting for CD166 [48, 55, 69]. In tissue recombination experiments with mouse UGM, human CD49f^{hi}Trop2^{hi} cells form clonal glands containing luminal and basal cells [11]. Mouse Sca-1$^+$, Lin$^-$Sca-1$^+$CD49f^{hi} and LSCT subpopulations repopulate clonal glands with increasing efficiency in recombination assays [33, 42, 48, 54, 76]. All subpopulations also undergo multilineage differentiation, in particular LSCT cells, which have been shown to produce neuroendocrine cells. Collectively, these studies demonstrate that a subset of basal cells has the capacity to proliferate extensively and repopulate fully differentiated epithelium after being isolated from adult prostate tissue.

6.1.4 Basal Cells as a Cell of Origin for Prostate Cancer

Basal cells can be a cell of origin for prostate cancer providing further insight into their hierarchical relationship with luminal and intermediate cells. Basal cells are typically lost in prostate cancer, except those surrounding intraductal carcinoma lesions, and rare p63$^+$ cells in high-grade tumours [77, 78]. It was once assumed that the absence of p63$^+$ in adenocarcinomas meant that basal cells could not initiate prostate cancer, but studies using new methods to isolate, tag and transplant basal cells haven proven otherwise. Benign human Trop2^{hi}CD49f^{hi} basal cells form tumours when they are transduced with ERG, AR and activated Akt expression vectors and grafted in tissue recombination experiments [11]. Similarly, mouse LSC^{hi} cells transduced with AR and activated Akt also form tumours when they are recombined with UGM [46], as do LSC^{hi} and LSC^{hi}CD166^{hi} basal cells from PTEN null mice [49, 55]. Basal cells are also susceptible to stromal-induced carcinogenesis when they are recombined with human cancer-associated fibroblasts or mouse UGM that over-expresses FGF10 or HMGA2, an epigenetic regulator [46, 79, 80]. Regardless of whether or not tumourigenesis is initiated by direct perturbation of basal cells or via the stroma, the resulting tumours in these recombination experiments are predominantly composed of luminal cells, with some intermediate cells also detected. This mimics the phenotype of human prostate cancer and indicates that adult basal cells are capable of differentiating into intermediate and luminal

cells when autocrine or paracrine signalling becomes aberrant. Indeed, lineage tracing experiments, without transplantation, indicate that the differentiation of basal cells is a rate-limiting step in carcinogenesis. When PTEN is deleted in $K5^+$ or $K14^+$ basal cells, tumour progression occurs very slowly compared to PTEN loss in $NKX3.1^+$ or $K8^+$ luminal cells and is always preceded by the appearance of atypical luminal cells [33, 64]. This confirms that initiated basal cells can differentiate into luminal cells. It also implies that tissue recombination accelerates this process, because tumours develop more rapidly.

6.1.5 Summary of Basal Cells and Stem Cells

The combined results from lineage tracing, castration–regeneration, cell culture and recombination studies demonstrate that the basal compartment contains a population of stem cells. Yet, the ability of basal cells to undergo multilineage differentiation varies depending on the context (Fig. 1c). During development, most basal cells produce luminal daughter cells, although there is also a subset of unipotent basal cells [28]. In contrast, adult basal cells rarely undergo multilineage differentiation in homeostasis or castration–regeneration assays, but readily differentiate in cell culture and tissue recombination experiments. A possible explanation for these discrepancies is that once the prostate matures, separate populations of unipotent and multipotent basal progenitors are activated under different circumstances. Alternatively, a single population of basal stem cells may exhibit different properties in different situations. A further complication, which will be discussed in the following sections of this chapter, is that not all prostate stem cells are necessarily basal cells. Nevertheless, the two opposing theories that the basal compartment contains either multipotent or unipotent progenitors have both now essentially been confirmed.

6.2 Luminal Cells

Until recently, prostatic stem cells have most commonly thought to be basal cells, but there has been lingering suspicion that the luminal compartment may also contain progenitors [34, 35, 81]. It has been difficult to test this hypothesis, because luminal cells exhibit poor survival in culture or when transplanted as isolated cells into hosts [82]. Yet, as the prostate stem cell field has moved towards other approaches to study stem cell activity, there has been increasing evidence that the luminal compartment also contains a population of stem cells.

6.2.1 Luminal Cells in Development

Some of the first evidence that progenitor activity may not be restricted to the basal compartment arose from studies of prostate development. It was noted that a greater proportion of luminal cells compared with basal cells were actively proliferating in

the early postnatal development of the prostate in rats [81]. This observation challenged the established idea that all luminal cells are post-mitotic cells arising from basal stem cells; it suggested that the luminal compartment may contain a subset of progenitors in its own right. This is supported by more recent studies of prostate development in transgenic mice. Lineage tracing of $K8^+$ and $K18^+$ cells during postnatal prostate development shows that they sustain a constant number of luminal cells, which is indicative of unipotent luminal progenitors [28]. Occasional $K5^+$ cells were also detected with $K8^+$ lineage tracing, perhaps from labelled intermediate cells, which also express K8. Luminal cells may also have a facultative ability to be self-sustaining in early prostate development. The prostate normally develops from $p63^+$ cells, but when p63 null UGS is transplanted under the kidney capsule of host mice, luminal and not basal cells arise in the grafts [83, 84]. Altogether, these studies suggest that the rapid expansion of cells in prostate development does not conform to a rigid hierarchy of basal to luminal differentiation, but that unipotent luminal progenitor cells co-exist with a variety of basal progenitors.

6.2.2 Luminal Cells in Castration

The castration and testosterone restoration model has provided substantial evidence that luminal progenitor cells also exist in the adult prostate. Initial studies showed that luminal cells proliferate extensively to help regenerate the prostatic epithelium when testosterone is re-administered after castration [34, 35]. A subset of quiescent, castrate-resistant luminal cells are also able to persist after multiple cycles of castration and regeneration according to BrdU labelling experiments [38]. Lineage tracing studies suggest that most of these cells are unipotent luminal progenitors. When PSA^+ or $K8^+$ luminal cells are tagged in intact mice, they only produce further luminal cells after multiple rounds of castration and testosterone restoration [64, 85]. However, a rare subpopulation of multipotent luminal stem cells has also been identified. In castrated mice, lineage tracing with the prostate-specific homeobox gene, Nkx3-1, labels CARNs (castrate-resistant NKX3.1-expressing cells) [39]. CARNs persist in a castrate milieu, are strictly luminal, and can give rise to basal, intermediate and neuroendocrine cells upon testosterone re-administration [39]. They also undergo self-renewal and can regenerate epithelium in tissue recombination assays, which was surprising given that luminal cells have notoriously low survival in transplant assays. Whether or not CARNs exist in intact tissue, or only after castration, has not been determined. Nevertheless, it is now clear that when testosterone is restored after castration, the luminal layer is regenerated by unipotent luminal progenitors and smaller populations of CARNs and multipotent basal cells.

6.3 Intermediate Cells as a Transit Amplifying Population

Intermediate cells are poorly characterised compared with basal and luminal cells. This is due to their scarcity in adult prostate tissue and the lack of a single marker

to distinguish them from basal and luminal cells, although K19 and prostate stem cell antigen (PSCA) may preferentially label intermediate cells [9]. There are two theories about where intermediate cells lie in the lineage hierarchy of prostate cells. One hypothesis is that adult intermediate cells are a small subpopulation of multipotent stem cells that persist from embryonic development when intermediate cells constitute the entire prostate epithelium [12]. Currently, there is no experimental evidence for this proposal, because there are no methods to specifically tag or isolate intermediate cells. The other theory is that intermediate cells represent transit amplifying or proliferating cells transitioning from multipotent basal stem cells to terminally differentiated luminal cells [66, 86–88]. In this case, intermediate cells represent a spectrum of cells that are gradually losing basal markers and gaining luminal markers. Intermediate cells may also differentiate into neuroendocrine cells, which have a similar keratin profile [89]. As transit amplifying cells, intermediate cells are also predicted to be highly proliferative, but have less capacity for self-renewal compared to basal stem cells [66].

Intermediate cells are rare in adult prostate tissue, but their increased abundance in actively growing prostate epithelium supports their role as transit amplifying cells. Lineage tracing experiments show that intermediate cells arise from $K14^+$ basal cells during postnatal development of the mouse prostate [28]. Intermediate cells were only observed in multipotent and not unipotent clones, confirming that they represent a transition between basal and luminal differentiation. Yet, intermediate cells were not detected in 75 % of multipotent clones, so it is possible that they are only transiently present before differentiating into luminal cells. This is supported by castration experiments in rats, where intermediate cells are abundant during the proliferative phase after testosterone is restored, but are scarce once regeneration is complete [90]. An alternative explanation for the relative scarcity of intermediate cells is that basal cells can bypass the intermediate phenotype and directly differentiate into luminal cells under some circumstances [33]. Intermediate cells are more stable in primary cell culture; they overtake basal cells after a few passages, confirming that intermediate cells are highly proliferative and are likely to arise from basal cells. Altogether, these experiments in development, regeneration and cell culture support the model where intermediate cells are proliferative transit amplifying cells derived from basal stem cells; however, the alternative hypothesis that intermediate cells are a subpopulation of multipotent stem cells in their own right cannot be eliminated.

6.4 Neuroendocrine Cells

Neuroendocrine cells are the least studied population of cells in the prostate epithelium. They are considered to be non-proliferating, fully differentiated cells; however, there has been some disagreement about the lineage from which they are derived. It was proposed that neuroendocrine cells comprise their own lineage, originating from neural crest ectoderm, because CGA positive cells migrate into the

UGS prior to glandular budding and differentiation of the developing human prostate [91, 92]. In contrast, it has also been suggested that neuroendocrine cells are endodermal, similar to the gastrointestinal tract, and share a common stem cell with basal and luminal cells [93]. Initially, this was based on the discovery of cells co-expressing CGA and either luminal or basal cell markers [63], which were thought to be possible intermediaries between a prostatic stem cell and the fully differentiated neuroendocrine population [93]. More recent studies provided functional evidence for this hypothesis. For example, clonal analysis of human prostate tissue identified neuroendocrine cells sharing COX mutations with adjacent basal and luminal cells that, therefore, must share a common multipotent progenitor [31]. This progenitor can be a basal cell; lineage tracing experiments show that $K5^+$ and $K14^+$ basal cells produce neuroendocrine cells in postnatal prostate development [28]. Similarly, in tissue recombination assays, $K5^+$ basal cells and the stem-enriched $CD117^+$ and LSCT populations all regenerate glands containing neuroendocrine cells [33, 47, 48]. Yet neuroendocrine cells may not exclusively arise from basal cells since luminal CARNs produce neuroendocrine cells in both lineage tracing and single cell transplantation assays [39]. These functional studies collectively suggest that neuroendocrine cells belong to the same lineage as other prostatic epithelial cells, although it is still possible that a subset of neuroendocrine cells is of neurogenic origin, especially in development.

7 Summary and Future Directions

There is emerging consensus that the prostate contains multiple populations of epithelial progenitor cells (Fig. 1b) but several questions remain unresolved. For instance, what is the relationship between stem cells in prostate development and normal adult homeostasis? The rapid expansion of the developing prostate is mediated by multipotent basal progenitors, unipotent luminal cells and, possibly, unipotent basal cells. It has been proposed that the unipotent cells are derived from multipotent progenitors in a hierarchy of cells with decreasing potential to differentiate [28]. These progenitors seem to persist into adulthood; however, their relative abundance or activity changes dramatically (Fig. 1c). Lineage tracing experiments show that multipotent cells become scarce in the adult prostate [33, 39]; indeed, they were not detected in some studies [64, 85]. Instead, basal and luminal cells become more self-sustaining populations, similar to the breast [94]. This decline in multipotent differentiation reflects physiological changes in the prostate from development, where there is a vast increase in the number of cells, to adulthood, where cells are slowly replaced over time. Further studies into these age-related differences are warranted because they may reveal the intrinsic mechanisms that regulate the fate of prostate stem cells as well as the role of the niche. Differences in the properties of progenitors in development and adulthood also serve as an important reminder that the characteristics of prostate stem cells may depend on the context.

Another important question is why basal cells exhibit different characteristics in different assays. Lineage tracing experiments demonstrate that basal cells are predominantly unipotent in the adult prostate microenvironment, although there is still evidence for multilineage differentiation [30, 31, 33, 64]. However, basal cells undergo multilineage differentiation much more readily when they are removed from the prostate and grown in cell culture or tissue recombination assays [33]. A similar observation has been reported in the breast where myoepithelial cells are unipotent in lineage tracing experiments, but multipotent in single cell transplantation assays [94]. This suggests that in situ homeostasis and ex situ regeneration are mediated by different basal stem cells or by a single stem cell displaying different properties.

The limitation of cell culture and transplantation assays is that cells are transferred from their normal niche, unlike lineage tracing experiments. This means that transplantation assays may reveal the potential of stem cells, but not necessarily their normal fate. Extreme examples are where stem cells are recombined with stroma from different tissues or implanted at different sites [95, 96]. In these cases, stem cells partially or completely differentiate into the new tissue, rather than their original lineage. Prostate stem cells are typically recombined with mouse UGM because it is more inductive than adult prostate stroma. This might accelerate the obligate activity of normal prostate stem cells or uncover a facultative capacity of other basal cells to undergo multilineage differentiation. Indeed, the rapid proliferation and differentiation of cells in cell culture and tissue recombination assays is probably more akin to development than normal homeostasis, perhaps explaining the activation of multipotent basal cells. Nevertheless, even if transplantation and cell culture assays favour the potential facultative properties of prostate cells over their usual fate, the results are still important. By comparing lineage tracing and transplantation assays, the factors that regulate basal to luminal differentiation, and possibly the obligate versus facultative properties of stem cells, may be identified. This is significant because basal cells must differentiate into luminal cells to be cells of origin for prostate cancer. Therefore, the real challenge for the future is to determine how stem cells identified in any assay relate to human disease, rather than simply defining the differences between basal stem cells based on specific assays.

The relationship between unipotent luminal cells and CARNs is another unresolved question. K8- and PSA-dependent lineage tracing, initiated in intact mice, did not detect CARNs [64, 85]. One possibility is that CARNs are always present in the normal adult prostate but are inefficiently labelled because they are extremely rare. This would be similar to the long-lived epidermal stem cells that are detected with some lineage tracing techniques, but not others [97]. An alternative possibility is that CARNs are facultative stem cells that only arise after castration, reminiscent of basal cells that may be activated by transplantation. Once again, this highlights that the assay used to study stem cells may influence their characteristics. Like transplantation, it has been questioned whether the castration–regeneration assay accelerates normal proliferation and differentiation or alters the lineage hierarchy [34]. Encouragingly though, unipotent luminal cells, unipotent basal cells and multipotent basal cells have all been observed in castration models as well as

homeostasis and development, suggesting that castration–regeneration may not be drastically different [28, 33, 64, 85] (Fig. 1c). CARNs are the exception, so it will be interesting to determine whether there are multipotent luminal cells in conditions other than castration.

8 Conclusion

An ongoing area of interest and debate is the characteristics of prostate stem cells in different conditions. As noted by Evans and Chandler, there is a "need to distinguish between different categories of stem cell, depending upon whether a cell exhibits its generative capacity normally ("functional" stem cell) or under conditions of damage and regeneration ("potential" or "clonogenic" stem cells) … these functional and potential stem cells may not be one and the same" [34]. Further research into these differences will help characterise the features of prostate stem cells more precisely and reveal the mechanisms that regulate their fate.

Whereas the emphasis of stem cell research in many tissues is regenerative medicine, the overall goal in the prostate is to determine whether stem cells have a role in the origin and progression of diseases like benign prostatic hyperplasia and prostate cancer. Studies of normal prostate stem cells have provided insight into the fundamental biology of the tissue, including different cell–cell interactions, possible hierarchies and important signalling molecules. Determining how these processes are perturbed may provide strategies for preventative medicine by uncovering the causes of disease and identify new therapeutic targets once disease has developed.

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References

1. Coffey DS (1992) The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In: Walsh PC, Retik AB, Stamey TA, Vaughan ED (eds) Campbell's urology, 6th edn. WB Saunders, Philadelphia, PA, pp 221–301
2. McNeal JE (1988) Normal histology of the prostate. Am J Surg Pathol 12(8):619–633
3. Shappell SB, Thomas GV, Roberts RL, Herbert R, Ittmann MM, Rubin MA, Humphrey PA, Sundberg JP, Rozengurt N, Barrios R, Ward JM, Cardiff RD (2004) Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res 64(6):2270–2305
4. Hayashi N, Sugimura Y, Kawamura J, Donjacour AA, Cunha GR (1991) Morphological and functional heterogeneity in the rat prostatic gland. Biol Reprod 45(2):308–321

5. Lawrence MG, Lai J, Clements JA (2010) Kallikreins on steroids: structure, function, and hormonal regulation of prostate-specific antigen and the extended kallikrein locus. Endocr Rev 31(4):407–446. doi:10.1210/er.2009-0034
6. El-Alfy M, Pelletier G, Hermo LS, Labrie F (2000) Unique features of the basal cells of human prostate epithelium. Microsc Res Tech 51(5):436–446
7. van Leenders G, Dijkman H, Hulsbergen-van de Kaa C, Ruiter D, Schalken J (2000) Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy. Lab Invest 80(8):1251–1258
8. Verhagen AP, Ramaekers FC, Aalders TW, Schaafsma HE, Debruyne FM, Schalken JA (1992) Colocalization of basal and luminal cell-type cytokeratins in human prostate cancer. Cancer Res 52(22):6182–6187
9. Hudson DL, Guy AT, Fry P, O'Hare MJ, Watt FM, Masters JR (2001) Epithelial cell differentiation pathways in the human prostate: identification of intermediate phenotypes by keratin expression. J Histochem Cytochem 49(2):271–278
10. Signoretti S, Waltregny D, Dilks J, Isaac B, Lin D, Garraway L, Yang A, Montironi R, McKeon F, Loda M (2000) p63 is a prostate basal cell marker and is required for prostate development. Am J Pathol 157(6):1769–1775
11. Goldstein AS, Huang J, Guo C, Garraway IP, Witte ON (2010) Identification of a cell of origin for human prostate cancer. Science 329(5991):568–571. doi:10.1126/science.1189992, 329/5991/568 [pii]
12. Wang Y, Hayward S, Cao M, Thayer K, Cunha G (2001) Cell differentiation lineage in the prostate. Differentiation 68(4–5):270–279
13. Xue Y, Smedts F, Verhofstad A, Debruyne F, de la Rosette J, Schalken J (1998) Cell kinetics of prostate exocrine and neuroendocrine epithelium and their differential interrelationship: new perspectives. Prostate Suppl 8:62–73
14. Abrahamsson PA, di Sant'Agnese PA (1993) Neuroendocrine cells in the human prostate gland. J Androl 14(5):307–309
15. Kellokumpu-Lehtonen P, Santti R, Pelliniemi LJ (1980) Correlation of early cytodifferentiation of the human fetal prostate and Leydig cells. Anat Rec 196(3):263–273
16. Prins GS, Putz O (2008) Molecular signaling pathways that regulate prostate gland development. Differentiation 76(6):641–659. doi:10.1111/j.1432-0436.2008.00277.x, DIF277 [pii]
17. Bieberich CJ, Fujita K, He WW, Jay G (1996) Prostate-specific and androgen-dependent expression of a novel homeobox gene. J Biol Chem 271(50):31779–31782
18. Mirosevich J, Gao N, Matusik RJ (2005) Expression of Foxa transcription factors in the developing and adult murine prostate. Prostate 62(4):339–352
19. Sugimura Y, Cunha GR, Donjacour AA (1986) Morphogenesis of ductal networks in the mouse prostate. Biol Reprod 34(5):961–971
20. Lowsley OS (1912) The development of the human prostate gland with reference to the development of other structures at the base of neck of the urinary bladder. Am J Anat 13:299–349
21. Trompetter M, Smedts F, van der Wijk J, Schoots C, de Jong HJ, Hopman A, de la Rosette J (2008) Keratin profiling in the developing human prostate. A different approach to understanding epithelial lineage. Anticancer Res 28(1A):237–243
22. Garraway LA, Lin D, Signoretti S, Waltregny D, Dilks J, Bhattacharya N, Loda M (2003) Intermediate basal cells of the prostate: in vitro and in vivo characterization. Prostate 55(3):206–218. doi:10.1002/pros.10244
23. Risbridger GP, Wang H, Frydenberg M, Cunha G (2001) The metaplastic effects of estrogen on mouse prostate epithelium: proliferation of cells with basal cell phenotype. Endocrinology 142(6):2443–2450
24. Bierie B, Nozawa M, Renou JP, Shillingford JM, Morgan F, Oka T, Taketo MM, Cardiff RD, Miyoshi K, Wagner KU, Robinson GW, Hennighausen L (2003) Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation. Oncogene 22(25):3875–3887. doi:10.1038/sj.onc.1206426, 1206426 [pii]
25. Wernert N, Seitz G, Achtstatter T (1987) Immunohistochemical investigation of different cytokeratins and vimentin in the prostate from the fetal period up to adulthood and in prostate carcinoma. Pathol Res Pract 182(5):617–626

26. Letellier G, Perez MJ, Yacoub M, Levillain P, Cussenot O, Fromont G (2007) Epithelial phenotypes in the developing human prostate. J Histochem Cytochem 55(9):885–890. doi:10.1369/jhc.7A7192.2007
27. Xue Y, Smedts F, Debruyne FM, de la Rosette JJ, Schalken JA (1998) Identification of intermediate cell types by keratin expression in the developing human prostate. Prostate 34(4):292–301
28. Ousset M, Van Keymeulen A, Bouvencourt G, Sharma N, Achouri Y, Simons BD, Blanpain C (2012) Multipotent and unipotent progenitors contribute to prostate postnatal development. Nat Cell Biol 14(11):1131–1138. doi:10.1038/ncb2600
29. Tunn S, Nass R, Ekkernkamp A, Schulze H, Krieg M (1989) Evaluation of average life span of epithelial and stromal cells of human prostate by superoxide dismutase activity. Prostate 15(3):263–271
30. Blackwood JK, Williamson SC, Greaves LC, Wilson L, Rigas AC, Sandher R, Pickard RS, Robson CN, Turnbull DM, Taylor RW, Heer R (2011) In situ lineage tracking of human prostatic epithelial stem cell fate reveals a common clonal origin for basal and luminal cells. J Pathol 225(2):181–188. doi:10.1002/path.2965
31. Gaisa NT, Graham TA, McDonald SA, Poulsom R, Heidenreich A, Jakse G, Knuechel R, Wright NA (2011) Clonal architecture of human prostatic epithelium in benign and malignant conditions. J Pathol 225(2):172–180. doi:10.1002/path.2959
32. Van Keymeulen A, Blanpain C (2012) Tracing epithelial stem cells during development, homeostasis, and repair. J Cell Biol 197(5):575–584. doi:10.1083/jcb.201201041
33. Wang ZA, Mitrofanova A, Bergren SK, Abate-Shen C, Cardiff RD, Califano A, Shen MM (2013) Lineage analysis of basal epithelial cells reveals their unexpected plasticity and supports a cell-of-origin model for prostate cancer heterogeneity. Nat Cell Biol. doi:10.1038/ncb2697
34. Evans GS, Chandler JA (1987) Cell proliferation studies in the rat prostate: II. The effects of castration and androgen-induced regeneration upon basal and secretory cell proliferation. Prostate 11(4):339–351
35. English HF, Santen RJ, Isaacs JT (1987) Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate 11(3):229–242
36. Sugimura Y, Cunha GR, Donjacour AA (1986) Morphological and histological study of castration-induced degeneration and androgen-induced regeneration in the mouse prostate. Biol Reprod 34(5):973–983
37. Isaacs JT (1987) Control of cell proliferation and cell death in the normal and neoplastic prostate: a stem cell model. In: Rodgers C, Coffey D, Cunha GR, Grayhack J, Hinman FJ, Horton R (eds) Benign prostatic hyperplasia. Government Printing Office, Washington, DC, pp 85–94
38. Tsujimura A, Koikawa Y, Salm S, Takao T, Coetzee S, Moscatelli D, Shapiro E, Lepor H, Sun TT, Wilson EL (2002) Proximal location of mouse prostate epithelial stem cells: a model of prostatic homeostasis. J Cell Biol 157(7):1257–1265
39. Wang X, Kruithof-de Julio M, Economides KD, Walker D, Yu H, Halili MV, Hu YP, Price SM, Abate-Shen C, Shen MM (2009) A luminal epithelial stem cell that is a cell of origin for prostate cancer. Nature 461(7263):495–500. doi:10.1038/nature08361, nature08361 [pii]
40. Hudson DL, O'Hare M, Watt FM, Masters JR (2000) Proliferative heterogeneity in the human prostate: evidence for epithelial stem cells. Lab Invest 80(8):1243–1250
41. Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT (2004) CD133, a novel marker for human prostatic epithelial stem cells. J Cell Sci 117(Pt 16):3539–3545
42. Lawson DA, Xin L, Lukacs RU, Cheng D, Witte ON (2007) Isolation and functional characterization of murine prostate stem cells. Proc Natl Acad Sci U S A 104(1):181–186
43. Xin L, Lukacs RU, Lawson DA, Cheng D, Witte ON (2007) Self-renewal and multilineage differentiation in vitro from murine prostate stem cells. Stem Cells 25(11):2760–2769
44. Lukacs RU, Memarzadeh S, Wu H, Witte ON (2010) Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. Cell Stem Cell 7(6):682–693. doi:10.1016/j.stem.2010.11.013, S1934-5909(10)00635-1 [pii]

45. Hayward SW (2002) Approaches to modeling stromal-epithelial interactions. J Urol 168(3):1165–1172
46. Lawson DA, Zong Y, Memarzadeh S, Xin L, Huang J, Witte ON (2010) Basal epithelial stem cells are efficient targets for prostate cancer initiation. Proc Natl Acad Sci U S A 107(6):2610–2615. doi:10.1073/pnas.0913873107, 0913873107 [pii]
47. Leong KG, Wang BE, Johnson L, Gao WQ (2008) Generation of a prostate from a single adult stem cell. Nature 456(7223):804–808. doi:10.1038/nature07427, nature07427
48. Goldstein AS, Lawson DA, Cheng D, Sun W, Garraway IP, Witte ON (2008) Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. Proc Natl Acad Sci U S A 105(52):20882–20887. doi:10.1073/pnas.0811411106, 0811411106 [pii]
49. Mulholland DJ, Xin L, Morim A, Lawson D, Witte O, Wu H (2009) Lin-Sca-1+CD49fhigh stem/progenitors are tumor-initiating cells in the Pten-null prostate cancer model. Cancer Res 69(22):8555–8562. doi:10.1158/0008-5472.CAN-08-4673, 0008-5472.CAN-08-4673 [pii]
50. Hayward SW, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR (1998) Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. Differentiation 63(3):131–140
51. Xin L, Ide H, Kim Y, Dubey P, Witte ON (2003) In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. Proc Natl Acad Sci U S A 100(Suppl 1):11896–11903. doi:10.1073/pnas.1734139100
52. Wang ZA, Shen MM (2010) Revisiting the concept of cancer stem cells in prostate cancer. Oncogene. doi:10.1038/onc.2010.530, onc2010530 [pii]
53. Banerjee PP, Banerjee S, Zirkin BR, Brown TR (1998) Lobe-specific telomerase activity in the intact adult brown Norway rat prostate and its regional distribution within the prostatic ducts. Endocrinology 139(2):513–519
54. Burger PE, Xiong X, Coetzee S, Salm SN, Moscatelli D, Goto K, Wilson EL (2005) Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. Proc Natl Acad Sci U S A 102(20):7180–7185
55. Jiao J, Hindoyan A, Wang S, Tran LM, Goldstein AS, Lawson D, Chen D, Li Y, Guo C, Zhang B, Fazli L, Gleave M, Witte ON, Garraway IP, Wu H (2012) Identification of CD166 as a surface marker for enriching prostate stem/progenitor and cancer initiating cells. PLoS One 7(8):e42564. doi:10.1371/journal.pone.0042564
56. Goto K, Salm SN, Coetzee S, Xiong X, Burger PE, Shapiro E, Lepor H, Moscatelli D, Wilson EL (2006) Proximal prostatic stem cells are programmed to regenerate a proximal-distal ductal axis. Stem Cells 24(8):1859–1868. doi:10.1634/stemcells.2005-0585
57. Bonkhoff H, Wernert N, Dhom G, Remberger K (1991) Relation of endocrine-paracrine cells to cell proliferation in normal, hyperplastic, and neoplastic human prostate. Prostate 19(2):91–98
58. Bonkhoff H, Stein U, Remberger K (1994) The proliferative function of basal cells in the normal and hyperplastic human prostate. Prostate 24(3):114–118
59. De Marzo AM, Meeker AK, Epstein JI, Coffey DS (1998) Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. Am J Pathol 153(3):911–919. doi:10.1016/S0002-9440(10)65632-5
60. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM, Campbell ML (1992) Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52(24):6940–6944
61. Kyprianou N, Tu H, Jacobs SC (1996) Apoptotic versus proliferative activities in human benign prostatic hyperplasia. Hum Pathol 27(7):668–675
62. Paradis V, Dargere D, Laurendeau I, Benoit G, Vidaud M, Jardin A, Bedossa P (1999) Expression of the RNA component of human telomerase (hTR) in prostate cancer, prostatic intraepithelial neoplasia, and normal prostate tissue. J Pathol 189(2):213–218. doi:10.1002/(SICI)1096-9896(199910)189:2<213::AID-PATH417>3.0.CO;2-A
63. Bonkhoff H, Stein U, Remberger K (1994) Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers. Hum Pathol 25(1):42–46

64. Choi N, Zhang B, Zhang L, Ittmann M, Xin L (2012) Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. Cancer Cell 21(2):253–265. doi:10.1016/j.ccr.2012.01.005, S1535-6108(12)00038-4 [pii]
65. Robinson EJ, Neal DE, Collins AT (1998) Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium. Prostate 37(3):149–160
66. Uzgare AR, Xu Y, Isaacs JT (2004) In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. J Cell Biochem 91(1):196–205
67. Brawer MK, Peehl DM, Stamey TA, Bostwick DG (1985) Keratin immunoreactivity in the benign and neoplastic human prostate. Cancer Res 45(8):3663–3667
68. Niranjan B, Lawrence MG, Papargiris MM, Richards MG, Hussain S, Frydenberg M, Pedersen J, Taylor RA, Risbridger GP (2013) Primary culture and propagation of human prostate epithelial cells. Methods Mol Biol 945:365–382. doi:10.1007/978-1-62703-125-7_22
69. Garraway IP, Sun W, Tran CP, Perner S, Zhang B, Goldstein AS, Hahm SA, Haider M, Head CS, Reiter RE, Rubin MA, Witte ON (2010) Human prostate sphere-forming cells represent a subset of basal epithelial cells capable of glandular regeneration in vivo. Prostate 70(5):491–501. doi:10.1002/pros.21083
70. Bhatia B, Jiang M, Suraneni M, Patrawala L, Badeaux M, Schneider-Broussard R, Multani AS, Jeter CR, Calhoun-Davis T, Hu L, Hu J, Tsavachidis S, Zhang W, Chang S, Hayward SW, Tang DG (2008) Critical and distinct roles of p16 and telomerase in regulating the proliferative life span of normal human prostate epithelial progenitor cells. J Biol Chem 283(41):27957–27972. doi:10.1074/jbc.M803467200
71. Barclay WW, Axanova LS, Chen W, Romero L, Maund SL, Soker S, Lees CJ, Cramer SD (2008) Characterization of adult prostatic progenitor/stem cells exhibiting self-renewal and multilineage differentiation. Stem Cells 26(3):600–610
72. Yamamoto H, Masters JR, Dasgupta P, Chandra A, Popert R, Freeman A, Ahmed A (2012) CD49f is an efficient marker of monolayer- and spheroid colony-forming cells of the benign and malignant human prostate. PLoS One 7(10):e46979. doi:10.1371/journal.pone.0046979
73. Missol-Kolka E, Karbanova J, Janich P, Haase M, Fargeas CA, Huttner WB, Corbeil D (2011) Prominin-1 (CD133) is not restricted to stem cells located in the basal compartment of murine and human prostate. Prostate 71(3):254–267. doi:10.1002/pros.21239
74. Burger PE, Gupta R, Xiong X, Ontiveros CS, Salm SN, Moscatelli D, Wilson EL (2009) High aldehyde dehydrogenase activity: a novel functional marker of murine prostate stem/progenitor cells. Stem Cells 27(9):2220–2228. doi:10.1002/stem.135
75. Brown MD, Gilmore PE, Hart CA, Samuel JD, Ramani VA, George NJ, Clarke NW (2007) Characterization of benign and malignant prostate epithelial Hoechst 33342 side populations. Prostate 67(13):1384–1396
76. Xin L, Lawson DA, Witte ON (2005) The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc Natl Acad Sci U S A 102(19):6942–6947
77. Parsons JK, Gage WR, Nelson WG, De Marzo AM (2001) p63 protein expression is rare in prostate adenocarcinoma: implications for cancer diagnosis and carcinogenesis. Urology 58(4):619–624. doi:10.1016/S0090-4295(01)01311-5
78. Montironi R, Scarpelli M, Cheng L, Lopez-Beltran A, Zhou M, Montorsi F (2012) Do not misinterpret intraductal carcinoma of the prostate as high-grade prostatic intraepithelial neoplasia! Eur Urol 62(3):518–522. doi:10.1016/j.eururo.2012.05.062
79. Zong Y, Huang J, Sankarasharma D, Morikawa T, Fukayama M, Epstein JI, Chada KK, Witte ON (2012) Stromal epigenetic dysregulation is sufficient to initiate mouse prostate cancer via paracrine Wnt signaling. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1217982109
80. Taylor RA, Toivanen R, Frydenberg M, Pedersen J, Harewood L, Australian Prostate Cancer B, Collins AT, Maitland NJ, Risbridger GP (2012) Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status. Stem Cells 30(6):1087–1096. doi:10.1002/stem.1094
81. Evans GS, Chandler JA (1987) Cell proliferation studies in rat prostate. I. The proliferative role of basal and secretory epithelial cells during normal growth. Prostate 10(2):163–178

82. Peehl DM (2005) Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer 12(1):19–47. doi:10.1677/erc.1.00795, 12/1/19 [pii]
83. Kurita T, Medina RT, Mills AA, Cunha GR (2004) Role of p63 and basal cells in the prostate. Development 131(20):4955–4964
84. Signoretti S, Pires MM, Lindauer M, Horner JW, Grisanzio C, Dhar S, Majumder P, McKeon F, Kantoff PW, Sellers WR, Loda M (2005) p63 regulates commitment to the prostate cell lineage. Proc Natl Acad Sci U S A 102(32):11355–11360
85. Liu J, Pascal LE, Isharwal S, Metzger D, Ramos Garcia R, Pilch J, Kasper S, Williams K, Basse PH, Nelson JB, Chambon P, Wang Z (2011) Regenerated luminal epithelial cells are derived from preexisting luminal epithelial cells in adult mouse prostate. Mol Endocrinol 25(11):1849–1857. doi:10.1210/me.2011-1081, me.2011-1081 [pii]
86. Schalken JA, van Leenders G (2003) Cellular and molecular biology of the prostate: stem cell biology. Urology 62(5 Suppl 1):11–20
87. van Leenders GJ, Schalken JA (2003) Epithelial cell differentiation in the human prostate epithelium: implications for the pathogenesis and therapy of prostate cancer. Crit Rev Oncol Hematol 46(Suppl):S3–S10
88. Isaacs JT, Coffey DS (1989) Etiology and disease process of benign prostatic hyperplasia. Prostate Suppl 2:33–50
89. Xue Y, Verhofstad A, Lange W, Smedts F, Debruyne F, de la Rosette J, Schalken J (1997) Prostatic neuroendocrine cells have a unique keratin expression pattern and do not express Bcl-2: cell kinetic features of neuroendocrine cells in the human prostate. Am J Pathol 151(6):1759–1765
90. Verhagen AP, Aalders TW, Ramaekers FC, Debruyne FM, Schalken JA (1988) Differential expression of keratins in the basal and luminal compartments of rat prostatic epithelium during degeneration and regeneration. Prostate 13(1):25–38
91. Aumuller G, Leonhardt M, Renneberg H, von Rahden B, Bjartell A, Abrahamsson PA (2001) Semiquantitative morphology of human prostatic development and regional distribution of prostatic neuroendocrine cells. Prostate 46(2):108–115
92. Aumuller G, Leonhardt M, Janssen M, Konrad L, Bjartell A, Abrahamsson PA (1999) Neurogenic origin of human prostate endocrine cells. Urology 53(5):1041–1048
93. Bonkhoff H (1998) Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. Prostate Suppl 8:18–22
94. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C (2011) Distinct stem cells contribute to mammary gland development and maintenance. Nature 479(7372):189–193. doi:10.1038/nature10573
95. Taylor RA, Wang H, Wilkinson SE, Richards MG, Britt KL, Vaillant F, Lindeman GJ, Visvader JE, Cunha GR, St John J, Risbridger GP (2009) Lineage enforcement by inductive mesenchyme on adult epithelial stem cells across developmental germ layers. Stem Cells 27(12):3032–3042. doi:10.1002/stem.244
96. Lu CP, Polak L, Rocha AS, Pasolli HA, Chen SC, Sharma N, Blanpain C, Fuchs E (2012) Identification of stem cell populations in sweat glands and ducts reveals roles in homeostasis and wound repair. Cell 150(1):136–150. doi:10.1016/j.cell.2012.04.045
97. Mascre G, Dekoninck S, Drogat B, Youssef KK, Brohee S, Sotiropoulou PA, Simons BD, Blanpain C (2012) Distinct contribution of stem and progenitor cells to epidermal maintenance. Nature 489(7415):257–262. doi:10.1038/nature11393

Adult Lung Stem Cells

Amy L. Firth, Ruby A. Fernandez, and Jason X.-J. Yuan

Abstract Over the past decade a wealth of information has been divulged on stem cells present in the lung both in the pulmonary vasculature and the respiratory tract. Cells have been identified with the capability of repopulating the lung and others that contribute to the pathogenesis or, conversely, have therapeutic benefit in pulmonary vascular disease. The isolation of a single-resident lung stem cell capable of repopulating any lung epithelium still remains elusive. What is currently known about stem and progenitor cells in the lung suggests that a non-classical stem cell hierarchy exists with a novel array of cellular mechanisms controlling proliferation and differentiation of such cells. This chapter serves to provide an up-to-date review of what is currently known about stem and progenitor cells within the lung.

Keywords Clara • Basal • Alveolar type II • Mesenchymal stem cells • Endothelial progenitor cells

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Abbreviations

$5\text{-}HT_{2B}$	5-hydroxytryptamine receptor 2B
ATI/ATII	Alveolar type I and type II cells
BADJ	Bronchoalveolar duct junction
BASC	Bronchiolar stem cell
BMP	Bone morphogenic protein
CCSP	Clara cell secretory protein (also CC10)
CGRP	Calcitonin gene-related peptide
COPD	Chronic obstructive pulmonary disease
CTEPH	Chronic thromboembolic pulmonary hypertension
CXCR4	c-x-c chemokine receptor 4
EGF/EGFR	Epidermal growth factor/epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EndMT	Endothelial mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
FGF	Fibroblast growth factor
FOXA2	Forkhead box protein A2
FOXJ1	Forkhead box protein J1
HSC	Hematopoietic stem cell
IL-13	Interleukin 13
IPF	Idiopathic pulmonary fibrosis
Krt	Cytokeratin
LDL	Acetylated low density lipoprotein
MSC/MPC	Mesenchymal stem/progenitor cell
NEB	Neuroepithelial body
OVA	Ovalbumin
PH	Pulmonary hypertension
PNE/PNEB	Pulmonary neuroendocrine cells/bodies
PO_2	Partial pressure of oxygen
RA	Retinoic acid
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)
SDF-1	Stromal-derived factor 1
Shh	Sonic hedgehog
SOX2	SRY (sex-determining region Y)-box 2
SPA/B/C/D	Surfactant protein A/B/C/D
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TTF-1	Thyroid transcription factor 1
VEGFR2	Vascular endothelial growth factor receptor 2

1 Introduction

The lung is an immensely complex organ consisting of a vast surface area of pulmonary epithelium for gas exchange and intertwined pulmonary vasculature transporting oxygenated blood to the heart to reach all cells in the body (Fig. 1). As such the lung is a vital organ which, under normal conditions, has a very slow turnover. In contrast, epithelial cells lining the airways, from the trachea to the most distal alveoli, experience a much more frequent cellular turnover. This is primarily due to the constant exposure to potentially toxic agents and pathogens being inhaled in the air. Such agents often cause injury to the epithelial cell layer; throughout life the cells in the airway need to be capable of quickly responding to such injury and rapidly establishing homeostasis again.

One should believe that a resident tissue-specific stem or progenitor cell would be responsible for this repair, being activated by signals from this injury and rapidly differentiating to the required cell type. To date no one specific stem cell has been identified within the respiratory tract capable of the regeneration of all the epithelial cells from the most proximal to the most distal epithelium. Utilization of mouse models and experimental procedures designed to induce specific lung injury has, however, led to the discovery of several progenitor cells identified in distinct anatomical regions of the lung (tissue-specific stem or progenitor cells). It is currently postulated that a non-classical stem cell hierarchy exists in the lung [1]. In this situation a normally quiescent differentiated cell is able to respond to injury by dividing

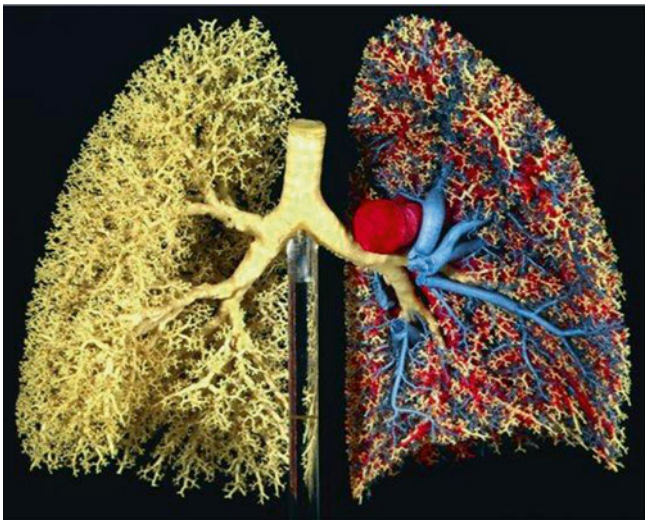

Fig. 1 Cast showing the structure of the lung airway and vasculature. Latex cast of the human airway (*white*), arterial tree (*red*), and venous tree (*blue*). Reproduced with kind permission from The American Physiological Society [162]

into one or more cell types and self-renewing; such a concept has been coined the term facultative stem cell.

To date the majority of studies investigating the origins of progenitor and stem cells in the lung are completed in mouse models. Mouse models are readily accessible; however, they do have definitive limitations. There are obvious size differences with the mouse and human lungs with the total lung capacity changing from 1 to 6,000 ml and major structural differences with mice having five smaller lobes compared to the humans having two larger lobes on the left side of the lung. Of more importance to those researching human lung disease may be differences in lung branching and airway generations (13–17 for mouse vs. 17–21 for human) and of the structure of the respiratory epithelium [2]. Mice have a thinner epithelial layer and a larger lumen to their airways to accommodate the more rapid respiratory rate. Sub mucosal glands are also a rarity in mice perhaps compensated for the increased presence of Clara cells [3]. Invariably there will be differences in the identity and localization of putative stem and progenitor cells between species. Due to the distinct differences in mouse and human airway biology, research must also be conducted utilizing reproducible in vitro models of the human airway epithelium.

Identification of lung progenitor and stem cells and thus understanding the origins of airway cell progenitors will provide valuable information pertaining to the differentiation and maintenance of mature airway cells. Ultimately, this may lead to novel therapeutic approaches for lung disease. This chapter summarizes the current knowledge regarding the identification of stem and progenitor cells within the lung, both in the respiratory tract and the pulmonary vasculature. We highlight strategies employed to identify and characterize putative stem cells and indicate the challenges of future research on lung injury and repair.

2 Structure and Function of the Adult Lung

2.1 Development of the Respiratory Tract

Distinct regional variation of epithelial cell type and function exists as you transect the proximodistal axis of the adult respiratory tract. The luminal surface of the airways comprises endoderm-derived epithelial cells supported by a network of mesenchymal derived cells such as airway smooth muscle cells, pulmonary fibroblasts, and vascular endothelium. In mice and humans, lung development has been defined into five stages; in humans the embryonic stage from weeks 0 to 5, the glandular from weeks 5 to 16, the canalicular from weeks 16 to 26, the saccular from week 26 to term, and finally, the alveolar stage occurring postnatal. Specification of the cells occurs early during embryogenesis where definitive endoderm ($FOXA2^+$, $SOX17^+$) gives rise to the first lung cells in the anterior foregut endoderm. Signals from the surrounding mesenchyme are involved in this process, including Wnt, bone morphogenic protein (BMP), fibroblast growth factor (FGF), sonic hedgehog (shh), and retinoic acid (RA) signaling pathways. Complex integration of each of these

signaling pathways is essential for proper lung formation. Longitudinal separation of this forms two tubes which will eventually generate the esophagus and trachea (dorsal and ventral, respectively) [4]. It was recently shown that conditional deletion of SOX2 in this ventral epithelial domain of the early anterior foregut results in defective trachea development; tracheas were stunted and grossly contained more mucous cells and fewer basal, ciliated, and Clara cells in mice [5]. TTF-1 or NKX2.1 is the earliest recognized marker of endodermal cells committed to the pulmonary ($FOXA2^+$) and thyroid ($PAX8^+$) lineages. At E9.5 in mice two buds form from the ventral foregut endoderm and develop into the splanchnic mesenchyme expressing NKX2.1 and surfactant protein C (SFTPC). From here a complex highly ordered branching morphogenesis occurs. This is driven by signaling through shh, BMP4, and FGF10 [6–9] and gives rise to the pulmonary tree. For comprehensive reviews of the lung morphogenesis, please refer to [10–12].

2.2 The Airways of the Adult Lung

As seen from the airway and pulmonary cast in Fig. 1, the lung is a complex three-dimensional structure. The cells which line the luminal epithelium have distinct differences as you move along the proximodistal axis. In the most proximal airways, from the trachea to the bronchi, a pseudostratified luminal epithelium exists. This predominantly consists of ciliated and mucous-secreting (goblet) columnar cells. Basal cells are flatter lying on the basal side of the epithelium and it is believed that these cells are more primitive (or less well differentiated) than the other cells in the proximal airways. Their proposed role as progenitor cells will be discussed later. The trachea branches into two primary bronchi which further branch into secondary then tertiary bronchi. Moving more distally the tertiary bronchi branch into large and then small bronchioles. Ciliated columnar cells exist in larger bronchioles and the emergence of nonciliated Clara cells in the smaller bronchioles. The final branches of the bronchioles are called terminal bronchioles again comprising of nonciliated columnar Clara cells and a limited number of ciliated cells. The terminal bronchioles divide to generate respiratory bronchioles leading to the respiratory portion comprising the alveolar ducts, alveolar sacs, and alveoli. Alveoli are formed from type I pneumocytes (95 % of the alveolar surface area) and type II pneumocytes (5 % of the alveolar surface area). Type I cells are larger flat cells thus having a thin diffusion barrier for gas exchange. Type II cells despite only covering 5 % of the surface account for around 60 % of the cell number. Their main function is to secrete surfactant proteins to decrease surface tension and prevent the alveoli from collapsing. The cells are connected by characteristic tight junctions which can be identified through the presence of occludin (ZO-1) located at the plasma membrane protein of tight junctions.

