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Alexander Birbrair *Editor*

# Stem Cells Heterogeneity in Different Organs

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# Stem Cells Heterogeneity in Different Organs

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# Preface

This book's initial title was *Stem Cells Heterogeneity*. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering stem cell biology under distinct circumstances. Therefore, the book was subdivided into three volumes entitled *Stem Cells Heterogeneity: Novel Concepts*, *Stem Cells Heterogeneity in Different Organs*, and *Stem Cells Heterogeneity in Cancer*.

This book, *Stem Cells Heterogeneity in Different Organs*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of stem cells to different organs biology in physiological and pathological conditions. Further insights into the biology of stem cells will have important implications for our understanding of organ development, homeostasis, and disease. The authors focus on the modern methodologies and the leading-edge concepts in the field of stem cell biology. In recent years, remarkable progress has been made in the identification and characterization of stem cells in several tissues using state-of-the-art techniques. These advantages facilitated identification of stem cell subpopulations and definition of the molecular basis of the role of stem cells within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of stem cells heterogeneity which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the stem cells in various tissues and under distinct pathophysiological conditions. Thirteen chapters written by experts in the field summarize the present knowledge about stem cells heterogeneity in different organs.

Rebecca A. Ihrie and colleagues from Vanderbilt University discuss the heterogeneity of neural stem cells in the subventricular zone. Mirjana Maletic-Savatic and colleagues from Baylor College of Medicine describe stem cells heterogeneity in the hippocampus. Matthias Brandenburger and Charli Kruse from Fraunhofer Research Institution for Marine Biotechnology and Cell Technology compile our understanding of sweat gland stem cells heterogeneity. Kiminori Sato from Kurume University School of Medicine updates us with what we know about heterogeneity

of stem cells in the human vocal fold mucosa. Carla Giordano and colleagues from the University of Palermo summarize current knowledge on stem cells heterogeneity in the thyroid. Kalpaj R. Parekh and colleagues from the University of Iowa address the importance of pulmonary stem cells heterogeneity. Rhiannon French and Giusy Tornillo from Cardiff University focus on the heterogeneity of mammary stem cells. Daniele Torella and colleagues from Magna Graecia University introduce our current knowledge about cardiac stem cells heterogeneity. Dong Seong Cho and Jason D. Doles from Mayo Clinic talk about the heterogeneity of skeletal muscle stem cells. Roland Jurecic from the University of Miami focuses on the hematopoietic stem cells heterogeneity. Deepa Bhartiya and colleagues from the National Institute for Research in Reproductive Health discuss the stem cells heterogeneity in the ovary. Hiroshi Kubota from Kitasato University describes spermatogonial stem cells heterogeneity. Finally, Frank G. Lyons and Tobias A. Mattei from Saint Louis University give an overview of the heterogeneity of human umbilical cord stem cells.

It is hoped that the chapters published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife, Veranika Ushakova, and to Mr. Murugesan Tamilsevan from Springer, who helped at every step of the execution of this project.

This book is dedicated to the memory of my grandfather, Pavel Sobolevsky, PhD, a renowned mathematician, who passed away during the creation of this piece.

**My grandfather Pavel Sobolevsky z"l, PhD (March 26, 1930–August 16, 2018)**

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# Chapter 1
# Heterogeneity of Neural Stem Cells in the Ventricular–Subventricular Zone


**Gabrielle V. Rushing, Madelyn K. Bollig, and Rebecca A. Ihrie**

**Abstract** In this chapter, heterogeneity is explored in the context of the ventricular–subventricular zone, the largest stem cell niche in the mammalian brain. This niche generates up to 10,000 new neurons daily in adult mice and extends over a large spatial area with dorso-ventral and medio-lateral subdivisions. The stem cells of the ventricular–subventricular zone can be subdivided by their anatomical position and transcriptional profile, and the stem cell lineage can also be further subdivided into stages of pre- and post-natal quiescence and activation. Beyond the stem cells proper, additional differences exist in their interactions with other cellular constituents of the niche, including neurons, vasculature, and cerebrospinal fluid. These variations in stem cell potential and local interactions are discussed, as well as unanswered questions within this system.

**Keywords** V-SVZ · Mouse · Human · Neural development · Brain · Stem cell Olfactory bulb · Transcription factor · Subependymal zone · Stem cell niche Neural stem cells

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## Introduction

The ventricular–subventricular zone (V-SVZ) produces an estimated 10,000 new neurons daily in the adult murine brain. Neural stem cells (NSCs, B1 cells) divide to form transit-amplifying progenitors (TAPs, C cells) that divide to form neuroblasts (NBs, A cells) that then migrate and differentiate into local interneurons in the olfactory bulb (OB) [1–3]. V-SVZ neural stem/progenitor cells (NSPCs) have a positional identity—their location within the niche determines the type and final location of the progeny they produce, thereby rendering the V-SVZ a heterogeneous mix of NSPC subtypes rather than a homogeneous group of equally plastic cell types. Here we discuss the origin of the V-SVZ and the diverse properties of its component cell types, considering both temporal and spatial influences. This chapter will focus on studies conducted in mice unless otherwise noted.