By way of chemical ablation of epithelial cells in the various lung compartments it has been shown that regenerative foci are maintained, postulated to be stem or progenitor cells in said compartments. A number of cells have been shown to

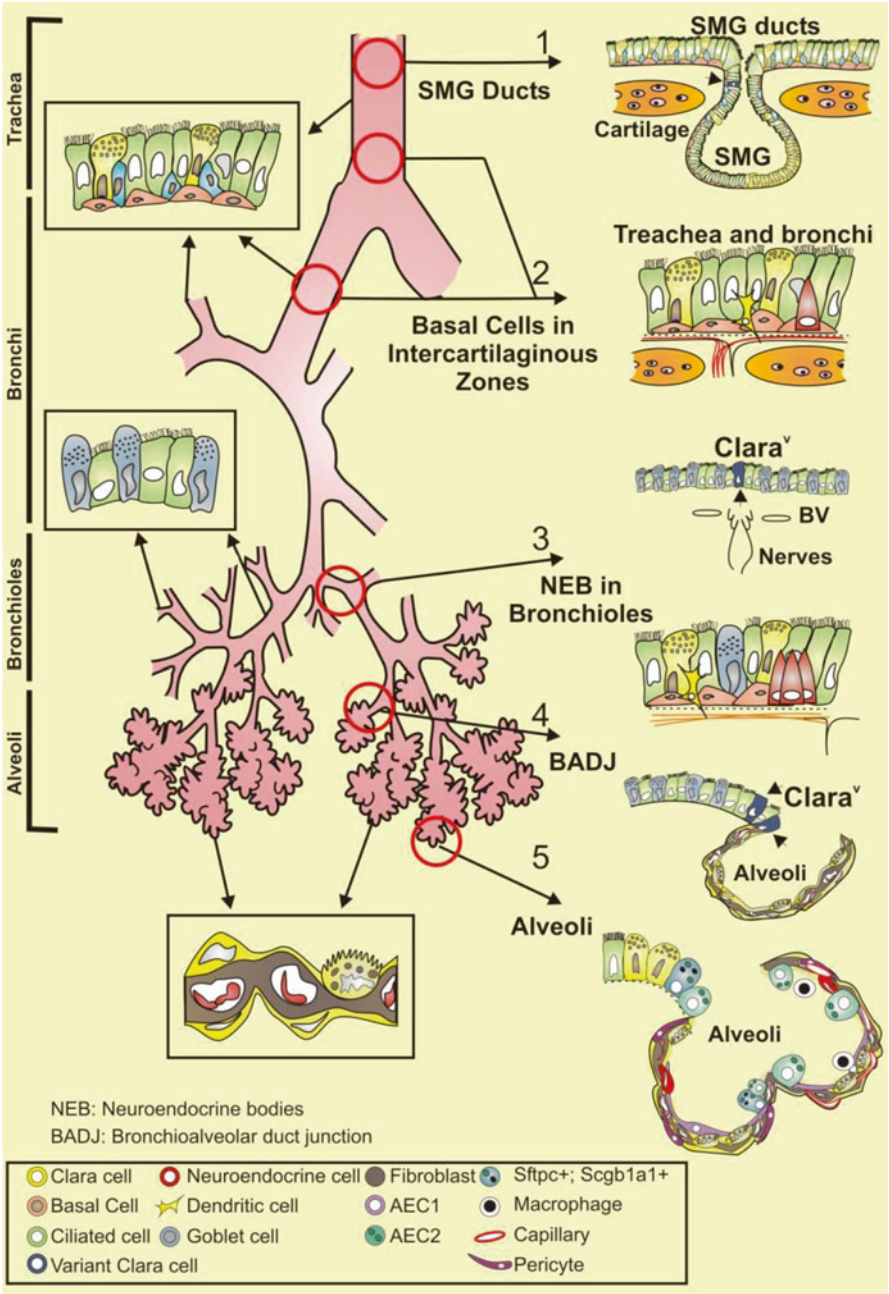

Fig. 2 Schematic showing the cells of the adult respiratory tract. The trachea, bronchi, and bronchioles for the three regions of conducting airways within the lung. The predominant cell types comprising the pseudostratified columnar tracheal and bronchial epithelium are basal, goblet, and ciliated cells. Neuroendocrine cells are less abundant and exist; less abundant neuroendocrine cells

demonstrate progenitor cell properties including basal cells in the proximal airways [13], Clara cells in the bronchioles [14, 15], and type II pneumocytes in the alveoli [16, 17]. The pulmonary neuroendocrine bodies (PNEB) may also contain a variant Clara cell possessing stem cell properties [18, 19]. More recently a putative stem cell has been identified in the bronchioalveolar duct junctions (BADJ) termed a bronchiolar stem cell or BASC, unlike all the other progenitor cells which have been identified, this cell has been shown to both self-renew and differentiate into multiple progeny [20]. A schematic representation of the cells present in the adult lung airways is featured in Fig. 2. The identification and isolation of these and other putative, progenitor cells will be discussed in detail throughout this chapter.

2.3 Pulmonary Circulation

The pulmonary system consists of a network of arteries, arterioles, and capillaries that transport deoxygenated blood from the heart to the lungs where, in the capillaries, oxygen diffuses out of the alveoli, re-oxygenating before being transported back to the heart for circulation around the body. The pulmonary vasculature is unique in its reaction to oxygen tension responding to decreased oxygen tension (PO_2) by contracting to divert the blood to the most oxygenated areas of the lung. As such, the cellular and molecular mechanisms controlling blood flow in the pulmonary circulation are often considered independent of systemic vasculature. The pulmonary vascular structure is reminiscent of the systemic arteries. The main pulmonary artery consists of a *tunica intima* formed from a single layer of endothelial cells lining the lumen and an internal elastic lamina; surrounding this is the *tunica media*, consisting of concentric layers of smooth muscle cells and an external elastic lamina; the outermost layer is the *tunica adventitia*, a strong layer of longitudinally arranged fibro elastic connective tissue. Figure 3 provides a schematic representation of the adult cells in the pulmonary vasculature in the lung.

The precise identity and roles of progenitor cells in the pulmonary vasculature remains somewhat poorly defined. There are no single markers that can successfully identify a stem or progenitor cell; so immunophenotyping, sorting, and functional characterization are still all essential [21]. Putative stem and progenitor cells in the pulmonary vasculature are either circulating hematopoietic stem cells (HSC),

Fig. 2 (continued) occur in the neuroendocrine bodies (NEB) in close proximity to bronchiole junctions. Submucosal glands (SMG) contain predominantly mucous and serous tubules and exist in the interstitium of the more proximal cartilaginous airways. As you move more distally through the airways, the epithelium contains mainly Clara and ciliated cells. Variant Clara cells are found in the bronchioalveolar duct junctions (BADJ) and are proposed to have stem cell properties. The most distal cells involved in gas exchange and pulmonary surfactant production in the alveoli are Type I and Type II pneumocytes (or alveolar cells, AT1 and AT2, respectively). Adapted from [163] with permission from Elsevier

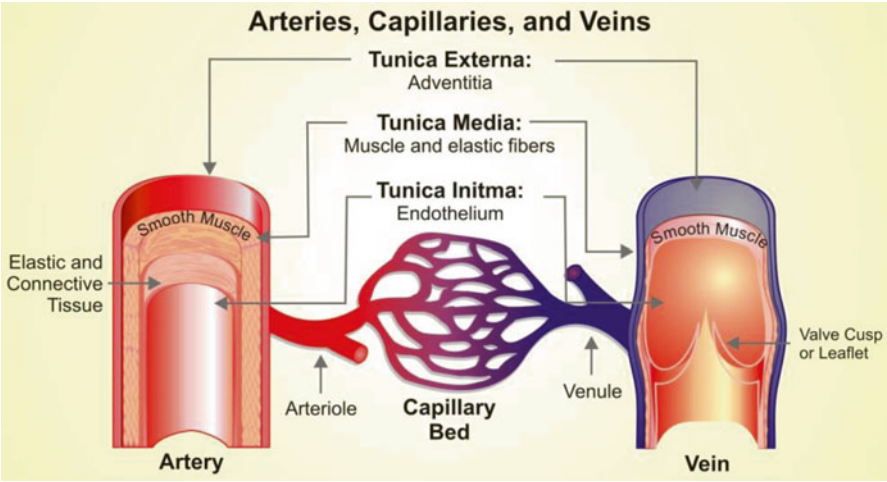

Fig. 3 Schematic showing the structure of the adult pulmonary vasculature. The pulmonary artery indicated on the left comprises the *Tunica Intima*, a single layer of endothelial cells, the *Tunica Media* formed from several layers of concentrically arranged smooth muscle cells and connective tissue, and the *Tunica Externa* comprising the adventitia. Veins transport the deoxygenated blood leaving the capillary bed back to the lungs, they are less muscular having a thinner *Tunica Media* and contain valves preventing backflow

endothelial progenitor cells (EPC), resident tissue stem cells and multipotential stem/progenitor cells (MSC/MPC). Each of these will be discussed in more detail later in the chapter.

3 Stem Cells in the Adult Lung: Respiratory Tract

3.1 Proximal Airways: Tracheal and Bronchial Stem Cells

Ciliated cells and mucous-secreting cells (goblet cells) act in concert to provide and innate defense system in the airways. Mucociliary clearance may be defective in patients with hereditary lung conditions such as asthma, COPD, cystic fibrosis, and ciliopathies or in response to viral infections or environmental stimuli (such as smoke). Goblet cell hyperplasia and hyper secretion of mucus is a defining pathophysiological feature of asthma and COPD [22, 23]. Goblet Cell hyperplasia in a patient with COPD is clearly depicted in the immunohistochemistry images shown in Fig. 4.

Understanding the mechanisms leading to this increase in goblet cell generation and thus excess mucus production may open the door for new therapeutic approaches. Knowledge of the progenitor cell type differentiating to goblet cells is essential in this process and is currently poorly understood. One of the most widely supported

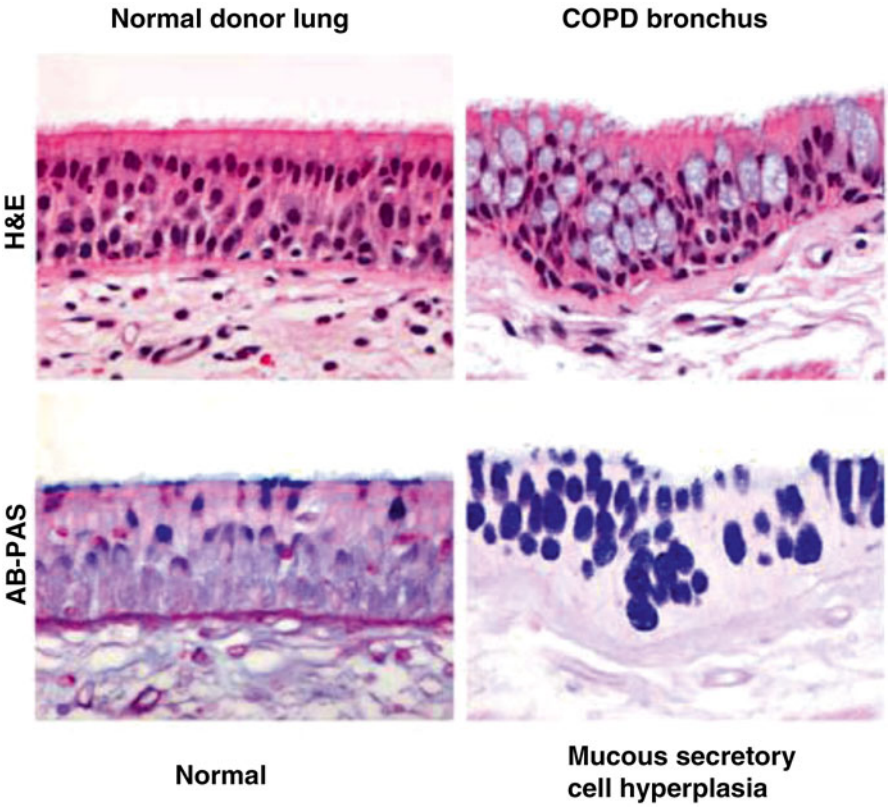

Fig. 4 Goblet cell hyperplasia. Representative images of normal and COPD bronchial epithelia. A marked expansion of mucous secretory cells in the COPD bronchus (*blue-stained material* in the *right panels*). *AB-PAS* alcian blue–periodic acid Schiff's stain, *H&E* hematoxylin and eosin. Reprinted with permission of the American Thoracic Society. Copyright © 2013 American Thoracic Society [161]

rational for goblet cell metaplasia suggests that Clara cells act as a goblet cell progenitor changing phenotype via a trans-differentiation process. Data from mouse models indicate that Clara cells are capable of undergoing a trans-differentiation to goblet cells. In an ovalbumin (OVA)-induced mouse model of allergic asthma, Hayashi and colleagues observed an increase of mucous-secreting (goblet) cells in the intrapulmonary bronchus and bronchioles compared to unchallenged mice. These cells resembled nonciliated epithelial cells which were similar to Clara cells of normal mice, containing homogeneous electron-dense secretory granules and mitochondria with poorly developed crista [24]. Notably there was a distinct lack of ciliated and basal cells and all indications suggested a trans-differentiation of Clara cells to a mucous-secreting goblet cell. Other studies verify this notion: in the same mouse model over a 22-day period decreases in Clara and ciliated cells by 75 and 25 %, respectively, were observed with a 70 % increased presence of mucous cells

in the proximal airways [25]. Again, in-depth analysis indicated a Clara cell metaplasia to mucous cells was occurring. Furthermore, after a single aerosol antigen challenge, Evans and colleagues noticed an increase in mucin secretion from a specific subset of Clara cells which also retained their expression of CCSP [3]. Other studies also support a direct differentiation of Clara cells to goblet cells via a mechanism involving activation of epidermal growth factor receptors (EGFR) in the tracheal epithelium and their direct role in the activation of CCSP [26]. EGFR was first noted for its role in the upregulation of cell proliferation in terminal bronchioles after naphthalene induced Clara cell injury [27]. In rat models EGFR stimulation by ligands EGF and TGF-α triggers MUC5AC expression, and in OVA sensitized rats, pretreatment with an EGFR tyrosine kinase inhibitor prevented the goblet cell hyperplasia observed in its absence [28].

The first study to investigate this phenomena in human airway reported a slightly different story. Using a cell lineage-tagging approach, Turner and colleagues investigated the generation of goblet cells from ciliated, FOXJ1-expressing, progenitor cells [29]. EGFP-labeled ciliated cells were generated using two lentiviral vectors, a FOXJ1 promoter-dependent CRE expression and a CMV-floxed EGFP. The fate of these FOXJ1-expressing cells in response to goblet cell driving interleukin-13 (IL-13) was monitored and data revealed an increase in EGFP positive goblet cells, suggesting the goblet cells were derived, at least in part, from FOXJ1 expressing progenitor cells [29].

In the pseudostratified airway epithelium, whether or not the Clara cells undergo a complete cell phenotype change to a goblet cell or just demonstrate a greater plasticity allowing for structural changes and greater secretory stimulation remains to be truly defined. In humans current evidence suggests that FOXJ1-expressing ciliated cell differentiation may account for the increased goblet cell expression; this may suggest different mechanisms occurring between the mouse and human airways.

In this same region of the airways, basal cells are also proposed to retain progenitor-like properties. In fact some of the earliest studies in a variety of animals indicated the presence of two distinct types of progenitor cells in the conducting airways: basal cells and Clara cells [30–33]. Basal cells have the fundamental role of anchoring the tracheobronchial epithelium to the basement membrane. They form a continuous monolayer becoming more clustered and individual in the smaller terminal bronchioles and represent 6–30 % of the cell population. A high percentage of basal and para-basal cells contribute to the proliferative fraction of the conducting airway in humans making them potential progenitor candidates [34].

An early study in Sprague–Dawley rats indicated that the nonciliated columnar cells and not the basal cells represented the progenitor cells [35]. Many subsequent studies provide evidence to the contrary. In rabbits two distinct cell populations were endowed with stem cell potential, namely, basal cells and bronchiolar Clara cells [36]. Using a xenograft model of proximal human airway, recombinant retrovirus reporter genes were used to infect human bronchial epithelial cells and seeded on to denuded rat trachea transplanted into athymic mice. Analysis of the clonal expansion revealed that basal and differentiated columnar cells were capable of self-renewal and multipotency [37]. In a more recent and similar study, basal cell marker-expressing cells (tetraspanin (CD151) and tissue factor (TF)) were isolated and used in the same

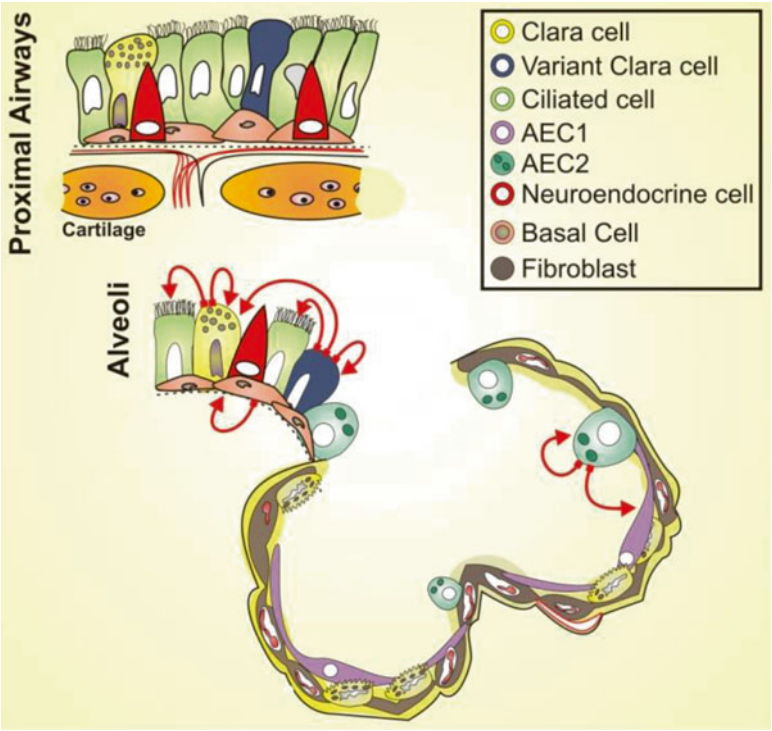

Fig. 5 Proposed stem cell hierarchy in the respiratory epithelium. The *red arrows* indicate the self-renewal and known progeny of proposed stem cells in the respiratory epithelium. Clara, basal, and variant clara cells are currently thought to be the predominant progenitor cells in the proximal airways and AEC Type II (AEC2) in the more distal airways

xenograft model in mice as well as in vitro in an air–liquid interface. These cells were capable of restoration of a fully differentiated functional airway epithelium [38]. Lineage tracing experiments also identify Trp-63 (p63), cytokeratin 5 (Krt5), or Krt14-expressing basal cells from both mouse and human trachea as being capable of self-renewal and multilineage differentiation [39, 40]. The role of basal cells as stem cells in the lung has recently been reviewed, please refer to the papers by Rock and colleagues for more information [40, 41]. The theoretical hierarchy of stem cells in the pseudostratified airway epithelium is shown in Fig. 5.

3.2 Bronchiolar Stem Cells

Moving to more distal-conducting airways, the bronchioles are a simple layer of epithelium comprised of Clara cells and ciliated cells. It is believed that the progenitor cells in this region are relatively quiescent and have the ability to respond to

injury by proliferation and/or differentiation. Again, most evidence supports a progenitor role for Clara cells in this region of the airways. More specifically a Clara cell resistant to chemically induced airway injury and closely associated with neuroepithelial bodies (NEB) seems to retain progenitor cell properties, this cell is commonly referred to as a variant Clara cell. An NEB comprises clusters of pulmonary neuroendocrine cells (PNE). PNE were originally considered to be terminally differentiated and mitotically inert therefore unable to possess the proliferative and self-renewing properties of a progenitor cell. Like Clara cells, however, PNE cells have been shown to proliferate in response to certain environmental stresses and both populations are now known to colocalize in the NEB [19].

Reynolds and colleagues were the first group to really demonstrate the existence of at least two epithelial cell variants with progenitor properties being maintained in the NEB environment [19]. One cell is a phenotypic intermediate of a Clara and PNE cell expressing calcitonin gene-related peptide (CGRP) and the other a variant Clara cell lacking immunoreactive CYP-2F2 protein. Both these cells are naphthalene resistant and when the ultrastructural characteristics of these cells in the NEB were investigated, a nonciliated epithelial cell or a modified/variant Clara cell was discovered; this cell lacks typical Clara cell features such as secretory vesicles and CYP-2F2, but are CCSP positive [19]. Subsequent studies by the same group indicated that the variant Clara cell was the most likely progenitor cell as CGRP expressing PNE, while able to proliferate, were unable to regenerate the airway after naphthalene injury [18]. Studies ensued to investigate the mechanisms by which these variant Clara cells were activated after lung injury and it was found that the Wnt/Fgf10 embryonic signaling cascade was again active in mature PSMCs (parabronchial SMC progenitors found in the distal mesenchyme) [42]. Subsequent to naphthalene-induced ablation of Clara cells, it was shown that Wnt7b expression was induced in surviving ciliated cells which were able to stimulate Fgf10 in PSMCs. Epithelial repair was induced by the secreted Fgf10 stimulating epithelial repair via Notch-induced Snai1 expression in variant Clara cells and a transient epithelial to mesenchymal transition (EMT) allowing them to become progenitors (Sftpc, Fgfr2b, and Snail1 positive) [42]. PSMCs should therefore be considered a putative stem cell niche important for epithelial repair post-lung injury. The mechanisms controlling the ability of lung epithelial cells to sense environmental insult and activate progenitor cells to repair the lung are still relatively poorly understood. It is becoming increasingly evident that this adaptability is reliant upon a reversible acquisition of mesenchymal features in the epithelial cells; it will be interesting to see if these observations from mouse models reflect changes in the human lung.

A very similar population of cells was also discovered at the bronchioalveolar duct junctions (BADJs). They were resistant to lung injury, exhibited clonal cell proliferation and multipotent potential in vitro and regenerated the respiratory epithelium in vivo [43, 44]. These cells, also known as bronchioalveolar stem cells (BASC), while capable of maintaining both bronchiolar Clara and alveolar cells are, however, proposed to be the precursor cells for adenocarcinomas [43, 45]. One of the most recent studies investigating this suggested that while Clara cells, the putative BASC, and alveolar type II cells (ATII) are cells of origin for K-Ras-induced

lung hyperplasia, it was only the ATII cells that progressed to the stage of adenocarcinoma [46]. Interestingly, these cells are potentially important in smoke-induced epithelial injury. De novo increases in Claudin-3 and CC10 are potential markers for early tobacco smoke-induced epithelial injury in alveolar duct epithelial cells [47].

Relatively little is known about BASC in terms of their specific identity; a recent study undertook a micro RNA (miRNA) screening and found a signature of miRNA in BASC but not control cells. This study was carried out in cells isolated from mouse lungs and BASC were sorted by flow cytometry gated on $CD31^{-}CD45^{-}CD34^{+}Sca\text{-}1^{+}$ and the control cells as $CD31^{-}CD45^{-}CD34^{-}Sca\text{-}1^{-}$. The miRNA profiling showed 56 upregulated and 60 downregulated in BASCs compared with the control cells suggesting a potential role for these miRNA in the self-renewing and differentiation capacity of BASC [48].

The presence and role of BASC is also somewhat controversial. A study by Rawlins and colleagues was the first to really follow the lineage fate of these postulated BASC [14]. In order to address the role of Clara cell and variant Clara cell populations (including BASC), Rawlins generated mice where CreER was "knocked into" the endogenous *Scgb1a1* locus; a gene expressed in all of these populations. This system allowed for lineage tracing of the labeled Clara type cells and there was no evidence for a significant contribution of the labeled cells to the alveoli. $Scgb1a1^{+}$ cells could function as long-term progenitors in the bronchioles where $Scgb1a1^{+}$ cells surviving naphthalene-induced lung injury could divide and regenerate the airway epithelium. In the trachea, however, most Clara cells appeared to be derived from a transiently activated population of $Scgb1a1^{-}$ basal cells during a steady state; after injury any surviving labeled Clara cells did show an increase proliferation and capability to generate ciliated cells [14]. The bronchiolar stem cell niche is shown in Fig. 6.

Among the most specific markers known to identify epithelial stem cell populations are members of the leucine-rich repeat-containing G protein-coupled receptor (Lgr) family [49, 50]. Oeztuerk-Winder and colleagues carried out an extensive study in human bronchiolar cells and gathered a novel $E\text{-}Cad^{+}/Lgr6^{+}$ signature [51]. In vitro they also expressed ATII (SP-C) and Clara (CC10); however, these markers were not evident in the in vivo studies. These cells were able to migrate and differentiate into bleomycin-injured human lungs and nude mice generating ATII, ATI, and Clara cells essentially regenerating the bronchiolar epithelium [51].

3.3 Alveolar Stem Cells

Flattened squamous and cuboidal pneumocytes (Type I and Type II pneumocytes, respectively) line the alveoli. For the longest time the type II pneumocyte (alveolar type II or ATII) has been indicated both in vivo and in vitro to be a progenitor for both type I and type II pneumocytes in the alveoli [52–54]. Type II cells comprise 15 % of all lung cells but only cover <5 % of the surface epithelium. Their primary role is surfactant protein production; however, no single surfactant component can

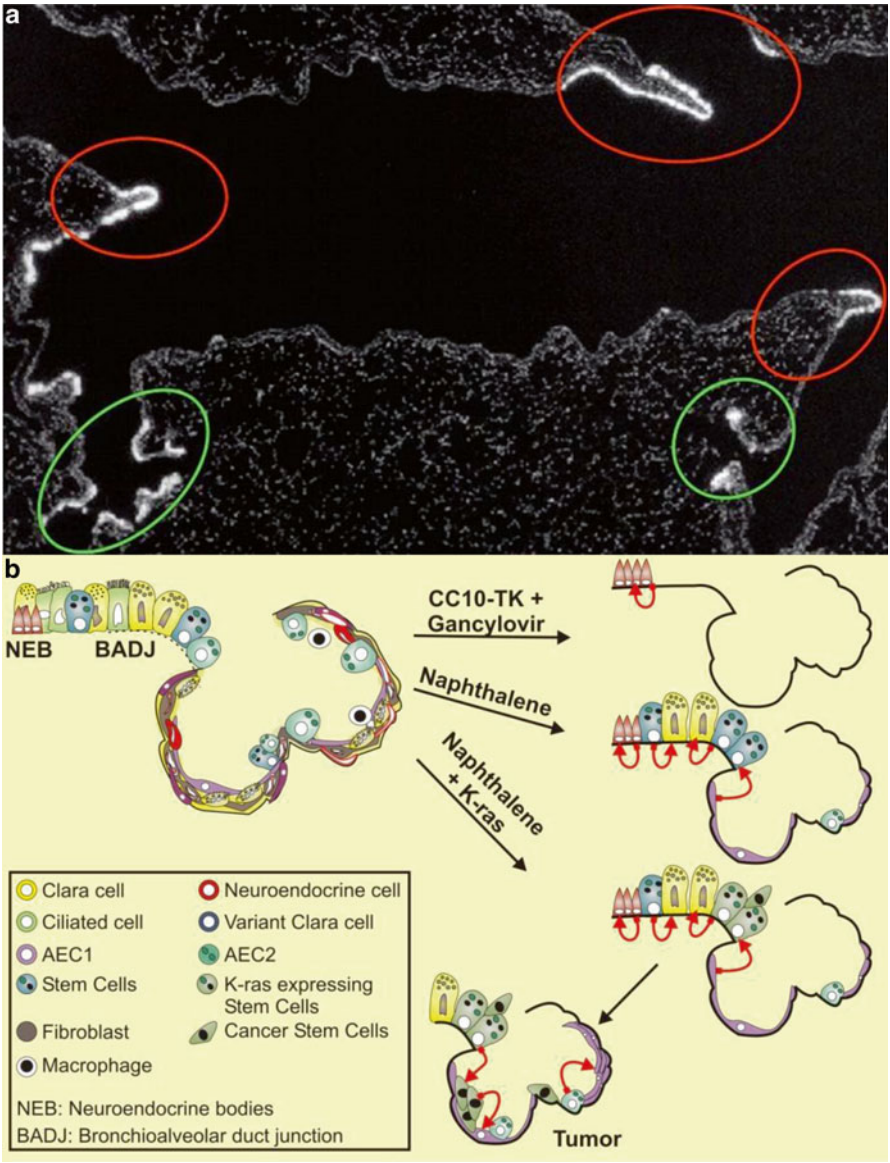

Fig. 6 Bronchoalveolar duct junction stem cells and lung injury. (**a**) The image indicates in situ mRNA levels of CC10 96 h post-naphthalene injury. The *red circles* highlight the regions of highest expression occurring in an orderly and progressive fashion at bifurcations. (**b**) The schematic shows a bronchiole neuroendocrine body (NEB). Variant clara cells are also termed bronchioalveolar stem cells (stem cells). Post-naphthalene-induced lung injury these stem cells are capable of repopulating the terminal bronchioles and alveolar surface. Notably, naphthalene treatment of conditional K-ras mice (in which K-ras is switched on by AdenoCre) induces enhanced proliferation of these stem cells but does not stimulate proliferation of lung alveolar cells. Panel **a** is reproduced with kind permission from the American Physiological Society [164] and panel **b** is adapted with kind permission from [165]

specifically identity type II cells. More distinct markers are the lamella bodies which contain surfactant and are readily observed by phase contrast microscopy.

ATII cells have long been established as being able to both proliferate and to differentiate into type I cells; they are capable of repopulation of the alveoli after injury [55, 56]. Such division and transformation/differentiation of the cells is also thought to occur in normal alveolar cell turnover. The earliest studies investigating the mechanisms of this transformation were complicated by extrapulmonary and humoral factors in vivo and the inability to sustain ATII cell phenotypes in vitro. Kinnard and colleagues were the first to really establish an in vitro lung explant system in which ATII cell proliferation and differentiation could be monitored [57]. The ability to monitor the differentiation from an ATII to ATI cell type is critically dependent upon specific identification of both cell types. Analysis of ultrastructural components, lamellar bodies, apical microvilli, cell to cell junctions, and cell shape allow for a good distinction [58]. The presence of cell surface markers is less precise as it is widely accepted that the cells pass through intermediate cell stages during the differentiation and marker expression varies with developmental/differentiation stage [59, 60]. A recent study made observations on the contrary where it was the ATI cells that had the potential to both proliferate and express markers of other differentiated airway epithelial cells in vitro. The in vitro plasticity of a pure population of RTI40 and aquaporin 5 expressing rat ATI cells was demonstrated by the induced expression of SP-C, a marker of the ATII cells and CC10, a marker of Clara cells [61]. Cell proliferation was evident in conditions that ATII cells do not proliferate. In response to infection in the lungs such as pneumonia (*Pseudomonas aeruginosa*), FoxM1 was demonstrated to be crucial for the proliferation and differentiation of ATII cells to ATI cells and thus restoration of alveolar homeostasis [62]. In a progenitor population isolated from piglet lungs, extensive self-renewal and the capacity for both ATI and ATII cell differentiation were evident. These cells were characterized by the presence of stem cell and epithelial markers and the absence of common mesenchymal and hematopoietic markers. (SSEA-1$^+$, pancytokeratin$^+$, krt-18$^+$, occludin$^+$, CD44$^-$, CD29 CD90$^-$, CD45$^-$). Interestingly these cells were able to support active replication of the influenza virus which subsequently caused cell lysis and a reduction in the progenitor population [63]. These cells may prove important in the development of novel therapeutic approaches for influenza. From the studies discussed above it is clear that there may be distinct differences related to the choice of animal model. A summary of the various stem cells identified in the lung is included in Table 1. It will be important to know how well the particular models reflect the progenitor populations in the human lung.

Until recently, there was no lung progenitor cell identified with the potential to generate ATII cells known in human lungs [16]. A clonogenic population of cells, coined alveolar epithelial type II progenitor cells or AEPCs, was isolated from adult human lungs that expressed both CD90 and vimentin (MSC markers) and pro-SPC, SPA, SPC, and SPD (ATII markers). The cells also expressed transcription factors found in the developing mesenchyme (Foxf1 and Tbx4) also indicating a mesenchyme origin of these AEPC. After in vitro differentiation the cells adopted a cuboidal-shaped morphology with lamellar bodies evident upon histological examination.

Table 1 Proposed stem/progenitor cells in the adult airways

Cell	Location	Markers	Progeny	Species	Reference
Clara	Trachea, main Bronchi, bronchioles	CCSP Scgb1a1	Goblet, Ciliated	Ms, Rt, Rb	[3, 14, 24–26, 28, 36]
Basal	Trachea, main bronchi, bronchioles	CD151, TF, TP63, Krt5, Krt14	Clara, Ciliated	Rb, Hu, Ms	[36–40]
Variant Clara	Close proximity to NEB	Scgb1a1, CYP-2F2, Sftpc, Fgfr2b, Snail1 E-Cad$^+$/Lgr6$^+$	ATII, ATI, Clara	Hu, Ms,	[14, 18, 19, 42, 51]
PNE	NEB	CGRP	Clara	Ms	[19]
BASC	BADJ	CD31$^-$CD45$^-$CD34$^+$ Sca-1$^+$	Clara, ATI, ATII	Ms	[43, 44, 48]
ATII	Alveoli	SPC, FOXM1, SSEA-1, Krt18, occludin, (CD44$^-$, CD29$^-$ CD90$^-$, CD45$^-$)	ATI	Ms, Pg, Rt	[52–57]
AEPC	No precise location	CD90, vimentin, pro-SPC, SPA, SPC, SPD	ATII	Hu	[16]
ATI	Alveoli	RTI40, AQP5	ATII	Rt	[61]
Ciliated	Trachea and main bronchi	FOXJ1	Goblet	Hu	[29]

CCSP Clara cell secretory protein, *FOXJ1* Forkhead box protein J1, *BASC* bronchioalveolar stem cell, *BADJ* bronchioalveolar duct junction, *NEB* neuroepithelial body, *PNE* pulmonary neuroendocrine cells, *ATI* alveolar type I, *ATII* alveolar type II. Species: *Ms* mouse, *Rt* rat, *Rb* rabbit, *Hu* human

The non-bold items are cells with less evidence for a role as an actual stem or progenitor cell

The expression of MSC markers was downregulated, while the expression of ATII markers was upregulated. Evidence suggests a role for the canonical wnt pathway in the transition from murine MSC to ATII [64]. Increasing knowledge about mesenchymal stem cells has led to several recent efforts to utilize them as novel cell-based therapeutic approaches for lung disease [65–68]. Preclinical data is particularly promising for the therapeutic benefit in acute lung injury [69, 70] and bronchopulmonary dysplasia and emphysema [71, 72]. In these studies several key features were noted including a preferential attraction of MSCs to the damaged lung versus normal lung, improved survival and attenuation of alveolar and lung vascular injury, weight gain, prevention of alveolar growth arrest, and suppression of inflammation of neonatal rats [72, 73]. It is important to note that the actual engraftment of exogenous MSC was particularly low in the lung; thus it is thought that the beneficial effects are mediated via a paracrine-mediated pathway. There is also potential use in the types of lung cancer. Using MSC as a vehicle to deliver IFN-alpha via i.v. injection in a mouse model of melanoma lung metastasis, a significant reduction in proliferation and angiogenesis and an increase in apoptosis reduced the growth of lung metastasis [74].

Going back to the ATII cells, their progenitor cell-like properties seem to make them targets for the development of lung cancer. ATII cells have long been associated with the development of type II cell carcinomas of varying glandular (acinar, adenoidcystic, or bronchioloalveolar) origin [46, 75–77]. While it is beyond the scope of this chapter to go into detail on this, it will be vital to decipher the molecular and cellular level on which this occurs. Furthermore, ATII cells are also targets of repeated lung injury leading to pulmonary fibrosis and lung cancer and again, little is known regarding the precise mechanisms governing this [78–80].

4 Stem Cells in the Adult Lung: Pulmonary Vasculature

The identity and role of stem and progenitor cells in the pulmonary vasculature are equally as complex as occurs in the airways. Such cells may be circulating or resident in the pulmonary vascular tissues themselves and recruitment of these cells in response to vascular stress or injury is routinely observed. What is somewhat more controversial is the role that those cells play once recruited: there is evidence for both pathogenic and restorative roles. Despite being seemingly contradictive is perfectly viable the stem and progenitor cells are indeed both. The identity and roles of such cells has recently been discussed in [21, 81, 82] and will be discussed briefly in the following sections of this chapter.

4.1 Endothelial Progenitor Cells

Endothelial progenitor cells or EPCs circulate within the bloodstream and have the ability to differentiate into mature endothelial cells. These cells are essential for adult neovascularization, both physiological and pathological [83]. EPC have also been shown to be both therapeutic and pathogenic having roles in the development of myocardial infarction, myocardial and limb ischemia, the healing of wounds, atherosclerosis, endogenous endothelial barrier repair, thrombosis, and vascularization of tumors [84–87].

The first concrete evidence for the existence of an EPC came in the late 1990s when the generation of mature endothelial cells from $CD34^+$ and von Willebrand factor (vWF^+) bone marrow-derived cells with a capacity for acetylated low-density lipoprotein (LDL) uptake was demonstrated [88, 89]. Prior to this discovery new blood vessel formation was thought to rise from the proliferation, migration, and remodeling of mature endothelial cells. Precise identification of an EPC is confounded by similarities to circulating endothelial cells and hematopoietic stem cells. A number of key features can be used to more precisely identify an EPC [90]: (1) they are circulating bone marrow-derived cells with a phenotype and function distinct from a mature endothelial cell; (2) they are capable of differentiation to fully functional endothelial cells; (3) they can undergo vasculogenesis; (4) common cell

surface markers include CD34, VEGFR2 (KDR, Flk-1), and CD133; and (5) they are able to uptake LDL [91–93].

EPC in the pulmonary vasculature have been extensively investigated. A hierarchy exists with subdivisions characterized by the cells' potential to divide and proliferate in a clonogenic nature [94, 95]. The terminology EPC is often used to refer to all of these cells when it should be restricted to those cells fitting the criteria outlined above. As mentioned above, the roles of EPCs in the pulmonary vasculature are paradoxical: physiological, having the ability to repair the pulmonary endothelium and pathological, where they can migrate and differentiate to mesenchymal cells contributing to intimal hyperplasia. The latter will be discussed in Sect. 4.1.1.

The physiological properties of EPC whereby the can restore function to the endothelial barrier has been extensively studied and, indeed, clinical trials are underway exploiting this property for the treatment of pulmonary arterial hypertension [96–98]. The Pulmonary Hypertension and eNOS Cell Therapy (PHACeT) trial is a phase 2a dose-ranging randomized trial started in 2007 comparing the effects of conventional therapy with or without the intravenous infusion of EPC in patients with IPAH; significant improvements in the mean walk test, mean pulmonary arterial pressure, pulmonary vascular resistance, and cardiac output in the EPC treated [96]. This trial is ongoing and highlights the potential for clinical use of EPC [98].

Data obtained in animal models of pulmonary hypertension (PH) have shown significant therapeutic potential for exogenous EPC. Transplantation of autologous EPC from peripheral blood caused neovascularization and a reduction in mean pulmonary arterial pressure (mPAP), cardiac output (CO), and pulmonary vascular resistance (PVR) in both canine and rat monocrotaline (MCT)-induced PH models [99, 100]. Observations from Zhao et. al further showed that in MCT rats delivery of fluorescently labeled endothelial-like progenitor cells lead to a marked improvement in survival, which was significantly greater in EPC transduced with eNOS [100]. EPC are mobilized in response to vascular injury; increased VEGF and shear stress are factors known to mobilize EPC and promote their differentiation to mature endothelial cells [101]. Once mobilized or transplanted how do these cells get recruited to the site of injury? Homing is most likely to involve chemokine receptor CXCR4 and the chemoattractant pull of SDF-1 released from EC and platelets. High levels of β2 integrins on EPC can interact with their ligands P-selectin, E-selectin, and ICAM-1 that are expressed on EC [102] and studies in mice indicate a role for erythropoietin/erythropoietin receptor (Epo/EpoR) system in recruiting EPC to the pulmonary [103]. It is known that a severe depletion of circulating EPCs correlates to the development of chronic lung disease, idiopathic pulmonary fibrosis (IPF), and PH [104, 105]. IPF patients developing secondary PH had a substantially worse depletion of EPC [105]; however, an increase in circulating endothelial cells was demonstrated recently in the most severe of IPF [106]. The number of circulating EPC is now known to correlate to cardiovascular risk and a disposable microfluidic platform capable of selectively capturing and enumerating EPC directly from human whole blood has been developed with the aim of being used in screening and monitoring of patients with PAH [107].

4.1.1 Endothelial Mesenchymal Transition

The ability of pulmonary endothelial cells to trans-differentiate to mesenchymal cells was first described in 1992 by Arciniegas et al. [108]. Cells are able to undergo a transformation from vWF-expressing endothelial cells to α-smooth muscle actin (α-SMA)-expressing smooth muscle-like mesenchymal cells (myofibroblasts). It is believed that such a transition is both important for embryonic development, in the pathogenesis of pulmonary hypertension by contributing to the significant vascular remodeling and in pathological lung fibrosis. The myofibroblast is believed to be an intermediary cell that is a key mediator of pulmonary fibrosis [109–112]. A myofibroblast may derive from resident mesenchymal cells and epithelial or endothelial cells via epithelial/endothelial–mesenchymal (EMT/EndMT) transition [113]. EndMT can be induced by transforming growth factor β (Tgf-β) signaling through Tgf-β receptor 1 (Tgf-βR1) in vitro [108, 114]. Marked increases in transcriptional repressors snail and slug are observed during EndMT and depletion of these factors has been shown to be sufficient to block the process, as was inhibition of the Tgf-βR1 [114, 115]. Caveolin-1 is known to be important for Tgf-βR1 signaling and studies in murine lung ECs also confirm a prominent role for caveolin-1 in EndMT [116]. In several animal models of pulmonary fibrosis (bleomycin induced), EndMT is evident as a major pathological process [110, 112]. In addition to EndMT, EPCs are known to substantially contribute to the development of plexiform lesions in PH [117] and to the fibrotic embolism in patients with CTEPH [118].

From both a clinical and experimental perspective it is particularly noteworthy that using imatinib mesylate (Gleevec®), a cAbl kinase inhibitor, or rottlerin, a PKC-δ inhibitor, was able to significantly block TGF-β-induced α-SMA expression and snail-1 induction thus preventing EndMT [115]. The activity of these small molecules in preventing EndMT may prove to be effective therapeutic approaches for pulmonary fibrosis. Unfortunately the first clinical trial using imatinib to treat idiopathic pulmonary fibrosis did not show very promising effects on survival or lung function [119]. A greater understanding of EndMT is necessary to develop novel therapeutic approaches for pulmonary fibrosis (Fig. 7).

4.2 Other Circulating Stem Cells

4.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells (multipotential stem cells or MSC) are also bone marrow-derived circulating stem cells. This cell population has been fairly extensively investigated; however, definitive cell surface markers still remain elusive. Currently they are characterized by a panel of cell surface markers ($CD29^+$, $CD105^+$, $CD73^+$, $CD44^+$, $CD90^+$, $CD166^+$, $CD45^-$, $CD14^-$, and $CD11b^-$ to name a few), their adherence and growth on plastic surfaces and their ability to

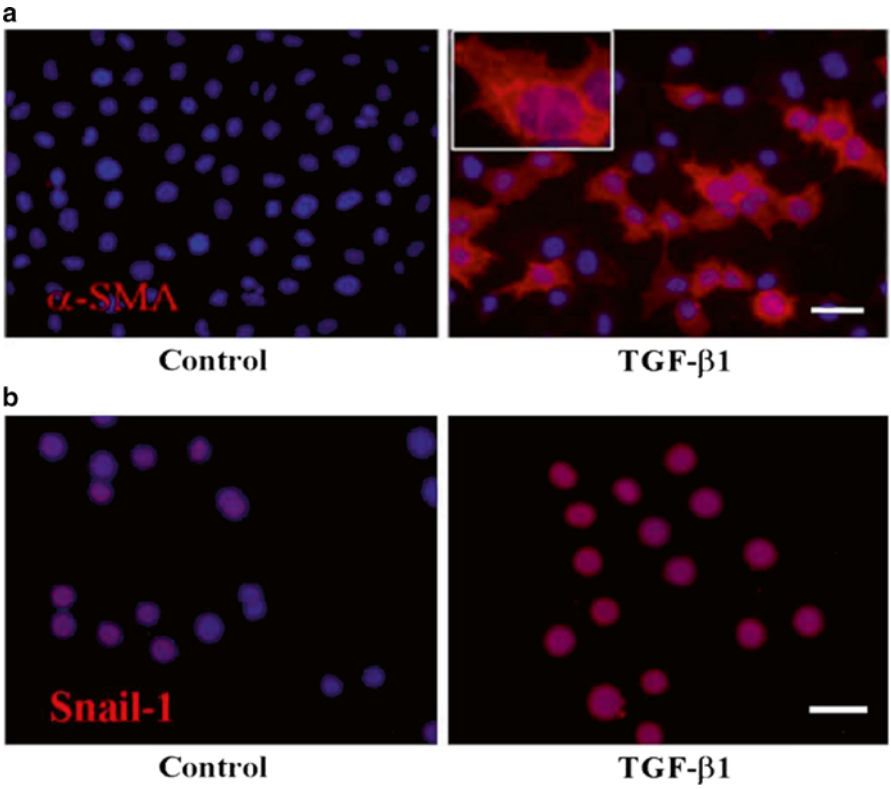

Fig. 7 Endothelial mesenchymal transition (EndMT). Primary pulmonary endothelial cells were treated with TGF-β1. (**a**) Endothelial cells acquire a fibroblast-like appearance and initiate expression of α-SMA. (**b**) Upregulation of Snail during EndMT. Reproduced with kind permission from [115]

differentiate into, at minimum, adipocytes, chondrocytes, and osteoblasts [120, 121]. MSC are known to be resident is a wide variety of postnatal perivascular tissues including the adventitial layer of the pulmonary vasculature [122, 123]. They are known to have the property of strong homing to the lung and thus have been widely investigated a therapeutic approach for pulmonary vascular disease. The mechanisms by which MSC preferentially home to the lung are largely unknown; however, it appears that circulating platelets can facilitate it [124]. The homing was prevented by use of an anti-P-selectin antibody and tirofiban, a glycoprotein (integrin) IIb/IIIa inhibitor, indicating the involvement of platelets in the homing process. Homing is also demonstrated through the stromal-derived factor-1 (SDF-1)/CXCR4 pathway [125, 126]. A number of studies are now utilizing ex vivo MSC derived from bone marrow, adipose tissues, and embryonic and induced pluripotent stem cells either as a direct cellular therapy or as a modality

to deliver specific genes to lung [124, 127–132]. It is beyond the scope of this particular chapter to discuss these in detail by readers are encouraged to look at recent reviews [21, 65, 98, 133, 134].