## V-SVZ Origins and Development

The V-SVZ develops from a subset of embryonic progenitor domains. In order to provide a framework to introduce similar features that may persist in the adult stem cell niche, we will review observations from early progenitor cells, including those that give rise to the cortex. The brain develops from the neuroepithelium, a sheet of neuroepithelial cells (NECs) derived from the ectoderm. NECs are responsible for forming the neural plate that subsequently folds inward from the ectodermal surface to form the neural tube, creating a polarized structure that eventually becomes the ventricular zone (VZ) [4]. Once the neural tube closes, NECs produce bipolar radial glia cells (RGCs) that serve as the progenitors for neurons and glia until early postnatal life [5–7]. Early transient stem cell niches, including the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE), possess a ventricular zone (VZ) at the ventricular surface, where most progenitor divisions occur. As brain development continues during embryonic days 11/12 (E11/12), cells begin to divide at a more basal location (farther away from the ventricular surface), thus initiating the formation of the subventricular zone (SVZ), the primary site of neurogenesis at E13/14 [8–10]. This increase in size and cell number coincides with the production of additional diverse progeny subtypes [11]. This transition reflects changes in RGC cell division—initially RGCs divide symmetrically to magnify the NSC pool and later divide asymmetrically producing an NSC that persists in the VZ and an intermediate progenitor cell (IPC) that migrates outwardly (basally) to develop the SVZ [12–14].

During embryonic development, RGCs give rise to astrocytic NSCs that remain relatively quiescent until postnatal activation [15]. However, shortly after birth, RGCS generate ependymal cells, neurons, oligodendrocytes, and the adult NSCs (B1 cells) of the V-SVZ [14, 16–20]. During this transformation of RGCs into adult NSCs and other progeny subtypes, the VZ compartment becomes displaced by the

ependymal lining and pushes progenitor cells away from the ventricular surface into the SVZ. Similar to earlier RGCs, adult NSCs retain contact with the ventricular surface through a primary cilium [21, 22], and thus, the adult niche is now referred to as the ventricular–subventricular zone (V-SVZ) [18, 23].

The postnatal and adult V-SVZ consists of NSCs derived from the embryonic telencephalic neuroepithelium, including cells from the spatially distinct regions of the pallium, MGE, and LGE [24–28]. These predecessor zones exhibit distinct transcriptional signatures and display a propensity to form specific subtypes of interneurons, with some progenitor populations possessing the capacity to form multiple subtypes [29–33]. Embryonic cortical development also exhibits cell fate restriction in temporal space—the cortex is formed "inside-out"—and an NSC's competence to produce progeny subtypes is restricted by its birthdate, emphasizing the cell-intrinsic contribution to identity within the VZ [34, 35]. The primary V-SVZ cell types in the postnatal brain were originally characterized using electron microscopy (see Table 1.1 for more information) [89, 90], and the primary cell type generated by B1 cells are immature neuroblasts. Broadly, neuroblasts from the murine V-SVZ converge at the anterior dorsal subregion and migrate in a network of chains through the rostral migratory stream (RMS) to add interneurons to the OB [1, 91, 92]. Depending on the position of its predecessor cell within the niche across the dorsal–ventral and rostral–caudal axes, the resulting interneuron integrates at variable distances from the OB core and will express distinct proteins as discussed further below (Fig. 1.1). An additional level of heterogeneity exists with respect to the planar organization within the niche, as several B1 cell "hotspots" (areas of higher B1 cell density and ventricular contact) have been observed [48, 53, 93].

## Transcriptional Heterogeneity Within the V-SVZ

Transcriptional heterogeneity exists during initial formation of the V-SVZ and is thought to persist through adulthood. While the majority of NSCs in the embryonic brain are transient, altering their potential over time, the NSCs found in the adult V-SVZ are maintained in a tightly organized spatial niche. Distinct transcription factor expression delineates the subpopulations of NSCs within the niche and contributes to a cell's positional identity [24, 27, 28, 53–62, 93–97]. Some of these transcription factors are likely critical for the maintenance of identity over time. One example is the ventrally expressed transcription factor NK2 Homeobox 1 (Nkx2.1)—embryonic removal of Nkx2.1 causes a re-specification of cells from a ventral to a dorsal fate [98]. Positional identity appears to be a cell-intrinsic feature, as NSC identity is retained following heterotopic transplantation and upon culture in vitro [61, 99]. Elegant lineage tracing experiments using retroviral libraries have shown that V-SVZ NSC identity is established as early as E11.5, with most "pre-B1" cells generated between E13.5 and 15.5 [15]. B1 NSCs formed embryonically remain quiescent until postnatal activation and share a common embryonic progenitor with cells that form the cortex, striatum, and septum. Additional studies supporting this finding show that most adult V-SVZ NSCs originate from a slowly dividing NSC population in the

**Table 1.1** Selected experiments revealing key properties of the V-SVZ and its subregions over time

| Key features of the V-SVZ | Brief experimental description | References |
|---|---|---|
| Derivation from earlier lineage: Neuroepithelial cells → Radial glia → B1 cells | 1. NECs → RGCs<br>NECs were shown to acquire expression of brain lipid-binding protein (BLBP) and glutamate aspartate transporter (GLAST). NECs also lose tight junctions and gain adherens junctions | [36–40 |
| | 2. RGCs → GFAP+ astrocytes<br>Fluorescent dyes were injected subpially into newborn *ferret* brains (a time when no GFAP+ cells exist)—Over time, the dye appeared in newborn parenchymal GFAP+ astrocytes. Similar experiments were also completed in rhesus monkeys, finding the same conclusions | [41–43] |
| | 3. RGCs → Postnatal cell types (ependymal cells, adult B1 cells, neurons, oligodendrocytes, astrocytes)<br>Neonatal (P0) injections of adenovirus carrying Cre into conditional fluorescent reporter animals and lineage tracing confirmed that RGCs transform into multiple cell types postnatally. This was also confirmed using time-lapse microscopy analyzing in vivo cell fate | [14, 16, 17] |
| | 4. RGCs → "pre-B1 cells" embryonically<br>A barcoded retroviral library consisting of >100,000 genetic tags (24-bp oligonucleotides), and green fluorescent protein was injected intraventricularly at different embryonic stages and progeny cells were mapped and collected using laser capture microdissection. This work showed that postnatal NSCs become regionally specified early in development and that postnatal and fetal forebrain NSCs share common progenitors in the early embryo | [15] |
| Identification of B1 cells as postnatal NSCs | Infusion of an antimitotic drug (cytosine-*B*-D-arabinofuranoside) into mouse lateral ventricles to eliminate dividing cells indicated type "A" and "C" cells were depleted<br>Subsequent [$^3$H]-thymidine injections (radioactive nucleoside analog incorporated into newly synthesized DNA during cell divisions) followed by electron microscopy revealed that the majority of labeled cells were B1 cells. The V-SVZ was regenerated from these cells within 14 days<br>Later experiments showed that ablation of GFAP-positive cells results in an overall decrease of BrdU incorporation in the V-SVZ as well as reduction in neuroblast generation and new neurons in the olfactory bulb. Long-term ablation prevents the formation of new neurons | [22, 44–46] |

| | | |
|---|---|---|
| Ultrastructural features of V-SVZ cell types | Electron microscopy<br>*B1 NSCs*: GFAP+, Vimentin+, Nestin+. Possess ultrastructural characteristics of astrocytes, including gap junctions, dense bodies, glycogen granules, thick bundles of intermediate filaments, and a light cytoplasm<br>*C cells (TAPs)*: Focal clusters closely associated with chains of type A cells. Large, irregularly shaped nuclei with deep invaginations<br>*A cells (Neuroblasts)*: PSA-NCAM+, Tuj1+, Nestin-. Oriented as chains parallel to lateral ventricle walls. No intermediate filaments but abundant microtubules. Abundant lax chromatin and elongated cell body. Dark cytoplasm with many free ribosomes<br>*E cells (ependymal cells)*: spherical, very light cytoplasm, no invagination of nuclei | [21, 22] |
| Anatomical organization | B1 cells' apical processes have a primary cilium that contacts the lateral ventricle at the center of pinwheel structures formed by ependymal cells, exposing them to signals within the CSF. The pinwheel center is the apical process of the B1 cell, and it is surrounded by multi-ciliated ependymal cells (E1 cells) and biciliated ependymal cells (E2 cells) | [21, 47–49] |
| Markers of V-SVZ cells | Varies temporally and spatially | Reviewed in [50] |
| Proliferative properties of NSCs | Radial glia—divide symmetrically early in development (in the VZ) to expand the population and later divide asymmetrically (in the SVZ). Intermediate progenitor cells—divide symmetrically in the SVZ to produce two daughter cells.<br>Retrovirus expressing GFP was injected into the LVs of E16 rat embryos. Twenty-four hours later, slice culture was completed to observe GFP+ NSPC divisions and migratory behavior in real time using confocal time-lapse microscopy<br>This was also confirmed using mice with GFP expressed under the Tis1 promoter (a marker of neural progenitors); this group observed that apical RG divided asymmetrically while basal RG underwent consuming symmetric divisions to produce two neurons | [12–14] |
| | Adult V-SVZ cell cycle times were determined using whole mount en face views of the lateral ventricular surface and a combination of thymidine analogs (CldU and EdU). Three analysis protocols were used: cumulative labeling, double analog, and percent of labeled mitoses<br>*B1 cells*—~8.6% proliferating at a given time with a total cell cycle length ($T_C$) = 17 h. S phase total time ($T_S$) = 4.5 h<br>*C cells*—~87% cycling; C cells divide on average three times before differentiating. $T_C$ = 18–25 h, $T_S$ = 12–17 h<br>*A cells* ~55% divide at least one time in the V-SVZ; $T_C$ = 18 h, $T_S$ = 9 h | [51] |
| | Short- and long-term lineage tracing methods along with thymidine analogs were used to assess NSC retention and lineage progression in the adult mouse V-SVZ. The majority of postnatal B1 cells (70–80%) were found to divide symmetrically, undergoing consuming divisions (the generation of TAPs while depleting B1 NSCs). 20–30% of postnatal B1 cells symmetrically self-renew and remain in the niche for several months prior to generating neurons | [52] |