The value of MSC as a tool for drug/gene delivery is demonstrated in experiments where considerable improvements in the pathogenesis of PH have been observed. Agents including angiopoietin-1 for acute lung injury [135], endothelial nitric oxide synthase (eNOS) for PAH-related right ventricular impairment [136], heme-oxygenase-1 for PH [137], CGRP in SMC proliferation [132], and prostacyclin-synthase for PH [138] have all been delivered by MSC cell therapy with positive therapeutic effects. On the other hand a distinct pathogenic role for these cells has also been widely demonstrated. Circulating fibrocytes (progenitor derivatives of MSC) contribute extracellular matrix deposition in pulmonary fibrosis [139]. They also contribute to pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension, along with MSC [140–142]. Here inhibition of the CXCR4/SDF-1-signaling pathway can prevent the mobilization of BM-MSC to the pulmonary vasculature [143]. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM α) may also act as a chemotactic agent for BM-MSC-mediated remodeling of the pulmonary vasculature [144]. The expression of 5-HT_{2B} receptors on BM-MSC is also implicated in the development of PAH in mice [145]. In this mouse study, restricted expression of 5-HT_{2B} receptors in bone marrow cells developed hypoxia or monocrotaline-induced PH; however, restricted expression of 5-HT_{2B} receptors conferred a complete resistance [145]. It should be noted that MSC-like cells have been shown to be recruited to the fibrotic embolism in chronic thromboembolic pulmonary hypertension (CTEPH) [146] and to be prominent in the development of hypoxia-induced pulmonary vascular remodeling [147]. The precise nature of the MSC recruitment to these areas is not clear. It is also interesting to note that intratracheal delivery of MSC in rats with CTEPH saw a substantial improvement in relevant clinical parameters [125]. The therapeutic benefit was both local and systemic, having notable effects on secondary liver fibrosis likely via reductions in caspase 3 cleavage and NFκB signaling [125]. It is seemingly evident that resident MSC or MSC-like cells can be recruited to sites of lung injury potentially contributing to the pathogenesis of the disease, whereas as exogenous delivery of MSC poses a significant therapeutic benefit.

4.2.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSC) are the most extensively studied and characterized stem cell population. They have the capacity for long-term self-renewal and differentiation into every cell type in the blood. In the lung their roles are less defined though HSC or HSC-like progenitor cells have been shown to be present in both the airways and the vasculature in the lung. Adult HSC reside primarily in the bone marrow and are round nonadherent cells with a high nucleus–cytoplasm ratio. They can be mobilized from the niche and the SDF-1/CXCL12)/CXCR4 axis is critical

for such mobilization and subsequent homing of HSC [148]. This mechanism has also been shown to be important for homing of c-Kit$^+$ hematopoietic progenitor cells to a perivascular pulmonary niche in mice [149]. In this study, antagonism of CXCR4 prevented PH and reduced the associated vascular remodeling and perivascular accumulation of hematopoietic progenitor cells [149].

Hematopoietic progenitor cells have also been shown to accumulate at sites of injury in the lung [150–152]. A recent study identified a clonogenic population of hematopoietic progenitor cells using a novel organotypic ex vivo pneumoexplant model of mammalian lung injury [153]. Repopulating CD45$^+$ and CD11b$^+$ cells, with subpopulations enriched in PECAM, AQP5, αSMA, SCGB1A1, and SPC, were identified in the free-floating anchorage-independent cells (termed AICs) [153]. The authors postulated that AICs could be resident stem cells in the lung interstitium which are activated/mobilized in response to lung injury. Further studies are necessary to fully characterize the regenerative potential and in vivo function of these cells. These cells have a signature very similar to that of cells referred to as fibrocytes characterized by the expression of both leukocytic markers (CD45, CD34, CD11b, CD14) and mesenchymal markers (α1-procollagen) [154]. Fibrocytes are known to be rapidly recruited to the adventitia in animals exposed to chronic hypoxia where they differentiate to myofibroblast [147]. Importantly, depletion of fibrocytes from the circulation could attenuate vascular remodeling. Fibrocytes and the pathogenesis of a variety of lung disease have been discussed in a number of recent reviews [155–159].

4.3 Resident Tissue-Specific Stem Cells

It is also possible the adventitia provides a niche where populations of dormant stem cells reside and can be activated in response to certain physiological changes in the vasculature. The diagram in Fig. 8 shows the potential niches for stem cells in the pulmonary vasculature. Indeed a subpopulation of adventitial fibroblasts are activated by a variety of stimuli, such as hypoxia, and differentiate into cells with a myofibroblast phenotype [160]. The accumulation of αSMA in the adventitial fibroblasts is the key phenotypic change during de-differentiation to a myofibroblast. It is possible that these progenitor cells are also a subpopulation of MSC [122]. In studies looking at the cellular mechanisms contributing to CTEPH, a large number of myofibroblasts were present in the fibrotic clot occluding the airway; these cells had the properties of a myofibroblast and were capable of multilineage differentiation like MSC [146]. It seems plausible that this adventitial-residing progenitor cell can trans-differentiate to a myofibroblast and contribute to the progression of many fibroblastic lung diseases.

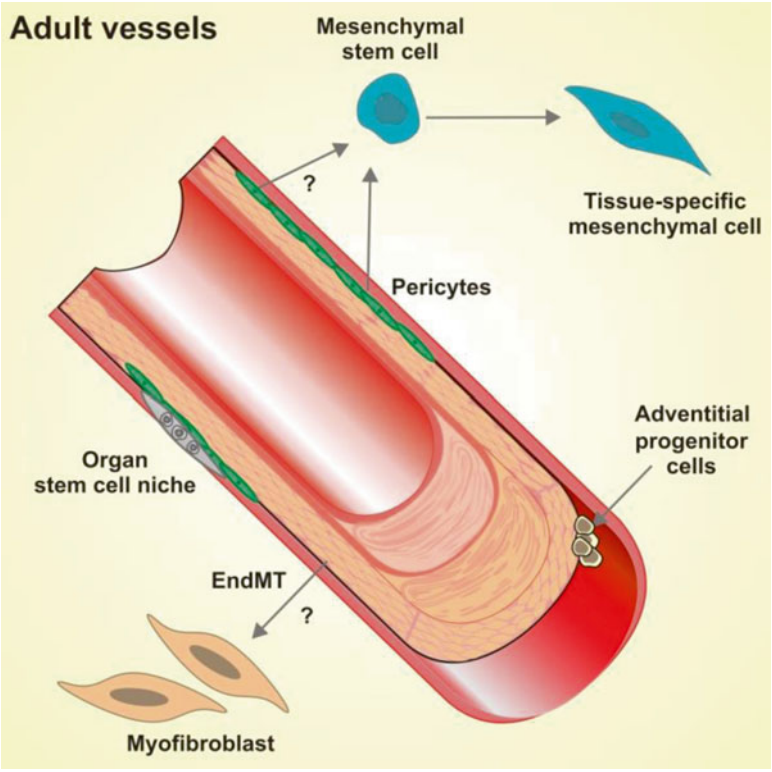

Fig. 8 Stem cell niches in the pulmonary vasculature. Adult blood vessels provide an environmental niche where organ-specific stem cells, pericytes, and endothelial cells can exist. Endothelial cells are able to undergo endothelial to mesenchymal transition (EndMT) and pericytes and other resident cells are able to give rise to mesenchymal progenitor cells contributing to the mesenchymal/myofibroblast cell pool. In addition circulating fibrocytes and endothelial progenitor cells are progenitors that exist circulating through the lumen of blood vessels

5 Conclusions

This chapter provides a recent update on the identification of stem cells within the airways and vasculature of the lung. While there has been an extensive amount of research into the identity and location of stem cells, it is evident that the precise identification of stem and progenitor cells is not easy. Furthermore, the roles of these stem cells in physiological and pathophysiological processes is complicated and likely confounded by the microenvironments in which they reside. On the other hand, exogenous application of EPC and MSC seems to have substantial therapeutic promise. It will be exciting to follow developments in the complicated field of stem cells in the adult lung as they emerge.

References

1. Stripp BR (2008) Hierarchical organization of lung progenitor cells: is there an adult lung tissue stem cell? Proc Am Thorac Soc 5:695–698
2. Plopper CG, Hyde DM (2008) The non-human primate as a model for studying COPD and asthma. Pulm Pharmacol Ther 21:755–766
3. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD et al (2004) Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. Am J Respir Cell Mol Biol 31:382–394
4. Que J, Choi M, Ziel JW, Klingensmith J, Hogan BL (2006) Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. Differentiation 74:422–437
5. Que J, Luo X, Schwartz RJ, Hogan BL (2009) Multiple roles for Sox2 in the developing and adult mouse trachea. Development 136:1899–1907
6. Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL (1997) Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 124:53–63
7. Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL (1997) Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 124:4867–4878
8. Weaver M, Dunn NR, Hogan BL (2000) Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. Development 127:2695–2704
9. Whitsett JA, Clark JC, Picard L, Tichelaar JW, Wert SE, Itoh N, Perl AK, Stahlman MT (2002) Fibroblast growth factor 18 influences proximal programming during lung morphogenesis. J Biol Chem 277:22743–22749
10. Maeda Y, Dave V, Whitsett JA (2007) Transcriptional control of lung morphogenesis. Physiol Rev 87:219–244
11. Jacobs IJ, Ku WY, Que J (2012) Genetic and cellular mechanisms regulating anterior foregut and esophageal development. Dev Biol 369:54–64
12. Morrisey EE, Hogan BL (2010) Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell 18:8–23
13. Gomperts BN, Belperio JA, Fishbein MC, Keane MP, Burdick MD, Strieter RM (2007) Keratinocyte growth factor improves repair in the injured tracheal epithelium. Am J Respir Cell Mol Biol 37:48–56
14. Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, Wang F, Hogan BL (2009) The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell 4:525–534
15. Plopper CG, Nishio SJ, Alley JL, Kass P, Hyde DM (1992) The role of the nonciliated bronchiolar epithelial (Clara) cell as the progenitor cell during bronchiolar epithelial differentiation in the perinatal rabbit lung. Am J Respir Cell Mol Biol 7:606–613
16. Fujino N, Kubo H, Suzuki T, Ota C, Hegab AE, He M, Suzuki S, Yamada M, Kondo T, Kato H et al (2011) Isolation of alveolar epithelial type II progenitor cells from adult human lungs. Lab Invest 91:363–378
17. Hollande E, Cantet S, Ratovo G, Daste G, Bremont F, Fanjul M (2004) Growth of putative progenitors of type II pneumocytes in culture of human cystic fibrosis alveoli. Biol Cell 96:429–441
18. Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR (2001) Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am J Respir Cell Mol Biol 24:671–681
19. Reynolds SD, Giangreco A, Power JH, Stripp BR (2000) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol 156:269–278

20. Kim CF (2007) Paving the road for lung stem cell biology: bronchioalveolar stem cells and other putative distal lung stem cells. Am J Physiol Lung Cell Mol Physiol 293:L1092–L1098
21. Firth AL, Yuan JX (2012) Identification of functional progenitor cells in the pulmonary vasculature. Pulm Circ 2:84–100
22. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G et al (2001) Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med 163:517–523
23. Maestrelli P, Saetta M, Mapp CE, Fabbri LM (2001) Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 164:S76–S80
24. Hayashi T, Ishii A, Nakai S, Hasegawa K (2004) Ultrastructure of goblet-cell metaplasia from Clara cell in the allergic asthmatic airway inflammation in a mouse model of asthma in vivo. Virchows Arch 444:66–73
25. Reader JR, Tepper JS, Schelegle ES, Aldrich MC, Putney LF, Pfeiffer JW, Hyde DM (2003) Pathogenesis of mucous cell metaplasia in a murine asthma model. Am J Pathol 162:2069–2078
26. Kim S, Shim JJ, Burgel PR, Ueki IF, Dao-Pick T, Tam DC, Nadel JA (2002) IL-13-induced Clara cell secretory protein expression in airway epithelium: role of EGFR signaling pathway. Am J Physiol Lung Cell Mol Physiol 283:L67–L75
27. Van Winkle LS, Isaac JM, Plopper CG (1997) Distribution of epidermal growth factor receptor and ligands during bronchiolar epithelial repair from naphthalene-induced Clara cell injury in the mouse. Am J Pathol 151:443–459
28. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA (1999) Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci U S A 96:3081–3086
29. Turner J, Roger J, Fitau J, Combe D, Giddings J, Heeke GV, Jones CE (2011) Goblet cells are derived from a FOXJ1-expressing progenitor in a human airway epithelium. Am J Respir Cell Mol Biol 44:276–284
30. Inayama Y, Hook GE, Brody AR, Cameron GS, Jetten AM, Gilmore LB, Gray T, Nettesheim P (1988) The differentiation potential of tracheal basal cells. Lab Invest 58:706–717
31. Brody JS, Joyce-Brady M, Paine R (1987) Lung cell differentiation. Mead Johnson Symp Perinat Dev Med (30):39–42
32. Moller PC, Partridge LR, Cox RA, Pellegrini V, Ritchie DG (1989) The development of ciliated and mucus cells from basal cells in hamster tracheal epithelial cell cultures. Tissue Cell 21:195–198
33. Johnson NF, Hubbs AF (1990) Epithelial progenitor cells in the rat trachea. Am J Respir Cell Mol Biol 3:579–585
34. Boers JE, Ambergen AW, Thunnissen FB (1998) Number and proliferation of basal and parabasal cells in normal human airway epithelium. Am J Respir Crit Care Med 157:2000–2006
35. Evans MJ, Shami SG, Cabral-Anderson LJ, Dekker NP (1986) Role of nonciliated cells in renewal of the bronchial epithelium of rats exposed to NO2. Am J Pathol 123:126–133
36. Nettesheim P, Jetten AM, Inayama Y, Brody AR, George MA, Gilmore LB, Gray T, Hook GE (1990) Pathways of differentiation of airway epithelial cells. Environ Health Perspect 85:317–329
37. Zepeda ML, Chinoy MR, Wilson JM (1995) Characterization of stem cells in human airway capable of reconstituting a fully differentiated bronchial epithelium. Somat Cell Mol Genet 21:61–73
38. Hajj R, Baranek T, Le Naour R, Lesimple P, Puchelle E, Coraux C (2007) Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. Stem Cells 25:139–148
39. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR (2004) Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am J Pathol 164:577–588

40. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL (2009) Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci U S A 106:12771–12775
41. Rock JR, Randell SH, Hogan BL (2010) Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech 3:545–556
42. Volckaert T, Dill E, Campbell A, Tiozzo C, Majka S, Bellusci S, De Langhe SP (2011) Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. J Clin Invest 121:4409–4419
43. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121:823–835
44. Giangreco A, Reynolds SD, Stripp BR (2002) Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am J Pathol 161:173–182
45. Yang YS, Yang MC, Weissler JC (2011) Pleiomorphic adenoma gene-like 2 expression is associated with the development of lung adenocarcinoma and emphysema. Lung Cancer 74:12–24
46. Xu X, Rock JR, Lu Y, Futtner C, Schwab B, Guinney J, Hogan BL, Onaitis MW (2012) Evidence for type II cells as cells of origin of K-Ras-induced distal lung adenocarcinoma. Proc Natl Acad Sci U S A 109:4910–4915
47. Cuzic S, Bosnar M, Kramaric MD, Ferencic Z, Markovic D, Glojnaric I, Erakovic Haber V (2012) Claudin-3 and Clara cell 10 kDa protein as early signals of cigarette smoke-induced epithelial injury along alveolar ducts. Toxicol Pathol 40:1169–1187
48. Qian S, Ding JY, Xie R, An JH, Ao XJ, Zhao ZG, Sun JG, Duan YZ, Chen ZT, Zhu B (2008) MicroRNA expression profile of bronchioalveolar stem cells from mouse lung. Biochem Biophys Res Commun 377:668–673
49. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ et al (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003–1007
50. de Visser KE, Ciampricotti M, Michalak EM, Tan DW, Speksnijder EN, Hau CS, Clevers H, Barker N, Jonkers J (2012) Developmental stage-specific contribution of LGR5(+) cells to basal and luminal epithelial lineages in the postnatal mammary gland. J Pathol 228:300–309
51. Oeztuerk-Winder F, Guinot A, Ochalek A, Ventura JJ (2012) Regulation of human lung alveolar multipotent cells by a novel p38alpha MAPK/miR-17-92 axis. EMBO J 31:3431–3441
52. Ward HE, Nicholas TE (1984) Alveolar type I and type II cells. Aust N Z J Med 14:731–734
53. Harris JB, Chang LY, Crapo JD (1991) Rat lung alveolar type I epithelial cell injury and response to hyperoxia. Am J Respir Cell Mol Biol 4:115–125
54. Miller BE, Hook GE (1990) Hypertrophy and hyperplasia of alveolar type II cells in response to silica and other pulmonary toxicants. Environ Health Perspect 85:15–23
55. Adamson IY, Bowden DH (1975) Derivation of type 1 epithelium from type 2 cells in the developing rat lung. Lab Invest 32:736–745
56. Evans MJ, Cabral LJ, Stephens RJ, Freeman G (1975) Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. Exp Mol Pathol 22:142–150
57. Kinnard WV, Tuder R, Papst P, Fisher JH (1994) Regulation of alveolar type II cell differentiation and proliferation in adult rat lung explants. Am J Respir Cell Mol Biol 11:416–425
58. Dobbs LG, Geppert EF, Williams MC, Greenleaf RD, Mason RJ (1980) Metabolic properties and ultrastructure of alveolar type II cells isolated with elastase. Biochim Biophys Acta 618:510–523
59. Joyce-Brady MF, Brody JS (1990) Ontogeny of pulmonary alveolar epithelial markers of differentiation. Dev Biol 137:331–348
60. Uhal BD (1997) Cell cycle kinetics in the alveolar epithelium. Am J Physiol 272:L1031–L1045

61. Gonzalez RF, Allen L, Dobbs LG (2009) Rat alveolar type I cells proliferate, express OCT-4, and exhibit phenotypic plasticity in vitro. Am J Physiol Lung Cell Mol Physiol 297:L1045–L1055
62. Liu Y, Sadikot RT, Adami GR, Kalinichenko VV, Pendyala S, Natarajan V, Zhao YY, Malik AB (2011) FoxM1 mediates the progenitor function of type II epithelial cells in repairing alveolar injury induced by Pseudomonas aeruginosa. J Exp Med 208:1473–1484
63. Khatri M, Goyal SM, Saif YM (2012) Oct4+ stem/progenitor swine lung epithelial cells are targets for influenza virus replication. J Virol 86:6427–6433
64. Liu AR, Liu L, Chen S, Yang Y, Zhao HJ, Guo FM, Lu XM, Qiu HB (2013) Activation of canonical wnt pathway promotes differentiation of mouse bone marrow-derived MSCs into type II alveolar epithelial cells, confers resistance to oxidative stress, and promotes their migration to injured lung tissue in vitro. J Cell Physiol 228(6):1270–1283
65. Gotts JE, Matthay MA (2011) Mesenchymal stem cells and acute lung injury. Crit Care Clin 27:719–733
66. Sun J, Han ZB, Liao W, Yang SG, Yang Z, Yu J, Meng L, Wu R, Han ZC (2011) Intrapulmonary delivery of human umbilical cord mesenchymal stem cells attenuates acute lung injury by expanding CD4+CD25+ Forkhead Boxp3 (FOXP3)+ regulatory T cells and balancing anti- and pro-inflammatory factors. Cell Physiol Biochem 27:587–596
67. Xue J, Li X, Lu Y, Gan L, Zhou L, Wang Y, Lan J, Liu S, Sun L, Jia L et al (2013) Gene-modified mesenchymal stem cells protect against radiation-induced lung injury. Mol Ther 21(2):456–465
68. Zhang X, Wang H, Shi Y, Peng W, Zhang S, Zhang W, Xu J, Mei Y, Feng Z (2012) Role of bone marrow-derived mesenchymal stem cells in the prevention of hyperoxia-induced lung injury in newborn mice. Cell Biol Int 36:589–594
69. Matthay MA, Goolaerts A, Howard JP, Lee JW (2010) Mesenchymal stem cells for acute lung injury: preclinical evidence. Crit Care Med 38:S569–S573
70. Matthay MA, Thompson BT, Read EJ, McKenna DH Jr, Liu KD, Calfee CS, Lee JW (2010) Therapeutic potential of mesenchymal stem cells for severe acute lung injury. Chest 138:965–972
71. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ, Galipeau J, Haromy A, Eaton F et al (2009) Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med 180:1131–1142
72. Zhang H, Fang J, Su H, Yang M, Lai W, Mai Y, Wu Y (2012) Bone marrow mesenchymal stem cells attenuate lung inflammation of hyperoxic newborn rats. Pediatr Transplant 16:589–598
73. Aslam M, Baveja R, Liang OD, Fernandez-Gonzalez A, Lee C, Mitsialis SA, Kourembanas S (2009) Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. Am J Respir Crit Care Med 180:1122–1130
74. Ren C, Kumar S, Chanda D, Chen J, Mountz JD, Ponnazhagan S (2008) Therapeutic potential of mesenchymal stem cells producing interferon-alpha in a mouse melanoma lung metastasis model. Stem Cells 26:2332–2338
75. TenHave-Opbroek AA, Hammond WG, Benfield JR, Teplitz RL, Dijkman JH (1993) Expression of alveolar type II cell markers in acinar adenocarcinomas and adenoid cystic carcinomas arising from segmental bronchi. A study in a heterotopic bronchogenic carcinoma model in dogs. Am J Pathol 142:1251–1264
76. Lin C, Song H, Huang C, Yao E, Gacayan R, Xu SM, Chuang PT (2012) Alveolar type II cells possess the capability of initiating lung tumor development. PLoS One 7:e53817
77. Ten Have-Opbroek AA, Benfield JR, van Krieken JH, Dijkman JH (1997) The alveolar type II cell is a pluripotential stem cell in the genesis of human adenocarcinomas and squamous cell carcinomas. Histol Histopathol 12:319–336
78. Garcia CK (2011) Idiopathic pulmonary fibrosis: update on genetic discoveries. Proc Am Thorac Soc 8:158–162

79. Banerjee ER, Henderson WR Jr (2012) Characterization of lung stem cell niches in a mouse model of bleomycin-induced fibrosis. Stem Cell Res Ther 3:21
80. Vaughan AE, Chapman HA (2013) Regenerative activity of the lung after epithelial injury. Biochim Biophys Acta 1832(7):922–930
81. Yeager ME, Frid MG, Stenmark KR (2011) Progenitor cells in pulmonary vascular remodeling. Pulm Circ 1:3–16
82. Gomez-Gaviro MV, Lovell-Badge R, Fernandez-Aviles F, Lara-Pezzi E (2012) The vascular stem cell niche. J Cardiovasc Transl Res 5:618–630
83. Masuda H, Kalka C, Asahara T (2000) Endothelial progenitor cells for regeneration. Hum Cell 13:153–160
84. Nuzzolo ER, Iachininoto MG, Teofili L (2012) Endothelial progenitor cells and thrombosis. Thromb Res 129:309–313
85. Liu J, Huang J, Yao WY, Ben QW, Chen DF, He XY, Li L, Yuan YZ (2012) The origins of vacularization in tumors. Front Biosci 17:2559–2565
86. Boos CJ, Lip GY, Blann AD (2006) Circulating endothelial cells in cardiovascular disease. J Am Coll Cardiol 48:1538–1547
87. Kolvenbach R, Kreissig C, Ludwig E, Cagiannos C (2007) Stem cell use in critical limb ischemia. J Cardiovasc Surg (Torino) 48:39–44
88. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR et al (1998) Evidence for circulating bone marrow-derived endothelial cells. Blood 92:362–367
89. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967
90. Mead LE, Prater D, Yoder MC, Ingram DA (2008) Isolation and characterization of endothelial progenitor cells from human blood. Curr Protoc Stem Cell Biol Chapter 2:Unit 2C 1
91. Voyta JC, Via DP, Butterfield CE, Zetter BR (1984) Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol 99:2034–2040
92. Hristov M, Erl W, Weber PC (2003) Endothelial progenitor cells: isolation and characterization. Trends Cardiovasc Med 13:201–206
93. Resch T, Pircher A, Kahler CM, Pratschke J, Hilbe W (2012) Endothelial progenitor cells: current issues on characterization and challenging clinical applications. Stem Cell Rev 8:926–939
94. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 104:2752–2760
95. Alvarez DF, Huang L, King JA, ElZarrad MK, Yoder MC, Stevens T (2008) Lung microvascular endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. Am J Physiol Lung Cell Mol Physiol 294:L419–L430
96. Wang XX, Zhang FR, Shang YP, Zhu JH, Xie XD, Tao QM, Chen JZ (2007) Transplantation of autologous endothelial progenitor cells may be beneficial in patients with idiopathic pulmonary arterial hypertension: a pilot randomized controlled trial. J Am Coll Cardiol 49:1566–1571
97. Jurasz P, Courtman D, Babaie S, Stewart DJ (2010) Role of apoptosis in pulmonary hypertension: from experimental models to clinical trials. Pharmacol Ther 126:1–8
98. Stewart DJ, Mei SH (2011) Cell-based therapies for lung vascular diseases: lessons for the future. Proc Am Thorac Soc 8:535–540
99. Takahashi M, Nakamura T, Toba T, Kajiwara N, Kato H, Shimizu Y (2004) Transplantation of endothelial progenitor cells into the lung to alleviate pulmonary hypertension in dogs. Tissue Eng 10:771–779
100. Zhao YD, Courtman DW, Deng Y, Kugathasan L, Zhang Q, Stewart DJ (2005) Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease. Circ Res 96:442–450

101. Obi S, Yamamoto K, Shimizu N, Kumagaya S, Masumura T, Sokabe T, Asahara T, Ando J (2009) Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol 106:203–211
102. Zampetaki A, Kirton JP, Xu Q (2008) Vascular repair by endothelial progenitor cells. Cardiovasc Res 78:413–421
103. Satoh K, Kagaya Y, Nakano M, Ito Y, Ohta J, Tada H, Karibe A, Minegishi N, Suzuki N, Yamamoto M et al (2006) Important role of endogenous erythropoietin system in recruitment of endothelial progenitor cells in hypoxia-induced pulmonary hypertension in mice. Circulation 113:1442–1450
104. Junhui Z, Xingxiang W, Guosheng F, Yunpeng S, Furong Z, Junzhu C (2008) Reduced number and activity of circulating endothelial progenitor cells in patients with idiopathic pulmonary arterial hypertension. Respir Med 102:1073–1079
105. Fadini GP, Schiavon M, Rea F, Avogaro A, Agostini C (2007) Depletion of endothelial progenitor cells may link pulmonary fibrosis and pulmonary hypertension. Am J Respir Crit Care Med 176:724–725, author reply 725
106. Smadja DM, Mauge L, Nunes H, d'Audigier C, Juvin K, Borie R, Carton Z, Bertil S, Blanchard A, Crestani B et al (2013) Imbalance of circulating endothelial cells and progenitors in idiopathic pulmonary fibrosis. Angiogenesis 16:147–157
107. Hansmann G, Plouffe BD, Hatch A, von Gise A, Sallmon H, Zamanian RT, Murthy SK (2011) Design and validation of an endothelial progenitor cell capture chip and its application in patients with pulmonary arterial hypertension. J Mol Med (Berl) 89:971–983
108. Arciniegas E, Sutton AB, Allen TD, Schor AM (1992) Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. J Cell Sci 103(Pt 2):521–529
109. Arciniegas E, Ponce L, Hartt Y, Graterol A, Carlini RG (2000) Intimal thickening involves transdifferentiation of embryonic endothelial cells. Anat Rec 258:47–57
110. Hashimoto N, Phan SH, Imaizumi K, Matsuo M, Nakashima H, Kawabe T, Shimokata K, Hasegawa Y (2010) Endothelial-mesenchymal transition in bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 43:161–172
111. Piera-Velazquez S, Li Z, Jimenez SA (2011) Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am J Pathol 179:1074–1080
112. Piera-Velazquez S, Jimenez SA (2012) Molecular mechanisms of endothelial to mesenchymal cell transition (EndoMT) in experimentally induced fibrotic diseases. Fibrogenesis Tissue Repair 5(Suppl 1):S7
113. Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 214:199–210
114. Diez M, Musri MM, Ferrer E, Barbera JA, Peinado VI (2010) Endothelial progenitor cells undergo an endothelial-to-mesenchymal transition-like process mediated by TGFbetaRI. Cardiovasc Res 88:502–511
115. Li Z, Jimenez SA (2011) Protein kinase Cdelta and c-Abl kinase are required for transforming growth factor beta induction of endothelial-mesenchymal transition in vitro. Arthritis Rheum 63:2473–2483
116. Li Z, Wermuth PJ, Benn BS, Lisanti MP, Jimenez SA (2013) Caveolin-1 deficiency induces spontaneous endothelial-to-mesenchymal transition in murine pulmonary endothelial cells in vitro. Am J Pathol 182(2):325–331
117. Toshner M, Voswinckel R, Southwood M, Al-Lamki R, Howard LS, Marchesan D, Yang J, Suntharalingam J, Soon E, Exley A et al (2009) Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. Am J Respir Crit Care Med 180:780–787
118. Yao W, Firth AL, Sacks RS, Ogawa A, Auger WR, Fedullo PF, Madani MM, Lin GY, Sakakibara N, Thistlethwaite PA et al (2009) Identification of putative endothelial progenitor cells (CD34+CD133+Flk-1+) in endarterectomized tissue of patients with chronic thromboembolic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 296:L870–L878
119. Daniels CE, Lasky JA, Limper AH, Mieras K, Gabor E, Schroeder DR (2010) Imatinib treatment for idiopathic pulmonary fibrosis: randomized placebo-controlled trial results. Am J Respir Crit Care Med 181:604–610

120. Mafi P, Hindocha S, Mafi R, Griffin M, Khan WS (2011) Adult mesenchymal stem cells and cell surface characterization – a systematic review of the literature. Open Orthop J 5:253–260
121. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317
122. Hoshino A, Chiba H, Nagai K, Ishii G, Ochiai A (2008) Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. Biochem Biophys Res Commun 368:305–310
123. da Silva Meirelles L, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
124. Jiang L, Song XH, Liu P, Zeng CL, Huang ZS, Zhu LJ, Jiang YZ, Ouyang HW, Hu H (2012) Platelet-mediated mesenchymal stem cells homing to the lung reduces monocrotaline-induced rat pulmonary hypertension. Cell Transplant 21(7):1463–1475
125. Jungebluth P, Luedde M, Ferrer E, Luedde T, Vucur M, Peinado VI, Go T, Schreiber C, Richthofen MV, Bader A et al (2011) Mesenchymal stem cells restore lung function by recruiting resident and non-resident proteins. Cell Transplant 20(10):1561–1574
126. Sordi V (2009) Mesenchymal stem cell homing capacity. Transplantation 87:S42–S45
127. Xie J, Hu D, Niu L, Qu S, Wang S, Liu S (2012) Mesenchymal stem cells attenuate vascular remodeling in monocrotaline-induced pulmonary hypertension rats. J Huazhong Univ Sci Technolog Med Sci 32:810–817
128. Zhang Y, Liao S, Yang M, Liang X, Poon MW, Wong CY, Wang J, Zhou Z, Cheong SK, Lee CN et al (2012) Improved cell survival and paracrine capacity of human embryonic stem cell-derived mesenchymal stem cells promote therapeutic potential for pulmonary arterial hypertension. Cell Transplant 21:2225–2239
129. Hansmann G, Fernandez-Gonzalez A, Aslam M, Vitali SH, Martin T, Mitsialis SA, Kourembanas S (2012) Mesenchymal stem cell-mediated reversal of bronchopulmonary dysplasia and associated pulmonary hypertension. Pulm Circ 2:170–181
130. Guan XJ, Song L, Han FF, Cui ZL, Chen X, Guo XJ, Xu WG (2012) Mesenchymal stem cells protect cigarette smoke-damaged lung and pulmonary funtion partly via VEGF-VEGF receptors. J Cell Biochem 114:323–335
131. Gupta N, Su X, Popov B, Lee JW, Serikov V, Matthay MA (2007) Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. J Immunol 179:1855–1863
132. Deng W, St Hilaire RC, Chattergoon NN, Jeter JR Jr, Kadowitz PJ (2006) Inhibition of vascular smooth muscle cell proliferation in vitro by genetically engineered marrow stromal cells secreting calcitonin gene-related peptide. Life Sci 78:1830–1838
133. Anversa P, Perrella MA, Kourembanas S, Choi AM, Loscalzo J (2012) Regenerative pulmonary medicine: potential and promise, pitfalls and challenges. Eur J Clin Invest 42:900–913
134. Sage EK, Loebinger MR, Polak J, Janes SM (2008) The role of bone marrow-derived stem cells in lung regeneration and repair. In: StemBook. Harvard Stem Cell Institute, Cambridge, MA
135. Xu J, Qu J, Cao L, Sai Y, Chen C, He L, Yu L (2008) Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. J Pathol 214:472–481
136. Kanki-Horimoto S, Horimoto H, Mieno S, Kishida K, Watanabe F, Furuya E, Katsumata T (2006) Implantation of mesenchymal stem cells overexpressing endothelial nitric oxide synthase improves right ventricular impairments caused by pulmonary hypertension. Circulation 114:I181–I185
137. Liang OD, Mitsialis SA, Chang MS, Vergadi E, Lee C, Aslam M, Fernandez-Gonzalez A, Liu X, Baveja R, Kourembanas S (2011) Mesenchymal stromal cells expressing heme oxygenase-1 reverse pulmonary hypertension. Stem Cells 29:99–107

138. Takemiya K, Kai H, Yasukawa H, Tahara N, Kato S, Imaizumi T (2010) Mesenchymal stem cell-based prostacyclin synthase gene therapy for pulmonary hypertension rats. Basic Res Cardiol 105:409–417
139. Strieter RM, Keeley EC, Hughes MA, Burdick MD, Mehrad B (2009) The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of pulmonary fibrosis. J Leukoc Biol 86:1111–1118
140. Nikam VS, Schermuly RT, Dumitrascu R, Weissmann N, Kwapiszewska G, Morrell N, Klepetko W, Fink L, Seeger W, Voswinckel R (2010) Treprostinil inhibits the recruitment of bone marrow-derived circulating fibrocytes in chronic hypoxic pulmonary hypertension. Eur Respir J 36:1302–1314
141. Li M, Riddle SR, Frid MG, El Kasmi KC, McKinsey TA, Sokol RJ, Strassheim D, Meyrick B, Yeager ME, Flockton AR et al (2011) Emergence of fibroblasts with a proinflammatory epigenetically altered phenotype in severe hypoxic pulmonary hypertension. J Immunol 187:2711–2722
142. Stenmark KR, Frid MG, Yeager ME (2010) Fibrocytes: potential new therapeutic targets for pulmonary hypertension? Eur Respir J 36:1232–1235
143. Yu L, Hales CA (2011) Effect of chemokine receptor CXCR4 on hypoxia-induced pulmonary hypertension and vascular remodeling in rats. Respir Res 12:21
144. Angelini DJ, Su Q, Kolosova IA, Fan C, Skinner JT, Yamaji-Kegan K, Collector M, Sharkis SJ, Johns RA (2010) Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM alpha) recruits bone marrow-derived cells to the murine pulmonary vasculature. PLoS One 5:e11251
145. Launay JM, Herve P, Callebert J, Mallat Z, Collet C, Doly S, Belmer A, Diaz SL, Hatia S, Cote F et al (2012) Serotonin 5-HT2B receptors are required for bone-marrow contribution to pulmonary arterial hypertension. Blood 119:1772–1780
146. Firth AL, Yao W, Ogawa A, Madani MM, Lin GY, Yuan JX (2010) Multipotent mesenchymal progenitor cells are present in endarterectomized tissues from patients with chronic thromboembolic pulmonary hypertension. Am J Physiol Cell Physiol 298:C1217–C1225
147. Frid MG, Brunetti JA, Burke DL, Carpenter TC, Davie NJ, Reeves JT, Roedersheimer MT, van Rooijen N, Stenmark KR (2006) Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. Am J Pathol 168:659–669
148. Sharma M, Afrin F, Satija N, Tripathi RP, Gangenahalli GU (2011) Stromal-derived factor-1/CXCR4 signaling: indispensable role in homing and engraftment of hematopoietic stem cells in bone marrow. Stem Cells Dev 20:933–946
149. Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian M, Renaud JF, Simonneau G, Lombet A, Humbert M (2011) Targeting of c-kit+ haematopoietic progenitor cells prevents hypoxic pulmonary hypertension. Eur Respir J 37:1392–1399
150. Giangreco A, Shen H, Reynolds SD, Stripp BR (2004) Molecular phenotype of airway side population cells. Am J Physiol Lung Cell Mol Physiol 286:L624–L630
151. Summer R, Kotton DN, Sun X, Fitzsimmons K, Fine A (2004) Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 287:L477–L483
152. Summer R, Kotton DN, Liang S, Fitzsimmons K, Sun X, Fine A (2005) Embryonic lung side population cells are hematopoietic and vascular precursors. Am J Respir Cell Mol Biol 33:32–40
153. Peter Y, Sen N, Levantini E, Keller S, Ingenito EP, Ciner A, Sackstein R, Shapiro SD (2013) CD45/CD11b positive subsets of adult lung anchorage-independent cells harness epithelial stem cells in culture. J Tissue Eng Regen Med 7(7):572–583
154. Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R (2004) Circulating fibrocytes: collagen-secreting cells of the peripheral blood. Int J Biochem Cell Biol 36:598–606
155. Mehrad B, Strieter RM (2012) Fibrocytes and the pathogenesis of diffuse parenchymal lung disease. Fibrogenesis Tissue Repair 5(Suppl 1):S22
156. Field JJ, Burdick MD, DeBaun MR, Strieter BA, Liu L, Mehrad B, Rose CE Jr, Linden J, Strieter RM (2012) The role of fibrocytes in sickle cell lung disease. PLoS One 7:e33702

157. Andersson-Sjoland A, Nihlberg K, Eriksson L, Bjermer L, Westergren-Thorsson G (2011) Fibrocytes and the tissue niche in lung repair. Respir Res 12:76
158. Herzog EL, Bucala R (2010) Fibrocytes in health and disease. Exp Hematol 38:548–556
159. Gomperts BN, Strieter RM (2007) Fibrocytes in lung disease. J Leukoc Biol 82:449–456
160. Stenmark KR, Gerasimovskaya E, Nemenoff RA, Das M (2002) Hypoxic activation of adventitial fibroblasts: role in vascular remodeling. Chest 122:326S–334S
161. Randell SH (2006) Airway epithelial stem cells and the pathophysiology of chronic obstructive pulmonary disease. Proc Am Thorac Soc 3:718–725
162. Glenny RW (2011) Emergence of matched airway and vascular trees from fractal rules. J Appl Physiol 110:1119–1129
163. Liu X, Driskell RR, Engelhardt JF (2004) Airway glandular development and stem cells. Curr Top Dev Biol 64:33–56
164. Stripp BR, Maxson K, Mera R, Singh G (1995) Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am J Physiol 269:L791–799
165. Berns A (2005) Stem cells for lung cancer? Cell 121:811–813

Adult Liver Stem Cells

Francesco Paolo Russo, Patrizia Burra, and Maurizio Parola

Abstract The liver possesses a remarkable ability to restore, through compensatory hyperplasia or regeneration, its original mass following partial or massive parenchymal cell loss. However, this ability is compromised in most relevant pathological conditions of clinical interest, with liver transplantation being at present the only resolutive treatment for severe acute liver failure (ALF), chronic inborn, or acquired end-stage liver diseases.

Replacing diseased hepatocytes and stimulating endogenous and exogenous regeneration by stem cells represent the main aims of liver-oriented cell therapy. Recent developments in stem cell technology have raised the hopes of identifying new expandable sources of liver cells for use in regenerative medicine and prompted studies on the best support for their growth.

In this chapter we will offer an overview of concept and data from available current literature by focusing the attention first on liver regeneration and the role of liver progenitor cells or adult liver stem cells and to then analyze current status of the therapeutic use of extrahepatic stem cells for liver diseases in either preclinical or clinical studies.

Keywords Hepatic adult stem cells • Bipotent hepatic progenitor cells • Acute liver failure • Chronic liver diseases • Hepatic regenerative medicine

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Abbreviations

AAT	α1-antitrypsin deficiency
ALF	Acute liver failure
α-FP	Alpha-fetoprotein
BDEC	Bile duct epithelial cells
BM	Bone marrow
BM-SCs	Bone marrow stem cells
CK-18	Cytokeratin 18
CLD	Chronic end-stage liver diseases
COX2	Cyclooxygenase type 2
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FGF-1	Fibroblast growth factor 1
FH	Familial hypercholesterolemia
GSD1	Glycogen storage disease type 1a
HAL	Hybrid-type artificial liver
HASC	Hepatic adult stem cells
HCC	Hepatocellular carcinoma
HCM	Hepatocyte culture medium
HCV	Hepatitis C virus
HF	Hollow fiber
HGF	Hepatocyte growth factor
HPCs	Hepatic progenitor cells
IFNγ	Interferon γ
IL-6	Interleukin 6
iPSCs	Induced pluripotent stem cells
LIF	Leukemia inhibitory factor
LPCs	Liver progenitor cells
MELD	Model for end-stage liver disease
MFs	Myofibroblast-like cells
MSCs	Mesenchymal stem cells
NAFLD	Nonalcoholic fatty liver disease
NOD-SCID mice	Non-obese diabetic-severe combined immune deficiency mice
NPCs	Non-parenchymal cells
OLT	Orthotopic liver transplantation
OSM	Oncostatin M
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
STAT3	Signal transducer and activator of transcription 3
TGFα	Transforming growth factor α
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
UCMSCs	MSCs from human umbilical cord

1 The "Clinical" Liver Scenario and the Need for Hepatic Regenerative Medicine

1.1 Acute and Chronic End-Stage Liver Diseases: Epidemiological Data

As it is well known, the liver possesses a remarkable ability to restore its original mass following partial or massive parenchymal cell loss by ensuing compensatory hyperplasia or regeneration. However, in most relevant pathological conditions of clinical interest orthotopic liver transplantation (OLT) is indeed the only resolutive treatment for severe acute liver failure (ALF), chronic inborn, or acquired end-stage liver diseases [1].

Along these lines, ALF is commonly defined as a multi-organ syndrome occurring in previously healthy subjects (that is, in the absence of underlying liver disease) which is characterized by severe hepatocellular dysfunction and often rapid progression to death. Major causes of ALF are represented by acetaminophen or non-acetaminophen drug-induced toxicity, prominent in Western countries, as well as by hepatitis viruses (mainly developing countries). Although the last two decades have been characterized by a consistent overall improvement in critical care and OLT, ALF is still associated with high mortality rate (30–100 %) and the USA data indicate that ALF has an incidence of 3.5 deaths per million population, then accounting for approx. 5–6 % of all OLT [2, 3].

Epidemiological data for chronic end-stage liver diseases (CLD) are more relevant and outline a global scenario dominated by an increasing worldwide prevalence of liver cirrhosis, mostly related to chronic infection by hepatitis C or B virus, alcohol consumption and nonalcoholic fatty liver disease (NAFLD) [4–8]. At present, approx. 170 million patients worldwide are estimated to be affected by a form of CLD and 25–30 % of these patients are expected to develop with time significant fibrosis and eventually cirrhosis and related complications. The latter scenario has a relevant clinical impact since, among disease of the GI tract, liver cirrhosis now represents the most common non-neoplastic cause of death in Europe and USA, as well as the seventh most common cause of death in Western countries. Moreover, particularly in Western countries, cirrhosis also represents the main predisposing cause for hepatocellular carcinoma (HCC), accounting for 85–90 % of primary liver cancers and representing the fifth most common human cancer and the third most common cause of cancer mortality worldwide [9].

The overall liver scenario, as for current epidemiological analysis, needs to take into account a peak for advanced CLD which is predicted to occur in the next decade, resulting then in a significant increase of the numbers of patients reaching end-stage disease and potentially requiring OLT that should face a predictable shortage of donor livers. Moreover, current knowledge suggests that OLT procedures can be further complicated by immunological incompatibilities and by the fact that OLT is not always effective. Clinical evidence has outlined the existence of a subset

of patients, particularly within those undergoing OLT for hepatitis C virus (HCV)-related cirrhosis, which can develop fulminant fibrotic progression to cirrhosis within a relatively short period of time (i.e., 2–3 years) [4–7]. Although OLT could be considered as an essentially successful surgical procedure, it should be emphasized that, in addition to the problem of the shortage of donor organs, OLT still suffers from operative damage. Therefore, recent attention has been focused on the ability to use cellular resources to bridge patients until transplantation or to restore liver mass and function [10].

1.2 The Need for Hepatic Regenerative Medicine: Introductory Remarks

Liver transplantation is the gold standard procedure for treating acute and chronic end-stage liver disease and the demand for treatment of end-stage liver disease will continue to rise and will drive development of alternatives [1]. Hepatocyte transplantation has been proposed to replace whole liver transplantation at least for selected cases of inherited liver disorders, but there are several limitations for the use of liver cell therapies. Studies on stem cells and on their potential sources have been intensified in recent years, given the promise of their clinical application, especially in regenerative medicine [11]. The behavior and composition of both multipotent and pluripotent stem cell populations are exquisitely controlled by a complex interplay of extracellular matrix and cell–cell interaction. An interesting review was recently published on developments of arrayed cellular environments and their contribution and potential in stem cells and regenerative medicine. Arrayed cellular environments provide a set of experimental elements with variation of one or several classes of stimuli across elements of the array with the capability to provide an understanding of the molecular and cellular events that underlie expansion and specification of stem cell and therapeutic cell populations [12].

Success for stimulating stem cells to differentiate into hepatocytes and other liver cell types has been reported; however, it appears that it is very difficult to obtain differentiated human hepatocytes from human cord blood or human cord mesenchymal stem cells. These cells only mimic the hepatocyte function and are usually called hepatocyte-like cells [13].

Replacing diseased hepatocytes and stimulating endogenous and exogenous regeneration by stem cells represent the main aims of liver-oriented cell therapy [14, 15]. Recent developments in stem cell technology have raised the hopes of identifying new expandable sources of liver cells for use in regenerative medicine [16] and prompted studies on the best support for their growth. Embryonic stem cells can be considered the best model of multipotency, but their use is limited due to ethical concerns [17] and the neoplastic risks after their in vivo use [18] have led to adult stem cells being considered a more acceptable source.

Adult stem cells have consequently been widely explored in recent years as a more acceptable source of cells, including the mesenchymal stem cells (MSCs),

a population of multipotent progenitors capable of differentiating towards adipogenic, osteogenic [19], and hepatogenic lineages [20, 21] with a low immunogenicity [22].

Cell transplantation is a practical procedure compared with organ transplantation. It can be performed with much less risk to the patient and much reduced cost for the healthcare system. Furthermore, given the little invasiveness of systemic administration, this method could be also applied to patients who are severely ill and would not be able to tolerate organ transplantation.

Recently, we have characterized a novel MSC population obtained from human umbilical cord (UCMSCs) and we have induced their differentiation towards hepatic lineages in vitro seeking the best cell support for this purpose. The main aim of our study was to evaluate the therapeutic potential of adult UCMSCs in a murine model of acute liver injury using carbon tetrachloride, a potent hepatotoxic chemical. Phenotypic analysis showed a profile compatible with MSCs and the simultaneous high expression of CD166, CD105, and CD73 demonstrated that our cells were a novel MSCs population. The morphological features, loss of MSC phenotype, gene expression changes, immune-cytochemical staining, albumin secretion, urea production, and glycogen storage all suggested that these cells can grow and differentiate into functional hepatocyte-like cells without any biological support [23].