(continued)

**Table 1.1** (continued)

| Key features of the V-SVZ | Brief experimental description | References |
|---|---|---|
| Positional identity | Positional identity has been assessed by<br>– the expression of transcription factors<br>– fate mapping using Cre recombinase driven by promoters of transcription factors known to inhabit embryonic progenitor regions<br>– electroporation of plasmids containing fluorescent proteins<br>– neonatal injections of Cre recombinase into fluorescent reporter animals | [24, 27, 28, 53–63] |
| | Dorsal regions were shown to express Emx1, Tbr2, Tbr1, and Pax6. Ventral regions express Gli1, Nkx2.1, and Nkx6.2. Subpallium regions express Dlx1/2/5 and Gsh1/2. Septal regions express Zic1/3. Glutamatergic projection neurons arise from Neurog2+ progenitors in the medial wall. These patterns also vary across the rostro-caudal extent of the niche | |
| | The intrinsic nature of positional identity was tested using heterotopic transplantation experiments. The movement of cells to another position within the niche is not sufficient to alter their intrinsic code to produce specific differentiated cell types | |
| NSC quiescence versus activation | Prospective identification of subtypes using FACS. GFAP::GFP mice (GFP under the control of human GFAP promoter) were used to permit co-localization studies<br>*qNSCs*—Nestin-; rarely form colonies in vitro; characterized as GFAP+CD133 + EGFR- (CD133 restricted to primary cilium)<br>*aNSCs*—Nestin+; activation of NSC yields upregulation of Nestin and EGFR; characterized as GFAP+CD133 + EGFR+ (diffuse CD133 staining on apical surface); robustly generate neurospheres | Mice: [64]<br>[65] |

| | | |
|---|---|---|
| Microenvironmental influences on NSC activity | CSF components | |
| | – Among other factors, BMP5 and IGF1 in LVCP conditioned media increase clone recruitment in neurosphere assays | [66] |
| | – ENaC channels enable CSF flow to regulate progenitors in contact with the ventricle; neurosphere cultures with siRNAs against ENaC had reduced cell counts, and removal of ENaC in vivo reduced the amount of proliferating cells. V-SVZ whole mounts were used to assess the contribution of CSF flow, which is required for the effects of ENaC channels | [67] |
| | Vasculature | |
| | – Confocal imaging and electron microscopy have shown that B1 NSCs and TAPs contact blood vessels | [68, 69] |
| | – Confocal whole-mount imaging shows that the majority of NSCs are within 20 µm of vasculature | [70] |
| | – Endothelial cells promote quiescence; this was shown by using endothelial cell conditioned media on NSCs | |
| | Neuronal innervation | |
| | – $CHaT^{+}$ neuron activity increases proliferation and neuroblast formation; to show this, in vivo stimulations and whole-cell recordings were conducted | [71] |
| | – Dopamine presence increases proliferation; loss of dopaminergic V-SVZ innervation in mice results in decreased proliferation, which can be rescued with the addition of external dopamine | [72] |
| | – Histological analysis and post-mortem retrograde tracing showed that orexigenic/hypocretin removal increases proliferation in adult V-SVZ | [73] |
| | – Transynaptic tracing showed that erotonergic activity regulates B1 cell proliferation | [74] |
| | – POMC neurons increase proliferation of anterior ventral NKX2.1+ cells; shown by intraventricular administration of POMC agonists followed by whole-mount immunostaining for MCM2 | [75] |
| | Cell–cell contact | |
| | – Neuroblasts provide negative feedback to NSCs through GABA; GABA antagonists | [76–80] |
| | – Notch1 promotes aNSC and TAP proliferation, while Notch3 promotes quiescence in V-SVZ NSCs; deletion of Notch1 results in selective loss of aNSCs, and Notch3 KO mice show a lower number of GFAP+ NSCs | [81–86] |

(continued)

**Table 1.1** (continued)

| Key features of the V-SVZ | Brief experimental description | References |
|---|---|---|
| Migration of neuroblasts from the V-SVZ | Neuroblasts move tangentially in chains from the V-SVZ through the RMS to the OB<br>This was determined using [$^3$H]-thymidine injections into the lateral walls of the lateral ventricles, followed by labeling cells over time. Migrating labeled cells moved more rostrally in a stream at a rate of ~30 μm/h | [87] |
| | CSF moves rostrally along dorsal edge into the anterior horn and then ventrally<br>This was determined by injecting a contrast agent into the LV and detection by real-time fluoroscopy. These results were confirmed by directly injecting India ink onto exposed surfaces of dissected LVs | [88] |
| | Neuroblasts follow the flow of cerebrospinal fluid in the adult brain | [88] |
| | Focal injections of retrovirus encoding alkaline phosphatase to label neuroblasts followed by measuring the orientation of neuroblasts' leading processes. Neuroblasts in the RMS were oriented toward the OB as expected and V-SVZ neuroblasts followed the direction of CSF flow within that region | |
| Temporal fate restriction of cortical cells | Heterochronic transplantation experiments in *ferret* embryos: Older VZ NSCs (destined for superficial layers) were transplanted into younger embryos (when deep layers are formed) and the transplanted cells produced only outer (superficial) layer neurons, suggesting that cellular competence is partially cell-intrinsic | [34] |