However, we had previously reported that stem cell differentiation can be stimulated by growth factors and extracellular matrix components used as a cell culture support. Using a homologous acellular matrix derived from surgical specimens represents an interesting tissue engineering approach since the matrix is biocompatible, contains adhesion molecules and growth factors, and is obtained from a healthy organ [24, 25].

Interestingly enough, more recently, the field of cell microencapsulation technology has opened many new perspectives. The immobilization of cells into polymeric scaffolds releasing therapeutic factors, such as alginate microcapsules, has been widely employed as a drug delivery system for numerous diseases for many years. Stem cells represent an ideal tool for cell immobilization and so does alginate as a biomaterial of choice in the elaboration of these biomimetic scaffolds [26].

Bone marrow (BM) is considered the main source of MSCs [27], but their number decreases significantly with age [28, 29] and this has led to the evaluation of alternative sources such as adipose tissue [30] and embryo-derived tissues, e.g., placenta [31], amniotic fluid [32], umbilical cord blood [33], and umbilical cord [34].

Moreover, regenerative medicine methods and technologies are currently being developed to manufacture different segments of the entire digestive tube [35]. The conveyance of these results into clinical practice would need to be considered with caution because more information is needed on cell behavior in vivo before any clinical applications can be hypothesized.

Continued research in this area and continued industry attention focused on developing liver support and cellular therapies should accelerate because of the ever pressing demand. It is this demand that has and will continue to drive us to push the limits, test new hypotheses, and take new risks [1].

In this chapter we will offer an overview of concept and data from available current literature by focusing the attention first on liver regeneration and the role of liver progenitor cells or adult liver stem cells and to then analyze current status of the therapeutic use of extrahepatic stem cells for liver diseases in either preclinical or clinical studies.

2 Liver Regeneration and Liver Progenitor Cells/Adult Liver Stem Cells

The liver has a remarkable capacity for regeneration [36]. This capacity is known since the ancient Greek myth of Titan Prometheus and his punishment for deceiving Zeus and protecting mankind. The myth of Prometheus is known to most members of the scientific community who study hepatic diseases, mainly because Prometheus's liver was the target of torture. The Myth of Prometheus is also known and cherished by many, because, according to one version, Prometheus created the first man. The ancient poet Hesiod (eighth century BC) [37] records that Prometheus twice tricked the gods. First, he offered mortals the best meat from a slaughtered cow and gave the fat and bones to the gods. Then, when an infuriated Zeus punished man by taking fire, Prometheus stole it back for mankind. Accordingly, Zeus punished Prometheus binding him on the mountain Caucasus. More explicitly, for students of the liver, an eagle fed from his liver each day, but the liver regenerated overnight [38, 39]. Self-renewal of hepatocytes is the main mechanism responsible for liver mass homeostasis and for liver regeneration after acute (moderate) liver injury and reduction of liver mass [40]. However, in conditions of chronic liver injury or submassive liver cell loss, such capacity for self-renewal is overwhelmed, exhausted, or impaired, leading to liver failure or insufficiency. In those conditions, hepatic progenitor cells (HPCs), which are dormant and found in periportal location in a healthy liver, actively proliferate and yield transit-amplifying cells (or oval cells). Since the 1950s, when Opie and Farber described a category of small hepatic cells that they called oval cells, emerging from the canal of Hering, where bile canaliculi connect with bile ducts, it has become a hackneyed term used to define a highly heterogeneous population of cells whose fate is classically bipotent giving rise to both hepatocytes and cholangiocytes at least in vitro and at least in rodents [41].

This reaction is known as ductular reaction in human beings or oval cell proliferation in rodents [42–44]. Although the initiating mechanisms of liver regeneration may be similar in rodents and humans, the time course of the process differs among species. Nevertheless, in rats and mice, the original liver mass is restored to approximately 100 % in 7–10 days. In humans, there is a very rapid increase in liver mass during the first 7 days after partial liver transplantation, leading to complete restoration by 3 months [45].

2.1 Cellular Mechanisms of Liver Regeneration

As already mentioned before, what is unique to the liver is that differentiated hepatocytes constitute the first line of response to injury or resection, while progenitor cells function as a reserve compartment. This differs from other tissues, such as skeletal muscle, in which differentiated myocytes do not replicate, but regeneration after injury can occur through the proliferation of precursor cells (satellite cells) [46] or the heart, in which there is little if any proliferation of differentiated myocytes or immature precursors [47].

Furthermore in highly proliferating tissues such as the skin and the gut, progenitor cells continuously produce transit-amplifying cells that differentiate and replenish short-lived mature cells [48, 49]. By contrast, the liver has very low levels of cell turnover, and it primarily relies on replication of highly differentiated parenchymal hepatocytes to regenerate in response to loss of liver mass [50–52].

Physiological turnover of liver parenchyma was originally proposed to follow the model of the "streaming liver," an hypothesis suggesting that young hepatocytes are formed in the portal area and then migrate towards the central vein to progressively replace older cells [53]. Although this concept has received some confirmation [54] other data do not support this hypothesis [43]. Whatever the mechanism or model involved, two concepts are widely acknowledged: (1) physiological hepatocyte turnover is slow, with a reported average life span of approx. 200–300 days, and (2) liver parenchyma turnover mostly depends on proliferation of adult hepatocytes and bile duct epithelial cells (BDEC), with a still debated, presumably minor, contribution by progenitor or stem cells [43].

Liver regeneration involves coordinated action of distinct cytokines and growth factors, which regulate three temporal stages of hepatocyte proliferation, namely, priming, DNA synthesis, and cell division, followed by growth termination. Tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) are critical priming factors, which facilitate G0 to G1 transition of hepatocytes, rendering them competent to respond to growth factors. Mice lacking TNF receptor 1 show delayed liver regeneration, which could be reversed by administration of IL-6, whereas IL-6 deficiency induces severe apoptosis because IL-6-induced STAT3 activation is essential for liver regeneration. Following priming, growth factors provide mitogenic signals that facilitate competent hepatocytes to progress through the cell cycle [55–57]. For example, under the standard experimental conditions of 2/3 partial hepatectomy, any hepatocyte may undergo one or two rounds of cell division (sustained by HGF, IL-6, TNF, TGFα, EGF) within 24–48 h. This is followed by wave of proliferation involving other hepatic cell populations called non-parenchymal cells (NPCs).

NPCs in the liver include stellate cells/myofibroblasts, which are the main producers of collagen; macrophages, which are involved in tissue remodeling and fibrosis resolution after extensive damage; endothelial cells, which are able to form new vessels; and other leukocytes recruited by local inflammation. NPCs produce cytokines and growth factors, like transforming growth factor β, that influence oval cells/HPCs and hepatocyte proliferation, but most of the signals they exchange with

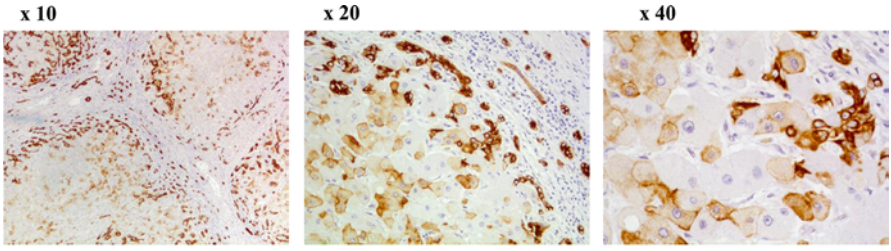

Fig. 1 Immune-histochemistry for cytokeratin 7 (CK-7, used as a marker to identify HPCs and cells derived from HPCs) in a human liver specimen obtained from a HCV-related cirrhotic patient. Positive stain is found in cholangiocytes and ductular-like structures (either typical or atypical) as well as in HPCs at the border of fibrotic septa or hepatocyte-like cells within the pseudo-lobule. Original magnification is indicated

the oval cell/HPC compartment and their role in regulating oval cell/LPC behavior has yet to be fully elucidated. Moreover, studies have demonstrated that in liver injury a proportion of myofibroblasts and macrophages are recruited from the BM. It has been claimed that oval cells are of BM origin; however, other studies have found that oval cells are intrinsic to the liver and not of BM origin [58–60].

2.2 Liver Progenitor Cells

Impairment of the replicative capacity of most remnant hepatocytes induces an alternate regenerative process from HPCs. These cells (also called oval cells in rodent) are located in the most peripheral branches of the biliary tree (canal of Hering).

Once activated, HPCs proliferate in the portal region and migrate into the hepatic lobule where they undergo further differentiation into hepatocytes or bile duct cells to repopulate the hepatic parenchyma (Fig. 1). This proliferative response characterized by the appearance of bile duct-like structures in humans is referred to as atypical ductular reaction [61].

While the term oval cell is widely used to describe liver progenitors, investigators do not agree on the phenotype and molecular signature of these cells. The terminal bile ductular system (i.e., the canal of Hering) is thought to be the main source of oval cells [62].

The oval cell compartment can probably not be attributed to a single cell type [63]. In order to avoid misunderstandings, the term oval cell activation is used to describe the heterogeneous cellular changes accompanying the appearance of progenitor cells, whereas the term oval cells refer to the progenitors themselves. Oval cells are considered bipotential transit-amplifying cells derived from normally quiescent "true stem cells" that reside in the biliary tree [62]. Proliferating oval cells constitute a heterogeneous population justifying the different names used to describe

them: ductular progenitor cells, atypical ductular cells, periductular liver progenitor cell, or individual progenies [63].

We summarize the current complexity of terminology as follows:

(a) Hepatic adult stem cells (HASC), normally quiescent and otherwise termed "oval" or HPC-precursor cells [43] that reside in portal areas within the canals of Hering, envisaged as the hepatic niche for progenitor cells.
(b) Activation of the HASC compartment (as in ALF or chronic liver injury) leads to the appearance of oval cells, described as bipotential transient-amplifying cells or as bipotential HPC.
(c) The classic view implies that during activation of the stem/progenitor compartment adult liver bipotential HPC can generate either hepatocytes or BDEC. A variant of this model hypothesizes the existence of different steps of maturation, with the most mature oval cell being bipotential and able to generate precursors of either hepatocytes or cholangiocytes, sometimes referred to as pre-hepatocytes or intermediate hepatocyte-like cells [58].

Furthermore oval cell activation can be envisaged as a process involving four distinct phases: [43] (1) activation, the phase in which the stem/precursor cell compartment is activated, leading to the emergence of oval cell or HPC, sustained and induced to proliferate by growth factors (Oncostatin M or OSM, IL-6, LIF) signaling through the JAK/STAT and other factors (TNF, TWEAK, IFNγ, SCF, COX-2); (2) oval cell population is further amplified by several polypeptide growth factors (TGFα, TGFβ, HGF, FGF-1, Sonic and Indian Hedgehog); (3) migration of progenitor cells, in response to the chemokine SDF-1 (CXCL12) or factors like uPA and tPA of the plasmin activator cascade; and (4) differentiation, the final step leading to either hepatocytes or BDEC in response again to LIF, OSM, or Dlk. This scenario has been recently implemented by an excellent in vivo study showing that in both human diseased liver and mouse models of the ductular reaction Notch and Wnt signaling are relevant in directing specification of HPCs via their interactions with activated myofibroblasts or macrophages [64]. In particular, during biliary regeneration, expression of Jagged 1 (a Notch ligand) by myofibroblasts promoted Notch signaling in HPCs and thus their biliary specification to cholangiocytes. Alternatively, during hepatocyte regeneration, macrophage engulfment of hepatocyte debris induced Wnt3a expression that resulted in canonical Wnt signaling in nearby HPCs, thus maintaining in these cells expression of the factor Numb and then promoting their specification towards hepatocyte phenotype. It is suggested that indeed these two pathways can regulate and/or promote adult parenchymal regeneration during chronic liver injury [64]. Another very recent experimental study has investigated in vivo the capacity of HPC to differentiate into hepatocytes and to contribute to liver regeneration. By performing lineage-tracing murine experiments (involving either regeneration and/or injury of liver parenchyma) in order to follow the fate of HPC and biliary cells, authors were able to show that hepatobiliary precursors do not contribute to liver mass homeostasis or to liver regeneration in the healthy liver [44]. By contrast, in conditions of chronic liver injury expanded transit-amplifying cells (HPC) were able to give rise to a small proportion of hepatocyte-like cells that were

shown to be well differentiated, polarized, and respond to pro-mitogenic stimuli as normal hepatocytes. Repopulation efficiency by HPC and/or biliary cells increased when extracellular matrix and laminin deposition were reduced.

However, it should be underlined that at present human HPCs have not been used in clinical trials. Although they can be differentiated in vitro into hepatocyte-like and cholangiocyte-like cells and effectively transplanted and engrafted into immune-compromised mice, HPCs exhibit phenotypical instability and in certain cases produce tumors in mice. Meanwhile, in vitro expansion of HPCs prior to the differentiation or transplantation into mice opens the prospect for development of liver stem cell therapy and exploitation of "humanized mouse models" [65].

3 Therapeutic Use of Extrahepatic Stem Cells in Liver Diseases: Preclinical Studies

According to the previous section, one can say that the application in clinical practice of hepatocyte transplantation as well as the use of hepatocytes in bio-artificial livers still poses considerable problems, including also the intrinsic difficulty of obtaining human hepatocytes as well as to maintain them viable and into the differentiate phenotype when cultured in vitro. These limitations have favored the alternative cell therapy approach consisting in the use of stem cells and growth factor. Indeed, a growing range of potential applications for therapeutic use of stem cells in liver diseases can be envisaged, with many pilot clinical studies already undertaken. As properly suggested in a recent editorial [66], one may identify a number of areas in which stem cell therapy could reasonably represent a future realistic aim, including the attempt: (a) to improve liver repopulation and reduce excess deposition of extracellular matrix and scarring by upregulating hepatic's own regenerative processes; (b) to inhibit immune-mediated liver injury; and (c) to obtain hepatocyte-like cells from stem cells and to employ them either for cell transplantation (i.e., to support or replace hepatocyte function) or in extracorporeal bio-artificial liver apparatus. The first available literature data in this field already suggest that a critical issue may be represented by the choice of therapeutic cell to be employed that may be tailored to the specific type of liver disease.

3.1 Stem Cells from Bone Marrow (BM-SCs) and Other Extrahepatic Sources

The rational suggestion to use autologous transplantation of BM-SCs as a putative strategy of intervention in liver diseases was initially proposed more than a decade ago on the basis of pioneer studies [67–69]. However, positive (although relatively modest) features from these initial experimental studies, some based on transplantation of hematopoietic stem cells and indicating recruitment to the injured liver of

these cells and their apparent differentiation into hepatocyte-like cells, were later shown to have resulted essentially from fusion between transplanted donor cells and resident recipient hepatocytes [70–72].

Having established this relevant point, several laboratories adopted a different strategy and reported successful "in vitro" differentiation of extrahepatic multipotent stem cells into hepatocyte-like cells (i.e., cells expressing defined hepatocellular antigens and functional properties). The list of multipotent cells employed for this purpose includes (1) so-called multipotent adult progenitor cells [73, 74], a unique population, originating from long-term culture of non-hematopoietic adherent cells (i.e., mesenchymal stem cells [MSC)]) from bone marrow, displaying the ability to differentiate into multiple lineages [75]; (2) MSC derived from either bone marrow [21, 76–78] as well as adipose tissue [79, 80], umbilical blood cord [23, 81], or even dental pulp [82]; and (3) multipotent stem cells from amniotic fluid and membranes [83, 84].

Whatever the source of multipotent stem cells, several laboratories reported successful "in vitro" differentiation of these extrahepatic cells into hepatocyte-like cells, that is, cells expressing defined hepatocellular antigens and functional properties that should include at least the following: (a) phenotypic changes leading to the acquisition of a polygonal (i.e., polarized) morphology; (b) expression of specific proteins like albumin, alpha-fetoprotein (α-FP), and cytokeratin 18 (CK-18); (c) the ability to synthetize urea as well as to synthetize and store glycogen; (d) acquisition of further antigens and/or functional activities such as expression of isoforms of cytochrome P450 and of drug metabolism-related enzymes; and (e) expression/activity of more selective proteins or enzymes, including glucose-6-phosphatase, tyrosine aminotransferase, triptophane-2,3-dioxygenase, hepatic nuclear factor 4, and canalicular antigen 9B2, to name just a few [58].

In most of these studies "in vitro" differentiation was based on a rather common scheme of experimental protocol which typically required first a so-defined differentiation step followed then by a maturation step. The protocol established for human MSCs by Lee and coworkers [21] may serve as a paradigm. In this study the differentiation step was sustained by treatment of MSCs with hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and nicotinamide; in the maturation cells were exposed to a medium containing oncostatin M (OSM), dexamethasone, insulin, transferrin, and selenium. Once hepatocyte-like cells were obtained, several laboratories transplanted in vivo these cells to test their efficiency in animal models of ALF or of CLD and most of these experimental studies have reported that transplanted cells can effectively engraft injured liver parenchyma [73, 84–90]. However, results in terms of repopulation were not numerically impressive, possibly depending on the specific protocol adopted [21, 73–79]. Best results were obtained in those experiments in which MSC-derived hepatocyte-like cells were transplanted. The overall scenario from these preclinical studies can be completed by a number of encouraging results reported in experimental protocols designed to prevent liver fibrosis [85, 86] or in studies that reported some improvement in parameters related to ALF [87–89]. Concerning prevention of liver fibrosis, however, it should be cautionary recalled that at least two studies could not document any significant

anti-fibrotic effect following transplantation of either murine MSC or human MSC (in NOD-SCID mice) [77, 90]. Moreover, at least three different laboratories have provided evidence indicating that BM-SCs engrafting the liver during the course of experimental model of chronic liver injury, in particular MSC [77, 91, 92] or fibrocytes [93], have the potential to differentiate into hepatic myofibroblast-like cells (MFs). As originally proposed by Forbes and coworkers, these results envisage a potential risk for these transplanted cells to contribute to liver fibrogenesis, although several authors believe that such a contribution should be considered as minor with hepatic stellates and portal fibroblasts being by far the most relevant sources of hepatic MFs [94–96].

3.2 The Use of Induced Pluripotent Stem Cells

A more recent approach for producing hepatocyte-like cells has taken advantage of induced pluripotent stem cells (iPSCs), one of the most exciting recent discoveries in the field of biology. iPSC cells by definition are somatic cells (of either murine or human origin) that are engineered (and then reprogrammed) in order to express combinations of defined transcription factors and to become pluripotent, remarkably resembling embryonic stem (ES) cells [97–100]. iPSCs are typically generated by retroviral induction of transcription factors such as Oct3/4, Sox2, KLF4, and c-Myc, in fibroblasts. Lentivirus and adenovirus induction, induction with other gene combinations and virus-free approaches such as using plasmids, small molecules, and recombinant proteins have also been reported (reviewed in [100]). In addition, it has been shown that iPSCs can be generated from a variety of cell types such as pancreatic cells, meningiocytes, keratinocytes, hematopoietic cells differentiated from ESCs, and primary human hepatocytes [101–103].

iPSCs are indeed very promising cells potentially able to overcome controversies and ethical concerns associated with the use of human embryonic stem (ES) cells and their availability has theoretically opened the way to their use for a number of perspectives and applications, including the possibility to use these cells in order to (1) design and test patient-customized (i.e., autologous) cell therapy with no need for immune suppression; (2) modeling inherited metabolic human diseases and investigate in detail pathogenic mechanisms; (3) drug discovery and testing, possibly patient customized. Unfortunately, at present the use of iPSCs for regenerative medicine is limited by two major and still unresolved concerns, the oncogenic potential of these cells and the so-called epigenetic transcriptional memory of somatic cells, that can affect the desired differentiation into the desired specific lineages [99, 100, 104, 105].

Whether the use of iPS cells in relation to liver diseases is concerned, different laboratories have used protocol similar to those designed for ES cells in order to obtain hepatocyte like cells from human [106–108] or murine [109, 110] somatic cells. This protocol usually requires from three to four steps with the following sequence: (a) endodermal differentiation step following exposure to activin A;

(b) hepatic specification step as for exposure to fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein-4 (BMP-4); (c) proliferation step elicited by HGF; and (d) maturation step by a specifically designed hepatocyte culture medium (HCM) containing oncostatin M (OSM) [111].

According to limitations for the use of iPS cells previously described, attempts to obtain engraftment and proliferation of hepatocytes-like cells from iPS cells led to limited and mostly disappointing results (reviewed in [111]) with just one apparent exception for a murine study [112]. In the latter study murine somatic cells were reprogrammed to iPSCs and when transplanted in vivo in the experimental model of mice carrying fumaryl-acetoacetate hydrolase deficiency (FAH −/− mice), these iPS cells apparently underwent normal ontogenic development into mature hepatocytes [111]. In particular, these murine iPSCs cells were injected into blastocysts of FAH −/− mice originating a generation of chimeric mice in which iPSC-derived hepatocytes proliferated (even responding to two/third partial hepatectomy) and repopulated the liver rescuing the chimeric mice. However, the FAH −/− mice is a rather unique and favorable model in the scenario of hepatic regenerative medicine and one should emphasize the fact that such a procedure (i.e., to inject iPS cells into blastocysts) is rather irrelevant for regenerative medicine if the final goal is to obtain safe and adult/mature hepatocyte-like cells to transplant under postnatal settings.

Literature indeed suggests that iPSC lines can be generated from patients suffering from specific diseases, providing a unique source for study and disease modeling. Along these lines, iPSC generation has been reported from individuals affected by several diseases, including neurodegenerative diseases, juvenile diabetes mellitus, muscular dystrophy, hematological diseases, Down syndrome, as well as ischemic heart failure [112]. In another recent study, human hepatocyte-like cells derived from iPSCs (obtained by reprogramming dermal fibroblasts) have been obtained from patients affected by inherited metabolic disorders like α1-antitrypsin deficiency (AAT), glycogen storage disease type 1a deficiency (GSD1), hereditary tyrosinemia type 1, Crigler–Najjar syndrome, and familial hypercholesterolemia (FH) [113]. Authors were able to generate a library of patient-specific human iPS cells to be then differentiated into hepatocyte-like cells and then characterized iPS cell-derived hepatocytes from patients affected by AAT, GSD1, and FH. Of interest, hepatocyte-like cells obtained in this way exhibited all phenotypic abnormalities of primary hepatocytes from patients carrying these diseases and, as also suggested by other researchers in a different study, potentially used in order to investigate disease pathogenesis and to test drugs in a patient-customized manner [114].

An even more interesting approach, with a potential future application in the field of ALF, is the one recently published in a study in which iPSCs as well as ESCs have been injected into hollow fiber (HF)/organoid culture in order to form organoid in the lumen of HF [115]. This study reported that the exposure of iPSCs and ESCs to agents able to promote differentiation resulted in upregulation of differentiation-related genes and a very efficient cell proliferation and organoid formation inside HFs characterized by a high cell density and promising results in terms of gain of liver-specific functions. This may represent a critical report implying the use of these cells as source for obtaining a hybrid-type artificial liver (HAL).

4 Therapeutic Use of Extrahepatic Stem Cells in Liver Diseases: Clinical Studies

There is an increasing range of potential applications of stem cells in liver diseases, with many clinical studies already undertaken. Whilst there have been advances in our understanding of the role of stem cells in liver damage and repair as well as encouraging results using stem cells as cell therapy in preclinical animal models, the precise mode of action and optimal cell usage has not been completely defined.

Cell therapy can be defined as "the use of living cells to restore, maintain, or enhance tissue and organ function" [116] and has several potential advantages when compared to OLT, since transplantable cells can be (a) expanded in vitro and cryopreserved, thus abolishing the limit of organ shortage; (b) genetically manipulated, to correct inborn errors of metabolism; c) cryopreserved for future use and infused without major surgery; and (d) obtained from the same patient, avoiding risk of rejection and need for lifelong immune-suppression [117].

Ideally, allogenic hepatocytes, ex vivo derived hepatocytes, or cells capable of hepatocyte differentiation could be administered directly and repopulate the failing liver. Allogenic hepatocyte transplantation has been explored as an alternative to OLT in ALF and metabolic liver diseases. However, difficulties in harvesting and storing sufficient quantities of hepatocytes and significant cell loss following transplantation have so far limited the potential of this therapy [118].

Given the right environment and stimuli, stem cells and certain progenitor cells can differentiate into hepatocytes. Stem cells are undifferentiated cells capable of proliferation, self-maintenance, and differentiation into functional progeny with flexibility or plasticity in these options [119]. Embryonic stem cells (ESCs) have pluripotency and unlimited capacity for self-renewal. In contrast, adult stem cells have a restricted differentiation capability and because of this they may be more correctly called progenitor cells. Despite the apparently limited differentiation capability of progenitor cells, given appropriate stimuli, progenitor cells can transdifferentiate into other cell lines. The ideal cell source to support hepatic regeneration must be reliably identifiable, be able to generate hepatocytes efficiently, evade the immune defenses, and behave predictably with a high safety profile.

Successful cell therapy depends on the innate clonogenicity of the administered cells or on the favorable condition in which transplanted cells have a selective growth advantage over the indigenous population. In the diseased human liver there may not be the substantial selective growth advantage for transplanted cells that pertains in many rodent models where it is possible to enrich for cells that continue to expand in the recipient liver in the absence of a major growth stimulus. Such cells might simply be fetal cells or a subpopulation of antigenically distinct adult cells [120].

Of the clinical studies published, the overwhelming data suggest that stem cell therapy is safe [121], although there are possible concerns regarding the route of delivery of cell therapy. Whilst no studies report superior outcomes when cells are directly injected into the liver (portal vein or hepatic artery), there have been complications such as hepatic artery dissection [122] and increased portal hypertensive

bleeding [123] following this approach. Furthermore, the intravenous administration of autologous BM mononuclear cells resulted in hepatic homing of the injected cells suggesting this easier, safer route may be an adequate option for cell delivery [124, 125]. Assuming that delivery to the liver is important for stem cell infusions to exert their optimal effect, then developing a better understanding of the mechanisms regulating their hepatic ingress may allow for further improvements to treatment protocols. Whilst patients with a wide range of disease severity have been included in clinical trials, the priority remains to irrefutably confirm the efficacy of cell/stem cell therapy. In this regard, choosing patients in which the benefit may be most reliably determined and of greatest value is important. Patients verging on the cusp of requiring a liver transplant (e.g., with MELD score approaching/just below 15) are good candidates as even a small percentage improvement in liver function may be sufficient to significantly delay or indeed remove altogether the need for liver transplant.

References

1. Burra P, Freeman R (2011) Trends in liver transplantation. J Hepatol 56(Suppl 1):S101–S111
2. Larson AM (2010) Diagnosis and management of acute liver failure. Curr Opin Gastroenterol 26:214–221
3. Bernal W, Auzinger G, Dhawan A, Wendon J (2010) Acute liver failure. Lancet 376:190–201
4. Poynard T, Bedossa P, Opolon P (1997) Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR and DOSVIRC groups. Lancet 349:825–832
5. Armstrong GL, Alter MJ, McQuillan GM et al (2000) The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. Hepatology 31:777–782
6. Wong JB, McQuillan GM, McHutchison JG et al (2000) Estimating future hepatitis C morbidity mortality, and costs in the United States. Am J Public Health 90:1562–1569
7. Pinzani M, Romanelli RG, Magli S (2001) Progression of fibrosis in chronic liver diseases – time to tally the score. J Hepatol 34:764–767
8. Parola M, Marra F, Pinzani M (2008) Myofibroblast-like cells and liver fibrogenesis: emerging concepts in a rapidly moving scenario. Mol Aspects Med 29:58–66
9. El-Serag HB, Rudolph KL (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132:2557–2576
10. Dalgetty DM, Medine CN, Iredale JP et al (2009) Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 297:G241–G248
11. Stocum DL (2001) Stem cells in regenerative biology and medicine. Wound Repair Regen 9:429–442
12. Titmarsh DM, Chen H, Wolvetang EJ, Cooper-White JJ (2012) Arrayed cellular environments for stem cells and regenerative medicine. Biotechnol J 8:822–834
13. Burra P, Bizzaro D, Ciccocioppo R et al (2011) Therapeutic application of stem cells in gastroenterology: an up-date. World J Gastroenterol 17:3870–3880
14. Burra P, Tomat S, Bizzaro D et al (2008) Stem cells in hepatology. Organs Tissues Cells 1:15–22
15. van Poll D, Parekkadan B, Cho CH et al (2008) Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. Hepatology 47:1634–1643

16. Burra P, Tomat S, Villa E et al (2008) Experimental hepatology applied to stem cells. Dig Liver Dis 40:54–61
17. Denker HW (2006) Potentiality of embryonic stem cells: an ethical problem even with alternative stem cell sources. J Med Ethics 32:665–671
18. Reya T, Morrison SJ, Clarke MF et al (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
19. Kern S, Eichler H, Stoeve J et al (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301
20. Campard D, Lysy PA, Najimi M et al (2008) Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. Gastroenterology 134:833–848
21. Lee KD, Kuo TK, Whang-Peng J et al (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284
22. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822
23. Burra P, Arcidiacono D, Bizzaro D et al (2012) Systemic administration of a novel human umbilical cord mesenchymal stem cells population accelerates the resolution of acute liver injury. BMC Gastroenterol 12:88
24. Burra P, Tomat S, Conconi MT et al (2004) Acellular liver matrix improves the survival and functions of isolated rat hepatocytes cultured in vitro. Int J Mol Med 14:511–515
25. Tomat S, Burra P, Gringeri E et al (2006) Metabolic activity of rat hepatocytes cultured on homologous acellular matrix and transplanted into Gunn rats. Int J Mol Med 18:837–842
26. Garate A, Murua A, Orive G et al (2012) Stem cells in alginate bioscaffolds. Ther Deliv 3:761–774
27. Bianco P, Riminucci M, Gronthos S et al (2001) Bone marrow stromal cells: biology and potential application. Stem Cells 19:180–192
28. Mareschi K, Ferrero I, Rustichelli D et al (2006) Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 97:744–754
29. Mendes SC, Tibbe JM, Veenhof M et al (2002) Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. Tissue Eng 8:911–920
30. Rodriguez AM, Elabd C, Amri EZ et al (2005) The human adipose tissue is a source of multipotent stem cells. Biochimie 87:125–128
31. Miao Z, Jin J, Chen L et al (2006) Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. Cell Biol Int 30:681–687
32. Yu SJ, Soncini M, Kaneko Y et al (2009) Amnion: a potent graft source for cell therapy in stroke. Cell Transplant 18:111–118
33. Lee OK, Kuo TK, Chen WM et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675
34. Wang HS, Hung SC, Peng ST et al (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 22:1330–1337
35. Orlando G, Garcia-Arras JE, Soker T et al (2012) Regeneration and bioengineering of the gastrointestinal tract: current status and future prospectives. Dig Liver Dis 44:714–720
36. Grisham J, Thorgeirsson S (1997) Liver stem cells. In: Potten CS (ed) Stem cells. Academic, London, pp 233–282
37. Hesiod (1999) Theogony-works and days. Oxford University Press, New York
38. Morfrod MPO, Lenardon RJ (2007) Classical mythology. Oxford University Press, New York
39. Smith W (2005) Dictionary of Greek and Roman biography and mythology. IB Tauris, London, p 1161
40. Fausto N, Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130
41. Gilgenkrantz H, Collin de l'Hortet A (2011) New insights into liver regeneration. Clin Res Hepatol Gastroenterol 35:623–629
42. Shafritz DA, Dabeva MD (2002) Liver stem cells and model systems for liver repopulation. J Hepatol 36:552–564

43. Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. Gastroenterology 137:466–481
44. Español-Suñer R, Carpentier R, Van Hul N et al (2012) Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 143:1564–1575
45. Russo FP, Parola M (2012) Stem cells in liver failure. Best Pract Res Clin Gastroenterol 26:35–45
46. Tanaka EM (2003) Regeneration: if they can do it, why can't we? Cell 113:559–562
47. Mathur A, Martin JF (2004) Stem cells and repair of the heart. Lancet 365:183–192
48. Slack JM (2008) Origin of stem cells in organogenesis. Science 322:1498–1501
49. Blanpain C, Fuchs E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol 10:207–217
50. Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60–66
51. Taub R (2004) Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5:836–847
52. Fausto N, Campbell JS, Riehle KJ (2006) Liver regeneration. Hepatology 43:S45–S53
53. Zajicek G, Oren R, Weinreb M Jr (1985) The streaming liver. Liver 5:293–300
54. Fellous TG, Islam S, Taudros PJ et al (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. Hepatology 49:1655–1663
55. Yamada Y, Kirillova I, Peschon JJ et al (1997) Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc Natl Acad Sci U S A 94:1441–1446
56. Li W, Liang X, Kellendonk C et al (2002) STAT3 contributes to the mitogenic response of hepatocytes during liver regeneration. J Biol Chem 277:28411–28417
57. Huh CG, Factor VM, Sanchez A et al (2004) Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A 101:4477–4482
58. Russo FP, Parola M (2011) Stem and progenitor cells in liver regeneration and repair. Cytotherapy 13:135–144
59. Chobert MN, Couchie D, Fourcot A (2012) Liver precursor cells increase hepatic fibrosis induced by chronic carbon tetrachloride intoxication in rats. Lab Invest 92:135–150
60. Lorenzini S, Bird TG, Boulter L et al (2010) Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. Gut 59:645–654
61. Zheng YW, Taniguchi H (2003) Diversity of hepatic stem cells in the fetal and adult liver. Semin Liver Dis 23:337–348
62. Shafritz DA, Oertel M, Menthena A (2006) Liver stem cells and prospects for liver reconstitution by transplanted cells. Hepatology 43:S89–S98
63. Dollé L, Best J, Mei J et al (2010) The quest for liver progenitor cells: a practical point of view. J Hepatol 52:117–129
64. Boulter L, Govaere O, Bird TC et al (2012) Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 18:572–579
65. Kisseleva T, Gigante E, Brenner DA (2010) Recent advances in liver stem cell therapy. Curr Opin Gastroenterol 26:395–402
66. Forbes SJ, Newsome PN (2012) New horizons for stem cell therapy in liver disease. J Hepatol 56:496–499
67. Petersen BE, Bowen WC, Patrene KD et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
68. Theise ND, Nimmakayalu M, Gardner R et al (2000) Liver from bone marrow in humans. Hepatology 32:11–16
69. Alison MR, Poulsom R, Jeffery R et al (2000) Hepatocytes from non-hepatic adult stem cells. Nature 406:257
70. Terada N, Hamazaki T, Oka M et al (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545

71. Wang X, Willenbring H, Akkari Y et al (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901
72. Vassilopoulos G, Wang PR, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904
73. Jiang Y, Jahagirdar BN, Reinhardt RL et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49
74. Schwartz RE, Reyes M, Koodie L et al (2002) Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 109:1291–1302
75. Reyes M, Verfaillie CM (2001) Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci 938:231–233
76. Lange C, Bassler P, Lioznov MV et al (2005) Hepatocytic gene expression in cultured rat mesenchymal stem cells. Transplant Proc 37:276–279
77. Valfrè di Bonzo L, Ferrero I, Cravanzola C et al (2008) Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential. Gut 57:223–231
78. Ishii K, Yoshida Y, Akechi Y et al (2008) Hepatic differentiation of human bone marrow-derived mesenchymal stem cells by tetracycline-regulated hepatocyte nuclear factor 3beta. Hepatology 48:597–606
79. Seo MJ, Suh SY, Bae YC et al (2005) Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 328:258–264
80. Aurich H, Sgodda M, Kaltwasser P et al (2009) Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. Gut 58:570–581
81. Hong SH, Gang EJ, Jeong JA et al (2005) In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. Biochem Biophys Res Commun 330:1153–1161
82. Ishkitiev N, Yaegaki K, Calenic B et al (2010) Deciduous and permanent dental pulp mesenchymal cells acquire hepatic morphologic and functional features in vitro. J Endod 36:469–474
83. De Coppi P, Bartsch G Jr, Siddiqui MM et al (2007) Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 25:100–106
84. Miki T, Lehmann T, Cai H et al (2005) Stem cell characteristics of amniotic epithelial cells. Stem Cells 23:1549–1559
85. Oyagi S, Hirose M, Kojima M et al (2006) Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4-injured rats. J Hepatol 44:742–748
86. Abdel Aziz MT, Atta HM, Mahfouz S et al (2007) Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. Clin Biochem 40:893–899
87. Banas A, Teratani T, Yamamoto Y et al (2008) IFATS collection: in vivo therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. Stem Cells 26:2705–2712
88. Kuo TK, Hung SP, Chuang CH et al (2008) Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells. Gastroenterology 134:2111–2121
89. Yan Y, Xu W, Qian H et al (2009) Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury in vivo. Liver Int 29:356–365
90. Carvalho AB, Quintanilha LF, Dias JV et al (2008) Bone marrow multipotent mesenchymal stromal cells do not reduce fibrosis or improve function in a rat model of severe chronic liver injury. Stem Cells 26:1307–1314
91. Forbes SJ, Russo FP, Rey V et al (2004) A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 126:955–963
92. Russo FP, Alison MR, Bigger BW et al (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology 130:1807–1821
93. Kisseleva T, Uchinami H, Feirt N et al (2006) Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. J Hepatol 45:429–438

94. Lee UE, Friedman SL (2011) Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol 25:195–206
95. Forbes SJ, Parola M (2011) Liver fibrogenic cells. Best Pract Res Clin Gastroenterol 25:207–217
96. Dranoff JA, Wells RG (2010) Portal fibroblasts: underappreciated mediators of biliary fibrosis. Hepatology 51:1438–1444
97. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
98. Yu J, Vodyanik MA, Smuga-Otto K et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
99. Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
100. Yamanaka S, Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. Nature 465:704–712
101. Okita K, Yamanaka S (2010) Induction of pluripotency by defined factors. Exp Cell Res 316:2565–2570
102. Yamanaka S (2009) A fresh look at iPS cells. Cell 137:13–17
103. Liu H, Ye Z, Kim Y et al (2010) Generation of endoderm-derived human induced pluripotent stem cells from primary hepatocytes. Hepatology 51:1810–1819
104. Yu J, Thomson JA (2008) Pluripotent stem cell lines. Genes Dev 22:1987–1997
105. Ben-David U, Benvenisty N (2011) The tumorigenicity of human embryonic and induced pluripotent stem cells. Nat Rev Cancer 11:268–277
106. Si-Tayeb K, Noto FK, Nagaoka M et al (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305
107. Song Z, Cai J, Liu Y et al (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242
108. Sullivan GJ, Hay DC, Park IH et al (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51:329–335
109. Gai H, Nguyen DM, Moon YJ et al (2010) Generation of murine hepatic lineage cells from induced pluripotent stem cells. Differentiation 79:171–181
110. Li W, Wang D, Qin J et al (2010) Generation of functional hepatocytes from mouse induced pluripotent stem cells. J Cell Physiol 222:492–501
111. Behbahan IS, Duan Y, Lam A et al (2011) New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes. Stem Cell Rev 7:748–759
112. Espejel S, Roll GR, McLaughlin KJ et al (2010) Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. J Clin Invest 120:3120–3126
113. Rashid ST, Corbineau S, Hannan N et al (2010) Modeling metabolic inherited disorders of the liver using human pluripotent stem cells. J Clin Invest 120:3127–3136
114. Soto-Gutierrez A, Tafaleng E, Kelly V et al (2011) Modeling and therapy of human liver diseases using pluripotent stem cells: how far we come ? Hepatology 53:708–711
115. Amimoto N, Mizumoto H, Nakazawa K et al (2011) Hepatic differentiation of mouse embryonic stem cells and induced pluripotent stem cells during organoid formation in hollow fibers. Tissue Eng Part A 17:2071–2078
116. Sipe JD (2002) Tissue engineering and reparative medicine. Ann N Y Acad Sci 961:1–9
117. Piscaglia AC, Campanale R, Gasbarrini G et al (2010) Stem cell-based therapies for liver diseases: state of the art and new perspectives. Stem Cells Int 2010:259461. doi:10.4061/2010/259461
118. Han B, Lu Y, Meng B et al (2009) Cellular loss after allogenic hepatocyte transplantation. Transplantation 87:1–5
119. Dan Y, Yeoh G (2008) Liver stem cells: a scientific and clinical perspective. J Gastroenterol Hepatol 23:687–698
120. Alison MR, Islam S, Lim S (2009) Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly. J Pathol 217:282–298

121. Houlihan DD, Newsome PN (2008) Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 135:438–450
122. Couto BG, Goldenberg RC, da Fonseca LM et al (2011) Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. Liver Int 31:391–400
123. Salama H, Zekri AR, Bahnassy AA et al (2010) Autologous CD34+ and CD133+ stem cells transplantation in patients with end stage liver disease. World J Gastroenterol 16:5297–5305
124. Terai S, Ishikawa T, Omori K et al (2006) Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 24:2292–2298
125. Lyra AC, Soares MB, da Silva LF et al (2010) Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. Eur J Gastroenterol Hepatol 22:33–34

Lineage-Committed Pancreatic Progenitors and Stem Cells

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Abstract Type 1 diabetes is an autoimmune disease where self-reactive T cells attack and destroy insulin-producing beta cells. The prevalence of type 1 diabetes is increasing worldwide, but therapeutic options to cure diabetes are presently restricted to transplantation of cadaveric insulin-producing (islet) cells. One of the limitations to success of islet transplantation therapy is the lack of donor pancreatic islets. An alternative is to generate insulin-producing (β-like) cells in the laboratory. Various sources of stem/progenitor cells, such as those from the umbilical cord blood, bone marrow, as well as embryonic stem (ES) cells/induced pluripotent stem (iPS) cells, have been tested for their potential to differentiate into an endocrine pancreatic lineage. These studies have confirmed that it is very difficult to generate a cell type that is able to produce physiologically significant amounts of insulin and secrete it in response to glucose, in a manner similar to that demonstrated by pancreatic beta cells. This chapter reviews the differentiation and commitment of adult pancreatic progenitor/stem cells to endocrine pancreatic lineage and discusses the practical difficulties towards using these for treatment of diabetes in humans.

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Abbreviations

EMT	Epithelial-to-mesenchymal transition
ES	Embryonic stem
Glp1R	Glucagon-like peptide 1 receptor
hIPCs	Human islet-derived progenitor cells
hUCB	Human umbilical cord blood
iPS	Induced pluripotent stem
PDX1	Pancreas-duodenal homeobox gene 1
ICAs	Islet-like Cell Aggregates

1 Introduction

The prevalence of type 1 diabetes is increasing worldwide, estimated to rise from 135 million in 1995 to 300 million in 2025 [1–3]. Recent estimates indicate that 6–27 % of the deaths in the world between 35- and 64-year-old individuals are attributable to diabetes [2]. Type 1 diabetes is characterized by loss of insulin-producing beta cells. The present therapy involves daily administration of insulin, which does not mimic regulation of glucose in real time. Transplantation of cadaveric human islets has been demonstrated as a successful therapy in treatment of diabetes [4–6]. However, increasing lack of donor islets and low efficiency of isolating islets from cadaveric pancreas limit this therapy from being available to millions of diabetic individuals worldwide [7, 8]. An alternative to using cadaveric islets is to produce β cells in the laboratory. Being able to create β cells is one of the "holy grails" of diabetes research. Until now, several laboratories have demonstrated generation of insulin-producing cells in the lab [9–25]. Some of these (and several not cited here due to space limitations) have tested the potential of non-pancreatic cells, such as those from the umbilical cord, cord blood mononuclear cells, bone marrow-derived mesenchymal cells as well as embryonic stem (ES) cells/induced pluripotent stem (iPS) cells, to differentiate into an endocrine pancreatic lineage. However, the efficiency of insulin expression that is generally achieved by using such cells is very low. Therefore, although understanding the differentiation and commitment of such stem cells to endocrine pancreatic lineage is scientifically intriguing, it would be practically difficult to use these for treatment of diabetes in humans.

2 Epigenetic Regulation of Lineage Commitment

Majority of the cells in our body undergo a process during embryonic (and/or postnatal) development, which leads to their differentiation to a specific cellular lineage. At the molecular level, this process is now known to involve multiple histone

modifications as well as DNA methylation. Histones are positively charged (basic) proteins around which mammalian DNA is wrapped so as to package this 2 m long DNA into a space (nucleus) that is generally smaller than 4 μm in diameter. Localized addition of a negative charge to histone tails (for example by acetylation of histones) results in looping out of the DNA in that region, thereby allowing for easier accessibility to gene transcription. Some methylation signatures (discussed below) can deliver such a localized positive or negative charge on the histones, thereby modulating the DNA–histone interactions and ultimately influencing the accessibility of DNA to transcriptional machinery. DNA methylation involves the methylation of only those cytosine residues that precede a guanine [26]. These CG dinucleotides are seen to be present mostly around gene promoters and may undergo covalent modifications wherein hydrogen H5 of the Cytosine is replaced by a methyl group. It is estimated that in mammalian genome, 60–90 % of CpGs are methylated [27]. DNA methylation regulates binding of transcription factors/proteins to target sites on DNA, thereby influencing gene expression and also chromatin organization [28, 29]. The regulation of these molecular events leads to generation of a specific spatial/physical structure that is inherited through cellular divisions/proliferation and successive generations of these cells. Such modifications are collectively referred to as "epigenetic" changes and are central to lineage commitment in development of the cellular phenotype.

During the past decade, we [13, 30–34] and others [35–38] proposed that mesenchymal-like stem cells derived by epithelial-to-mesenchymal transition (EMT) of human pancreatic islets are the most efficient stem cells for differentiation to endocrine pancreatic lineage since they retain the epigenetic memory that defines an active insulin promoter region [33, 36]. These studies also conclusively demonstrated by lineage-tracing analysis [12, 31, 33, 35, 36] that human pancreatic beta cells proliferate in vitro to generate a population of lineage-committed human islet-derived progenitor cells (hIPCs). Until now, several progenitor cell types have been proposed to be useful for replacement therapy in diabetes (few outlined in Fig. 1a). It was demonstrated [39–43] that forced expression of "reprogramming factors" (also known as the "Yamanaka factors") allowed for conversion of fibroblasts or somatic cells to an embryonic stem cell-like state (Fig. 1a). These cells, referred to as induced pluripotent stem (iPS) cells are popular since they can be derived from adult somatic cells, including those from diabetic patients [25, 44], and could be potentially used for autologous transplantation in diabetes, *if efficient differentiation is achieved*. Another study [24, 45] also demonstrated that forced expression of pancreatic transcription factors (Pdx1, Ngn3, and MafA) induced insulin expression in exocrine pancreatic (acinar) cells (Fig. 1a). We and others have also demonstrated [32, 33, 35, 46] the role of several regulators (Fig. 1a) in generation of islet-derived progenitors (via EMT) and their conversion to insulin-producing cells (via reverse EMT or MET). We believe that it is presently difficult to achieve efficient differentiation of iPS or exocrine pancreatic cells to insulin-producing lineage unless accompanied by genetic manipulation. This is a big limitation that needs to be overcome by newer efficient technologies using, say, small regulatory molecules, which could achieve generation of such cell types without genetic manipulation. The ability to efficiently transcribe insulin gene depends on several factors that limit accessibility of transcriptional machinery to the insulin promoter region. Some of these regulatory factors are the enzymes that directly

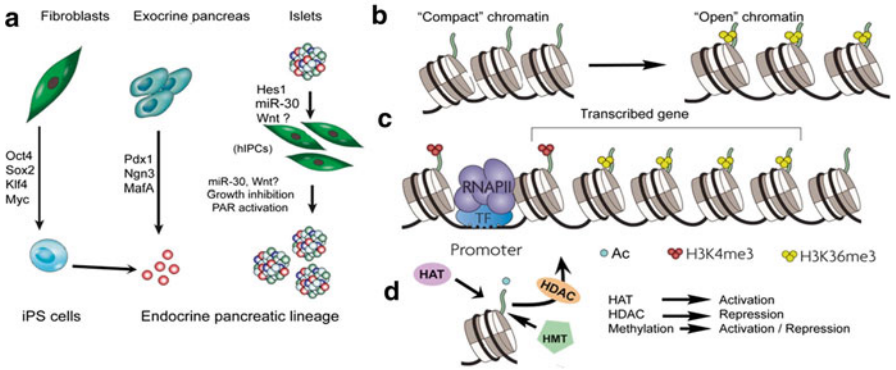

Fig. 1 Epigenetic regulation of cellular plasticity. It has been demonstrated that forced expression of master regulatory genes (shown in **a**) can induce the potency of fibroblasts or other cell types. Conversion of islets to hIPCs is now a well-known mechanism for generation of lineage-committed pancreatic stem cells. Chromatin conformation (**b**) decides for the accessibility of transcriptional machinery to physically bind the gene promoter (**c**) and read through (transcribe) the gene of interest. A number of different histone-modifying enzymes are known to play an important role in achieving an open or compact chromatin structure

influence the open or compact structure of chromatin (Fig. 1b). An open chromatin conformation would allow the transcriptional machinery to "sit" or physically occupy a binding site upstream of the insulin promoter region and "read" through the entire gene (Fig. 1c). The open or compact conformations of chromatin are regulated during embryonic development, when cells commit to specific lineages. Recruitment of specific enzymes (Fig. 1d) that methylate or acetylate histones is one of the regulatory events that decide the accessibility of gene promoters and, therefore, the efficiency of gene expression.

Stem/progenitor cells (discussed above) are thought to be alternative cells for replacement therapy in diabetes. Although studies on ES cells have shown to mimic pancreas development in vitro, thereby leading to differentiation into insulin-producing cells [47–49], several issues related to safety as well as efficiency of the protocol with other ES/iPS cells need to be addressed before thinking of their role for cell therapy in treatment of human diabetes. Other important sources of progenitors are mesenchymal stem cells derived from various adult tissues such as the bone marrow. Several different strategies have been used to induce differentiation of these progenitors into beta-like insulin-producing cells. Apart from these, transdifferentiation of liver cells or acinar cells or alpha cells into beta cells is also considered as an important source of obtaining insulin-producing cells. These cells have been used based on their ontogenic relation to beta cells; all being derived from developing endoderm. Most of these differentiated cells were also able to reverse experimental diabetes in rodent animal models. However, the amount of insulin produced by them is far less than that seen in adult human islet β cells. As of now, the best replacement for human pancreas are the pancreatic islet cells themselves, which is unequivocally proved through several transplantations using isolated islets from cadaveric human

donors [50, 51]. However, their availability and yield remain to be some of the major constraints. In order to overcome this problem, attempts were made to expand/grow β cells in vitro. Pancreatic β cells have a fairly long life (predicted at least ~4 months) and they do not proliferate rapidly in vivo. Although there are observations supporting in vivo proliferation of β cells [52–54], in vitro proliferation of β cells is difficult to achieve unless allowing for their dedifferentiation. We demonstrated earlier [13, 30, 33, 34] that pancreatic β cells rapidly lose their identity in culture and dedifferentiate into mesenchymal-like cells through a process identified as epithelial-to-mesenchymal transition (EMT). However, there has been a debate regarding EMT of human β cells in cultures [31]. Reports from mouse pancreatic islets show that mouse beta cells may not proliferate as mesenchymal progenitors in cultures. However, in the last few years, we and others have independently demonstrated that human pancreatic β cells proliferate in vitro and also undergo EMT to generate proliferative population of mesenchymal progenitor cells. We believe that human islet-derived progenitor cells (hIPCs) are better islet cell progenitors than other embryonic stem/pluripotent cells, as they are derived from cells that know (epigenetic memory) how to produce insulin. Such hIPCs retain an active chromatin conformation at the insulin promoter region [33, 55] even after thousand fold expansion in vitro. As hIPCs have epigenetic memory of producing insulin, they can be differentiated into insulin-producing cells in vitro with better efficiency as compared to other progenitor cells that have either a globally open chromatin conformation (such as embryonic stem cells) or inactive chromatin conformation at insulin promoter region (such as skin cells). Embryonic stem cells are said to have a "ground state" chromatin conformation where most of the chromatin is transcriptionally active. Such an open chromatin is accessible to various external signals and transcription factors that eventually direct the differentiation of these embryonic stem cells and their progenies. Embryonic stem cells therefore require passing through several stages involving a delicate balance of different transcription factors and signaling molecules before differentiating into insulin-producing cells. It is therefore proposed that pancreatic islet-derived progenitors need fewer steps to commit again to insulin-producing cells [56].

3 Cell Replacement Therapy in Diabetes

Diabetes affects ~300 million individuals globally and is a major health concern worldwide. Type 1 diabetes is characterized by hyperglycemia due to loss of functional beta cell mass, while type 2 diabetes is characterized by insulin resistance and ultimately may involve exogenous insulin administration. Although daily insulin injections can regulate blood glucose concentrations, the long-term complications of diabetes, affecting other body organs, are unavoidable. Islet transplantation under immunosuppressive regimen has been shown to be successful for up to 5 years post-transplantation and is presently used as a cell-based therapy for diabetes [8, 57]. However scarcity of available pancreas and low yields of isolated islets make it necessary to look for alternate sources of insulin-producing cells.

Stem cells from both adult and embryonic sources offer a promising resource to generate a wide range of tissue types for the treatment of a variety of degenerative and autoimmune diseases, such as Parkinson's disease, spinal cord injuries, myocardial infarction, and diabetes [58–65]. However, as discussed above, there is now increasing evidence and rationale to believe that adult stem cells hold greater promise for cell replacement therapy in diabetes.

4 Adult Stem Cells

A stem cell is an undifferentiated cell that can be distinguished by its ability to proliferate through asymmetric cell division; a process wherein one of the daughter cells commits to a specialized lineage while the other continues as an undifferentiated stem cell retaining the capacity to undergo multilineage differentiation [66]. This unique property of asymmetric division makes stem cells a potential key therapeutic source for restoring cells and tissues and to treat a wide range of diseases, including diabetes [60, 67]. Although asymmetric division is believed to be a property of embryonic stem cells, it is now well agreed that even adult stem cells show asymmetric division to produce a differentiated cell type while retaining their own stem cell population. Adult stem cell research is becoming increasingly popular as it does not raise the common controversies that embryonic stem cells do. Adult stem cells can be obtained from different tissues, including the umbilical cord, bone marrow, adipose tissue, and pancreatic cells. This chapter describes the stem cells derived from these different adult tissues and discusses their potential as a source for cell replacement therapy in diabetes.

5 Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells

We demonstrated that mesenchymal-like cells obtained from umbilical cord blood are lineage-committed pancreatic progenitor cells [17]. We find that human umbilical cord blood (hUCB)-derived mesenchymal cells naturally express two important pancreas-specific gene transcripts: pancreas-duodenal homeobox gene 1 (PDX1) and glucagon-like peptide 1 receptor (Glp1R) [17]. Pdx1 is a homeodomain transcription factor expressed by cells lining the primitive gut tube, which eventually give rise to the pancreas [68]. Presence of PDX1 in the nucleus of hUCB-derived mesenchymal cells indicates that pdx1 is transcribed, translated, phosphorylated, and translocated to the nucleus of these cells. Although PDX1 is critical for pancreas development, it is also known to be necessary for efficient insulin gene transcription [69]. Glp1R is an important cell surface receptor that controls expression of pdx1 during early development and also controls the beta-cell function in adult life [70].

Expression of pdx1 along with glp1r is shown to promote growth and differentiation during embryonic development as well as improved glucose homeostasis [71]. The expression of pancreas-specific genes by hUCB-derived mesenchymal cells is indeed intriguing. The potential of hUCB-derived mesenchymal cells to differentiate into endocrine pancreatic lineage is therefore very high. However, one of the limiting factors that we observe is the number of such cells obtained from human umbilical cord blood. When we attempted to grow these hUCB-derived mesenchymal cells in vitro, pancreas-specific transcription factors decreased in abundance indicating that endocrine pancreatic progenitors either die and/or get diluted out or change/dedifferentiate further, as these cell populations proliferate in vitro. A few studies have shown the potential of MNCs to differentiate in NOD/SCID β-2 microglobulin^{null} mice following intravenous infusion [72] and exhibit improvement in prediabetic status in experimental animals [73, 74]. Their role in alleviation of diabetic symptoms is attributed to the high T(reg) cell population present within MNCs [75]. In order to test their potential to cure diabetes, hUCB-derived mesenchymal cells were transplanted under the kidney capsule of NOD/SCID mice or encapsulated in a commercially available immunoisolatory device (Theracyte™) and implanted subcutaneously in immunocompetent (FVB/Nj) mice [17]. Here, 50 % of the pancreas was removed following partial pancreatectomy to develop transient diabetes in a group of transplant recipients. Partial pancreatectomy is a well-established model [76, 77] to study regenerative biology of the pancreas, even in diabetic mice. Mice were sacrificed at predefined time intervals and animal sera and transplant grafts were collected. Quantitative estimation of insulin transcripts and serum insulin concentration data suggests that hUCB-derived mesenchymal cell grafts synthesize, process, and secrete insulin. Interestingly, the (pro-) insulin transcript abundance in grafts and mature human insulin in circulation were found tenfold and fivefold more, respectively, in the pancreatectomized group as compared to the sham-operated mice. These data indicate that mononuclear cells do have potential to differentiate to insulin-producing cells upon transplantation in immune-compromised as well as immune-competent mice. Furthermore, factors secreted during pancreatic regeneration may help in achieving better differentiation/maturation of transplanted hUCB-MNCs into insulin-producing cells. Identification of such molecular regulators will allow us to devise protocols for efficient differentiation of these pancreatic progenitor cells in vitro.

Identification of potential pancreatic progenitors is becoming increasingly important to generate more “transplant friendly” source of cells for replacement therapy in diabetes. The discovery of endocrine pancreas-specific transcription factors and cell surface markers in hUCB-derived mesenchymal cells is very exciting. Although we remain unaware of the exact molecular mechanisms that are turned on during the development of blood cells, it is important to learn their developmental biology, so as to identify and develop approaches to expand these in culture, and optimize suitable differentiation protocols that would make further use of stored umbilical cord blood samples for treatment of diabetes.

6 Bone Marrow-Derived Stem Cells

The bone marrow is one of the most common primitive progenitor/mesenchymal cell type derived for treating a wide range of diseases [67, 78–83]. The bone marrow contains a number of distinct stem cells such as hematopoietic stem cells, mesenchymal stems cells, endothelial progenitor cells, and fibrocytes [84, 85]. Mesenchymal stem cells (MSCs) are a class of adult progenitor cells capable of differentiation to multiple lineages. They can be isolated from many tissues, with the bone marrow being the most common site for isolation of MSCs [86]. In order to isolate MSCs, needle aspirates are obtained through a relatively painful procedure from the bone marrow and then mononucleated cells are isolated and grown prior to using these in any differentiation procedures. Although such MSCs can be made available in large numbers, several studies presented until now have failed to achieve efficient differentiation of bone marrow-derived MSCs to insulin-producing beta cells. Some studies have failed to achieve any differentiation of bone marrow cells to express insulin [87, 88]. A study by Lechner et al. [88] did not find bone marrow-derived cells to contribute to regeneration of pancreatic endocrine cells. A study by Choi et al. [87], which applied GFP-tagged bone marrow cells into irradiated mice with also the addition of low-dose streptozotocin (STZ), a beta-cell toxin, indicated that these cells cannot endure any insulin expression even with low exposure to STZ. Further clarification would require understanding the potential of bone marrow stem cells to be a source in contributing to or for differentiating into functional insulin-producing cells.

It is generally observed that human bone marrow-derived mesenchymal cells (hBMCs) isolated from fetal, young adult, or elder donors show homogenous expression of mesenchymal markers and also exhibit plasticity when induced to differentiate into hematopoietic, adipogenic, chondrogenic, osteogenic, or neural lineage [18]. However, these studies also indicate that hBMCs from fetal and young adult donors could be expanded over billion fold in vitro whereas those from elder donors were difficult to expand beyond 2,000-fold. These observations suggest that human bone marrow cells may offer best expansion potential (unlike cord blood-derived mesenchymal cells), when isolated from younger donors. It has been demonstrated that hBMCs play a role in organ-specific repair and regeneration [89]. We find that hBMCs, during initial phase of expansion, express islet-specific transcription factors such as Hlxb9, Nkx6.1, Pax6, Isl1, Brn-4 (Pou3F4), as well as Gcg along with Gata4 and Gata6. Our in vitro differentiation studies reveal that hBMCs transition into epithelial lineage following induction of differentiation. NeuroD was not detected in the differentiated cells in vitro possibly due to the histone deacetylases 1 and 3 [90] expression in the hBMC-derived islet-like cell aggregates/ICAs [18]. Although we were able to achieve increased abundance of pancreas-specific transcription factors (ngn3, brn4, nkx6.1, pax6, and isl1) as well as endocrine pancreatic (pro-) hormone transcripts (gcg and sst) were observed, pro-insulin transcript expression was not seen indicating the inefficiency of this protocol to induce

differentiation into mature insulin-producing cells. We recently reported that hBMCs derived from chronic diabetic patients expressed C-peptide and insulin [91]. Insulin expression, albeit at very low levels, suggests the potential of hBMCs to differentiate into endocrine pancreatic lineage. Our studies suggest that paracrine factors and the in vivo niche may actually be critical in promoting the differentiation of hBMCs in vivo. We therefore transplanted lineage-committed hBMCs (hBMCs that have undergone mesenchymal–epithelial transition) under the kidney capsule of NOD/SCID mice that were pancreatectomized (as discussed earlier in hUCB-derived cells). Significant increases in pro-insulin transcripts were seen in transplanted hBMCs [18] as compared to in vitro differentiated hBMCs [92]. Ai et al. [93] demonstrated that human fetal BMCs successfully mature into insulin-secreting islet-like clusters in fetal pancreatic microenvironment. Transfection of three genes, Pdx-1, NeuroD1, and Ngn3 in hBMCs followed by in vitro differentiation, created cells that were less glucose responsive in vitro but then became more efficient after transplantation [94]. Since transplantation of ICAs in pancreatectomized mice model significantly enhanced the expression of the pancreatic hormones, it is speculated that certain key factors from the pancreatic niche are essential for maturation of hBMC-derived ICAs. Studies carried out in our laboratory demonstrate that in vitro expanded hBMCs show significantly higher H3K9-me2 and H3K27-me2 (inactive marks/compact chromatin) at the insulin promoter region, as compared to human pancreatic islet cells [18]. As discussed earlier (Fig. 1), acetylation or deacetylation of histone terminal domains is known to regulate gene expression. We therefore hypothesized that paracrine factors secreted from regenerating pancreas may target localized acetylation and/or methylation at insulin promoter region. To test this, we modified our in vitro differentiation protocol so as to assess the potential of HDAC inhibitors and DNA methyl transferase inhibitors. hBMCs were grown in cell culture media containing DNA methylation inhibitor (5-aza-2′-deoxycytidine) along with a histone deacetylase (HDAC) inhibitor (Trichostatin A), prior to inducing differentiation using our conventional protocol [18]. We observe significant increase in the levels of all endocrine gene transcripts [18] including Pdx1 and glucokinase in these clusters. These data demonstrate that DNMTase and HDAC inhibitors can significantly enhance in vitro differentiation of hBMCs [18]. Recently Haumaitre et al. [95] demonstrated that exposure of murine embryonic pancreas to trichostatin A and sodium butyrate enhanced the pool of β cells within the embryonic pancreas. It appears that the in vivo niche possibly works through induction of chromatin modulators by recruiting specific methyl transferases and/or demethylases, so as to bring about changes in gene expression. In summary, hBMCs represent a class of pancreatic progenitors that express mesenchymal gene transcripts as well as key pancreatic transcription factors that are known to be necessary for normal development of endocrine pancreas. The use of a xeno protein-free media in our protocol [18, 91, 96] further helps in optimization of expansion protocols for human bone marrow-derived mesenchymal stem cells. Further studies will help in optimizing methods for efficient differentiation of hBMCs to endocrine pancreatic lineage for replacement therapy in diabetes.

7 Hepatic and Biliary Stem Cells

The liver is the central organ for homeostasis, responsible for balancing multiple metabolisms such as for carbohydrate, lipid, and glycogen storage [97]. Liver stem cells have the capacity to trans-differentiate into functional endocrine cells such as insulin-secreting cells [98–103]. Forced expression of pdx1 (the key regulator of pancreatic development) in liver cells has been identified to be sufficient for inducing insulin expression in liver cells [15, 22, 99, 100]. However, no small molecules or other non-genetic manipulation methodologies have been identified as yet to mimic this effect. Genetic manipulation of cells is a major concern in cellular therapies for human trials and therefore more and more emphasis is now given to developing our understanding about small regulatory molecules and other naturally occurring insulin-producing cells. One of the most interesting findings from our group and others in the past decade was the identification of insulin-producing cells in the biliary duct and the gallbladder [19, 104–108]. Human gallbladder/biliary duct epithelial cells express islet-specific hormones and transcription factors, albeit at very low levels, in their natural state [104]. We observe that human gallbladder or biliary duct epithelial cells contain insulin-producing cells [19, 104] and proliferate in vitro when exposed to growth-promoting medium. In fact, the proliferating population of cells originates from the native insulin expressing cells in the gallbladder epithelium (unpublished data and personal communications). Since human gallbladder epithelial cells produce insulin and other pancreatic hormones in their normal state, it is becoming increasingly interesting to use these cells for generation of pancreatic progenitors. These cells have at least two major advantages: first, there are a large number of human gallbladders available from cholecystectomies (surgical removal of gallbladders), thereby making it easier to have these available in plenty, and second, they do not need any genetic manipulation for insulin production. However, the major limitation is that the level of insulin produced by these cells is very low as compared to adult human islets. Nonetheless, these are at par with (or better than) the level of insulin reported in most differentiated stem cells. Further studies are therefore needed to understand the molecular mechanisms that allow for enhancing the expression of insulin in human gallbladder epithelial cells. Identification of such regulatory mechanisms will aid in development of endocrine pancreatic lineage-committed cells for treatment of diabetes.

8 Human Pancreatic Stem Cells

Pancreatic stem cells have been extensively studied for their potential to regenerate islet cells [84, 109–114]. Pancreatic islets, ducts, and acinar cells are believed to harbor stem cells that have the potential to differentiate into insulin-producing cells. The potency of these cells (uni-, multi-, or pluripotency) is a matter of debate. With the recent progress in generation of iPS cells, it would be possible to convert

these into pluripotent cells with the possibility of retaining their epigenetic state [115]. These findings demonstrated that the differentiation potential of iPS cells may depend on their origin and iPS cells derived from pancreatic beta cells would have several significant advantages over conventional pluripotent cells.

Pancreatic "stem cells" have been isolated and expanded from adult human pancreatic ducts and induced to differentiate into insulin-producing cells [10, 34, 116–118]. Studies carried out in mice with experimentally induced diabetes (using STZ) suggest that mouse pancreatic duct precursor cells retain the capacity to regenerate the entire pancreas following surgical resection and give rise to functional insulin-producing cells in vivo [76, 119, 120]. Although these studies carried out in the intact animal as well as studies carried out in vitro [121] indicate that pancreatic duct cells are capable of differentiation to endocrine pancreatic lineage, even in the diabetic condition, these studies lack the lineage studies that could confirm the role of pancreatic duct cells in restoration of beta-cell mass in diabetic mice, following pancreatectomy.

As discussed earlier (Fig. 1a), the exocrine pancreas is one of the most interesting sites to look for pancreatic stem cells. The exocrine pancreas is a bag full of enzymes and the islets (interestingly located within the pancreas) face risk of being autolyzed following exposure to the cocktail of digestive enzymes produced by the exocrine pancreas. However, studies presented and discussed above (Fig. 1a) demonstrate that although these exocrine cells are functionally very diverse to the endocrine pancreas, forced expression of a combination of pancreas-specific transcription factors (such as Pdx1, MafA, and Ngn3) can lead to induction of insulin expression in these cells. A model of efficient insulin gene expression is presented in Fig. 2. As shown here, Pdx1 and MafA—the two most important transcription factors essential for efficient production of insulin—are localized to chromosomes other than those containing the insulin gene. It is believed that the epigenetic state of pancreatic beta cells allows for the chromatin to be in a physical conformation that allows for these (and/or other) transcription factors to loop in to a single transcription factory (dotted arrow to the left in Fig. 2), so as to enhance insulin gene transcription. Since ES cells or other pluripotent cells may lack this physical conformation, these cell types would be mostly able to synthesize very low levels of insulin. An endocrine pancreatic lineage-committed cell would be one that is generated from insulin-producing cells, such as the gallbladder epithelial cells, or ideally, from human pancreatic islet beta cells.

Human islet-derived progenitor cells (hIPCs) have been widely discussed until now. Studies presented by the group of Prof. Shimon Efrat [35, 36] as well as our group [30–33] indicate using lineage-tracing studies that human pancreatic beta cells proliferate and give rise to mesenchymal cells in vitro. However, one of the disadvantages reported [13] was that the efficiency of differentiation of these hIPCs decreased with increasing passage numbers. This may potentially happen for two reasons: (1) the epigenetic marks that define the insulin-promoter region in pancreatic beta cells may change following exposure to different conditions and following over million-fold expansion and (2) pancreatic beta cells may proliferate at a slower rate than other pancreatic islet cells in culture, thereby leading to increased dilution

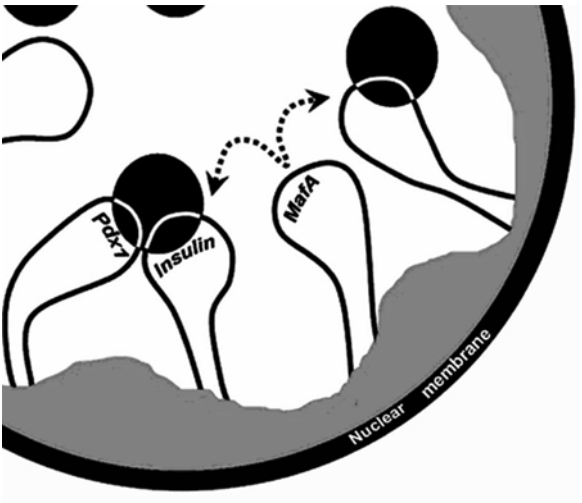

Fig. 2 A dynamic model of chromatin loop interactions in lineage-committed cells. It is well known that the mammalian genome is well organized inside the nucleus and that there are chromosomal territories that are retained by specific cell types. The densely staining regions of the nucleus observed after exposure to a nuclear dye such as DAPI or Hoechst 33372 indicates the heterochromatin (inactive) regions. It is now well known that chromosomal loops "swing" into the euchromatin region to form a part of transcriptional factories (*black circles*). The insulin, Pdx1, and MafA genes are localized on different chromosomes and are believed to loop in to a highly efficient insulin-producing factory, in pancreatic beta cells. The presence or absence of these transcription factors and their physical conformation would play a major role in deciding the fate of insulin production in such stem/progenitor cells

of beta-cell progenies in the subsequent passages. This can be overcome by sorting for beta cells using highly specific cell surface markers. However, efficient technologies for retaining chromatin conformation, DNA methylation, and cellular transcriptome need to be developed in order to identify beta-cell proliferation strategies for generation of insulin-producing beta-like cells.

9 Conclusions

A stem cell can be derived from the embryo or in case of adult stem cells; these can be found in umbilical cord blood, adipose tissue, and other tissues as discussed above. In general, a stem cell is believed to have the ability for self renewal as well as differentiation/lineage commitment. Depending on their potency (uni-, multi-, or pluripotency), they are also capable of forming any type of tissue or organ in the body. ES cells originate from the inner cell mass of blastocysts. They are pluripotent and capable of differentiating to any lineage. Adult stem cells are generally restricted to differentiate into a specific lineage, often restricted to the tissue that these are

derived from. For example, a "stem" (precursor) cell isolated from the fat pads in mouse limbs can be expanded/propagated and differentiated to produce more adipocytes/fat storing cells. However, it may be difficult to turn such cells into neurons. Lineage commitment is the checkpoint in development, which is difficult to be reversed by conventional growth and propagation techniques. ES cells are pluripotent as they have not committed to any specific lineage. However, when we speak of cell replacement therapy for diabetes, the question that we really need to ask is: do we need to start with pluripotent cells? There are several limitations as we discussed earlier. One of these is the ability to achieve efficient and homogeneous differentiation in all the ES/pluripotent cells that are used. This is often an uphill task with little success achieved in the differentiation protocols across the world. The major issue that marks a red flag to research on embryonic stem cells is their potential to form teratomas in vivo. With the heterogeneity that is achieved in differentiation of cell types, it is easy to imagine that a few ES cells may remain undifferentiated within a cluster of seemingly differentiated, insulin-producing cells. However, a few ES cells can proliferate in vivo and form tumors. This is a major limitation of most pluripotent cells.

Restricting the lineage to pancreatic lineage has several advantages. The major advantage is that the insulin gene would be in a physical conformation that is poised to transcribe insulin. Epigenetic regulation of gene expression is well recognized as a critical factor in cellular differentiation. Heterochromatinization of DNA through histone methylation as well as DNA methylation of CpGs are mechanisms of gene silencing. DNA methylation is believed to present a more stable form of gene repression. During ES cell differentiation, histone modifications at Oct-3/4 (a pluripotency-associated gene) occur prior to DNA methylation. Interestingly, heterochromatinization alone was seen to be able to inhibit the reactivation of Oct-3/4 expression when the process of differentiation was terminated, but this alone was not enough to prevent reprogramming of the differentiated cells. However, DNA methylation at Oct-3/4 promoter was shown to be capable of ensuring that differentiated cells would not easily return to the pluripotent state [122]. Epigenetic signatures such as DNA methylation and histone modifications thus play an important role in defining the differentiation potential of adult stem cells. As discussed above, adult stem cells that are committed to endocrine pancreatic lineage hold great promise for cell therapy. Understanding molecular mechanisms that can help in increasing the levels of insulin gene expression in these naturally occurring tissues (such as the gallbladder) or in lineage-committed stem cells (such as hIPCs) would help in generating substantial numbers of clinically important stem cells for cell replacement therapy in diabetes.

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References

1. King H, Aubert RE, Herman WH (1998) Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 21(9):1414–1431
2. Roglic G, Unwin N, Bennett PH, Mathers C, Tuomilehto J, Nag S, Connolly V, King H (2005) The burden of mortality attributable to diabetes: realistic estimates for the year 2000. Diabetes Care 28(9):2130–2135
3. Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27(5):1047–1053
4. Ricordi C, Hering BJ, Shapiro AM (2008) Beta-cell transplantation for diabetes therapy. Lancet 372(9632):27–28, author reply 29–30
5. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC et al (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355(13):1318–1330
6. Shapiro AM, Ryan EA, Lakey JR (2001) Diabetes. Islet cell transplantation. Lancet 358(Suppl):S21
7. Harlan DM, Rother KI (2004) Islet transplantation as a treatment for diabetes. N Engl J Med 350(20):2104, author reply 2104
8. Rother KI, Harlan DM (2004) Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus. J Clin Invest 114(7):877–883
9. Bonner-Weir S, Inada A, Yatoh S, Li WC, Aye T, Toschi E, Sharma A (2008) Transdifferentiation of pancreatic ductal cells to endocrine beta-cells. Biochem Soc Trans 36(Pt 3):353–356
10. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 97(14):7999–8004
11. Burke ZD, Shen CN, Tosh D (2004) Bile ducts as a source of pancreatic beta cells. Bioessays 26(9):932–937
12. Efrat S (2008) In vitro expansion of human beta cells. Diabetologia 51(7):1338–1339
13. Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B, Raaka BM (2004) Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science 306(5705):2261–2264
14. Hardikar AA, Wang XY, Williams LJ, Kwok J, Wong R, Yao M, Tuch BE (2002) Functional maturation of fetal porcine beta-cells by glucagon-like peptide 1 and cholecystokinin. Endocrinology 143(9):3505–3514
15. Horb ME, Shen CN, Tosh D, Slack JM (2003) Experimental conversion of liver to pancreas. Curr Biol 13(2):105–115
16. Kikugawa R, Katsuta H, Akashi T, Yatoh S, Weir GC, Sharma A, Bonner-Weir S (2009) Differentiation of COPAS-sorted non-endocrine pancreatic cells into insulin-positive cells in the mouse. Diabetologia 52(4):645–652
17. Parekh VS, Joglekar MV, Hardikar AA (2009) Differentiation of human umbilical cord blood-derived mononuclear cells to endocrine pancreatic lineage. Differentiation 78(4): 232–240
18. Phadnis SM, Joglekar MV, Dalvi MP, Muthyala S, Nair PD, Ghaskadbi SM, Bhonde RR, Hardikar AA (2011) Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage in vivo. Cytotherapy 13(3):279–293
19. Sahu S, Tosh D, Hardikar AA (2009) New sources of beta-cells for treating diabetes. J Endocrinol 202(1):13–16
20. Weir GC, Bonner-Weir S (2004) Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 53(Suppl 3):S16–S21
21. Xu PX, Zhang X, Heaney S, Yoon A, Michelson AM, Maas RL (1999) Regulation of Pax6 expression is conserved between mice and flies. Development 126(2):383–395
22. Zalzman M, Anker-Kitai L, Efrat S (2005) Differentiation of human liver-derived, insulin-producing cells toward the beta-cell phenotype. Diabetes 54(9):2568–2575

23. Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci U S A 100(12):7253–7258
24. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455(7213):627–632
25. Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL, Melton DA (2009) Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci U S A 106(37):15768–15773
26. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5(3):309–314
27. Brena RM, Huang TH, Plass C (2006) Toward a human epigenome. Nat Genet 38(12): 1359–1360
28. Biran A, Meshorer E (2012) Concise review: chromatin and genome organization in reprogramming. Stem Cells 30(9):1793–1799
29. Fisher CL, Fisher AG (2011) Chromatin states in pluripotent, differentiated, and reprogrammed cells. Curr Opin Genet Dev 21(2):140–146
30. Joglekar MV, Hardikar AA (2012) Isolation, expansion, and characterization of human islet-derived progenitor cells. Methods Mol Biol 879:351–366
31. Joglekar MV, Hardikar AA (2010) Epithelial-to-mesenchymal transition in pancreatic islet beta cells. Cell Cycle 9(20):4077–4079
32. Joglekar MV, Patil D, Joglekar VM, Rao GV, Reddy DN, Mitnala S, Shouche Y, Hardikar AA (2009) The miR-30 family microRNAs confer epithelial phenotype to human pancreatic cells. Islets 1(2):137–147
33. Joglekar MV, Joglekar VM, Joglekar SV, Hardikar AA (2009) Human fetal pancreatic insulin-producing cells proliferate in vitro. J Endocrinol 201(1):27–36
34. Hardikar AA, Marcus-Samuels B, Geras-Raaka E, Raaka BM, Gershengorn MC (2003) Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. Proc Natl Acad Sci U S A 100(12):7117–7122
35. Russ HA, Ravassard P, Kerr-Conte J, Pattou F, Efrat S (2009) Epithelial-mesenchymal transition in cells expanded in vitro from lineage-traced adult human pancreatic beta cells. PLoS One 4(7):e6417
36. Russ HA, Bar Y, Ravassard P, Efrat S (2008) In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes 57(6):1575–1583
37. Efrat S (2008) Beta-cell replacement for insulin-dependent diabetes mellitus. Adv Drug Deliv Rev 60(2):114–123
38. Efrat S (2008) Ex-vivo expansion of adult human pancreatic beta-cells. Rev Diabet Stud 5(2):116–122
39. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
40. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
41. Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 321(5889):699–702
42. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26(1):101–106
43. Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313–317
44. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA (2008) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26(11):1269–1275
45. Zhou Q, Melton DA (2008) Extreme makeover: converting one cell into another. Cell Stem Cell 3(4):382–388

46. Bar Y, Russ HA, Knoller S, Ouziel-Yahalom L, Efrat S (2008) HES-1 is involved in adaptation of adult human beta-cells to proliferation in vitro. Diabetes 57(9):2413–2420
47. Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, Bhoumik A, Cepa I, Cesario RM, Haakmeester C et al (2012) A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 7(5):e37004
48. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24(11):1392–1401
49. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23(12):1534–1541
50. Gill RG, Bishop NH (2012) Clinical islet transplantation: where immunity and metabolism intersect? Curr Opin Endocrinol Diabetes Obes 19(4):249–254
51. Robertson RP (2010) Islet transplantation a decade later and strategies for filling a half-full glass. Diabetes 59(6):1285–1291
52. Van de Casteele M, Leuckx G, Baeyens L, Cai Y, Yuchi Y, Coppens V, De Groef S, Eriksson M, Svensson C, Ahlgren U et al (2013) Neurogenin 3(+) cells contribute to beta-cell neogenesis and proliferation in injured adult mouse pancreas. Cell Death Dis 4:e523
53. Gargani S, Thevenet J, Yuan JE, Lefebvre B, Delalleau N, Gmyr V, Hubert T, Duhamel A, Pattou F, Kerr-Conte J (2013) Adaptive changes of human islets to an obesogenic environment in the mouse. Diabetologia 56(2):350–358
54. Plank JL, Frist AY, LeGrone AW, Magnuson MA, Labosky PA (2011) Loss of Foxd3 results in decreased beta-cell proliferation and glucose intolerance during pregnancy. Endocrinology 152(12):4589–4600
55. Mutskov V, Raaka BM, Felsenfeld G, Gershengorn MC (2007) The human insulin gene displays transcriptionally active epigenetic marks in islet-derived mesenchymal precursor cells in the absence of insulin expression. Stem Cells 25(12):3223–3233
56. Gershengorn MC, Geras-Raaka E, Hardikar AA, Raaka BM (2005) Are better islet cell precursors generated by epithelial-to-mesenchymal transition? Cell Cycle 4(3):380–382
57. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343(4):230–238
58. Aguayo-Mazzucato C, Bonner-Weir S (2010) Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol 6(3):139–148
59. Brignier AC, Gewirtz AM (2010) Embryonic and adult stem cell therapy. J Allergy Clin Immunol 125(2 Suppl 2):S336–S344
60. Dominguez-Bendala J, Lanzoni G, Inverardi L, Ricordi C (2012) Concise review: mesenchymal stem cells for diabetes. Stem Cells Transl Med 1(1):59–63
61. Halban PA (2004) Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. Nat Cell Biol 6(11):1021–1025
62. Lerou PH, Daley GQ (2005) Therapeutic potential of embryonic stem cells. Blood Rev 19(6):321–331
63. Mimeault M, Batra SK (2008) Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. Stem Cell Rev 4(1):27–49
64. Redi CA, Monti M, Merico V, Neri T, Zanoni M, Zuccotti M, Garagna S (2007) Stem cells. Endocr Dev 11:145–151
65. Serakinci N, Keith WN (2006) Therapeutic potential of adult stem cells. Eur J Cancer 42(9):1243–1246
66. Hardikar AA, Gershengorn MC (2004) In: LeRoith D, Taylor SI, Olefsky JM (eds) Stem cells diabetes mellitus: a fundamental and clinical text, Lippincott Williams & Wilkins, 3rd edn. Part V(Chapter 51): 773–781
67. Togel F, Westenfelder C (2007) Adult bone marrow-derived stem cells for organ regeneration and repair. Dev Dyn 236(12):3321–3331

68. Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371(6498):606–609
69. Babu DA, Deering TG, Mirmira RG (2007) A feat of metabolic proportions: Pdx1 orchestrates islet development and function in the maintenance of glucose homeostasis. Mol Genet Metab 92(1–2):43–55
70. Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48(12):2270–2276
71. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ (2005) beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. Diabetes 54(2):482–491
72. Yoshida S, Ishikawa F, Kawano N, Shimoda K, Nagafuchi S, Shimoda S, Yasukawa M, Kanemaru T, Ishibashi H, Shultz LD et al (2005) Human cord blood-derived cells generate insulin-producing cells in vivo. Stem Cells 23(9):1409–1416
73. Ende N, Chen R, Reddi AS (2004) Effect of human umbilical cord blood cells on glycemia and insulitis in type 1 diabetic mice. Biochem Biophys Res Commun 325(3):665–669
74. Ende N, Chen R, Reddi AS (2004) Transplantation of human umbilical cord blood cells improves glycemia and glomerular hypertrophy in type 2 diabetic mice. Biochem Biophys Res Commun 321(1):168–171
75. Haller MJ, Viener HL, Wasserfall C, Brusko T, Atkinson MA, Schatz DA (2008) Autologous umbilical cord blood infusion for type 1 diabetes. Exp Hematol 36(6):710–715
76. Hardikar AA, Karandikar MS, Bhonde RR (1999) Effect of partial pancreatectomy on diabetic status in BALB/c mice. J Endocrinol 162(2):189–195
77. Joglekar MV, Parekh VS, Mehta S, Bhonde RR, Hardikar AA (2007) MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. Dev Biol 311(2):603–612
78. Marappagounder D, Somasundaram I, Dorairaj S, Sankaran RJ (2013) Differentiation of mesenchymal stem cells derived from human bone marrow and subcutaneous adipose tissue into pancreatic islet-like clusters in vitro. Cell Mol Biol Lett 18(1):75–88
79. Iskovich S, Goldenberg-Cohen N, Stein J, Yaniv I, Fabian I, Askenasy N (2012) Elutriated stem cells derived from the adult bone marrow differentiate into insulin-producing cells in vivo and reverse chemical diabetes. Stem Cells Dev 21(1):86–96
80. Cai S, Pan Y, Han B, Sun TZ, Sheng ZY, Fu XB (2011) Transplantation of human bone marrow-derived mesenchymal stem cells transfected with ectodysplasin for regeneration of sweat glands. Chin Med J (Engl) 124(15):2260–2268
81. Yamada Y, Ito K, Nakamura S, Ueda M, Nagasaka T (2011) Promising cell-based therapy for bone regeneration using stem cells from deciduous teeth, dental pulp, and bone marrow. Cell Transplant 20(7):1003–1013
82. Sheng Z, Fu X, Cai S, Lei Y, Sun T, Bai X, Chen M (2009) Regeneration of functional sweat gland-like structures by transplanted differentiated bone marrow mesenchymal stem cells. Wound Repair Regen 17(3):427–435
83. Korbling M, Estrov Z, Champlin R (2003) Adult stem cells and tissue repair. Bone Marrow Transplant 32(Suppl 1):S23–S24
84. Godfrey KJ, Mathew B, Bulman JC, Shah O, Clement S, Gallicano GI (2012) Stem cell-based treatments for type 1 diabetes mellitus: bone marrow, embryonic, hepatic, pancreatic and induced pluripotent stem cells. Diabet Med 29(1):14–23
85. Rankin SM (2012) Chemokines and adult bone marrow stem cells. Immunol Lett 145(1–2):47–54
86. Pittenger MF (2008) Mesenchymal stem cells from adult bone marrow. Methods Mol Biol 449:27–44
87. Choi JB, Uchino H, Azuma K, Iwashita N, Tanaka Y, Mochizuki H, Migita M, Shimada T, Kawamori R, Watada H (2003) Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. Diabetologia 46(10):1366–1374

88. Lechner A, Yang YG, Blacken RA, Wang L, Nolan AL, Habener JF (2004) No evidence for significant transdifferentiation of bone marrow into pancreatic beta-cells in vivo. Diabetes 53(3):616–623
89. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117(Pt 14):2971–2981
90. Liu WD, Wang HW, Muguira M, Breslin MB, Lan MS (2006) INSM1 functions as a transcriptional repressor of the neuroD/beta2 gene through the recruitment of cyclin D1 and histone deacetylases. Biochem J 397(1):169–177
91. Phadnis SM, Ghaskadbi SM, Hardikar AA, Bhonde RR (2009) Mesenchymal stem cells derived from bone marrow of diabetic patients portrait unique markers influenced by the diabetic microenvironment. Rev Diabet Stud 6(4):260–270
92. Karnieli O, Izhar-Prato Y, Bulvik S, Efrat S (2007) Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. Stem Cells 25(11):2837–2844
93. Ai C, Todorov I, Slovak ML, Digiusto D, Forman SJ, Shih CC (2007) Human marrow-derived mesodermal progenitor cells generate insulin-secreting islet-like clusters in vivo. Stem Cells Dev 16(5):757–770
94. Zhao M, Amiel SA, Ajami S, Jiang J, Rela M, Heaton N, Huang GC (2008) Amelioration of streptozotocin-induced diabetes in mice with cells derived from human marrow stromal cells. PLoS One 3(7):e2666
95. Haumaitre C, Lenoir O, Scharfmann R (2008) Histone deacetylase inhibitors modify pancreatic cell fate determination and amplify endocrine progenitors. Mol Cell Biol 28(20): 6373–6383
96. Phadnis SM, Joglekar MV, Venkateshan V, Ghaskadbi SM, Hardikar AA, Bhonde RR (2006) Human umbilical cord blood serum promotes growth, proliferation, as well as differentiation of human bone marrow-derived progenitor cells. In Vitro Cell Dev Biol Anim 42(10): 283–286
97. Tanaka M, Itoh T, Tanimizu N, Miyajima A (2011) Liver stem/progenitor cells: their characteristics and regulatory mechanisms. J Biochem 149(3):231–239
98. Yang LJ, Li SW, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 99(12):8078–8083
99. Ber I, Shternhall K, Perl S, Ohanuna Z, Goldberg I, Barshack I, Benvenisti-Zarum L, Meivar-Levy I, Ferber S (2003) Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem 278(34):31950–31957
100. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N et al (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6(5):568–572
101. Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, Bussolati B, Camussi G (2006) Isolation and characterization of a stem cell population from adult human liver. Stem Cells 24(12):2840–2850
102. Bonner-Weir S, Weir GC (2005) New sources of pancreatic beta-cells. Nat Biotechnol 23(7):857–861
103. Lechner A, Habener JF (2003) Stem/progenitor cells derived from adult tissues: potential for the treatment of diabetes mellitus. Am J Physiol Endocrinol Metab 284(2):E259–E266
104. Sahu S, Joglekar MV, Dumbre R, Phadnis SM, Tosh D, Hardikar AA (2009) Islet-like cell clusters occur naturally in human gall bladder and are retained in diabetic conditions. J Cell Mol Med 13(5):999–1000
105. Coad RA, Dutton JR, Tosh D, Slack JM (2009) Inhibition of Hes1 activity in gall bladder epithelial cells promotes insulin expression and glucose responsiveness. Biochem Cell Biol 87(6):975–987

106. Dutton JR, Chillingworth NL, Eberhard D, Brannon CR, Hornsey MA, Tosh D, Slack JM (2007) Beta cells occur naturally in extrahepatic bile ducts of mice. J Cell Sci 120(Pt 2): 239–245
107. Nagaya M, Katsuta H, Kaneto H, Bonner-Weir S, Weir GC (2009) Adult mouse intrahepatic biliary epithelial cells induced in vitro to become insulin-producing cells. J Endocrinol 201(1):37–47
108. Sumazaki R, Shiojiri N, Isoyama S, Masu M, Keino-Masu K, Osawa M, Nakauchi H, Kageyama R, Matsui A (2004) Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. Nat Genet 36(1):83–87
109. Gong J, Zhang G, Tian F, Wang Y (2012) Islet-derived stem cells from adult rats participate in the repair of islet damage. J Mol Histol 43(6):745–750
110. Bouwens L (1998) Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. Microsc Res Tech 43(4):332–336
111. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429(6987):41–46
112. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 42(12):1715–1720
113. Bonner-Weir S (2000) Life and death of the pancreatic beta cells. Trends Endocrinol Metab 11(9):375–378
114. Holland AM, Gonez LJ, Harrison LC (2004) Progenitor cells in the adult pancreas. Diabetes Metab Res Rev 20(1):13–27
115. Bar-Nur O, Russ HA, Efrat S, Benvenisty N (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell 9(1):17–23
116. Wu L, Cai X, Dong H, Jing W, Huang Y, Yang X, Wu Y, Lin Y (2010) Serum regulates adipogenesis of mesenchymal stem cells via MEK/ERK-dependent PPARgamma expression and phosphorylation. J Cell Mol Med 14(4):922–932
117. Pisania A, Weir GC, O'Neil JJ, Omer A, Tchipashvili V, Lei J, Colton CK, Bonner-Weir S (2010) Quantitative analysis of cell composition and purity of human pancreatic islet preparations. Lab Invest 90(11):1661–1675
118. Yatoh S, Dodge R, Akashi T, Omer A, Sharma A, Weir GC, Bonner-Weir S (2007) Differentiation of affinity-purified human pancreatic duct cells to beta-cells. Diabetes 56(7): 1802–1809
119. Hardikar AA (2004) Generating new pancreas from old. Trends Endocrinol Metab 15(5): 198–203
120. Hardikar AA, Bhonde RR (1999) Modulating experimental diabetes by treatment with cytosolic extract from the regenerating pancreas. Diabetes Res Clin Pract 46(3):203–211
121. Finegood DT, Weir GC, Bonner-Weir S (1999) Prior streptozotocin treatment does not inhibit pancreas regeneration after 90% pancreatectomy in rats. Am J Physiol 276(5 Pt 1):E822–E827
122. Epsztejn-Litman S, Feldman N, Abu-Remaileh M, Shufaro Y, Gerson A, Ueda J, Deplus R, Fuks F, Shinkai Y, Cedar H et al (2008) De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. Nat Struct Mol Biol 15(11):1176–1183

Adult Stromal (Skeletal, Mesenchymal) Stem Cells: Advances Towards Clinical Applications

Abbas Jafari, Linda Harkness, Walid Zaher, and Moustapha Kassem

Abstract Mesenchymal Stem Cells (MSC) are non-hematopoietic adult stromal cells that reside in a perivascular niche in close association with pericytes and endothelial cells and possess self-renewal and multi-lineage differentiation capacity. The origin, unique properties, and therapeutic benefits of MSC are under intensive investigation worldwide. Several challenges with regard to the proper source of clinical-grade MSC and the efficacy of MSC-based treatment strategies need to be addressed before MSC can be routinely used in the clinic. Here, we discuss three areas that can potentially facilitate the translation of MSC into clinic: Generation of MSC-like cells from human pluripotent stem cells, strategies to enhance homing of MSC to injured tissues, and targeting of MSC in vivo.