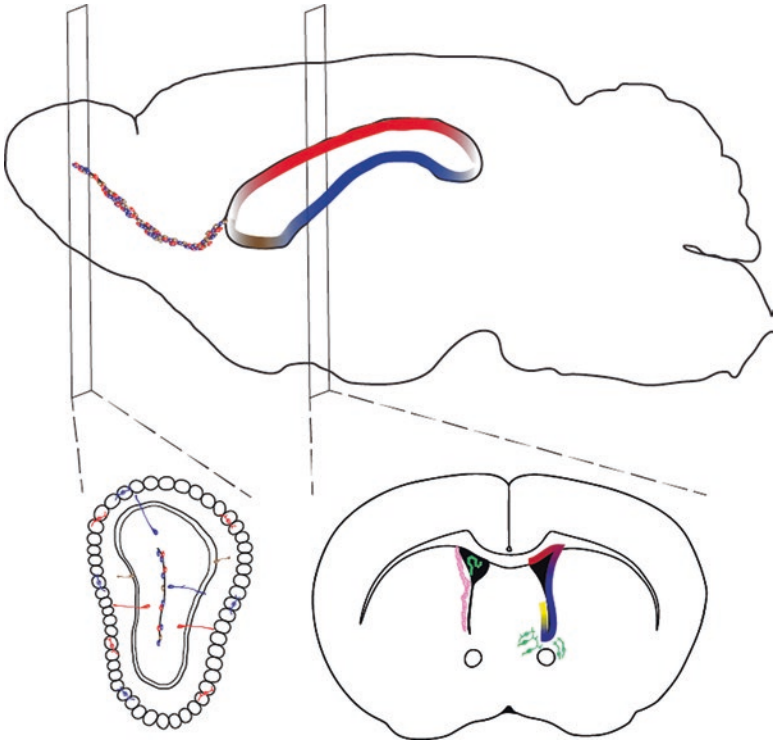

**Fig. 1.1** A sagittal section of the adult mouse brain is shown at the top with cross sections through the olfactory bulb and V-SVZ below. The lateral ventricles are shown in black. Color coding indicates a selection of the subregions from which specific interneuron types (shown, not to scale) are derived. Within the V-SVZ, additional local and global factors regulating stem cell activity are shown, including ventrally innervating neurons (green, at right), lateral ventricle choroid plexus (green, at left), and vasculature (pink, at left)

embryonic ganglionic eminence [100]. These results illustrate that the majority of adult NSCs are specified early in embryonic development and are programmed to make specific neuronal subtypes in the OB. Future work is needed to assess the limitations on the multipotency of adult NSCs generated early in development.

The influx of V-SVZ-derived interneurons into the olfactory bulb during postnatal life is required for plasticity during the processing of olfactory information [101–103]. Local inhibitory circuits likely shape odor representations in the OB, as GABAergic interneurons vastly outnumber principal neurons [104–106]. The majority of interneurons formed from V-SVZ neuroblasts in the OB are either granule cells (GC) or periglomerular cells (PGCs), although specific V-SVZ subdomains have been shown to contribute additional types to the mitral cell layer [60]. GCs predominantly present as four subtypes defined by the location of their cell bodies and projections after integration and maturation—superficial, intermediate, or deep within the granule cell layer—and by their expression of calretinin ($CalR^+$) [107]. PGCs can also be subdivided into three separate types of interneurons: those that express $CalR^+$, calbindin ($CalB^+$), or tyrosine hydroxylase ($TH^+$) [108, 109]. The

specific interneuron subtype generated is dependent on its progenitor's positional identity (the subdomain in the V-SVZ in which the parental B1 cell resides). For example, the ventral subdomain produces deep GCs and CalB+ PGCs, but the dorsal subdomain generates superficial GCs and TH+ PGCs [25, 28, 57, 58, 61, 94, 109]. Further analysis has revealed additional small progenitor domains in the anterior V-SVZ extending just ~100–300 μm along the dorsal–ventral axis and ~400–800 μm along the rostral–caudal axis, comprising less than 5% of the V-SVZ surface area [60]. These smaller progenitor domains are partially delineated by expression of the Nkx6.2 and Zic family transcription factors and produce four additional interneuron subtypes (deep-branching GCs, shrub GCs, perimitral cells, and satellite cells) that were previously unappreciated. This characterization suggests that additional microdomains may exist that remain to be explored. Intriguingly, in early postnatal brain, there is a preferential integration of newborn neurons into the deep portion of the granule cell layer of the OB (progenitors originating from the ventral V-SVZ), indicating an additional layer of V-SVZ heterogeneity exists across both spatial and temporal domains [102, 110].