Keywords Mesenchymal stem cells • MSC-like cells • Pluripotent stem cells • Homing • In vivo targeting

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Abbreviations

AMD	Age-related macular degeneration
AMI	Acute myocardial infarction
Bzb	Bortezomib
CCR1	C-C Chemokine receptor type 1
CXCR4	C-X-C Chemokine receptor type 4
EB	Embryoid body
FAK	Focal adhesion kinase
GMP	Good manufacturing practice
hESC	Human embryonic stem cells
HLA	Human leukocyte antigen
hPSC	Human pluripotent stem cells
ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSC	Mesenchymal stem cells
Runx2	Runt-related transcription factor 2
SCID	Severe combined immunodeficiency
SDF-1α	Stromal cell-derived factor-1
siRNA	Small-interfering RNA

1 Introduction

Mesenchymal stem cells (MSC) are multipotent cells that were first identified by Friedenstein as bone marrow osteogenic stem cells [1]. The term "mesenchymal stem cell" was coined by Caplan to describe a population of cells that are involved in the formation of bone and cartilage during embryonic development, bone turnover, and repair throughout adulthood [2]. However, the term "mesenchymal" is contentious and not generally accepted [3]. Other names also exist for MSC including multipotent mesenchymal stromal cells, skeletal stem cells, adult stromal stem cells, and bone marrow stromal cells [4, 5]. MSC are defined as non-hematopoietic, plastic adherent multipotent stem cells that are present in the bone marrow stroma and can differentiate into cells of mesodermal lineage including osteoblasts, adipocytes, and chondrocytes. In ex vivo culture, MSC are positive for a number of CD markers: CD105, CD106, CD90, CD73, CD140b, CD166 and negative for CD31, CD45, CD34, CD14, CD133 and the major histocompatibility complex (MHC) class II markers [4, 6]. In addition to their presence in bone marrow, MSC-like cell populations have been isolated from the stromal compartment of adipose tissue, umbilical cord, dental pulp, skeletal muscle, synovium, and periodontal ligament [7–13]. While MSC-like cell populations share a common molecular signature with

bone marrow MSC, they exhibit differences in their molecular phenotype and differentiation potential characteristic for their tissue of origin [14]. A common in vivo location of MSC in the bone marrow and in other tissues is in a perivascular niche in close association with pericytes and endothelial cells [15].

2 Towards Clinical Use

MSC hold a great promise for clinical use in tissue regeneration in a large number of clinical conditions. 379 clinical trials, worldwide, are currently undergoing investigations into the therapeutic benefits of MSC (http://clinicaltrials.gov). These range from enhancing hematopoiesis following hematopoietic stem cell transplantation to enhancing tissue regeneration for cardiomyopathies, nerve tissue, bone and cartilage repair following injury and chronic disease.

Several factors limit the clinical use of MSC [16], including the inability to obtain the large number of MSC required for clinical transplantation due to in vitro replicative senescence [17], heterogeneity of ex vivo cultured MSC with respect to their differentiation capacity, and lack of specific markers that identify MSC prospectively and are predictive of their in vivo phenotype. In the current review, we will discuss progress in studies related to three areas that received a lot of attention due to their possible use to facilitate clinical use of MSC: (1) use of human pluripotent stem cells as a source for generation of an unlimited number of MSC, (2) development of approaches to enhance in vivo migration of MSC into injured tissues, and (3) novel strategies for targeting MSC in vivo with the aim of enhancing bone formation.

3 Generation of MSC-Like Cells from Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSC) are a group of specialized cells that have the unique ability to differentiate into cells of the mesoderm, endoderm, and ectoderm lineages and are thus termed pluripotent. There are two major sources of hPSC: human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). hESC are generated through isolation of the inner cell mass (ICM) from a 5- to 6-day-old human blastocyst [18]. Since the derivation of hESC by Thomson in 1998 [18] much effort has been focused to develop protocols for differentiation of hESC into lineage-specific cell types [19]. The creation of induced pluripotent stem cells (iPSC) from adult somatic cells [20–22] has added a new dimension to the field of regenerative medicine by offering the possibility of generating autologous pluripotent stem cells [23]. ESC and iPSC are similar in their expression of the self-renewal markers and ability to differentiate into the three basic cell lineages: ectoderm, endoderm, and mesoderm [20–22, 24, 25]. Pluripotent stem cells offer much promise

within the field of regenerative medicine due to their unlimited proliferation ability, scalability, and differentiation capacity.

A number of methods have been reported for derivation of functional MSC-like cells from PSC, using embryoid body formation (EB), monolayer differentiation, coculture, selective isolation of spontaneously differentiated cells, and cultures using biomaterials [23, 26–29].

EB Formation: Standard methods demonstrate that EBs can be formed spontaneously from small clumps of pluripotent cells that are passaged either mechanically or using enzymatic methods and cultured in suspension using low adhesion plastic vessels. This method allows spontaneous nondirected differentiation or directed differentiation of PSC, through addition of growth factors/morphogens/cytokines. EBs imitate the structure of the early embryo and recapitulate many of the early embryonic developmental events like gastrulation, polarization, and primitive streak formation [26, 30]. However, a disadvantage of the EB method is that it provides heterogeneous populations of MSC-like cells. In an attempt to reduce cellular heterogeneity, a number of alternative approaches have been developed including methods of synchronized growth and differentiation through forced aggregation by centrifugation [31], bioreactor cultures [32], and stirred suspension cultures [33] and recently Son et al. [34] published data demonstrating a simple method using periodic passaging of hEBs to maintain uniformity on size and proliferation whilst preserving their differentiation potential.

Monolayer Differentiation: 2-D PSC cultures have an advantage over EB-based differentiation as it is possible to visualize the progression of ex vivo differentiation. However, this technique fails to recapitulate the gastrulation-like processes apparent in EB formation. Development of MSC-like cells has been obtained by using induction media that allows synchronized differentiation, e.g. adding Rock inhibitor Y27632 [35], by selection by continuous subculture over a number of weeks to select for stromal (MSC-like) cells [36, 37], or by cell sorting based on specific surface markers, e.g., the selection of a $CD105^{+}/CD24^{-}$ cell population [38].

Coculture: A number of groups have used coculture of hESC with differentiated cells to induce differentiation into an MSC-like phenotype. Barberi et al. employed coculture with murine OP9 cells followed by sorting for $CD73^{+}$ MSC-like cells [39]. This method of induced differentiation presupposes that secreted factors from the differentiated cells can supply microenvironmental cues necessary for differentiation, but the nature of these factors is not known.

Spontaneous Differentiation: Spontaneous differentiation into MSC-like cells often occurs at the edges of the hPSC colonies, obtained when hPSC are cultured in a feeder-free system. In the “raclure” method, the cells at the edges of the colonies are manually scrapped [40, 41], or cells can be enriched through adherence to selective extracellular matrix components such as hyaluronic acid (HA)-coated plates [28],

or through forced differentiation through overgrowth of cultures [42]. In our laboratory, we found that selection of MSC-like cells based on selective adherence to HA-coated culture plates resulted in obtaining a morphologically homogeneous cell population with a similar phenotype to bone marrow-derived MSC [28]. In addition, Liu et al. demonstrated that hESC and iPSC differentiated into MSC-like cells through plating of single cells on a fibrillar type 1 collagen matrix [29].

The MSC-like cells derived from hESC or iPSC using the above-mentioned approaches exhibit a phenotypic profile comparable to MSC as defined by CD markers and differentiation ability into one or more of the osteoblastic, chondrocytic, or adipocytic lineages. While most of the reported differentiation capacities are based on in vitro data, a number of groups have demonstrated the ability of hPSC-derived MSC-like cells to form bone in vivo following osteogenic induction ex vivo or through direct implantation of the cells in osteoinductive scaffolds [28, 43, 44].

4 Concerns of Using hPSC-Derived MSC-Like Cells in Cellular Therapy

For clinical use, hPSC-derived MSC-like cells should be obtained from GMP (good manufacture practice) compliant hPSC lines. There have been an increasing number of reported hESC lines [45–48] and iPSC lines [49] derived under GMP standards. Additionally, clinical-grade derivation protocols for MSC-like cells have been reported [50]. The necessity for extensive ex vivo culture, which would be required for clinical therapy, has raised concerns about the possibility of genetic changes and the development of a transformed phenotype. A number of reports have highlighted the issue of karyotypic stability during routine maintenance of hESC cultured ex vivo where gains in chromosomes 12, 17, and X have been reported [51–55]. Of additional concern is the unintentional transplantation of undifferentiated hPSC in conjunction with their differentiated progeny that may lead to teratoma formation upon transplantation. As the purity of hPSC differentiated cultures is variable, attempts are being made to deplete undifferentiated hPSC within the cultures either by using cytotoxic agents, mechanically removing undifferentiated cells [56, 57], or separating out undifferentiated cells using fluorescent tags which identify undifferentiated cells [58, 59]. Thus, before hPSC-derived MSC are considered for cellular therapy safety criteria are needed to be instituted [60, 61].

MSC-like cells derived from iPS cells should be compatible with their recipient and thus will not elicit an immunological rejection reaction. Interestingly, differentiated cells derived from hESC may be hypoimmunegenic. Drukker et al. [62] demonstrated absence of the MHC class II molecules and the presence of low levels of class I molecules in hESC. Additionally, normal irradiated mice transplanted with bone marrow from immune compromised (SCID) mice were transfused with human peripheral blood mononuclear cells to test the possible immunological reaction or

rejection of transplanted hESC. Over the course of a month, transplanted hESC did not demonstrate significant rejection [62]. More recently Araki et al. demonstrated limited or no immune response in differentiated mouse ESC and iPSC [63].

It is envisaged that off-the-shelf MSC-like cells should be available in stem cell banks that contain hPSC lines that cover the majority of the Western European population tissue types. It has been estimate that 150 hESC cell lines in Europe [64] and 170 cell lines for the Japanese population [65] would be needed to obtain an acceptable degree of HLA matching which would only require a minimum of immune suppressor therapy.

iPSC were initially derived using a combination of four transcription factors (OCT4, SOX2, KLF4, and cMyc) to reprogram the somatic cells to their pluripotent status [20]. Whilst the success of reprogramming was a huge step forward towards generation of cells for therapy, standard methods used to generate iPSC may result in cells not suitable for therapy due to the use of viral vectors. New strategies are being developed to overcome these concerns using plasmids [66], recombinant proteins [67], or RNA molecules [68, 69].

5 Directing MSC to Injured Tissues

Current thinking vis-a-vis the clinical use of MSC in therapy is modeled on the hematopoietic stem cell transplantation model where HSC are infused intravenously and consequently home to the bone marrow where they establish hematopoiesis [70]. Although homing of MSC to sites of injury and their involvement in healing and/or regeneration of defected tissues is a natural repair mechanism, it was observed that this endogenous ability can be further enhanced by exogenously administered MSC [71, 72]. Systemic infusion of MSC for treatment of tissue injury represents a more attractive procedure for clinical applications. In addition, studies on MSC migration to injured tissues have been shaped by concepts related to leukocyte recruitment from the circulation to inflammation sites, through a coordinated multistep biological process termed “cell homing” that includes infused cell rolling/adhering onto sinusoidal endothelial cells followed by their firm adhesion preventing their back movement to circulation, resulting in transmigration to their destined tissues [73]. Employing this model for MSC has been supported by evidence for the presence of osteoprogenitors or MSC-like cells in the circulation that can home to bone marrow or inflammatory sites [74].

Following injury, damaged cells secrete a number of chemokines that act as attractants to cells participating in tissue repair [75]. However, one of the major challenges facing MSC-based cell therapy is the observed low and inefficient homing of systemically infused MSC to non-injured tissues [76]. Several groups have demonstrated successful but limited homing after systemic delivery to ischemic, irradiated, or otherwise injured skeletal tissues in which only a small fraction of transplanted MSC can be found in the target tissue [77–79].

5.1 Novel Approaches to Enhance Homing of MSC into Injured Tissues

It is well known that adhesion and integrin molecules are important key players in determining the potential of cellular homing [80]. For example, the CD44 antigen is a cell surface glycoprotein involved in cell adhesion and migration [81]. A specialized glycoform of CD44 called hematopoietic cell E-/L-selectin ligand (HCELL) is an E-selectin ligand expressed on human cells [82]. Using real-time confocal microscopy cell trafficking was monitored in immune-compromised mouse calvaria. These results indicated that overexpression of HCELL E-selectin on MSC caused, within hours, enhanced osteotropic migration to the bone marrow [83]. Recently, modification of MSC cell surface integrins to enhance homing of MSC to bone surfaces was achieved by attaching a synthetic ligand (LLP2A) against integrin α4β1 on the MSC surface to a bisphosphonate (alendronate, Ale). Upon administration in in vivo animal models the LLP2A-ALE-modified MSC showed enhanced homing to bone surfaces with improved bone formation at the endo-cortical, trabecular, and periosteal surfaces when compared to non-modified MSC [84]. In another study, cell adhesion molecules were chemically attached to the cell surface to improve rolling efficiency of MSC. This chemical approach involved introduction of biotin groups to the cell surface by treatment with sulfonated biotinyl-*N*-hydroxy-succinimide, the addition of streptavidin, and attachment of a biotinylated cell rolling ligand (sialyl Lewisx (SLeX)) found on the surface of leukocytes [85]. This approach can be used to potentially target P-selectin expressing endothelium in the bone marrow or at sites of inflammation [86].

Another hypothesized explanation for the poor homing capacity of MSC is their inadequate expression of homing-associated chemokines. For example, CXCR4, a homing signaling molecule known for its interaction with its cognate ligand Stromal cell-derived factor-1 (SDF-1α), is expressed at low levels on the MSC cell surface [87, 88]. In a number of in vivo studies, homing of transplanted MSC to tumors [89], myocardium [90], and bone marrow [78] has been improved by overexpression of CXCR4 on the MSC surface. For example, MSC overexpressing CXCR4 were infused intravenously 24 h after coronary occlusion in a rat model of AMI and were found to home to the infarcted myocardium resulting in better recovery of left ventricular function as compared to rats infused with control (low CXCR4 expressing) cells [87]. In another study, C3H10T1/2 cells, a multipotent mouse stem cell line, overexpressing CXCR4 were injected intravenously in immune-competent glucocorticoid-induced osteoporotic mice. These cells had enhanced homing efficiency to the bone marrow and increased bone mass in the osteoporotic mice [79]. Another member of the chemokine family is the C-C chemokine receptor type 1 (CCR1), known to be involved in the recruitment of immune cells to sites of inflammation, e.g., injured myocardium [91]. Mouse bone marrow MSC, overexpressing CCR1, were injected intra-myocardially in a mouse model of AMI. One hour post coronary artery ligation, MSC overexpressing CCR1 had accumulated in the

infarcted myocardium at significantly higher levels than control MSC. This led to significant reduction in infarct size, reduced cardio-myocyte apoptosis, increased capillary density, and restoration of cardiac function via enhancement of transplanted cells' viability and engraftment [92].

All these studies demonstrate work in progress towards developing a clinically relevant protocol for intravenous infusion of the MSC to patients in need of enhanced tissue regeneration.

6 Targeting of MSC In Vivo

Therapeutic strategies that employ ex vivo cultured cells are associated with some limitations such as the need for substantial number of cells requiring extensive ex vivo cell expansion, the need for GMP facilities, as well as development of robust methods for differentiation induction [16]. Targeting of the endogenous MSC populations, using small molecules, small-interfering RNA (siRNA), or MicroRNA (miRNA), is an attractive alternative and is suitable for treatment of diseases where the mature cell populations, which are to be targeted by the drug, are depleted or do not respond to standard treatment. An example of such a clinical setting is the use of osteoblast-targeting anabolic therapies for treatment of bone loss, in which osteoblasts are decreased in number and activity [93].

Small molecules are very attractive agents to be used in clinical applications, due to the opportunity of fine-tuning their chemical structure using traditional chemistry techniques, high stability, adaptability to large-scale production leading to substantial reduction of the treatment costs, and a potential for oral delivery [39, 94, 95]. Some examples of these approaches have been recently reported.

Bortezomib (Bzb) is a small molecule proteasome inhibitor that is used in the clinic for treatment of multiple myeloma [96]. It has been shown that Bzb targets bone marrow MSC in vivo and induces their differentiation toward the osteoblastic lineage through regulation of runt-related transcription factor 2 (Runx2), known as a master regulator of osteogenesis [97, 98]. Intraperitoneal (i.p.) administration of Bzb to mice for 3 weeks increased bone mass, trabecular bone connectivity, trabecular number, serum osteocalcin, as well as bone formation rate demonstrating enhanced in vivo osteoblastic bone formation activity. Moreover, it was shown that in contrast to MSC, osteoprogenitors and osteoclasts did not respond to Bzb treatment [98].

siRNA can specifically silence the synthesis of any desired protein by base paring to its mRNA sequence [99]. To date, more than 20 siRNA-based drugs are under clinical investigation for treatment of a variety of conditions including solid tumors, acute kidney injury, age-related macular degeneration (AMD), diabetic macular edema, hepatitis C, AIDS-associated lymphoma, and respiratory syncytial virus infection [100]. Administration of siRNA and silencing the synthesis of a gene of interest can be used to alter the differentiation fate of MSC in vivo [101]. However, the large therapeutic doses of systematically administered siRNA that is needed to exert the desired clinical outcome may lead to activation of immune response, as

well as adverse effects on other tissues. Thus, the development of novel systems that deliver siRNA specifically to the cell population of interest is highly desirable. Recently, a novel targeting system has been developed that delivers siRNA to the bone-forming surfaces enriched for MSC and osteoprogenitors [102]. This system involves dioleoyl trimethylammonium propane (DOTAP)-based cationic liposomes attached to six repetitive sequences of aspartate, serine, serine ((AspSerSer)6). This system has been used for in vivo systemic delivery of siRNA targeting Plekho1 (a negative regulator of bone formation) in rats and led to significant enhancement of bone formation, enhanced the bone micro-architecture, and increased the bone mass in both healthy and osteoporotic rats [102]. In addition to siRNA, miRNAs have potential use in therapy. miRNAs are endogenous, short, noncoding RNAs that regulate diverse biological processes mostly through translational repression of their target genes [103]. miRNAs can be employed to modulate the differentiation fate of MSC in vitro and in vivo [103]. Exogenous supplementation or ectopic expression of miRNAs as well as using anti-miRs to antagonize the effect of miRNAs are promising strategies to be employed for treatment of different clinical conditions [104]. In our group, we have demonstrated that miR-138 negatively regulates in vitro osteoblast differentiation and in vivo bone formation of MSC, by targeting focal adhesion kinase (FAK), a kinase playing a central role in promoting osteoblast differentiation [105]. Using a preclinical in vivo bone formation model, we showed that pharmacological inhibition of miR-138 by antimiR-138 increased ectopic bone formation and thus it is possible to develop antimiR-138 into a novel strategy for treatment of bone loss conditions [105].

7 Conclusions and Future Perspectives

Regenerative medicine holds promise to restore normal tissue functions in the body using stem cell transplantation or ex vivo grown tissues and organs generated through a combination of stem cells and biomaterials, i.e., tissue engineering approaches. The transition from the laboratory to the clinic has proven to be difficult and currently there is no standard stem cell-based therapy for non-cancer indications. Conversely, a large number of clinical trials testing the ability of different types of stem cells including MSC in a number of disease conditions are being conducted and include conditions such as nonunion fractures, ulcerative colitis, type 1 diabetes mellitus, liver cirrhosis, idiopathic dilated cardiomyopathy, multiple sclerosis, spinal cord injury, acute and chronic graft-versus-host disease, middle cerebral artery infarct, osteoarthritis, relapsed/refractory severe acquired aplastic anemia, chronic critical limb ischemia, Parkinson's disease, acute myocardial infarction, hematological malignancies, Crohn's disease, acute leukemia, lupus nephritis, and non-healing wounds (please see: http://clinicaltrials.gov). It is hoped that these trials will establish the efficacy of stem cells and MSC in therapy and their place among other current treatment modalities.

References

1. Friedenstein AJ, Chailakhyan RK, Gerasimov UV (1987) Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 20(3):263–272
2. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9(5):641–650
3. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ et al (2013) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19(1):35–42
4. Garcia-Gomez I, Elvira G, Zapata AG, Lamana ML, Ramirez M, Castro JG et al (2010) Mesenchymal stem cells: biological properties and clinical applications. Expert Opin Biol Ther 10(10):1453–1468
5. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2(4):313–319
6. Sivasubramaniyan K, Lehnen D, Ghazanfari R, Sobiesiak M, Harichandan A, Mortha E et al (2012) Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. Ann N Y Acad Sci 1266:94–106
7. Akiyama K, Chen C, Gronthos S, Shi S (2012) Lineage differentiation of mesenchymal stem cells from dental pulp, apical papilla, and periodontal ligament. Methods Mol Biol 887:111–121
8. Asakura A, Komaki M, Rudnicki M (2001) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 68(4–5): 245–253
9. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP (2001) Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44(8):1928–1942
10. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J et al (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364(9429):149–155
11. Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19(3):180–192
12. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228
13. Kermani AJ, Fathi F, Mowla SJ (2008) Characterization and genetic manipulation of human umbilical cord vein mesenchymal stem cells: potential application in cell-based gene therapy. Rejuvenation Res 11(2):379–386
14. Al-Nbaheen M, Vishnubalaji R, Ali D, Bouslimi A, Al-Jassir F, Megges M et al (2013) Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential. Stem Cell Rev 9(1):32–43
15. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3(3):301–313
16. Aldahmash A, Zaher W, Al-Nbaheen M, Kassem M (2012) Human stromal (mesenchymal) stem cells: basic biology and current clinical use for tissue regeneration. Ann Saudi Med 32(1):68–77
17. Stenderup K, Justesen J, Clausen C, Kassem M (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 33(6):919–926
18. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391): 1145–1147
19. Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. Stem Cells 19(3):193–204
20. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5): 861–872

21. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K et al (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448(7151):318–324
22. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858): 1917–1920
23. Lian Q, Zhang Y, Zhang J, Zhang HK, Wu X, Lam FF et al (2010) Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. Circulation 121(9):1113–1123
24. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA et al (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451(7175):141–146
25. Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T et al (2009) iPS cells produce viable mice through tetraploid complementation. Nature 461(7260):86–90
26. Mahmood A, Harkness L, Abdallah BM, Elsafadi M, Al-Nbaheen MS, Aldahmash A et al (2012) Derivation of stromal (skeletal and mesenchymal) stem-like cells from human embryonic stem cells. Stem Cells Dev 21(17):3114–3124
27. Inanc B, Elcin AE, Elcin YM (2007) Effect of osteogenic induction on the in vitro differentiation of human embryonic stem cells cocultured with periodontal ligament fibroblasts. Artif Organs 31(11):792–800
28. Harkness L, Mahmood A, Ditzel N, Abdallah BM, Nygaard JV, Kassem M (2011) Selective isolation and differentiation of a stromal population of human embryonic stem cells with osteogenic potential. Bone 48(2):231–241
29. Liu Y, Goldberg AJ, Dennis JE, Gronowicz GA, Kuhn LT (2012) One-step derivation of mesenchymal stem cell (MSC)-like cells from human pluripotent stem cells on a fibrillar collagen coating. PLoS One 7(3):e33225
30. ten Berge D, Koole W, Fuerer C, Fish M, Eroglu E, Nusse R (2008) Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell 3(5):508–518
31. Ng ES, Davis RP, Azzola L, Stanley EG, Elefanty AG (2005) Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. Blood 106(5):1601–1603
32. Yirme G, Amit M, Laevsky I, Osenberg S, Itskovitz-Eldor J (2008) Establishing a dynamic process for the formation, propagation, and differentiation of human embryoid bodies. Stem Cells Dev 17(6):1227–1242
33. Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H (2012) Bioprocess development for mass production of size-controlled human pluripotent stem cell aggregates in stirred suspension bioreactor. Tissue Eng Part C Methods 18(11):831–851
34. Son MY, Kim HJ, Kim MJ, Cho YS (2011) Physical passaging of embryoid bodies generated from human pluripotent stem cells. PLoS One 6(5):e19134
35. Wu R, Gu B, Zhao X, Tan Z, Chen L, Zhu J et al (2013) Derivation of multipotent nestin+/CD271-/STRO-1- mesenchymal-like precursors from human embryonic stem cells in chemically defined conditions. Hum Cell 26(1):19–27
36. Arpornmaeklong P, Brown SE, Wang Z, Krebsbach PH (2009) Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cells Dev 18(7):955–968
37. Karlsson C, Emanuelsson K, Wessberg F, Kajic K, Axell MZ, Eriksson PS et al (2009) Human embryonic stem cell-derived mesenchymal progenitors – potential in regenerative medicine. Stem Cell Res 3(1):39–50
38. Lian Q, Lye E, Suan Yeo K, Khia Way Tan E, Salto-Tellez M, Liu TM et al (2007) Derivation of clinically compliant MSCs from CD105+, CD24- differentiated human ESCs. Stem Cells 25(2):425–436
39. Aravamudhan A, Ramos DM, Nip J, Subramanian A, James R, Harmon MD et al (2013) Osteoinductive small molecules: growth factor alternatives for bone tissue engineering. Curr Pharm Des 19(19):3420–3428
40. Olivier EN, Rybicki AC, Bouhassira EE (2006) Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem Cells 24(8):1914–1922

41. Olivier E, Bouhassira E (2011) Differentiation of human embryonic stem cells into mesenchymal stem cells by the raclure method. In: Nieden NI (ed) Embryonic stem cell therapy for osteo-degenerative diseases. Humana, New York, pp 183–193
42. Trivedi P, Hematti P (2008) Derivation and immunological characterization of mesenchymal stromal cells from human embryonic stem cells. Exp Hematol 36(3):350–359
43. Mahmood A, Harkness L, Schroder HD, Abdallah BM, Kassem M (2010) Enhanced differentiation of human embryonic stem cells to mesenchymal progenitors by inhibition of TGFb/activin/nodal signaling using SB-431542. J Bone Miner Res 25(6):1216–1233
44. Tremoleda JL, Forsyth NR, Khan NS, Wojtacha D, Christodoulou I, Tye BJ et al (2008) Bone tissue formation from human embryonic stem cells in vivo. Cloning Stem Cells 10(1):119–132
45. Crook JM, Peura TT, Kravets L, Bosman AG, Buzzard JJ, Horne R et al (2007) The generation of six clinical-grade human embryonic stem cell lines. Cell Stem Cell 1(5):490–494
46. Sidhu KS, Walke S, Tuch, BE (2008) Derivation and Propagation of hESC Under a Therapeutic Environment. Current Protocols in Stem Cell Biology 6:1A.4.1–1A.4.31
47. Skottman H, Dilber MS, Hovatta O (2006) The derivation of clinical-grade human embryonic stem cell lines. FEBS Lett 580(12):2875–2878
48. Unger C, Skottman H, Blomberg P, Sirac Dilber M, Hovatta O (2008) Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet 17(R1): R48–R53
49. Ohmine S, Dietz A, Deeds M, Hartjes K, Miller D, Thatava T et al (2011) Induced pluripotent stem cells from GMP-grade hematopoietic progenitor cells and mononuclear myeloid cells. Stem Cell Res Ther 2(6):46
50. Han G, Jing Y, Zhang Y, Yue Z, Hu X, Wang L et al (2010) Osteogenic differentiation of bone marrow mesenchymal stem cells by adenovirus-mediated expression of leptin. Regul Pept 163(1–3):107–112
51. Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J et al (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22(1):53–54
52. Maitra A, Arking DE, Shivapurkar N, Ikeda M, Stastny V, Kassauei K et al (2005) Genomic alterations in cultured human embryonic stem cells. Nat Genet 37(10):1099–1103
53. Mitalipova MM, Rao RR, Hoyer DM, Johnson JA, Meisner LF, Jones KL et al (2005) Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 23(1): 19–20
54. Pera MF (2004) Unnatural selection of cultured human ES cells? Nat Biotechnol 22(1):42–43
55. Spits C, Mateizel I, Geens M, Mertzanidou A, Staessen C, Vandeskelde Y et al (2008) Recurrent chromosomal abnormalities in human embryonic stem cells. Nat Biotechnol 26(12):1361–1363
56. Bieberich E, Silva J, Wang G, Krishnamurthy K, Condie BG (2004) Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. J Cell Biol 167(4):723–734
57. Schriebl K, Satianegara G, Hwang A, Tan HL, Fong WJ, Yang HH et al (2012) Selective removal of undifferentiated human embryonic stem cells using magnetic activated cell sorting followed by a cytotoxic antibody. Tissue Eng Part A 18(9–10):899–909
58. Tang C, Lee AS, Volkmer JP, Sahoo D, Nag D, Mosley AR et al (2011) An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. Nat Biotechnol 29(9):829–834
59. Wang YC, Nakagawa M, Garitaonandia I, Slavin I, Altun G, Lacharite RM et al (2011) Specific lectin biomarkers for isolation of human pluripotent stem cells identified through array-based glycomic analysis. Cell Res 21(11):1551–1563
60. Fong CY, Gauthaman K, Bongso A (2010) Teratomas from pluripotent stem cells: a clinical hurdle. J Cell Biochem 111(4):769–781

61. Knoepfler PS (2009) Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells 27(5):1050–1056
62. Drukker M (2008) Immunological considerations for cell therapy using human embryonic stem cell derivatives. In: StemBook [Internet]. Cambridge (MA): Harvard Stem Cell Institute. Available from: http://www.ncbi.nlm.nih.gov/books/NBK27031/
63. Araki R, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S et al (2013) Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature 494(7435):100–104
64. Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA (2005) Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366(9502):2019–2025
65. Isomoto S, Hattori K, Ohgushi H, Nakajima H, Tanaka Y, Takakura Y (2007) Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells. J Orthop Sci 12(1):83–88
66. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science 322(5903):949–953
67. Lin T, Ambasudhan R, Yuan X, Li W, Hilcove S, Abujarour R et al (2009) A chemical platform for improved induction of human iPSCs. Nat Methods 6(11):805–808
68. Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y et al (2011) Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 8(4):376–388
69. Judson RL, Babiarz JE, Venere M, Blelloch R (2009) Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 27(5):459–461
70. Magnon C, Frenette PS (2008) Hematopoietic stem cell trafficking. StemBook. Cambridge, MA
71. Grauss RW, Winter EM, van Tuyn J, Pijnappels DA, Steijn RV, Hogers B et al (2007) Mesenchymal stem cells from ischemic heart disease patients improve left ventricular function after acute myocardial infarction. Am J Physiol Heart Circ Physiol 293(4):H2438–H2447
72. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J et al (2003) Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 5(12):1028–1038
73. Lawrence MB, Springer TA (1991) Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 65(5):859–873
74. Pignolo RJ, Kassem M (2011) Circulating osteogenic cells: implications for injury, repair, and regeneration. J Bone Miner Res 26(8):1685–1693
75. Wu Y, Zhao RC (2012) The role of chemokines in mesenchymal stem cell homing to myocardium. Stem Cell Rev 8(1):243–250
76. Bentzon JF, Stenderup K, Hansen FD, Schroder HD, Abdallah BM, Jensen TG et al (2005) Tissue distribution and engraftment of human mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene. Biochem Biophys Res Commun 330(3): 633–640
77. Bobis-Wozowicz S, Miekus K, Wybieralska E, Jarocha D, Zawisz A, Madeja Z et al (2011) Genetically modified adipose tissue-derived mesenchymal stem cells overexpressing CXCR4 display increased motility, invasiveness, and homing to bone marrow of NOD/SCID mice. Exp Hematol 39(6):686–696
78. Devine MJ, Mierisch CM, Jang E, Anderson PC, Balian G (2002) Transplanted bone marrow cells localize to fracture callus in a mouse model. J Orthop Res 20(6):1232–1239
79. Lien CY, Chih-Yuan HK, Lee OK, Blunn GW, Su Y (2009) Restoration of bone mass and strength in glucocorticoid-treated mice by systemic transplantation of CXCR4 and cbfa-1 co-expressing mesenchymal stem cells. J Bone Miner Res 24(5):837–848
80. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD (1998) Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. Proc Natl Acad Sci U S A 95(24):14423–14428

81. Zhu H, Mitsuhashi N, Klein A, Barsky LW, Weinberg K, Barr ML et al (2006) The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. Stem Cells 24(4):928–935
82. Sackstein R (2012) Glycoengineering of HCELL, the human bone marrow homing receptor: sweetly programming cell migration. Ann Biomed Eng 40(4):766–776
83. Sackstein R, Merzaban JS, Cain DW, Dagia NM, Spencer JA, Lin CP et al (2008) Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. Nat Med 14(2):181–187
84. Guan M, Yao W, Liu R, Lam KS, Nolta J, Jia J et al (2012) Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass. Nat Med 18(3):456–462
85. Sarkar D, Vemula PK, Teo GS, Spelke D, Karnik R, Wee lY et al (2008) Chemical engineering of mesenchymal stem cells to induce a cell rolling response. Bioconjug Chem 19(11):2105–2109
86. Sarkar D, Zhao W, Gupta A, Loh WL, Karnik R, Karp JM (2011) Cell surface engineering of mesenchymal stem cells. Methods Mol Biol 698:505–523
87. Cheng Z, Ou L, Zhou X, Li F, Jia X, Zhang Y et al (2008) Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. Mol Ther 16(3):571–579
88. Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE et al (2004) A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 104(9):2643–2645
89. Song C, Li G (2011) CXCR4 and matrix metalloproteinase-2 are involved in mesenchymal stromal cell homing and engraftment to tumors. Cytotherapy 13(5):549–561
90. Zhang D, Fan GC, Zhou X, Zhao T, Pasha Z, Xu M et al (2008) Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. J Mol Cell Cardiol 44(2):281–292
91. Hodgkinson CP, Gomez JA, Mirotsou M, Dzau VJ (2010) Genetic engineering of mesenchymal stem cells and its application in human disease therapy. Hum Gene Ther 21(11):1513–1526
92. Huang J, Zhang Z, Guo J, Ni A, Deb A, Zhang L et al (2010) Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ Res 106(11):1753–1762
93. Kassem M, Marie PJ (2011) Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. Aging Cell 10(2):191–197
94. Sachsenmaier C (2001) Targeting protein kinases for tumor therapy. Onkologie 24(4): 346–355
95. Via MC (2011) Kinase-targeted therapeutics: development pipelines, challenges, and opportunities, August. http://www.insightpharmareports.com/Kinase-Targeted-Therapeutics-Report.aspx
96. Field-Smith A, Morgan GJ, Davies FE (2006) Bortezomib (Velcadetrade mark) in the treatment of multiple myeloma. Ther Clin Risk Manag 2(3):271–279
97. Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J et al (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16(3):307–310
98. Mukherjee S, Raje N, Schoonmaker JA, Liu JC, Hideshima T, Wein MN et al (2008) Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 118(2):491–504
99. Andersen MO, Nygaard JV, Burns JS, Raarup MK, Nyengaard JR, Bunger C et al (2010) siRNA nanoparticle functionalization of nanostructured scaffolds enables controlled multilineage differentiation of stem cells. Mol Ther 18(11):2018–2027
100. Burnett JC, Rossi JJ, Tiemann K (2011) Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol J 6(9):1130–1146
101. Takayama K, Suzuki A, Manaka T, Taguchi S, Hashimoto Y, Imai Y et al (2009) RNA interference for noggin enhances the biological activity of bone morphogenetic proteins in vivo and in vitro. J Bone Miner Metab 27(4):402–411

102. Zhang G, Guo B, Wu H, Tang T, Zhang BT, Zheng L et al (2012) A delivery system targeting bone formation surfaces to facilitate RNAi-based anabolic therapy. Nat Med 18(2):307–314
103. Taipaleenmaki H, Bjerre Hokland L, Chen L, Kauppinen S, Kassem M (2012) Mechanisms in endocrinology: micro-RNAs: targets for enhancing osteoblast differentiation and bone formation. Eur J Endocrinol 166(3):359–371
104. Lares MR, Rossi JJ, Ouellet DL (2010) RNAi and small interfering RNAs in human disease therapeutic applications. Trends Biotechnol 28(11):570–579
105. Eskildsen T, Taipaleenmaki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY et al (2011) MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. Proc Natl Acad Sci U S A 108(15):6139–6144

Regeneration After Injury: Activation of Stem Cell Stress Response Pathways to Rapidly Repair Tissues

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Abstract Stem cells play key roles in the development of tissues and maintain tissue homeostasis. Because of these properties a great deal of research is focused on exploiting tissue stem cells as a means to treat degenerative diseases. In fact recent advances in the derivation of tissue stem cell populations from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells hold great promise for the development of new therapies. Unfortunately much of this promise has not been fulfilled. An alternative approach is to examine the mechanisms by which tissues respond to injury and regenerate. In this chapter, we will discuss a number of different strategies that stem cells use to repair injured tissue that differ from the mechanisms that regulate homeostatic maintenance of the tissue. Although this discussion only touches on a few examples, each situation has direct implications for therapy development, which would suggest that tissue regeneration may be more complicated than transplanting ES- or iPS-derived stem cells into patients.

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Abbreviations

b-gal	Beta-galactosidase
BMP4	Bone morphogenetic protein 4
BrdU	Bromo-deoxyuridine
Epo	Erythropoietin
ES	Embryonic stem
GM-CSF	Granulocyte macrophage colony-stimulating factor
H2B-CreA	Histone 2B CreA fusion protein
H2B-YFP	Histone 2B YFP fusion protein
HH	Hedgehog
HSC	Hematopoietic stem cell
Ifng	Interferon gamma
IL-7Ra	Interleukin 7 receptor alpha
iPS	Induced pluripotent stem
KSL	Kit+Sca1+Lineage–
LPS	Lipopolysaccharide
LRC	Label-retaining cell
M-CSF	Macrophage colony-stimulating factor
SCF	Stem cell factor
TLR	Toll-like receptor
TNFa	Tumor necrosis factor alpha
YFP	Yellow fluorescent protein

1 Introduction

Stem cell based therapies represent the promise of regenerative medicine. The ability to expand and purify stem cell populations that can be used to repair damaged organs and treat degenerative disease is a highly sought after goal. Unfortunately, for the most part this promise is still unfulfilled. Although in principle this strategy is amenable to many tissues that harbor adult stem cell populations, in practice stem cell transplants are only utilized to treat hematopoietic diseases [1, 2]. Even in these cases transplants are limited by the difficulty in finding compatible donors. The recent development of protocols to generate induced pluripotent stem cells (iPS cells) suggests that in the future organ-specific stem cells can be generated from a patient's own cells that can be transplanted without complications [3–6].

However, the generation and expansion of stem cell populations from iPS or embryonic stem cells have proved difficult. In proof-of-principle experiments with iPS cells, the threat of tumorigenesis was evident [7]. An alternative approach may be to consider how stem cells resident in tissues are mobilized to repair damage to organs and gear therapies to exploit the natural mechanisms of tissue repair. This chapter will focus on the changes in stem cell responses to injury that drive the switch from homeostasis to the generation of mature cells that regenerate the tissue.

The ability of stem cell populations to maintain homeostasis is well recognized. Through interactions with the microenvironment stem cells are induced to proliferate and commit to lineage-specific differentiation. This overall process is precisely regulated to maintain organ homeostasis as terminally differentiated cells age and are lost to apoptosis or active mechanisms of removal. In contrast to homeostasis, regeneration after injury represents a different problem to a stem cell population. New cells which may include multiple cell lineages need to be replaced for normal organ function. Often the number of cells required to repair the organ is much greater than the numbers of cells normally produced during homeostasis. Therefore regenerative processes must encompass mechanisms that not only allow for large numbers of cells to be produced rapidly in response to injury, but also regulate the specific cell types produced. For the purposes of this discussion, we will examine two mechanisms that are utilized by stem cell populations to repair damaged tissue. The first mechanism relies on the property that stem cell populations are heterogeneous and often include actively cycling and quiescent subpopulations [8, 9]. Several investigators have reported that distinct subpopulations of stem cells maintain homeostasis while other populations act to regenerate tissues after injury. This example has implications for regenerative cell therapies to treat disease in that if we are transplanting stem cells to affect organ repair it is important to first understand the relationship between the different populations of stem cells to ensure that the transplanted stem cells are capable of repair rather than homeostasis. In contrast, the second mechanism proposes that stem cells respond to injury by changing the output of terminally differentiated progeny. Simplistically, this can be thought of as a rapid expansion and differentiation of different lineages, which is skewed toward the cell types that are required to confront and repair the injury. A corollary to this second mechanism is the production by homeostatic stem cells of a novel progenitor cell whose sole purpose is to rapidly generate new cells required repair the injury. This mechanism also has implications for therapy. Pharmacological interventions that mimic the responses of stem cells to injury and stimulate the production terminally differentiated cells to repair a tissue might be more efficacious in the short term than stem cell transplant. In tissues where stem cells generate novel progenitors that act to rapidly generate new cells to repair injury, the transplant of these progenitors rather than stem cells may represent a viable treatment. Although this chapter will discuss these two alternative mechanisms more possibilities exist which suggests that development of in vitro-derived stem cell populations for transplant to repair damaged organs may be naïve and more work is needed to understand regenerative processes before we move them into the clinic.

2 Distinct Population of Stem Cells That Respond to Injury: An Example from the Intestinal Epithelium

The development and maintenance of the intestinal epithelium rely on the action of stem cells in the glandular crypts located at the base of the villi. This stem cell population is marked by the expression of Lgr5 [10, 11]. The homeostasis of the epithelium is maintained by this rapidly dividing population of stem cells that generates proliferative progenitors which terminally differentiate. The terminally differentiated cells are short lived (4–5 days). As they move to the tips of the villi, they are eventually sloughed off and must be continuously replaced. The identification and development of these stem cell populations have been extensively reviewed (see [12]). For this discussion, we will focus on a recent paper from Buczacki et al. which analyzes an additional stem cell population in the intestinal epithelium [13]. Previous work had identified a quiescent cell population, which occupied a different position in the crypt-villus unit and expressed stem cell marker genes [14–16]. These cells were originally characterized by their ability to retain the DNA label, BrdU. Buczacki et al. show that this population is also Lgr5+. These cells referred to as label-retaining cells (LRCs) give rise to mature Paneth cells, a long-lived intestinal epithelial cell that functions in host defense to infection [17]. The differentiation of LRCs into Paneth cells does not require cell division. The novel finding in this work however is the demonstration that LRCs also function as stem cells to regenerate the intestinal epithelium in response to injury.

Buczacki et al. use a combination of experimental techniques to investigate the role of LRCs and establish their role as stem cells. Instead of using nucleotide analogs such as BrdU to label LRCs, they used an inducible transgene which expressed a histone 2b-YFP (H2B-YFP) fusion protein [18]. After a short induction, H2B-YFP is incorporated into the chromatin of dividing cells, but as the cells continue to divide it is diluted out and the labeling is lost. YFP+ cells are rapidly lost in a few days because of the rapid cell cycling and turnover of cells in the intestinal epithelium. Small populations of cells retain the YFP label longer—Paneth cells and the cells that correspond to the previously identified BrdU+ LRCs [15]. Gene expression analysis of the LRC population showed that they were a distinct population that differed from Paneth cells and Lgr5+ stem cells based on principal component analysis. Furthermore, this transcriptome analysis showed that LRCs expressed genes associated with both secretory cells and stem cells. One major caveat of the label-retaining experiments is that although many stem cells are LRCs, not all label-retaining cells are stem cells. Clearly the long-term labeling of Paneth cells in these experiments illustrates this point. In order to show that LRCs in fact can act as stem cells they have to exhibit the ability to contribute to multiple cell lineages. Buckzacki et al. addressed this question in two ways. Previous work demonstrated that the rapidly dividing Lgr5+ crypt stem cell population could be cultured in vitro where individual stem cells generate crypt-villus units [19]. Sorted YFP+ cells which lacked the Paneth cell marker UEA were plated in vitro in organoid cultures. LRCs were compared to Lgr5+ crypt stem cells for their ability to generate intact

crypt-villus units. In this in vitro test of regeneration, LRCs were equivalent to Lgr5+ stem cells. The surprising finding came when they analyzed the ability of LRCs to function as stem cells in vivo. To address this question they used lineage tracing in novel way that incorporated aspects of label retention with lineage-tracing experiments. They used a split Cre recombinase system. Cre is split into two proteins CreA and CreB, which are each fused to the ligand binding domain of FKHB. The two proteins only function as a recombinase when FKHB ligand dimerizes CreA and CreB [20, 21]. The label-retaining aspect of this experimental system comes from the fact that CreA is expressed as a H2B fusion protein from an inducible promoter, where it is retained in cells much like H2B-YFP. CreB is expressed from a constitutive transgene promoter. Experimentally, H2B-CreA is induced and then at different times after induction the dimerizing FKHB ligand is added. A cell expressing the CreA/B dimer will catalyze recombination resulting in constitutive expression of a b-gal transgene in a cell and all of its progeny. Three weeks after induction, intestines are harvested and stained for b-gal+ cells. Treatment with FKHB ligand within 3 days of H2B-CreA induction led to multiple clones of b-gal+ cells. However, if the FKHB ligand treatment was done after 3 days post-H2B-CreA induction no b-gal-labeled clones were observed. These data support the idea that LRCs do not contribute to the homeostasis of the intestinal epithelium. The surprising result came when they treated H2B-CreA-induced mice with FKHB ligand greater than 3 days post-induction and then injured the epithelium with hydroxyurea, irradiation, or doxorubicin. This combination of treatments led to multiple b-gal+ clones of cells in the intestine. These data showed that despite the fact that LRCs were present and would be labeled by b-gal in this experimental system, the progeny of LRCs do not maintain the homeostasis of the intestinal epithelium but rather only generate intestinal epithelial cells in response to injury.

3 Changes in Stem Cell Output in Response to Injury: Examples from Hematopoiesis

Much of what we know about stem cell populations comes from studies on hematopoiesis. Hematopoietic stem cells (HSCs) are the best understood tissue stem cell population [9, 22, 23]. Early studies defined assays for stem cells based on their ability to completely reconstitute all hematopoietic lineages following transplant and self-renew as measured in serial transplant assays. The accessibility of HSCs for isolation and the ease of transplant allowed for the progressive delineation of stem cell identity using cell surface markers and dye efflux properties measured by flow cytometry [24, 25]. HSCs are now defined as a heterogeneous population based on cell surface markers, their ability for long-term or short-term reconstitution [26], cycling status, and recently whether they exhibit myeloid or lymphoid skewing in their development [27, 28]. The ease with which hematopoietic cells can be isolated and cultured in vitro has also led to the identification of specific progenitor

populations that give rise to the different hematopoietic cell lineages [29–31]. This groundwork has established an experimental system that can be exploited to understand the response of HSCs and the hematopoietic system to injury. In this section we will focus on two types of hematopoietic "injury." The first type of injury is infection. Although many times infection does not directly lead to pathogen-induced destruction of hematopoietic cells, the hematopoietic system must respond to infection to prevent tissue injury and elements of the hematopoietic system (primarily macrophages and platelets) aid the repair of damaged tissues.

Innate immunity is the first active line of defense to infection. Work over the last 15–20 years established a paradigm by which innate immune cells recognize pathogen-specific molecular patterns [32–35]. Molecules such as lipopolysaccharide (LPS) and flagellin are recognized by a complex of mammalian Toll-like receptors (TLRs). Activation of TLR and other pattern recognition receptors leads to the induction of inflammation and recruitment of effector cells to the site of infection. Much work has gone into understanding the mechanisms that regulate inflammatory responses and the recruitment and action of innate immune cells at the site of infection. In addition to the responses at the site of infection, the mobilization of the innate immune cells dramatically changes the output of the hematopoietic system [36, 37]. For example, infection of mice with the murine malaria parasite *Plasmodium chabaudi* causes a major shift in hematopoiesis as the immune system attempts to resolve the infection [38]. There is a rapid loss in erythroid potential and myeloid colony-forming cells decrease in the first week post-infection. There is also a loss of common lymphoid progenitors and decrease in B and T cell potential in malaria-infected bone marrow. Despite this loss in potential, bone marrow hematopoiesis on the whole is not depressed. Hematopoietic production shifts to produce a new myeloid progenitor population characterized by the IL-7Ra^{+}Kit^{hi} cell surface phenotype. The IL-7Ra^{+}Kit^{hi} cells produce phagocytes that are capable of phagocytosing *P. chabaudi*-infected erythrocytes in vitro. Belyaev et al. showed that adoptive transfer of IL-7Ra^{+}Kit^{hi} cells into *P. chabaudi*-infected mice had a profound effect in the progression of the disease. Infected mice receiving transfers exhibited less severe anemia and resolved the parasitemia much faster than untreated controls. This novel progenitor population is induced by malaria infection. Inflammation caused by the infection leads to an upregulation of interferon gamma (Ifng) [39, 40]. Signaling by Ifng is a critical determinant in the expansion of these progenitors as Ifng receptor mutant mice fail to expand these cells in response to infection. These data provide an excellent example of how infection leads to a change in hematopoiesis from homeostasis to the production of specialized progenitors that are required to resolve the infection.

Infection and inflammation change the output of bone marrow hematopoiesis. Mechanistically, this can happen at several levels. First pro-inflammatory cytokines have dramatic effects on hematopoiesis. In addition to the example above, others have shown that Ifng and TNFa both inhibit steady-state bone marrow erythropoiesis, but stimulate the production of myeloid effector cells needed to fight infection [41, 42]. Pathogen-specific molecules themselves can also skew hematopoiesis [43]. Nagai et al. showed that HSCs express Toll-like receptors (TLRs) and respond to TLR ligands [44].

Treatment with LPS leads to the rapid development of Mac1+F4/80+ cells that develop without transiting through the intervening stages of myeloid development. In addition the response to TLR ligands does not require the action of lineage-specific growth factors like GM-SCF or M-CSF. These data demonstrate that the primitive cells of the HSC compartment can sense and respond directly to infection. The cells rapidly commit to specific myeloid lineages bypassing the normal homeostatic differentiation pathways. This response to TLR ligands is mediated by the direct induction of myeloid-specific transcription factors which explains the skewed production of myeloid effector cells to fight infection. In response to infection this skewing of HSC output plays a role in clearing the pathogen, but inflammation can also be caused by autoimmune mechanisms that lead to significant pathology in the absence of infection. KRNxG7 mice develop autoimmune arthritis [45, 46]. They exhibit a preferential production of Gr1+ and Mac1+ myeloid effector cells that contribute to the disease phenotype. Using this model of autoimmune arthritis, Oduro et al. showed that the skewed production of myeloid effector cells is a result of a cell intrinsic change in the most primitive compartment of the bone marrow [47]. Indeed transcriptome analysis showed that HSCs (KSL) from arthritic mice exhibit a significant increase the expression of genes associated with myeloid differentiation. The myeloid skewing of the HSCs isolated from arthritic animals was evident upon transplant, but the effect was lost over time such that by 5 weeks post-transplant the myeloid skewing is no longer observed. This observation suggested that the bone marrow microenvironment in arthritic mice maintains the myeloid skewed phenotype. Older mice naturally exhibit myeloid skewing of hematopoiesis as they age [28]. Competitive repopulation experiments using older (20 months) recipient mice showed that arthritic HSCs preferentially contributed to the myeloid population where contribution to lymphoid cells was similar between arthritic and control HSCs. These data support the hypothesis that inflammation alters the microenvironment to maintain the myeloid skewing phenotype. Arthritic HSCs exhibit a myeloid inflammation gene signature. One of the genes overexpressed in this signature is S100a8 (also known as Mrp8). This protein along with its partner S100a9 or Mrp14 binds to TLR4 [48, 49]. During infection these proteins are released by the activation of phagocytes where they amplify the response to LPS. S100a8 and S100a9 are found in the joints of arthritis patients associated with phagocytes at the site of maximal cartilage destruction [48, 50]. This observation is consistent with these proteins contributing to the pathology in inflamed joints. In contrast, expression in the bone marrow of arthritic mice may elicit a different effect—stimulating myeloid differentiation through TLR4.

These two examples demonstrate that disease, caused by infection or autoimmunity, leads to an alteration of hematopoiesis which skews the production of terminally differentiated cells towards myeloid effector cells that in the case of malaria infection help to resolve the infection, but in the arthritis model they exacerbate the pathology of the disease. Therapies geared to exploit the production of myeloid effector cells would be beneficial in treating malaria. In contrast, therapies to treat arthritis would need to do the opposite: inhibit myeloid differentiation induced by inflammation.

4 Establishment of a Stress Response Compartment: Stress Erythropoiesis During the Recovery from Anemia

Clearly infection and inflammation can skew hematopoietic development. Another mechanism by which the hematopoietic system deals with environmental stress is to establish a dedicated stress response compartment that is maintained as a quiescent population of cells that can rapidly respond to a loss in homeostasis and generate new cells to restore equilibrium. The best example of this response is stress erythropoiesis, which rapidly generates new erythrocytes in response to anemic stress [51]. Early work in the field suggested that stress erythropoiesis was regulated by tissue hypoxia [52, 53]. Loss of erythrocytes due to hemorrhage or disease would lead to drop in oxygen delivery to the peripheral tissues. Once tissue hypoxia reached a certain level such that it was sensed in the kidney, the interstitial cells in the kidney would start to make erythropoietin (Epo) [54, 55]. The sudden rise in serum Epo concentration would in turn act on erythroid progenitors in the bone marrow and stimulate their proliferation and differentiation. The expansion of progenitor cells far exceeded the capacity of the bone marrow cavity and it was proposed that progenitors migrated to the spleen where they finished their proliferation and terminally differentiated. This idea was consistent with the observations that the spleen increased in size during the recovery from anemia. This model was based on several assumptions. The first assumption is that Epo was the primary signal that drives stress erythropoiesis. The second assumption was that the progenitors that respond to anemic stress are the same progenitors that maintain homeostatic erythropoiesis. These progenitors were thought to have excess capacity that was activated by the increased serum Epo during the recovery from anemia.

Although this model explains many of the changes in erythropoiesis that occurred in response to anemia, analysis of several mouse mutants with defects in stress erythropoiesis led to the development of a new model [56–60]. In contrast to the earlier work, this analysis showed that bone marrow erythroid progenitors did not expand during the recovery from anemia. The major expansion of the erythroid progenitors occurred in the spleen. The stress erythroid progenitors in the spleen exhibited properties that were distinct from bone marrow steady-state erythroid progenitors. The expansion of stress erythroid progenitors in the spleen is driven by the combination of bone morphogenetic protein 4 (BMP4), stem cell factor (SCF), and hypoxia [61]. These factors promote the expansion of progenitors that terminally differentiate in response to high serum Epo present during the recovery from anemia. The requirement for hypoxia in this process limits the activation of stress erythropoiesis to times of anemic stress [62]. Stress erythroid progenitors exhibit ideal properties in that they rapidly generate large numbers of new erythrocytes much faster than bone marrow steady-state erythroid progenitors.

Perry et al. showed that the activation of stress erythropoiesis results in a complete mobilization of stress erythroid progenitors [63]. Mice that have recovered from an experimentally induced anemia are unable to immediately respond to a second anemic challenge. It takes two additional weeks of recovery before a mouse can respond to

a second challenge like an untreated mouse. This observation suggested that the stress erythroid progenitors in the spleen needed to be replenished from another source. Transplant experiments showed that bone marrow cells could give rise to new stress erythroid progenitors in the spleen. However, bone marrow erythroid progenitors do not respond to BMP4, SCF, and hypoxia-like spleen stress progenitors. Perry et al. showed that signals in the spleen microenvironment, Hedgehog (HH) ligands, were sufficient to induce the development of stress erythroid progenitors from bone marrow. Mutations that blocked HH signaling blocked the development of stress erythroid progenitors in the spleen [63].

In addition to recovery from anemia, stress erythropoiesis is required during bone marrow transplant to generate new erythrocytes in the immediate post-transplant period [64]. These progenitors referred to as erythroid short-term radioprotective cells maintain erythroid homeostasis until HSCs can engraft and start producing new erythrocytes. Using the erythroid recovery after bone marrow transplant as an assay, Harandi et al. showed that the bone marrow progenitor that gives rise to the stress erythroid progenitors in the spleen is actually an HSC (CD34+Kit+Sca1+Lin−) [26, 64]. These cells migrate into the spleen within the first 12 h after transplant where they rapidly expand. Using flow cytometry to analyze stress erythroid progenitors in the spleen after transplant, Harandi et al. demonstrated that there were three distinct populations of stress erythroid progenitors. The most immature population referred to as Population I expressed both stem cell markers (Kit+Sca1+) [22] and erythroid lineage markers ($CD71^{lo}$ and TER119+/−) [65]. These cells respond to BMP4, SCF, and hypoxia and contain all erythroid colony-forming activity in the spleen. Because these cells expressed stem cell markers, Harandi et al. tested their ability to give rise to multiple lineages in transplant experiments. Surprisingly, transplant of purified Population I cells into lethally irradiated mice leads to a wave of donor-derived erythropoiesis, which maintains the survival of the recipient until endogenous HSCs that have survived irradiation can repopulate the mouse. The donor stress erythroid progenitors are erythroid restricted and do not contribute to other lineages. In addition to providing erythroid radioprotection, the donor stress erythroid progenitors established a stress response compartment that generated new erythrocytes in response to subsequent anemic challenges. Furthermore, donor stress erythroid progenitors (Population I) can be serially transplanted which supports the idea that these cells self-renew. Based on these observations, it was proposed that stress erythropoiesis relies on the presence of a self-renewing population of erythroid-restricted stress progenitors.

Stress erythropoiesis illustrates the idea that the hematopoietic system can respond to a loss of homeostasis by generating a population of stress response progenitors which are only activated at times of severe stress. In the case of stress erythropoiesis the regulation comes from the requirement for tissue hypoxia [59, 61]. Stress erythroid progenitors have enormous proliferative potential and in the context of a transplant situation they are able to self-renew like stem cells and establish a distinct donor-derived stress response compartment [64]. The identification of this population has distinct implications for the treatment of anemia. Could these progenitors be used as cellular therapy for hereditary anemia or could pharmacological

therapies activate stress erythropoiesis in anemic individuals? In addition to erythrocytes, megakaryocytes, which make platelets and neutrophils, must be made in the immediate post-transplant period to prevent the risk of hemorrhage and infection prior to donor stem cell engraftment [66–69]. Are there similar types of stress progenitors for these hematopoietic lineages that could exhibit self-renewal and enormous proliferative capacity if the right conditions were identified? Future work will be needed to address this possibility.

5 Summary

The primary goal of tissue-specific stem cells is to maintain homeostasis. In response to injury, however, tissue resident stem cells use a variety of mechanisms designed to repair the injury and reestablish homeostasis. We have discussed a small number of the different mechanisms in this chapter. The one lesson that is clear is that the ability of stem cells to repair damage may require (1) different stem cell populations that are dedicated to regenerative processes or (2) distinct differentiation schemes that lead to the production of large numbers of specific cell lineages which can effectively restore the tissues. In many cases these mechanisms will be distinct from the homeostatic mechanisms. This difference is illustrated in stress erythropoiesis. Hedgehog signaling plays an essential role in stress erythropoiesis [63]. In contrast, blocking Hedgehog signaling has no effect on bone marrow steady-state erythropoiesis [70, 71]. In future, new experiments will be needed to identify stem/progenitor populations and the signals that promote regeneration rather than maintenance of tissues. Experimental systems that mimic stress and injury situations will need to be developed so that these processes can be examined in a rigorous nature. A better understanding of the molecular mechanisms that regulate tissue regeneration will lead to development of new therapies for degenerative disease.

References

1. Bryder D, Rossi DJ, Weissman IL (2006) Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol 169(2):338–346
2. Weissman I (2012) Stem cell therapies could change medicine… if they get the chance. Cell Stem Cell 10(6):663–665
3. Okano H et al (2013) Steps toward safe cell therapy using induced pluripotent stem cells. Circ Res 112(3):523–533
4. Yamanaka S (2012) Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 10(6):678–684
5. Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1(1):39–49
6. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
7. Hanna J et al (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318(5858):1920–1923

8. Cheshier SH et al (1999) In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A 96(6):3120–3125
9. Copley MR, Beer PA, Eaves CJ (2012) Hematopoietic stem cell heterogeneity takes center stage. Cell Stem Cell 10(6):690–697
10. Barker N, van de Wetering M, Clevers H (2008) The intestinal stem cell. Genes Dev 22(14):1856–1864
11. Barker N et al (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449(7165):1003–1007
12. Barker N, van Oudenaarden A, Clevers H (2012) Identifying the stem cell of the intestinal crypt: strategies and pitfalls. Cell Stem Cell 11(4):452–460
13. Buczacki SJ et al (2013) Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature 495(7439):65–69
14. Munoz J et al (2012) The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J 31(14):3079–3091
15. Takeda N et al (2011) Interconversion between intestinal stem cell populations in distinct niches. Science 334(6061):1420–1424
16. Sangiorgi E, Capecchi MR (2008) Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 40(7):915–920
17. Bevins CL, Salzman NH (2011) Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol 9(5):356–368
18. Lopez-Garcia C et al (2010) Intestinal stem cell replacement follows a pattern of neutral drift. Science 330(6005):822–825
19. Sato T et al (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(7244):262–265
20. Jullien N et al (2007) Conditional transgenesis using Dimerizable Cre (DiCre). PLoS One 2(12):e1355
21. Jullien N et al (2003) Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. Nucleic Acids Res 31(21):e131
22. Weissman IL, Shizuru JA (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. Blood 112(9):3543–3553
23. Doulatov S et al (2012) Hematopoiesis: a human perspective. Cell Stem Cell 10(2): 120–136
24. Ergen AV et al (2013) Isolation and characterization of mouse side population cells. Methods Mol Biol 946:151–162
25. Mayle A et al (2013) Flow cytometry analysis of murine hematopoietic stem cells. Cytometry A 83(1):27–37
26. Osawa M et al (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273(5272):242–245
27. Dykstra B et al (2007) Long-term propagation of distinct hematopoietic differentiation programs in vivo. Cell Stem Cell 1(2):218–229
28. Benz C et al (2012) Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell Stem Cell 10(3):273–283
29. Traver D et al (2001) Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. Blood 98(3):627–635
30. Akashi K et al (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404(6774):193–197
31. Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91(5):661–672
32. Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30(1):16–34
33. Kumar H, Kawai T, Akira S (2009) Toll-like receptors and innate immunity. Biochem Biophys Res Commun 388(4):621–625
34. Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

35. Medzhitov R, Janeway CA Jr (1999) Innate immune induction of the adaptive immune response. Cold Spring Harb Symp Quant Biol 64:429–435
36. King KY, Goodell MA (2011) Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. Nat Rev Immunol 11(10):685–692
37. Baldridge MT, King KY, Goodell MA (2011) Inflammatory signals regulate hematopoietic stem cells. Trends Immunol 32(2):57–65
38. Belyaev NN et al (2010) Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. Nat Immunol 11(6):477–485
39. Young HA, Hardy KJ (1995) Role of interferon-gamma in immune cell regulation. J Leukoc Biol 58(4):373–381
40. Su Z, Stevenson MM (2000) Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection. Infect Immun 68(8):4399–4406
41. Libregts SF et al (2011) Chronic IFN-gamma production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. Blood 118(9):2578–2588
42. Felli N et al (2005) Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis. J Immunol 175(3):1464–1472
43. Boiko JR, Borghesi L (2012) Hematopoiesis sculpted by pathogens: Toll-like receptors and inflammatory mediators directly activate stem cells. Cytokine 57(1):1–8
44. Nagai Y et al (2006) Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity 24(6):801–812
45. Kouskoff V et al (1996) Organ-specific disease provoked by systemic autoimmunity. Cell 87(5):811–822
46. Ma YD et al (2009) Defects in osteoblast function but no changes in long-term repopulating potential of hematopoietic stem cells in a mouse chronic inflammatory arthritis model. Blood 114(20):4402–4410
47. Oduro KA Jr et al (2012) Myeloid skewing in murine autoimmune arthritis occurs in hematopoietic stem and primitive progenitor cells. Blood 120(11):2203–2213
48. Ehrchen JM et al (2009) The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 86(3):557–566
49. Vogl T et al (2007) Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 13(9):1042–1049
50. Youssef P et al (1999) Expression of myeloid related proteins (MRP) 8 and 14 and the MRP8/14 heterodimer in rheumatoid arthritis synovial membrane. J Rheumatol 26(12):2523–2528
51. Paulson RF, Shi L, Wu DC (2011) Stress erythropoiesis: new signals and new stress progenitor cells. Curr Opin Hematol 18(3):139–145
52. Hara H, Ogawa M (1976) Erythropoietic precursors in mice with phenylhydrazine-induced anemia. Am J Hematol 1:4530458
53. Hara H, Ogawa M (1977) Erythropoietic precursors in mice under erythropoietic stimulation and suppression. Exp Hematol 5(2):141–148
54. Bunn HF (2013) Erythropoietin. Cold Spring Harb Perspect Med 3(3):a011619
55. Ebert BL, Bunn HF (1999) Regulation of the erythropoietin gene. Blood 94(6):1864–1877
56. Cole R, Regan T (1976) Haematopoietic progenitor cells in the prenatal conegenitally anaemic “Flexed-tail” (f/f) mice. Br J Haematol 33:387–394
57. Gruneberg H (1942) The anaemia of the flexed-tail mice (Mus musculus L.) II. Siderocytes. J Genet 44:246–271
58. Gruneberg H (1942) The anaemia of the flexed-tail mouse (Mus musculus L.) I. Static and dynamic haematology. J Genet 43:45–68
59. Lenox L, Perry J, Paulson R (2005) BMP4 and Madh5 regulate the erythroid response to acute anemia. Blood 105:2741–2748
60. Mixter R, Hunt H (1933) Anemia in the flexed tailed mouse, Mus musculus. Genetics 18:367–387

61. Perry J, Harandi O, Paulson R (2007) BMP4, SCF and hypoxia cooperatively regulate the expansion of murine stress erythroid progenitors. Blood 109:4494–4502
62. Wu DC, Paulson RF (2010) Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia. PLoS One 5(6):e11303
63. Perry JM et al (2009) Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. Blood 113(4):911–918
64. Harandi OF et al (2010) Murine erythroid short-term radioprotection requires a BMP4-dependent, self-renewing population of stress erythroid progenitors. J Clin Invest 120(12): 4507–4519
65. Zhang J et al (2003) Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. Blood 102(12): 3938–3946
66. Paquette R, Dorshkind K (2002) Optimizing hematopoietic recovery following bone marrow transplantation. J Clin Invest 109(12):1527–1528
67. Na Nakorn T et al (2002) Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J Clin Invest 109(12):1579–1585
68. BitMansour A et al (2005) Single infusion of myeloid progenitors reduces death from Aspergillus fumigatus following chemotherapy-induced neutropenia. Blood 105(9):3535–3537
69. Slayton WB et al (2002) The spleen is a major site of megakaryopoiesis following transplantation of murine hematopoietic stem cells. Blood 100(12):3975–3982
70. Hofmann I et al (2009) Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. Cell Stem Cell 4(6):559–567
71. Gao J et al (2009) Hedgehog signaling is dispensable for adult hematopoietic stem cell function. Cell Stem Cell 4(6):548–558

Molecular and Endocrine Mechanisms Underlying the Stem Cell Theory of Aging

Daniel L. Coutu and Jacques Galipeau

Abstract The aging process is associated with broad systemic and cellular malfunction. The cellular theory of aging put forward by Hayflick in the 1960s suggests that cell-intrinsic defects such as telomere erosion cause cellular senescence and eventually tissue dysfunction leading to the aging phenotype. Further studies in nematodes and flies identified systemic factors that promote aging and whose inhibition prolongs life span, observations that were reproduced in mammals. The link between cell-extrinsic and cell-intrinsic aging pathways and how they affect tissue homeostasis however remain speculation. Since many tissues are constantly renewed by somatic (adult) stem cells, a stem cell theory of aging has been proposed where senescence of adult stem cells but not differentiated cells is the cause of aging. We here review the experimental evidence supporting that hypothesis and suggest that not only are stem cells the mediators of aging but are also the best cellular system to study the causes and consequences of aging in mammals.

Keywords Stem cells • Aging • Self-renewal • Cell cycle • Senescence

Abbreviations

AGM	Aorta-gonad-mesonephros
Akt	RAC-alpha serine/threonine-protein kinase, protein kinase B

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Arf	Cyclin-dependent kinase inhibitor 2A isoform p19 (mouse) or p14 (humans)
Atm	Ataxia telangiectasia mutated
Atr	Ataxia telangiectasia and Rad3-related
Bmi1	Bmi1 polycomb ring finger oncogene
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitors
CNS	Central nervous system
Fgf	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Foxo	Class O forkhead box transcription factors
GH	Growth hormone
Hh	Hedgehog
Hmga2	High-mobility group AT-hook 2
HSC	Hematopoietic stem cell
Igf	Insulin-like growth factor
Igf1R	Insulin-like growth factor 1 receptor
Igfbp	Insulin-like growth factor binding-protein
Ink4a	Cyclin-dependent kinase inhibitor 2A isoform p16
Insr	Insulin receptor
KO	Knockout
Mapk	Mitogen-activated protein kinase
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NSC	Neural stem cell
PI3K	Phosphoinositide 3-kinase
Rb	Retinoblastoma
ROS	Reactive oxygen species
SAM	Senescence-accelerated mice
Scf	Stem cell factor
SOD	Superoxide dismutase
TF	Transcription factor
Tgfβ	Transforming growth factor beta
Tpo	Thrombopoietin
TSH	Thyroid-stimulating hormone

1 Introduction

Aging is a slow, systemic degenerative process affecting a majority of known multicellular organisms and eventually results in death caused by a single or multiple organ failure. Humans have been concerned about aging and have sought ways to extend their individual life span since time immemorial, as evidenced by early mythology and literature. For instance in the Epic of Gilgamesh (from about 2000 BC

Mesopotamia and one of the earliest known works of literature), the hero Gilgamesh is so troubled by the death of his friend Endiku that he sets on a quest for eternal life. In most civilizations and cultures, the search for immortality (that is to defeat aging) would remain within the realm of myth and folklore for the next millennia.

The scientific study of the causes and consequences of aging (gerontology) can however be traced back to the early twentieth century. For an exhaustive list of early references, we suggest reading Baker and Achenbaum [1] and its appendix listing the major works compiled by the distinguished researcher Nathan W. Shock. In the early 1900s most of the studies on aging focused on simple organisms such as Planarians because they were easier to handle in the laboratory (e.g., see [2, 3]). However, Alexis Carrel, one of the first mammalian cell culturist, already drew analogies between the death of cells and tissues in vitro and the aging process and also observed differences in the cell supporting activity of serum derived from young or old chickens [4, 5]. However, because of technological and scientific limitations most research of the first half of the last century studied aging from a physiological perspective trying to link aging to environmental causes, metabolism, and endocrine dysfunctions (e.g., [6, 7]). Starting around 1950, a series of scientific breakthroughs revolutionized biological research and brought aging research to a new level.

First, works by White, Morgan, Eagle, Ham, and others [8–10] allowed biologists to culture mammalian cells in vitro over extended periods of time using defined media. These techniques would allow studying aging of cells in vitro and would lead Hayflick to propose his cellular theory of aging a few years later [11]. Next, Watson and Crick published the structure of DNA [12]. This discovery was soon to be followed by a series of seminal papers by Jacob and Monod unraveling the mechanisms of gene regulation and expression as well as elucidating how DNA was transcribed into mRNA which was then translated into proteins [13–19]. Altogether, these studies allowed studying aging from a molecular and genetic perspective. Then in 1963 Becker et al. [20] provided the first experimental proof of the existence of adult stem cells [in this case hematopoietic stem cells (HSCs)], which would eventually lead to the stem cell theory of aging (see below). Finally, the advent of mouse genetic techniques (including the generation of the first genetically engineered mouse line by Kuehn et al. [21] and the complete sequencing of the mouse and human genomes) now allow researchers to fully exploit modern technologies to study aging.

Given what has just been described one can rightly ask: so where are we now, what is the current status in the field, what is known and what is still unknown, and most importantly can we envision increasing human life span or increasing the quality of life in old age in the foreseeable future? Unfortunately, there are still today no straight answers to most of these questions. It is quite clear from the literature that aging is a multifactorial process involving (non-exhaustively) environmental stress, genetic susceptibility, epigenetics, and metabolism. All these contribute to deregulation of several molecular and cellular mechanisms in various tissues, affecting cross talk between different systems and eventually accumulating to result in what

is known as aging. Thus, it is essential to study aging using systemic approaches and a good knowledge of general physiology is mandatory. But where do all these factors converge and how can we study them in regard to what is known of mammalian physiology?

As briefly mentioned above, an intriguing hypothesis that started to emerge in the last decade is the stem cell theory of aging, which is firmly rooted in Hayflick's cellular theory of aging [22]. The latter is based on the observation that human diploid cells have a limited replication capacity in vitro and after a given number of divisions they enter a state called cellular senescence (a state that is now widely attributed to telomere erosion, DNA damage, oxidative stress, and/or ionizing radiation exposure) [11, 23, 24]. Hayflick suggested that if this process also occurred in vivo then it could contribute and even be the main cause of aging. There is now robust experimental evidence that senescent cells indeed accumulate in many tissues during aging. However, it is still debated whether cellular senescence in tissues is a direct cause of aging or vice versa, whether the aging process itself induces cellular senescence in tissues [25]. It must be noted here that the systemic physiological changes that occur during aging and the cellular changes that reflect cellular senescence have mainly been studied separately. How do those cell nonautonomous and cell autonomous processes are linked is still unclear, although as we will see there is accumulating evidence that they are indeed linked at the molecular level. So where do stem cells enter this picture?

Since the pioneering discovery by Till and McCulloch of HSCs, still today the prototypical adult stem cell population, adult stem cells have been identified in almost all tissues (bone, brain, skin, intestine, muscle, fat, heart, liver, pancreas). These cells are known (in some cases) or thought to produce replacement cells for their respective tissue during homeostasis and repair. Because aging typically affects tissues that are mitotically active or have a high metabolic demand (those same tissues that are thought to be maintained by adult stem cells) and because of the decreasing regenerative potential of these tissues during aging, one intriguing hypothesis is that aging might be caused by loss or entry into senescence of stem cells [26].

This chapter will thus review what is currently known about the relationship between the aging process and adult stem cells. We will first describe various animal models available to study aging in mammals (although many nonmammalian systems also exist and have proven useful to identify key gene and pathways involved in aging, reviewing them would exceed the scope of this chapter; we will nevertheless mention some of them as appropriate). We will next discuss the main cellular and molecular mechanisms that have been linked to aging and cellular senescence using animal models and in vitro assays. Finally, we will review our current knowledge of how aging affects adult stem cells but also how stem cells might affect aging. We will see that certain populations of stem cells might in fact be useful models to study and unravel the mechanisms of aging in mammals and how manipulating adult stem cells might potentially help to alleviate diseases associated with aging in the future.

2 Animal Models

The study of aging in animal models is not recent. As mentioned above, rodents were already used to study the physiological process of aging in the first half of the last century. It is also interesting to note that the life span extending effects of caloric restriction in rodents have been described as early as 1946 [27]. However, most of the early studies on the molecular and cellular mechanisms of aging were conducted in simpler organisms such as *C. elegans* and *Drosophila*. The advantages of using these organisms are quite evident: in the case of *C. elegans*, its short life span (about 17 days) and a simple cellular organization (959 cells make up the total organism) make it a great tool to rapidly screen various genes for the capacity to extend or shorten life span as well as to study their systemic effects. Intriguingly, many of the genes identified in *C. elegans* to be associated with aging have homologs in mice and humans which have also been linked to aging in these organisms. Mice share about 99 % of their genes with humans and because their physiology is closer to humans than that of nematodes, murine models are currently preferred in aging research.

We will here review various spontaneous and genetically engineered mouse models that have been used in aging research. This section will describe general models of aging. Many gene-specific knockouts or overexpression models, as well as models affecting only specific cell types, will be treated in the following sections. For a more complete review on genetically engineered mouse models of aging, see [28].

2.1 Inbred Mouse Strains

It has long been known, although often overlooked, that inbred strains of mice differ in their average life span [29]. These differences can be quite dramatic when comparing the short-lived AKR/J strain (median life span of 251 days for females) with the long-lived strain WSB/EiJ (median life span of 964 days for females). The widely used C57Bl/6J ranks amongst the longest lived at 866 and 901 days media life span for females and males, respectively. These differences in the speed of aging have been exploited by some groups to identify genes or gene loci responsible for life span extension.

An elegant example of how to exploit these differences comes from the study of BXD mice [30]. These mice were generated by intercrossing the F_1 progeny of C57Bl/6 (long-lived) and DBA/2 mice (short-lived). The crossing-over patterns generated by these matings were bred to homozygosity so that all strains have a unique mosaic pattern of C57Bl/6 and DBA/2 genes (strain distribution patterns). Importantly, these mice are commercially available. Because the genomic chimerism has been mapped by geneticists, it is possible to compare how various BXD strains differ for a certain phenotype and identify the genomic locus or loci responsible for that trait (e.g., [31, 32] and see below).

2.2 Telomerase-Deficient Mice

As we briefly mentioned above, cellular senescence of human diploid cells in vitro is thought to be caused by telomere erosion, which occurs at every cell division due to the structural impossibility of the DNA polymerase to completely duplicate the end of linear DNA molecules [33]. To circumvent this issue, rare cell populations such as some adult and germinal stem cells as well as some cancer cells have the capacity to elongate telomeres, the DNA structures capping the end of chromosomes. To do this they express a gene encoding a different DNA polymerase called telomerase. In the absence of this enzyme, chromosomes shorten at every cell division until genomic instability ensues resulting in recombination events between chromosomes.

To test whether telomere erosion and the resulting cellular senescence are involved in aging, telomerase knockout (KO) mice have been used [34–36]. However, it must be mentioned that murine telomerase regulation is different than in humans. Also, mice have very long telomeres compared to humans. For this reason, there is no apparent phenotype in mice lacking telomerase until the sixth generation, where telomeres reach a critical length. These mice have a shortened life span, are infertile, and show signs reminiscent of aging in various high turnover tissues including skin and blood. Histologically, the tissues appear normal in terms of cell numbers and types. However, they appear deficient in their capacity to respond to stress and injury [30].

Although telomerase KO mice are an appealing model to study the role of cellular senescence in aging, the above-mentioned fact that the regulation and expression of telomerase in mice may not reflect the situation in humans makes it difficult to draw definitive conclusions. Moreover, since the phenotype is only observable after six generations, this model is costly and time-consuming to study and requires a large mouse colony to be maintained.

2.3 Senescence-Accelerated Mice

Senescence-accelerated mice (SAM or senescence prone mice, SAMP) are progeroid animals that were created in Japan by selective inbreeding of the short-lived AKR/J mice originally provided by the Jackson laboratory [37]. Several of these lines have been created (as well as their counterparts, the senescence-resistant mice SAMR) and display some sign of accelerated aging very early. These mice have an increased accumulation of mutations in somatic tissues, suggesting that DNA damage in normal cells is indeed linked to longevity. A few studies also examined adult stem cell populations in these mice (see below) with intriguing findings. However, the use of SAMs as animal models of normal aging is controversial and the genetic causes of their accelerated aging are not clear [38]. Moreover, not all strains of SAMs display the same phenotypes and penetration is not 100 %. However, the use of a genetic strategy such as that used with the BXD mice mentioned above could lead to the identification of potential genes regulating longevity.

2.4 Dwarf Mice

Ames and Snell dwarf mice carry mutations in transcription factors required for pituitary development. These mice are smaller than normal mice and are long-lived (about 30–50 % increased life span). This extension of life span has been attributed to decreased growth hormone (GH) levels (although there is also a deficiency in prolactin and TSH) which subsequently causes a reduction in circulating Igf1 (reviewed in [30, 39]). Indeed, genetic ablation of the GH-receptor binding protein or GH-releasing hormone receptor also leads to decreased Igf1 levels with accompanying increased life span. Moreover, Igf1-receptor heterozygous knockout mice are also long-lived (the homozygosity being perinatally lethal). Interestingly, wild mice typically grow more slowly than their laboratory counterparts, have low circulating Igf1 levels, and live longer. Furthermore, studies in nematodes and flies also identified homologs and/or orthologs of the insulin/Igf-PI3K-Akt-Foxo1 pathways as key regulators of longevity and aging (DAF-2, AGE-1, AKT, and DAF-16 in worms; insulin receptor, PI3K, AKT, and FOXO in flies). Collectively, these observations provide an interesting link between organism growth rate, longevity, and Igf signaling. It may also be worth mentioning that in dogs and humans, smaller individuals tend to live longer.

2.5 Klotho-Deficient Mice

The klotho-deficient mouse was initially created serendipitously by Kuro-o et al. [40]. While attempting to create a transgenic line they inadvertently mutated a gene locus (rendering it hypomorphic) which they named klotho, based on the Greek goddess who spins the thread of life and determines the life span of mortals. Indeed, these mice display a progeria syndrome (accelerated aging) and die at around 3 months (klotho knockout mice with the same phenotype have since been generated) [41, 42]. Klotho-deficient mice suffer from hypogonadism, premature thymic atrophy, osteopenia, ectopic calcification, skin atrophy, pulmonary emphysema, neurodegeneration, hearing loss, and aberrant blood chemistry with 100 % penetrance. Overexpression of klotho in the knockout background rescues the phenotype whereas in normal mice it increases life span by about 20–30 %. As we will discuss in the next section, klotho exists as a transmembrane protein or secreted, circulating form and appears to act by modulating Fgf and Igf signaling at the receptor level.

3 Molecular Pathways and Mechanisms

The animal models described above suggest a link between genetics, metabolism, cell cycle, as well as telomere shortening and aging. With the exception of telomere erosion, the pathways mentioned in the previous section act in a cell

nonautonomous or endocrine manner to modulate the aging process systemically. However, the cellular and stem cell hypotheses of aging favor a cell autonomous mechanism of aging that incorporates modulation of cell cycle components by DNA damage, oxidative stress, and telomere shortening leading to cellular senescence. As we will see in this section, these cell-extrinsic and intrinsic mechanisms are not exclusive and are indeed intricately linked through signaling pathways and the cell cycle machinery (see also Fig. 1).

3.1 Cell Nonautonomous Pathways

3.1.1 Insulin-IGF1/AKT/FOXO1 Axis

As mentioned above, studies in nematodes, flies, and dwarf mice were the first to suggest a link between insulin/Igf1 signaling and longevity [28, 33, 39, 43]. In mammals, the main source of circulating Igf1 is the liver which secretes it as an endocrine hormone important for development and postnatal growth. Its production is stimulated by growth hormone and inhibited by caloric restriction. Igf1 effects are mediated by a receptor tyrosine kinase, Igf1r, and its activity is modulated by various Igf-binding proteins (Igfbps). Insulin is produced by the pancreas and has a well-established function in glucose and fat metabolism but may also have other less understood functions in other tissues than liver, muscle, and adipose tissues, as suggested by the expression of the insulin receptor (Insr, another receptor tyrosine kinase) by other cell types. However, these functions have been poorly studied due to the difficulty of uncoupling them from general metabolic disturbances. However, caloric restriction in mammals decreases insulin levels in the context of normal glucose levels and increases life span. Furthermore, fat-specific deletion of insulin receptor in mice (FIRKO mice) also increases life span [39, 44]. Interestingly, these mice are resistant to high-fat diet or age-induced obesity and protected against diabetes. These observations suggest an intriguing relationship between metabolism, insulin, and adipose tissue that regulates the aging process of the whole organism. Potential mediators of this effect are leptin, adiponectin, and steroid hormones, but further work is required to better understand this phenomenon [39].

Both Igf1r and Insr share many signaling pathways including the PI3K-Akt axis. Akt appears to be a key player in transmitting the pro-aging signals from Igf1 and insulin. Indeed, it leads to activation of both D-type cyclins-CDK4/6 and cyclinE/A-CDK2 complexes promoting entry into cell cycle and cell cycle progression, respectively (see below). Cell cycle control is important in controlling cellular senescence, stem cell quiescence and differentiation, as well as oncogenic transformation [45]. It is also intrinsically linked to telomere shortening, as discussed. Thus, by stimulating cell cycle entry Igf1 signaling may cause loss of adult stem cells and aging.

Another target of PI3K-Akt signaling downstream of Igf1r and Insr are class O forkhead box (Foxo) transcription factors (TFs) which are thought to play an essential role in aging and are inhibited by Akt [46]. When phosphorylated by Akt, Foxo

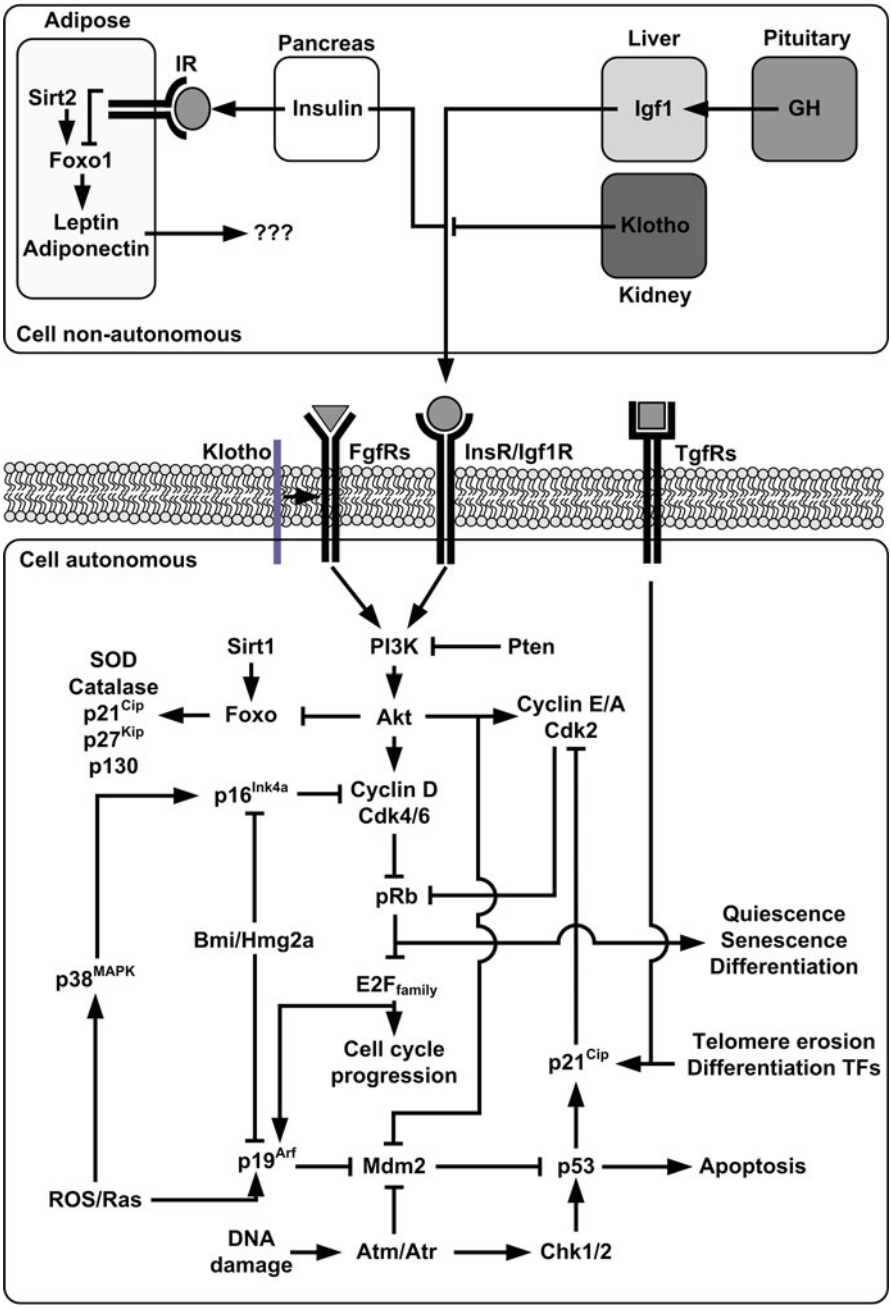

Fig. 1 Link between the main pathways regulating aging in a cell nonautonomous and cell autonomous manner. The main pathways that have so far been identified to regulate aging in a cell nonautonomous manner are the insulin and Igf pathways. These act in an endocrine and systemic manner to affect cells in distant tissues. Their main effect is to stimulate metabolism and proliferation, both of which are deleterious to longevity, and to inhibit the Foxo transcription factors essential to promote longevity. At the cell autonomous level, pathways regulating cellular proliferation, stem cell self-renewal, and cellular senescence converge on the regulation of cell cycle mediators and their inhibitors

proteins are sequestered to the cytoplasm while unphosphorylated proteins translocate to the nucleus. Foxo (or DAF-16 in worms) activates transcription of longevity genes while inhibiting pro-aging genes [44, 47, 48]. In lower organisms, these include reactive oxygen species (ROS) detoxifying genes (superoxide dismutase [SOD], catalase) as well as genes involved in oxidative stress response [33]. The role of Foxo TFs in promoting longevity in mammals is less well established. However, genes directly targeted by Foxo include factors involved in cell cycle inhibition ($p21^{Cip}$, $p27^{Kip}$, p130), DNA damage repair (Gadd45, Ddb1), metabolism (G6Pase, Pepck), and ROS detoxification (MnSOD, catalase) [49, 50]. In addition to inhibition by Akt-mediated phosphorylation, Foxo family members are activated by sirtuins (Sirt1 and 2), which are protein deacetylases. This posttranslational modification appears to antagonize Akt-mediated phosphorylation of Foxo1, but the effects of Sirt1/2 might be tissue specific. Of note, overexpression or activators of sirtuins also prolong life span in lower organisms.

3.1.2 Klotho/Fgf23 Axis

The klotho gene encodes a transmembrane protein with two extracellular domains sharing homology with glycosidases, although it lacks glycosidase activity [51]. Klotho is expressed mainly in the brain choroid plexus and kidney distal tubules; however, through alternative splicing or proteolytic cleavage at the membrane it also exists as a secreted and circulating protein. As mentioned earlier, klotho deficiency leads to a progeroid syndrome whereas its overexpression or intraperitoneal administration increases life span in mice, qualifying it as an anti-aging hormone. Overexpression of klotho leads to insulin resistance with increased insulin levels but normal glucose levels. This insulin resistance is thought to be caused by inhibition of insulin and Igf1 signaling at the receptor level [41]. To further support the antagonizing function of klotho on insulin signaling, the progeria of klotho-deficient mice is partially rescued by knocking out one allele of insulin receptor substrate 1 [39].

Klotho-deficient mice also display elevated calcium, phosphate, and vitamin D levels. Worth mentioning is the fact that feeding these mice with a vitamin D poor diet also partially rescues the progeroid syndrome (it accelerates growth, inhibits ectopic calcification, and increases life span) [41, 42]. The effects of klotho on calcium and phosphate homeostasis are mediated by Fgf23 [52]. Indeed, Fgf23 deficiency partially phenocopies klotho deficiency [53]. Moreover, membrane klotho increases the affinity of various Fgf receptors in the kidney towards the bone-derived Fgf23, triggering phosphate retention.

Finally, preliminary observations suggest that klotho might delay cellular senescence of primary human fibroblasts and HUVECs in vitro through inhibition of the $p53/p21^{Cip}$ pathway [51], which are involved in cell cycle arrest as we will see below.

3.1.3 Fgf/Fgfrs Axes

Fibroblast growth factors (Fgfs) are a large family of secreted proteins (23 members) that play key roles in embryonic development, postnatal homeostasis, and tissue

repair [54]. These factors, which activities are regulated by heparan sulfate proteoglycans, signal through five receptor tyrosine kinases (Fgfr1–5) that are expressed as multiple splice variants [55, 56]. Each receptor isoform possess affinity towards a limited spectrum of ligands. As a general rule, mesoderm-derived Fgfs bind preferentially to ectoderm-expressed Fgfrs and vice versa. Thus, Fgfs are key mediators of cross talk between tissues. The high redundancy in expression and activity of the various Fgfs has made it difficult to study their various biological effects using genetic techniques due to compensation mechanisms. At the receptors level, Fgfr1 and Fgfr2 knockout mice are embryonic lethal, and as such, conditional and/or inducible knockouts are required to study their roles in a tissue-specific manner [57–59]. Fgfr3 knockout results in a skeletal phenotype of osteopenia whereas Fgfr4 knockout mice have no noticeable phenotype [60, 61]. Fgfr5 is only poorly studied as of yet.

Fgf signaling induces many intracellular signaling pathways including MAP kinases and PI3/K-Akt. However, accumulating evidence suggests that Fgf signaling prevents cellular senescence of human and murine adult stem cells and stimulates their self-renewal [62, 63]. Apart from the role of Fgf23 in mediating some anti-aging properties of klotho, the molecular mechanisms underlying Fgfs modulation of stem cell self-renewal and bypass of senescence are still mostly unclear and may be cell type and developmental stage specific.

3.1.4 Cell Autonomous Pathways

All of the environmental, physiologic, cellular, and molecular factors involved in aging that were described above share in common the downstream regulation of cell cycle components. This is expected since deregulation of cell cycle mechanisms leads to growth arrest, cellular senescence, and apoptosis, or conversely their activation promotes neoplasic transformation. A basic understanding of the cell cycle and its regulation is thus important to fully grasp how extrinsic pro-aging factors are linked to intrinsic cellular senescence. We will here describe the main players regulating cell cycle and how they are regulated as well as the key role of the Ink4/Arf locus in mediating cell cycle arrest in various situations.

3.1.5 Cell Cycle Machinery

As just mentioned, the cell cycle machinery requires tight control to prevent apoptosis and senescence as well as cancer progression while remaining capable of responding to physiological cues in normal growth or tissue repair situations [64]. This is accomplished by exquisitely complex regulatory feedback loops (both positive and negative) involving various players, by their levels of expression as well as their activation state. The number of molecules involved is high and their interactions may sometimes be difficult to grasp. For this reason, we have illustrated in Fig. 1 the major proteins involved in cell cycle regulation and their mutual interactions. We will here describe these pathways in more details.

Most of the cell cycle activating and inhibiting pathways converge to the pRb (retinoblastoma) protein. pRb was first characterized as the cause of the inheritable retinoblastoma tumors (in which it is mutated) and along with p53 constitute the prototypical tumor suppressor genes that are both inactivated by DNA tumor viruses (by SV40 T antigen, human papilloma virus E6 and 7 antigens, and adenovirus E1A and B antigens). pRb is the founding member of the Rb family of proteins that also include p130 and p107, whose expression levels vary during cell cycle (p130 being expressed in G0, p107 in S-phase, and pRb in all phases) [65]. The main function of pRb is to prevent progression from G0 through G1 and into S-phase. It accomplishes this in its non- or hypo-phosphorylated form by binding to and inhibiting the E2F family of TFs which regulate proliferation-associated genes [66].

Upon mitogen stimulation, signaling through PI3K, Akt, and the mammalian target of rapamycin (mTOR) downstream of receptors leads to the activation of D-type cyclins and cyclin-dependent kinases 4 and 6 (CDK4/6). The cyclinD-CDK4/6 complex then starts to phosphorylate pRb, causing exit from G0 and progression through G1. Subsequent activation of cyclin E-CDK2 complex further phosphorylates pRb, alleviating E2F TFs inhibition [67]. The latter stimulate transcription of S-phase-associated genes, thus promoting the G1 to S-phase transition. Activation of the PI3K-Akt pathway also inactivates Foxo family members, as seen before, further pushing the cell towards cell cycle progression (Foxos upregulating the transcription of cyclin-dependent kinase inhibitors [CKIs] such as $p21^{Cip}$, $p27^{Kip1}$, and $p57^{Kip2}$) [68]. Given the central role of pRb in driving first the G0–G1 and then the G1–S-phase transitions, it is not surprising that in different cellular contexts it controls various states of growth arrest including terminal differentiation, cellular senescence, and quiescence.