Embryonically, the V-SVZ also produces cortical interneurons, but the contribution of positional identity and transcriptional heterogeneity remains elusive. As mentioned above, studies have determined that transient progenitor zones give rise to specific interneuron subtypes with ~65–70% of cortical interneurons originating from the MGE, ~30% from the CGE, and 5–10% in the preoptic area [111–113]. However, the wide dispersal of these cells and the challenges of uniting per cell and bulk lineage tracing data have left unresolved whether original cell positioning in the embryo influences the spatial distribution of the differentiated progeny cells within the cortex [29–32]. The contribution of NSC lineage as a predictor of cortical interneuron fate is still debated, as spatially organized interneuron clusters (nonrandom dispersion) have also been reported [29].

Transcriptional heterogeneity also contributes to the transient formation of oligodendrocytes. In postnatal and adult brain, V-SVZ NSCs primarily contribute to new interneurons to the olfactory bulb (OB) [21, 24, 114–117]; however, oligodendrocytes and astrocytes are also produced within a defined period of time postnatally [118–125]. In vivo experiments illustrate that adult-born oligodendrocytes and astrocytes largely integrate into the corpus callosum and are likely derived from dorsal V-SVZ progenitor cells [122–125]. Some in vitro work has described distinct neuronal and oligodendroglial lineage-committed NSCs, but in vivo work has yet to confirm the existence of these distinct progenitor pools [123]. One factor that has been extensively used to trace V-SVZ lineages is the transcriptional activator GLI1, which acts downstream of the SHH morphogen. *Gli*1 is consistently expressed in the ventral V-SVZ [58, 126–130], but it is also transiently expressed in the dorsal V-SVZ [125]. *Gli*1 in the subcallosal zone is high at birth through postnatal day 7, lessened by postnatal day 14, and absent by postnatal day 21. This transient expression in the dorsal V-SVZ is important for the production of oligodendrocytes that inhabit the corpus callosum. Intriguingly, recent studies in an injury model suggest that GLI1 is transiently expressed during oligodendrocyte generation after damage, raising the question of whether the early postnatal program is reawakened in a subgroup of V-SVZ cells [131]. This work further highlights the diversity of V-SVZ progenitors.

## Heterogeneity in Human V-SVZ

The magnitude of neurogenesis in the adult human brain is still debated, and studies involving human tissue must be carefully considered. Some groups report extensive proliferation in adult human specimens [132], while others find that fewer than 1% of V-SVZ cells are dividing in adults [133]. Many challenges exist in the study of human samples, including differences in tissue processing, age and postmortem interval of samples, the inability to lineage trace, and the absence of conclusive markers to measure neurogenesis in static, fixed tissue samples. Despite these limitations, many works have attempted to understand key differences between V-SVZ NSPCs in humans and those in other species [132–142]. Positional identity may exist in humans, and thus, studies of earlier progenitor populations may allow us to infer NSC features that could persist in the adult niche. A major difference is that humans have an expanded area called the outer SVZ (OSVZ) that is not observed in rodents. The OSVZ consists of outer RG (oRG) cells that likely contribute to the increased size and complexity of the human brain [143]. Transcriptionally, human oRG cells are distinct from RG found in mice [134], with some transcripts lacking mouse orthologs. During the late first and early second trimester in fetal human brain, the ganglionic eminences expand causing thinning of the VZ and enlargement of the SVZ, forming the OSVZ in the MGE around post-conception week (PCW) 8 and a defined OSVZ in cortical areas between PCWs 10 and 12 [136, 143]. By PCW 14, over 50% of cortical interneurons are derived from CGE progenitors, which preferentially migrate into the upper layers of the human fetal cortex, in contrast to rodent brain, where ~30% of interneurons are CGE-derived [111, 136]. Migration patterns are similar to those of mice in that MGE-derived cortical interneurons move into the caudate to populate the developing striatum as well as into the developing globus pallidus [98, 136, 144–146]. Interestingly, the tangential migration of MGE progenitors that has been well studied in mice [147] is also observed in humans, although this migratory path appears to pass through the LGE OSVZ. Importantly, OSVZ progenitor cells within the GEs resemble the nearby VZ areas where the progenitor cells originated, suggesting that positional identity may also be present in humans.

Single-cell mRNA sequencing (scRNA-seq) conducted on human cortical and MGE samples during neurogenesis has shown similarities to that done in mice, including the strong influence of embryonic positioning on neuron diversity during cortical development [148]. Findings in humans exhibit differences between dorsal and ventral RG as observed in mice, with one example being the contrasting expression of the patterning of TFs *Emx*1 and *Nkx*2.1. Additionally, newborn neuron clusters exhibit progressive enrichment patterns across developmental time points reminiscent of temporal switches in the types of cortical neurons being generated. Overall, these findings suggest that the transcriptional states present in early development influence the region-specific features of differentiated cells, a feature also observed in the mouse brain. A human-specific difference was observed in the oRG cells—these cells exhibited increased expression of mammalian target of rapamycin (mTOR) regulators as compared to other RG

cells, a feature previously unappreciated [148]. These distinctions emphasize the heterogeneity of V-SVZ subpopulations and their predecessor cells in humans.

There are additional structural and phenotypic differences between human and rodent V-SVZ in both the pre- and postnatal periods. For example, in fetal human brain, radial glia cells (RGCs) express vimentin and glial fibrillary acidic protein (GFAP), with adjacent cells expressing doublecortin (DCX) and B-III tubulin (TUBB3) [141]. In rodents, RGCs do not express GFAP but rather express RC1, RC2, and vimentin among others [36, 50, 141, 149–154]. The infant human V-SVZ shows extensive migration of immature neurons prior to 18 months of age; after 6 months, this process sharply declines and is almost absent in adult tissue [139]. Additionally, in adult V-SVZ, there is a gap layer lacking nuclei but filled with GFAP-positive processes containing intermediate filaments and gap junctions [133]. This gap layer separates ependymal cells from a ribbon of astrocytes that contains the V-SVZ NSCs. Thus, both rodent and human V-SVZ NSCs can be considered "disguised as astrocytes" [133, 155].

Most strikingly, additional V-SVZ-derived migratory streams exist in young human brain, which do not exist in mice. The medial migratory stream (MMS) branches off the RMS and into the ventromedial prefrontal cortex but is only observed between 4 and 6 months of age [139]. An additional population of young neurons, termed the "Arc," is evident between 2 and 5 months of age. The Arc collects around the anterior body of the lateral ventricle, and the neuroblasts within it appear to be migrating into the infant frontal lobe [156]. These immature neurons are likely derived from the ventral forebrain, as they express transcription factors reminiscent of the MGE (Nkx2.1) and CGE (Sp8). The existence of additional migratory streams highlights the increased regional complexity observed in humans.

## Functional Heterogeneity Within the V-SVZ

### *Transcriptional Variations Across the NSC Lineage*

In *humans*, the increased mTOR signature observed in oRG cells suggests that during neurogenesis, oRG cells could be particularly susceptible to mutations in the mTOR pathway, a pathway associated with many developmental disorders including autism [157]. During early development of the *murine* forebrain, mTORC1 is downregulated along with c-MYC at E10.5 when compared to E8.5, accompanying a reduction in transcripts encoding protein biosynthetic machinery including ribosomal proteins (Rpl24), translation factors (Eif4e), and genes involved in ribosome biogenesis [158]. This dynamic regulation of protein biosynthesis is likely to direct cell transition states during early forebrain development as it does in other tissue types [158–160]. mTOR is also critical in the postnatal mouse V-SVZ as a regulator of the TAP population—without adequate mTORC1 signaling, B1 NSCs adopt a quiescent-like state that is reversible [161]. This transcriptional variation is likely coupled to differential protein expression within the lineage and the many markers of V-SVZ cell subtypes, which have been extensively reviewed [50].

## *Cell Cycle Heterogeneity*

Over developmental time, NSC behavior differs with respect to the frequency and patterning of cell division. In the embryo, RG cells principally exhibit asymmetric self-renewal [13, 162]. However, in both the juvenile and adult mouse brains, the vast majority of V-SVZ NSCs (GFAP+) divide symmetrically, with a small fraction (~20%) serving to maintain the NSC pool by symmetric self-renewal and the remaining fraction (~80%) symmetrically generating TAPs [52]. Alterations in cell cycle length may also contribute to cell fate within the V-SVZ and its predecessor niches [11]. Early in development (~E10.5), cell cycle length is slightly extended in MGE progenitors as compared to LGE progenitors [163], and LGE progenitors display a longer cell cycle than cortical progenitors [164]. Cell cycle length is important for the regulation of proliferation and differentiation within these transient stem cell niches, and alterations in these kinetics have been shown to alter the type of progeny produced [164–168]. Specifically, during embryonic development, progenitors experiencing neuron-generating divisions have a significantly longer cell cycle than progenitors undergoing proliferative divisions [165]. Furthermore, extending the length of mitosis in embryonic radial glia leads to preferential production of neuronal or apoptotic progeny at the expense of progenitor production [167]. Variations in cell cycle time have also been observed within the V-SVZ lineage—once a B1 cell is activated, it gives rise to C cells that divide on average three times prior to differentiating into A cells (neuroblasts) that may divide one to two more times within the V-SVZ as they migrate toward the OB [51]. Differences in total cell cycle time as well as the length of S phase have been observed along these transitions, with C cells exhibiting the most variability (see Table 1.1 for details). This variability may be dependent on whether a C cell is generated from a B1 or a C cell. Attempts to assess regional differences in proliferation have found opposing results; however, these studies analyzed subregions along the anterior–posterior and dorsal–ventral axes without addressing the potential contributions of small microdomains (mouse [51], rat [93]). Future work is necessary to evaluate potential proliferative differences across these tightly organized V-SVZ microdomains.