3.1.6 Pleiotropic Roles of the INK4/ARF Locus

The Ink4/Arf gene locus has a complex architecture and encodes three proteins: $p15^{Ink4b}$, $p16^{Ink4a}$, and $p19^{Arf}$ as well as a long intragenic noncoding RNA transcribed in antisense direction from the Arf promoter (reviewed in [69]). Arf and $p16^{Ink4a}$ share two exons, but they are read in alternative reading frames, generating two structurally unrelated proteins that are however functionally related as the both play a role in cell cycle inhibition. The Ink4/Arf locus indirectly regulates pRb and p53 and is one of the most frequently deleted gene loci in various cancers.

The Ink4 family of proteins (which also includes $p18^{Ink4c}$ and $p19^{Ink4d}$) are CKIs that antagonize cyclin D/CDK4–6 complexes [70]. The function of $p19^{Arf}$ on the other hand is to sequester and inhibit the E3 ubiquitin ligase Mdm2, preventing it from targeting p53 for proteasomal degradation [71, 72]. The Ink4a/Arf locus is silenced by the high-mobility group AT-hook 2 (Hmg2a) protein and the polycomb group protein Bmi1 (itself regulated by hedgehog [Hh] signaling) in fetal and young age, respectively [69, 73–75]. The expression of the locus is typically low in young animals and gradually increases during life span. Its transcription can be induced by hyper-mitogenic signals, DNA damage (which induces p16 but not Arf),

constitutively active Ras (which activates both p16 and Arf in mice but only p16 in humans), and ROS. DNA damage can also induce growth arrest through a p16-independent mechanism involving Atm/Atr which activates the p53/p21^{Cip} pathway by activating CHK1/2 and inhibiting Mdm2 [34, 67]. The fact that p16 transcription is not acutely responsive to mitogens suggests that it undergoes a gradual epigenetic modification during aging or cellular stress and that prolonged or repetitive stress is required for its expression [69]. On the other hand, the Arf-Mdm2-p53 axis appears to be important in monitoring the levels of mitogenic signals.

Because of their growth inhibitory properties and their activation by cellular stress and aging, p16 and Arf are considered the hallmarks of cellular senescence [76]. However, in humans the role of Arf does not appear to be as important whereas induction of p21^{Cip} by telomere erosion is more significant [77].

4 Adult Stem Cells and Aging

We have seen in the previous sections that the systemic (metabolic and endocrine) as well as cell-intrinsic molecular regulation of the aging process is multifactorial and regulated through complex feedback loops. However when considered globally, we can see that both cell autonomous and cell non-autonomous pathways converge on the regulation of the cell cycle machinery. This is in retrospect not surprising since tight regulation of cell cycle is central to phenomena like tumor suppression, terminal differentiation of cells, apoptosis, cellular senescence, and stem cell quiescence and self-renewal. In many experimental settings, it has become increasingly evident that cell cycle progression (active proliferation) causes an accumulation of DNA damage and mutations. For instance in an elegant study, Dollé and colleagues demonstrated that a LacZ reporter gene integrated in the genome of mice was subjected to more mutations in a highly proliferative tissue (the liver) than a tissue with slow turnover (the brain) [78].

Adult stem cells are mainly quiescent or slow dividing in mammals and the currently accepted hypothesis is that this protects them for DNA damage insults caused by sustained proliferation and ensuing rapid telomere shortening (at least in humans) [79]. Indeed in tissues with high cellular turnover, senescence or apoptosis of terminally differentiated cells caused by these insults is relatively benign since these cells can be replaced by stem and progenitor cells. However, loss of stem cell function would rapidly result in tissue dysfunction. This hypothesis is the main building ground for the stem cell theory of aging, wherein aging of adult stem cells within tissues leads to tissue dysfunction and the phenotypes associated with old age.

As we will see in this section, there is increasing evidence linking deregulation of the various pathways described above in stem cells and the aging phenotype. To illustrate this, we will focus on three types of adult stem cells. First the prototypical adult stem cells, the HSC, which is the best characterized and most studied adult stem cell and the only one used clinically in a standard manner. Second the neural stem cell (NSC), which is probably the next best characterized and studied

population of adult stem cells with definite therapeutic potential. Finally the mesenchymal stem cell (MSC) (we will here focus our discussion on bone- or marrow-derived MSCs, or skeletal stem cells), which although still poorly characterized at the fundamental level to date, is the most widely used non-hematopoietic adult stem cell in clinical trials for a wide variety of disease conditions.

4.1 Hematopoietic Stem Cells

HSCs are responsible for the generation of all blood and immune cells throughout the life span of an organism. In normal homeostatic hematopoiesis, the adult human body produces over 10^{11} of these blood cells daily. The HSCs are the only cells capable of generating all lineages of blood and immune cells while maintaining themselves in a multipotent state by the process of self-renewal [80, 81]. This process is still poorly understood at the molecular and cellular level but is thought to involve either symmetric or asymmetric cell divisions, where a dividing stem cell gives rise to two daughter stem cells (stem cell expansion) or one stem cells and one differentiated progeny (stem cell maintenance), respectively. Moreover, this self-renewal is thought to be controlled by ill-defined niches in bone marrow that may include osteoblasts, marrow stroma, vasculature, neurons, extracellular matrix, soluble factors, and endocrine signaling [82–85].

In mice, HSCs-like cells, which are thought to be derived from ventral mesoderm, first arise in the yolk sac around E7–7.5 to produce the erythrocytes and endothelial cells necessary to carry oxygen to the growing embryo [86]. These cells, termed hemangioblasts, then undergo a nomadic journey that will successively take them to the aorta-gonad-mesonephros (AGM), the placenta, and the fetal liver before settling in the forming bone marrow shortly before birth [87]. Whereas functional (transplantable) and phenotypically detectable HSCs are first seen in the AGM, the fetal liver is the first site where all blood lineages are produced. The fetal liver HSCs are highly proliferative with 95–100 % of them cycling within 24 h and undergo stem cell expansion. This proliferative phenotype is maintained in the bone marrow until about 4 weeks of age in mice. At this point, most HSCs adopt a quiescent phenotype (characterized by G0 or G1 arrest) although label retention assays suggest that there may be a small subpopulation of actively cycling HSCs (accounting for about 5 % of all HSCs) [68, 88]. It is not known whether this switch from proliferative to quiescent phenotype reflects a change in the HSC niche, a cell-autonomous change, or simply a regulatory feedback loop instructing the HSCs that homeostasis has been reached (a combination of these is likely to be involved). However, it is known that fetal but not adult HSCs depend on Sox17 expression for their maintenance and that the CKI $p18^{Ink4c}$ is highly expressed in adult but not fetal HSCs (see [68] and references therein). Similarly, the Ink4a/Arf locus may not be silenced by the same repressors in fetal and postnatal adult stem cells (Hmga2 and Bmi1, respectively). Interestingly, fetal liver and adult bone marrow HSCs, as well as more differentiated progenitors, all transit through the cell cycle with the same kinetic (about 14 h). It thus appears that the

quiescent phenotype of HSCs reflects differences in how they enter the cell cycle rather than how they progress through it.

In aged mice, the numbers of phenotypically identifiable HSCs are strain specific. They are increased in C57Bl/6 but reduced in DBA/2 mice compared with younger mice [89]. Independently of the size of the HSC pool, their proliferation rate is decreased and they display reduced self-renewal upon transplantation [90–95]. Importantly, old HSCs are also lineage biased towards a myeloid fate. Transplantation of old HSCs into young mice does not rescue these age-associated changes in HSCs, suggesting a cell-autonomous defect or, alternatively, a systemic or niche-related change that induced an irreversible aging phenotype in the HSCs.

A series of seminal studies by Van Zant, de Haan, and colleagues suggest a strong link between HSCs' cycling state and aging [32, 89, 96–102]. They first reported that the proliferation rates of HSCs vary up to tenfold in various inbred mouse strains and subsequently that this proliferation rate was inversely correlated with the life span in these strains. Furthermore, they noticed strain differences in the number of HSCs during aging. Indeed, HSC numbers tend to decrease in aged, short-lived mouse strains, whereas it increases during aging of long-lived strains. An aggregation experiment producing C57Bl/6 (long-lived, slow proliferating) and DBA/2 (short-lived, rapidly proliferating) chimeras then demonstrated that in aged animals the peripheral blood contained only C57Bl/6-derived cells. Intriguingly, the marrow of these mice still contained DBA/2-derived HSCs as assessed by transplantation assays, but these cells contributed only transiently to hematopoiesis in recipient mice. Their contribution was even lessened upon secondary transplantation. Further studies using BXD mice attempted to map gene loci that were linked to both HSC numbers and cycling behavior and life span. Various genetic linkage analyses identified quantitative trait loci linking HSC proliferation and life span as well as HSC numbers and cytokine responsiveness (kit ligand, flt3 ligand, thrombopoietin, and TGFβ) (reviewed in [103]). These studies suggest that organismal aging is reflected by the cycling behavior of HSCs. Furthermore, the aggregation study suggest that hematopoietic decline in aged animals is not caused by an aging niche but rather a cell-autonomous defect.

Proliferation and quiescence of HSCs (and purportedly all somatic and germinal stem cells) are thought to be controlled by a balance of positive and negative regulators of proliferation provided by the niche but also by systemic cues and epigenetic changes within the HSCs that render them responsive or not to external cues. For instance, it is thought that stem cell factor (Scf) and thrombopoietin (Tpo) signaling in HSCs trigger lipid raft clustering on the plasma membrane [68, 104, 105]. This is believed to enhance growth factor receptor activation and signaling, leading to downstream signaling pathway activation (such as MAPK and PI3K). As we have seen, activation of PI3K leads to proliferation and also to inactivation of Foxo family members (mainly Foxo3a in HSCs) which results in downregulation of longevity genes. However, other niche components such as TGFβ inhibit lipid raft clustering and stimulate $p21^{Cip}$ activity, resulting in growth arrest. Since the HSC niche is as of yet poorly described, it is difficult to study age-related changes in the HSC niche. However, since HSCs can be transplanted as single cells, it is possible to uncouple niche effects from stem cell effects in mutant mice using transplantation assays.

To decipher the molecular basis underlying different HSC (and other adult stem cell types) behaviors in fetal, young, and old organisms remains challenging primarily because of the high redundancy in cell cycle regulators. This means that in some instances, double or triple knockouts need to be studied and HSCs from fetal, young, and old mice compared. Furthermore, to uncouple cell-autonomous defects from niche effects, conditional knockouts are preferred. Nevertheless, and although much work remains to be accomplished, our understanding of HSC biology, aging, cell cycle, and cellular senescence and the accessibility of mouse genetic techniques provide a strong basis for unraveling this process. Not surprisingly, the regulation of HSC proliferation, quiescence, and aging is mainly controlled by CKIs of the Ink4 and Cip/Kip families [104, 105], by their transcriptional repression or expression (through epigenetic modifications implicating Bmi1 and Hmga2) and activation state at different developmental stages (see Table 1). Describing in detail the vast number of studies evaluating stem cell function in knockout mice is beyond the scope of this chapter, but excellent recent reviews already exist [68, 69, 79, 88, 106, 107]. However it can be seen in Table 1 that generally, increasing HSC proliferation by knocking out cell cycle inhibitors results in decreased HSC numbers, blunted self-renewal, and lineage bias, eventually leading to a phenotype resembling aging.

One notable exception to this is $p16^{Ink4a}$, the inactivation of which increases self-renewal in old but not young HSCs (consistent with its absence in young HSCs and age-dependent upregulation). The increase of $p16^{Ink4a}$ in aged HSCs is believed to inhibit their infrequent cell division, and although the mechanism underlying this upregulation is not known, it probably serves to limit oncogenic transformation due to accumulating DNA damage. To support this, overexpression of $p16^{Ink4a}$ decreases HSC function similar to age, and its inactivation increases neoplasms. Furthermore, inactivation of genes involved in DNA damage response (such as Atr and Atm) also causes HSC functional defects similar to aging whereas caloric restriction in a $p16^{Ink4a}$-deficient background increases HSC function in aged mice. It must be noted that silencing of the $p16^{Ink4a}$ gene (and thus prevention of cellular senescence) is mostly regulated by Bmi1 and Hmga2 at various levels during development and aging whereas cycling and quiescence are likely mediated by other members of the Ink4 family as well as Cip/Kip family of CKIs [68, 88]. However, the precise regulation and interactions between these cell cycle inhibitors during development and aging are not fully understood yet.

The role of insulin/Igf signaling in HSCs has been poorly studied, but Igfbp2-null mice have low HSC numbers and are defective in supporting HSC self-renewal [108]. On the other hand, the role of Fgf signaling has been extensively studied a few decades ago, but since our definition of HSCs is much evolved since, it remains difficult to interpret these data in light of what is currently known about HSC biology (reviewed in [62, 63]).

From what has been described, it appears that tight control of the cell cycle is primordial for maintaining HSCs throughout life span. During fetal development and early in postnatal life, HSCs are cycling rapidly to support the increasing needs of the growing organism. They accomplish this by silencing the Ink4a/Arf locus and downregulating most CKIs through Polycomb group proteins (Bmi1, Ezh2) and

Table 1 Phenotype and expression of key factors in adult stem cells of fetal, young, and old mice

	HSCs				NSCs				MSCs			
Marker/behavior	F	Y	O	KO/inhibition	F	Y	O	KO/inhibition	F	Y	O	KO/inhibition
Proliferation	+	–	+/–	N/A	+	+/–	–	N/A	+	+	–	N/A
Self-renewal	+	+	–	N/A	+	+	–	N/A	+	+	–	N/A
Differentiation	MP	MP	LB	N/A	MP	MP	LB	N/A	MP	MP	?	N/A
pRb_{family}	+	+	+	#/cycling ↑ SR ↓ LB: myeloid (triple CKO)	+	+	+	Neurogenesis ↓ Apoptosis	+	+	+	LB: adipo #/cycling ↑ Calvarial defects
PI3K	+	+	+	Embryonic lethal Inhibition: ↓ cycling	+	+	+	Embryonic lethal Inhibition: ↓ cycling	+	+	+	Embryonic lethal Inhibition: ↓ cycling
Pten	+	+	+	Cycling ↑ #/SR ↓ MPN (o)	+	+	+	?	+	+	+	?
$p27^{Kip1}$	?	?	?	Gigantism Neoplasms	?	?	?	?	?	+	?	Adipo ↓ Cycling ↑ Normal/↑ osteo
$p57^{Kip2}$	+	+	?	Cycling ↑ #/SR/Quiescence ↓	?	?	?	Oligodendrogenesis ↓	?	?	?	?
$p21^{Cip1}$	–	–	+	Cycling ↓ (o)	+	+	++	?	?	+	?	Cycling/SR ↑ Osteogenesis ↑
$p19^{Arf}$	–	+/–	+	No rescue of Bmi1 KO (only with Ink4a KO)	–	–	+	Rescues Bmi1 KO	?	+/–	+	?
$p16^{Ink4a}$	–	–	+	#/SR ↑ (o) Normal (y)	–	–	+	#/SR ↑ (o) (SVZ)	?	+/–	+	Cycling/SR ↑ Senescence ↓
$p18^{Ink4c}$	–	+	?	#/cycling ↑ Normal SR	?	?	?	?	?	?	?	Adipo ↓
Bmi1	+	+	+	#/SR ↓ (y/o) BM failure (lethal) (y) LB: B-lympho	+	+	+	#/SR ↓ (y/o) (SVZ) Neuro abnormal. LB—glial/astro	?	+	?	SR ↓ Osteogenesis ↓ Osteopenia

(continued)

Table 1 (continued)

	HSCs				NSCs				MSCs			
Marker/behavior	F	Y	O	KO/inhibition	F	Y	O	KO/inhibition	F	Y	O	KO/inhibition
Hmga2	+	+/−	−	?	+	+/−	−	#/SR ↓ (y)	?	?	?	SR ↓
Foxo	−	+	?	Cycling ↑ (o)	+	+	?	SR ↓ Cycling ↑	?	+	?	Osteogenesis ↓ Chondrogenesis ↑ Senescence ↑
Klotho	−	−	−	# ↓	−	−	−	Neurodegeneration	−	−	−	Senescence ↑ Osteopenia

F, fetal; Y, young; O, old; KO, knockout; MP, multipotent; LB, lineage biased; N/A, not applicable; #, numbers; SR, self-renewal; CKO, conditional knockout; MPN, myeloproliferative neoplasms; SVZ, subventricular zone; +, expressed; −, not expressed

high mobility group proteins (Hmga2) [74, 88, 109–111]. However, once homeostasis is reached at around 4 weeks of age, HSCs enter quiescence (probably through $p18^{Ink4c}$) to avoid potential genotoxic insults during proliferation. Accumulation of DNA damage during life then leads to a gradual de-repression of $p16^{Ink4a}$, ensuing cellular senescence in the HSC compartment, and an aging phenotype.

4.2 Neural Stem Cells

During development, NSCs arise from neuroepithelium near the dorsal midline of the embryo. After folding of the neural tube, these multipotent cells proliferate in a self-renewing manner [88]. Various patterning events lead to the specification of the many compartments of the central and peripheral nervous systems. In the central nervous system (CNS) at the onset of neurogenesis, NSCs adopt a radial glia morphology with a short cellular process containing a single cilium contacting the ventricular cavity and a long process extending to the pial surface of the cortex [112, 113]. The cells are thought to generate most of the neurons and glial cells of the central nervous system during development. Later in development, NSCs become restricted to specific areas of the CNS: the cerebellum, the subgranular zone of the dentate gyrus (in the hippocampus), and the subventricular zone. NSCs in the cerebellum persist only a few weeks postnatally [114, 115]. On the other hand, NSCs in the dentate gyrus will contribute new excitatory granule neurons important for learning and memory throughout life. Similarly, NSCs in the subependymal zone (formerly the subventricular zone) generate neuroblasts that migrate along the rostral migratory stream to the olfactory bulb (an important site of adult neurogenesis) to generate interneurons important for odor discrimination (at least in rodents) [116]. These NSCs adopt an astroglial identity in the adult subependymal zone. It is worth mentioning that neuroblasts migrating through the rostral migratory stream can diverge in response to injury to participate in tissue repair.

It is well described that aging is accompanied with deficits in cognition, odor discrimination (in rodents), and regenerative capacity (reviewed in [117]). The fact that the number, self-renewal, and cycling of NSCs decreases with age is also well described. Senescence markers also increase in the subependymal zone with aging and the number of glial cells decreases in the cerebellum, hippocampus, subependymal zone, and olfactory bulb. An age-associated decline in subependymal zone area is also correlated with decreased olfactory bulb neurogenesis. Thus, age-associated deficits in cognition and regeneration correlate with decreased NSC function in aged animals.

It is somewhat intriguing that the molecular pathways regulating NSC cycling, self-renewal, senescence, and aging significantly overlap with those regulating HSCs. Indeed, inactivating $p16^{Ink4a}$ partially rescues the loss of NSCs numbers and self-renewal in old animals whereas it has little effects in juvenile animals, consistent with its low expression at a young age. On the other hand, Hmga2 inactivation causes an increase in $p16^{Ink4a}$/$p19^{Arf}$ expression in fetal and young NSCs with

ensuing decrease in their number and self-renewal capacity [118]. This can be rescued by inactivating $p16^{Ink4a}/p19^{Arf}$ in fetal or young animals. The role of Bmi1 in NSC maintenance is more controversial. Its expression (and transcriptional repressor activity on Ink4a/Arf and $p21^{Cip1}$) nevertheless appears required for NSC self-renewal [119–123].

Similar to HSCs, the role of insulin/Igf signaling in NSCs has been poorly described. However, it appears that Igf1 and Igf2 may signal through Igf1r and Insr in neural progenitor cells and NSCs, respectively, to promote proliferation [124–126]. In line with this, Foxo1/3/4-deficient mice show an initial increase in proliferation during embryogenesis which is followed by premature NSCs exhaustion [127]. Regarding Fgf signaling, we and others have already reviewed its role in NSCs [62, 63]. Briefly, it is well known that Fgf signaling induces self-renewing proliferation of NSCs in vitro. In vivo, at least 10 (out of 23) Fgf ligands are expressed in early CNS development. Fgf receptors (Fgfrs, mainly Fgfr1) are expressed as early as E8.5 in murine CNS and are restricted to the subgranular and subependymal zones at later stages. The age-associated decline in NSCs number is mirrored by a reduction in Fgfrs+ glial cells at these sites. Furthermore, administration of Fgf2 induces NSCs proliferation in vivo (although it is not clear if it is a self-renewing proliferation) and appears to protect SAMP mice against age-related decline in memory. Finally, the fact that klotho is highly expressed in the choroid plexus and that its inactivation causes neurodegeneration could indicate that it has a direct effect on the subependymal NSCs, potentially by potentiating Fgf signaling.

4.3 Mesenchymal Stem Cells

MSCs, or probably more appropriately called skeletal stem cells, were first characterized by Friedenstein in the late 1960s and early 1970s [128–131]. These cells were, and are still in most cases, isolated from bone marrow by a sinple plastic adherence protocol. MSCs have received considerable attention in the last 2 decades due to their capacity to generate bone, cartilage, and adipose tissues and also because of their apparent capacity to modulate immune responses (but positively and negatively, depending on the context) and to stimulate tissue repair after injury [132]. However it must be noted that MSCs, as they are currently defined, possess these properties only after ex vivo culture expansion, which is known to alter their phenotype. The fundamental biology of these cells as well as their true physiological role in vivo is still a matter of debate and more basic studies are urgently required to clarify the identity of MSCs in vivo, what is their exact function, and how it relates to normal bone development, homeostasis, and repair [133–135]. This would greatly help harnessing their power and designing better MSC-based therapies.

There are 209 different skeletal elements in the adult human body and each varies in form, function, and developmental origin. For instance, many bones in the skull (whose main functions are to protect the brain) are derived from neuroectoderm and formed through the process of intramembranous ossification [136].

Most other bones are mesoderm derived, serve a structural and mechanical role, and are formed through endochondral ossification. Moreover, different genetic developmental programs are thought to exist between the axial and appendicular skeleton, although the main signaling pathways are conserved [137]. Appendicular skeleton development is probably the best described and understood. Mesenchymal cells from the lateral plate mesoderm migrate towards the prospective limb bud where upon receiving signals from the apical ectodermal ridge, they form a mesenchymal condensation [138, 139]. Cells in the center of the condensation differentiate into chondrocytes while peripheral (perichondrial) cells remain undifferentiated. This cartilage template expands through chondrocyte proliferation and patterning signals induces branching and segmentation of this template. Eventually, cells in the middle of the template undergo hypertrophy, start to mineralize the extracellular matrix, and secrete angiogenic factors. Vascularization of the perichondrium triggers bone collar (cortical bone) formation and vascular invasion of hypertrophic chondrocytes (which by now undergo apoptosis) is accompanied by osteo-stromal-progenitor cells' invasion that migrate along the blood vessels [140]. These cells will eventually form trabecular bone as well as marrow stroma. How bones are maintained and where skeletal stem or progenitor cells (MSCs) are located in postnatal life are not as well described, although many hypotheses have been proposed [135]. However, it is well known that aged bones display blunted regenerative capacity and homeostatic maintenance (e.g., osteoporosis, which may however be caused by increased osteoclastic resorption and not decreased mineral apposition) which is mirrored by a decrease in isolatable MSCs and their self-renewal and differentiation potential [141]. It might be important to note that contrary to the hematopoietic system or the central nervous system where development occurs at the fetal or early postnatal stages, the skeleton continues to grow and mature until adulthood (approximately 5 months in mice, 18 years in humans) at which point the growth plate closes (becomes mineralized) and the bones stop growing. Thus, MSCs may need to be maintained in a fetal-like state much longer than other adult stem cells.

As mentioned before very little is known about the in vivo counterpart of culture expanded MSCs. In addition, no true functional assay currently allows discriminating MSCs from more differentiated progenitors. Moreover in the literature on MSCs, proliferation is typically indiscriminately associated with self-renewal whereas in most adult stem cell populations, proliferation is associated with transient amplifying clones rather than with the stem cell itself. For these reasons, one must be cautious in interpreting the published data on MSCs and in vivo data is preferred to data generated on ex vivo cultured cells. It is nevertheless interesting to note that the same players involved in HSCs and NSCs maintenance, self-renewal, and aging are also active in MSCs. Indeed, $p16^{Ink4a}$ inactivation also increases MSCs cycling while avoiding cellular senescence [142]. Furthermore, Bmi1 KO mice (who display elevated $p16^{Ink4a}$ levels) are osteopenic with a decrease in MSC self-renewal and osteogenesis [143]. Hmga2 inactivation also appears to decrease MSC self-renewal whereas modulation of various CKIs or cell cycle inhibitors causes lineage bias in MSCs [144–149]. Foxo and klotho deficiencies also cause increased MSC senescence, decreased osteogenesis (osteopenia), and lineage bias [41, 150].

Igf1 is one of the most abundant growth factors in the bone matrix and is thought to stimulate differentiation of MSCs towards osteoblasts [151]. Furthermore, Foxo deficiency was shown to increase MSC senescence resulting in decreased osteogenesis. We and others have previously shown that Fgf signaling is required for the self-renewal of MSCs in vitro and that it inhibits both differentiation and cellular senescence (by inhibiting Mdm2) [152–156]. Fgfrs are expressed as early as the mesenchymal condensation stage in the developing embryo and important Fgfs-Fgfrs cross talk exists between the developing appendicular skeleton and apical ectodermal ridge [136]. Importantly, conditional inactivation of Fgfr1 and 2 demonstrated that Fgf signaling has stage-specific effects and differentially affects skeletal stem/progenitor cells and differentiated osteoblasts [57–59, 157].

5 Conclusion

We have seen throughout this chapter that aging is a multifactorial process that affects each cell in the body individually and that those alterations in cellular physiology can have systemic effects. It is obvious from what has been described that metabolic changes involving insulin and Igf signaling can promote aging. These changes induce cell-intrinsic aging through stimulation of proliferation (by the activation of D-type cyclins for instance) and downregulation of cell cycle inhibitors (CKIs) and ROS detoxifying enzymes. This in turn leads to accumulation of DNA damage and gradual epigenetic changes within cells, resulting in upregulation of senescence-associated genes such as the Ink4a/Arf locus. It is reasonable to assume that these changes affect all cells in the organism. However, somatic cells within a tissue with a high turnover such as blood, skin, intestine, liver, and bone are continually replaced by adult stem cells. Thus, their increased senescence in age may not have a big impact on general aging. On the other hand, aging and cellular senescence of the adult stem cells would have considerable consequences on the homeostatic and regenerative properties of a given tissue.

There is strong evidence that adult stem cells age and become senescent with time and that this is linked to the blunted regenerative capacity of tissues. Furthermore, it appears that the frequency of stem cell cycling is highly correlated with their aging speed. The redundancy of pathways regulating adult stem cells proliferation, quiescence, senescence, and self-renewal suggests a strong link between these mechanisms and the aging process in most tissues. However, more exhaustive studies exploring the various functions of all cell cycle regulators of the Ink4 and Cip/Kip families at different developmental stages might be required to fully understand how we can uncouple anti-aging mechanisms from pro-oncogenic stimuli.

It is probable that aging in a specific tissue might lead to systemic changes affecting other tissues. Nevertheless, the aging process will always be best studied from a cell autonomous perspective. Thus, adult stem cells provide a useful tool to study the molecular mechanisms of aging. Indeed, deregulation of pro- and anti-aging

genes in adult stem cells rapidly translates into identifiable tissue defects. Moreover, the use of well-described populations of stem cells such as HSCs and NSCs makes the use of conditional knockout or knockin technology readily practicable. Finally, the feasibility to transplant single HSCs into recipient mice and the use of parabiosis model systems allow uncoupling of stem cell-intrinsic genetic defects from niche or systemic associated defects. Hopefully, further research along this line might help to alleviate suffering and the decreasing quality of life associated with aging in the near future.

References

1. Baker GT, Achenbaum WA (1992) A historical perspective of research on the biology of aging from Nathan W. Shock. Exp Gerontol 27(3):261–273
2. Child CM (1911) A study of senescence and rejuvenescence based on experiments with Planaria dorotocephala. Arch Entwickl Mech Org 31:537–616
3. Child CM (1913) The asexual cycle in Planaria velata in relation to senescence and rejuvenescence. Biol Bull 25(3):181–203
4. Carrel A (1912) On the permanent life of tissues outside of the organism. J Exp Med 15(5):516–528
5. Carrel A, Ebeling A (1923) Antagonistic growth principles of serum and their relation to old age. J Exp Med 38(4):419–425
6. Boothby WM, Berkson J, Dunn HL (1936) Studies of the energy metabolism of normal individuals. Am J Physiol 116(2):468–484
7. Solomon DH, Shock NW (1950) Studies of adrenal cortical and anterior pituitary. J Gerontol 5(4):302–313
8. Eagle H (1956) The salt requirements of mammalian cells in tissue culture. Arch Biochem Biophys 61(2):356–366
9. Eagle H (1971) Buffer combinations for mammalian cell culture. Science 174(4008):500–503
10. Rizzino A, Rizzino H, Safo G (1979) Defined media and the determination of nutritional and hormonal requirements of mammalian cells in culture. Nutr Rev 37(12):369–378
11. Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636
12. Watson JD, Crick F (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. Nature 171(4356):737–738
13. Jacob F, Perrin D, Sanchez C, Monod J (1960) L'opéron: groupe de gènes à expression coordonnée par un opérateur. C R Hebd Seances Acad Sci 250:1727–1729
14. Pardee AB, Jacob F, Monod J (1958) Sur l'expression et le rôle des allèles "inductibles" et "constitutifs" dans la synthèse de la beta-galactosidase chez les zygotes d'Escherichia Coli. C R Hebd Seances Acad Sci 246(21):3125–3128
15. Jacob F, Monod J (1961) Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol 3:318–356
16. Pardee AB, Jacob F, Monod J (1959) The genetic control and cytoplasmic expression of "inducibility" in the synthesis of beta-galactosidase by E. Coli. J Mol Biol 1:165–178
17. Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6(4):306–329
18. Jacob F, Ullman A, Monod J (1964) Le promoteur, l'élément génétique nécessaire à l'expression d'un opéron. C R Hebd Seances Acad Sci 258:3125–3128
19. Jacob F, Monod J (1959) Gènes de structure et gènes de régulation dans la biosynthèse des protéines. C R Hebd Seances Acad Sci 249:1282–1284

20. Becker AJ, McCulloch EA, Till JE (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452–454
21. Kuehn MR, Bradley A, Robertson EJ, Evans MJ (1987) A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature 326:295–298
22. Sharpless NE, Depinho RA (2004) Telomeres, stem cells, senescence, and cancer. J Clin Invest 113(2):160–168
23. Hayflick L, Moorhead PS (1961) The serial cultivation of human cell strains. Exp Cell Res 621:585–621
24. Watts G (2011) Leonard Hayflick and the limits of ageing. Lancet 377(9783):2075
25. Smith JA, Daniel R (2012) Stem cells and aging: a chicken-or-the-egg issue? Aging Dis 3(3):260–268
26. Vijg J, Campisi J (2008) Puzzles, promises and a cure for ageing. Nature 454(7208): 1065–1071
27. Carlson AJ, Hoelzel F (1946) Apparent prolongation of the life span of rats by intermittent fasting. J Nutr 31:363–375
28. Ladiges W et al (2009) Lifespan extension in genetically modified mice. Aging Cell 8(4):346–352
29. Yuan R et al (2009) Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels. Aging Cell 8(3):277–287
30. Van Zant G, de Haan G (2004) Genetic control of lifespan: studies from animal models. Expert Rev Mol Med 1(20). doi:10.1017/S1462399499001441
31. Gelman R, Watson A, Bronson R, Yunis E (1988) Murine chromosomal regions correlated with longevity. Genetics 118(4):693–704
32. de Haan G, Gelman R, Watson A, Yunis E, Van Zant G (1998) A putative gene causes variability in lifespan among genotypically identical mice. Nat Genet 19(2):114–116
33. Johnson FB, Sinclair DA, Guarente L (1999) Molecular biology of aging. Cell 96:291–302
34. Sperka T, Wang J, Rudolph KL (2012) DNA damage checkpoints in stem cells, ageing and cancer. Nat Rev Mol Cell Biol 13(9):579–590
35. Liu L, Rando TA (2011) Manifestations and mechanisms of stem cell aging. J Cell Biol 193(2):257–266
36. Tümpel S, Rudolph KL (2012) The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems. Ann N Y Acad Sci 1266:28–39
37. Takeda T, Hosokawa M, Higuchi K (1997) Senescence-accelerated mouse (SAM): a novel murine model of senescence. Exp Gerontol 32(1–2):105–109
38. Harrison D (1994) Potential misinterpretations using models of accelerated aging. J Gerontol 49(6):B245–B246
39. Russell SJ, Kahn CR (2007) Endocrine regulation of ageing. Nat Rev Mol Cell Biol 8(9):681–691
40. Kuro-o M et al (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390(6655):45–51
41. Kuro-o M (2009) Klotho and aging. Biochim Biophys Acta 1790(10):1049–1058
42. Kuro-o M (2010) Klotho. Pflugers Arch 459(2):333–343
43. Brunet A (2012) Bien vieillir – La voie de signalisation insulin-FOXO et la longévité. Médecine/Sciences 28:316–328
44. Kuningas M et al (2008) Genes encoding longevity: from model organisms to humans. Aging Cell 7(2):270–280
45. Campisi J, Di Fagagna FDA (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8(9):729–740
46. Daitoku H, Fukamizu A (2007) FOXO transcription factors in the regulatory networks of longevity. J Biochem 141(6):769–774
47. Henderson ST, Johnson TE (2001) daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr Biol 11:1975–1980
48. Murphy CT et al (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature 424(6946):277–283

49. van der Horst A, Burgering BMT (2007) Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 8(6):440–450
50. Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24(50):7410–7425
51. Wang Y, Sun Z (2009) Current understanding of klotho. Ageing Res Rev 8(1):43–51
52. Razzaque MS (2009) The FGF23-Klotho axis: endocrine regulation of phosphate homeostasis. Nat Rev Endocrinol 5(11):611–619
53. Deluca S et al (2008) Amelioration of the premature ageing-like features of Fgf-23 knockout mice by genetically restoring the systemic actions of FGF-23. J Pathol 216:345–355
54. Itoh N, Ornitz DM (2011) Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J Biochem 149(2):121–130
55. Ornitz DM (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays 22(2):108–112
56. Zhang X et al (2006) Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem 281(23):15694–15700
57. Verheyden JM, Lewandoski M, Deng C, Harfe BD, Sun X (2005) Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning. Development 132(19):4235–4245
58. Eswarakumar VP et al (2002) The IIIc alternative of Fgfr2 is a positive regulator of bone formation. Development 129(16):3783–3793
59. Yu K et al (2003) Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth [Internet]. Development 130(13):3063–3074
60. Valverde-Franco G et al (2004) Defective bone mineralization and osteopenia in young adult FGFR3-/- mice. Hum Mol Genet 13(3):271–284
61. Xu X, Weinstein M, Li C, Deng C (1999) Fibroblast growth factor receptors (FGFRs) and their roles in limb development. Cell Tissue Res 296(1):33–43
62. Coutu DL, Galipeau J (2011) Roles of FGF signaling in stem cell self-renewal, senescence and aging. Aging 3(10):920–933
63. Yeoh JS, de Haan G (2007) Fibroblast growth factors as regulators of stem cell self renewal and aging. Mech Ageing Dev 128(1):17–24
64. Beausejour CM, Campisi J (2006) Balancing regeneration and cancer. Nature 443:404–405
65. De Falco G, Comes F, Simone C (2006) pRb: master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription. Oncogene 25(38):5244–5249
66. Fajas L, Blanchet E, Annicotte J-S (2010) CDK4, pRB and E2F1: connected to insulin. Cell Div 5(1):6
67. Vermeulen K, Van Bockstaele DR, Berneman ZN (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 36(3):131–149
68. Pietras EM, Warr MR, Passegué E (2011) Cell cycle regulation in hematopoietic stem cells. J Cell Biol 195(5):709–720
69. Sherr CJ (2012) Ink4-Arf locus in cancer and aging. Wiley Interdiscip Rev Dev Biol 1(5):731–741
70. Cánepa ET et al (2007) INK4 proteins, a family of mammalian CDK inhibitors with novel biological functions. IUBMB Life 59(7):419–426
71. Meek DW, Knippschild U (2003) Posttranslational modification of MDM2. Mol Cancer Res 1(14):1017–1026
72. Meek DW, Hupp TR (2010) The regulation of MDM2 by multisite phosphorylation – opportunities for molecular-based intervention to target tumours? Semin Cancer Biol 20(1):19–28
73. Tzatsos A, Bardeesy N (2008) Ink4a/Arf regulation by let-7b and Hmga2: a genetic pathway governing stem cell aging. Cell Stem Cell 3(5):469–470
74. Park I, Morrison S, Clarke M (2004) Bmi1, stem cells, and senescence regulation. J Clin Invest 113(2):175–179
75. Jiang L, Li J, Song L (2009) Bmi-1, stem cells and cancer. Acta Biochim Biophys Sin 41(7):527–534

76. Collins CJ, Sedivy JM (2003) Involvement of the INK4a/Arf gene locus in senescence. Aging Cell 2(3):145–150
77. Itahana K, Campisi J, Dimri GP (2004) Mechanisms of cellular senescence in human and mouse cells. Biogerontology 5(1):1–10
78. Dollé M, Giese H, Hopkins C (1997) Rapid accumulation of genome rearrangements in liver but not in brain of old mice. Nature 17:431–434
79. Sharpless NE, DePinho RA (2007) How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol 8(9):703–713
80. Schroeder T (2010) Hematopoietic stem cell heterogeneity: subtypes, not unpredictable behavior. Cell Stem Cell 6(3):203–207
81. Bryder D, Rossi DJ, Weissman IL (2006) Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol 169(2):338–346
82. Lymperi S, Ferraro F, Scadden DT (2010) The HSC niche concept has turned 31. Has our knowledge matured? Ann N Y Acad Sci 1192:12–18
83. Purton LE, Scadden DT, Vincent S (2008) The hematopoietic stem cell niche. StemBook 1–14
84. Scadden DT (2006) The stem-cell niche as an entity of action. Nature 441(7097):1075–1079
85. Adams GB, Scadden DT (2006) The hematopoietic stem cell in its place. Nat Immunol 7(4):333–337
86. Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132(4):631–644
87. Mikkola HK, Orkin SH (2006) The journey of developing hematopoietic stem cells. Development 133(19):3733–3744
88. Levi BP, Morrison SJ (2008) Stem cells use distinct self-renewal programs at different ages. Cold Spring Harb Symp Quant Biol 73:539–553
89. de Haan G, Nijhof W, Van Zant G (1997) Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. Blood 89(5):1543–1550
90. Dykstra B, Olthof S, Schreuder J, Ritsema M, de Haan G (2011) Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. J Exp Med 208(13):2691–2703
91. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissmann IL (1996) The aging hematopoietic stem cells. Nat Med 2(9):1011–1016
92. Rossi DJ, Jamieson CHM, Weissman IL (2008) Stems cells and the pathways to aging and cancer. Cell 132(4):681–696
93. Beerman I, Maloney WJ, Weissmann IL, Rossi DJ (2010) Stem cells and the aging hematopoietic system. Curr Opin Immunol 22(4):500–506
94. Kuranda K et al (2011) Age-related changes in human hematopoietic stem/progenitor cells. Aging Cell 10(3):542–546
95. Pang W, Price E (2011) Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci U S A 108(50):20012–20017
96. Phillips RL, Reinhart AJ, Van Zant G (1992) Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. Proc Natl Acad Sci U S A 89(23):11607–11611
97. De Haan G, Van Zant G (1999) Genetic analysis of hemopoietic cell cycling in mice suggests its involvement in organismal life span. FASEB J 13(6):707–713
98. Van Zant G, Eldridge PW, Behringer RR, Dewey MJ (1983) Genetic control of hematopoietic kinetics revealed by analyses of allophenic mice and stem cell suicide. Cell 35(3 Pt 2): 639–645
99. Van Zant G, Holland B (1990) Genotype-restricted growth and aging patterns in hematopoietic stem cell populations of allophenic mice. J Exp Med 171(5):1547–1565
100. de Haan G, Van Zant G (1999) Dynamic changes in mouse hematopoietic stem cell numbers during aging. Blood 93(10):3294–3301
101. Van Zant G, Scott-Micus K, Thompson BP, Fleischman RA, Perkins S (1992) Stem cell quiescence/activation is reversible by serial transplantation and is independent of stromal cell genotype in mouse aggregation chimeras. Exp Hematol 20(4):470–475

102. de Haan G, Van Zant G (1997) Intrinsic and extrinsic control of hemopoietic stem cell numbers: mapping of a stem cell gene. J Exp Med 186(4):529–536
103. Gerrits A, Dykstra B, Otten M, Bystrykh L, de Haan G (2008) Combining transcriptional profiling and genetic linkage analysis to uncover gene networks operating in hematopoietic stem cells and their progeny. Immunogenetics 60(8):411–422
104. Matsumoto A et al (2011) P57 is required for quiescence and maintenance of adult hematopoietic stem cells. Cell Stem Cell 9(3):262–271
105. Zou P et al (2011) p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell 9(3):247–261
106. Sahin E, Depinho RA (2010) Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464(7288):520–528
107. He S, Nakada D, Morrison SJ (2009) Mechanisms of stem cell self-renewal. Annu Rev Cell Dev Biol 25:377–406
108. Huynh H et al (2011) IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. Blood 118(12):3236–3243
109. Kamminga LM et al (2006) The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. Blood 107(5):2170–2179
110. Janzen V et al (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443(7110):421–426
111. Lessard J, Sauvageau G (2003) Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423(6937):255–260
112. Götz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6(10):777–788
113. Beckervordersandforth R et al (2010) In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. Cell Stem Cell 7(6):744–758
114. Miller FD, Gauthier-Fisher A (2009) Home at last: neural stem cell niches defined. Cell Stem Cell 4(6):507–510
115. Weinandy F, Ninkovic J, Götz M (2011) Restrictions in time and space – new insights into generation of specific neuronal subtypes in the adult mammalian brain. Eur J Neurosci 33(6):1045–1054
116. Costa MR, Götz M, Berninger B (2010) What determines neurogenic competence in glia? Brain Res Rev 63(1–2):47–59
117. Conover JC, Shook BA (2011) Aging of the subventricular zone neural stem cell niche. Aging Dis 2(1):49–63
118. Nishino J, Kim I, Chada K, Morrison SJ (2008) Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. Cell 135(2):227–239
119. Fasano CA et al (2007) shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell 1(1):87–99
120. He S et al (2009) Bmi-1 over-expression in neural stem/progenitor cells increases proliferation and neurogenesis in culture but has little effect on these functions in vivo. Dev Biol 328(2):257–272
121. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R (2005) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev 19(12):1432–1437
122. Wang Y et al (2010) Bmi-1 regulates self-renewal, proliferation and senescence of human fetal neural stem cells in vitro. Neurosci Lett 476(2):74–78
123. Yadirgi G et al (2011) Conditional activation of Bmi1 expression regulates self-renewal, apoptosis, and differentiation of neural stem/progenitor cells in vitro and in vivo. Stem Cells 29(4):700–712
124. Aberg MA, Aberg ND, Hedbäcker H, Oscarsson J, Eriksson PS (2000) Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci 20(8): 2896–2903

125. Bracko O et al (2012) Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. J Neurosci 32(10): 3376–3387
126. Ziegler AN et al (2012) IGF-II promotes stemness of neural restricted precursors. Stem Cells 30(6):1265–1276
127. Paik J et al (2009) FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell 5(5):540–553
128. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16(3):381–390
129. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968) Heterotopic transplants of bone marrow. Transplantation 6(2):230–247
130. Friedenstein A, Kuralesova AI (1971) Osteogenic precursor cells of bone marrow in radiation chimeras. Transplantation 12(2):99
131. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues: cloning in vitro and retransplantation in vivo. Transplantation 17(4):331
132. Caplan AI, Correa D (2011) The MSC: an injury drugstore. Cell Stem Cell 9(1):11–15
133. Bianco P, Robey PG, Saggio I, Riminucci M (2010) "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. Hum Gene Ther 21(9):1057–1066
134. Bianco P et al (2013) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19(1):35–42
135. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2(4):313–319
136. Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev 16(12):1446–1465
137. Karsenty G, Kronenberg HM, Settembre C (2009) Genetic control of bone formation. Annu Rev Cell Dev Biol 25:629–648
138. Caplan AI, Pechak DG (1987) The cellular and molecular embryology of bone formation. In: Peck WA (ed) Bone and mineral research. Elsevier, New York, p 458
139. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423(6937):332–336
140. Maes C et al (2010) Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell 19(2):329–344
141. Kassem M, Marie PJ (2011) Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. Aging Cell 10(2):191–197
142. Cakouros D et al (2012) Twist-1 induces Ezh2 recruitment regulating histone methylation along the Ink4A/Arf locus in mesenchymal stem cells. Mol Cell Biol 32(8):1433–1441
143. Zhang H-W et al (2010) Defects in mesenchymal stem cell self-renewal and cell fate determination lead to an osteopenic phenotype in Bmi-1 null mice. J Bone Miner Res 25(3):640–652
144. Aikawa T, Segre GV, Lee K (2001) Fibroblast growth factor inhibits chondrocytic growth through induction of p21 and subsequent inactivation of cyclin E-Cdk2. J Biol Chem 276(31):29347–29352
145. Calo E et al (2010) Rb regulates fate choice and lineage commitment in vivo. Nature 466(7310):1110–1114
146. Drissi H, Hushka D, Aslam F, Nguyen Q (1999) The cell cycle regulator p27kip1 contributes to growth and differentiation of osteoblasts. Cancer Res 59:3705–3711
147. Gutierrez GM et al (2008) Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. Proc Natl Acad Sci U S A 105(47):18402–18407
148. Kang JW et al (2008) The effects of cyclin-dependent kinase inhibitors on adipogenic differentiation of human mesenchymal stem cells. Biochem Biophys Res Commun 366(3):624–630

149. Yew T-L et al (2011) Knockdown of p21(Cip1/Waf1) enhances proliferation, the expression of stemness markers, and osteogenic potential in human mesenchymal stem cells. Aging Cell 10(2):349–361
150. Tseng P-C et al (2011) Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. J Bone Miner Res 26(10):2552–2563
151. Xian L et al (2012) Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. Nat Med 18(7):1095–1101
152. Coutu DL, François M, Galipeau J (2011) Inhibition of cellular senescence by developmentally regulated FGF receptors in mesenchymal stem cells. Blood 117(25):6801–6812
153. Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R (1997) Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 138(10):4456–4462
154. Kuznetsov SA, Friedenstein AJ, Robey PG (1997) Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 97(3):561–570
155. Tsutsumi S et al (2001) Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun 288(2):413–419
156. Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF (2010) Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue Eng Part A 16(3):1009–1019
157. Jacob AL, Smith C, Partanen J, Ornitz DM (2006) Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation. Dev Biol 296(2):315–328

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