## *Microenvironmental Influences in the V-SVZ and Its Subregions*

The unique microenvironment of the V-SVZ contributes to its heterogeneity by affecting NSC proliferation and exposing V-SVZ subregions to distinct signaling milieus [23, 169–171]. Through varying proximity to the ventricles, vasculature, and other cellular components of the V-SVZ niche, NSCs may have differing access to extrinsic factors that affect their behavior [47]. One large contributor to the niche is the cerebrospinal fluid (CSF), which is produced by the choroid plexus within the lateral ventricles (LVCP) [66, 172, 173]. The CSF provides many secreted factors

that B1 NSCs access through their primary cilium [70]. Experiments using these factors in isolation indicate that they are often capable of increasing NSC proliferation [66]. Recent findings have suggested that epithelial sodium channels (ENaC) with a αENaC subunit also enable CSF flow in the ventricle to regulate proliferation of NSCs in contact with the ventricle [67], potentially coupling a mechanical stimulus to biological outcome. This regulation occurs by the induction of intracellular sodium and calcium signals, which lead to the phosphorylation of extracellular signal-regulated kinases (ERK) and downstream proliferative signaling. These soluble signals also vary during organismal lifetime. For example, the addition of conditioned media generated using choroid plexi (LVCPsec) from aged mice results in a senescence-like phenotype when added to aNSCs from young mice. However, exposure to young LVCPsec appeared to reverse senescence effects in aNSCs from aged mice [66]. Moreover, Alonso et al. [174] have shown that addition of CSF from the embryonic brain can activate qNSCs in the adult, further highlighting the importance of CSF factors in the maintenance of the V-SVZ niche.

During development, secreted morphogens are central to establishing cell fate/positional identity (for review, see [175]). A particularly relevant example within the telencephalon is the opposing gradients of WNT (dorsal) and SHH (ventral) morphogens, similar to those that appear in the developing spinal cord [176]. After birth, these signaling molecules are more restricted and diffuse within a smaller area—for example, SHH is thought to be locally released by a small subset of neurons located near the ventral V-SVZ [58]. Interestingly, a primary cilium is necessary for a small population of anterior ventral B1 cells in order for SHH to mediate its effects on proliferation; without the cilium, proliferation in this region is reduced [177], while other V-SVZ subregions are largely unaffected. Proliferation is further regulated in specific subpopulations of the V-SVZ by innervation from other brain regions and the consequent presence of various neurotransmitters. Specifically, selective innervation of the anterior ventral V-SVZ by proopiomelanocortin (POMC) neurons from the hypothalamus causes increased proliferation in a subpopulation of V-SVZ cells expressing NKX2.1 [75]. Innervation by choline acetyltransferase (ChAT) neurons, serotonergic, and orexigenic/hypocretin projections may further contribute to directing and modifying localized V-SVZ neurogenesis and proliferation [71, 73, 74]. Additionally, many neurotransmitters and other factors have the capacity to modify NSC proliferative behavior en masse, although subregion-specific effects have not yet been detailed [72, 178, 179]. For example, dopamine acts through D2-like receptors to increase proliferation, while the absence of dopamine reduces NSC proliferation [72].

The vasculature surrounding the V-SVZ similarly plays a major role in NSC proliferation and migration. With a much greater density of vascularization than that of the surrounding areas [180, 181], the vasculature presents as a key factor influencing NSC activity in the V-SVZ. Over half of all V-SVZ nuclei can be found within 20 μm of blood vessel surfaces [70]. Proliferative C cells are closely associated with the vasculature [70], and B1 NSCs establish direct blood vessel contact through a long basal process, likely enabling access to secreted factors carried in the bloodstream [48, 68, 70, 182]. Direct contact with endothelial cells promotes quies-

cence through joint EphrinB2 and Jagged1 activity, thus preventing NSCs from entering the cell cycle [183]. Soluble factors secreted by endothelial cells also limit embryonic and adult V-SVZ differentiation, emphasizing the role of the vasculature in promoting NSC self-renewal [69]. Furthermore, migration of neuroblasts from the V-SVZ to the olfactory bulb occurs in chains following both the orientation of the blood vessels and the direction of CSF flow [88, 184].

The extracellular matrix (ECM) contributes to the regulation of the V-SVZ NSC niche, particularly to the balance between activated (aNSCs) and quiescent NSCs (qNSCs). aNSCs express Syndecan-1, a cell surface proteoglycan that interacts with the ECM and growth factors [185]. Additionally, the ECM contains fractones, specialized ECM structures composed of laminin, which intercalate with NSCs and regulate their proliferation by mediating growth factor availability [186–191]. Contact between cells enables further nuanced regulation of NSCs within the V-SVZ. Through negative feedback mechanisms, more differentiated cells are hypothesized to limit the differentiation of progenitors within the adult V-SVZ [81, 192, 193]. For example, neuroblasts release gamma-aminobutyric acid (GABA), which helps preserve the quiescent state of NSCs; upon neuroblast removal (and thus removal of GABA), NSCs enter a more proliferative state [76–80]. Cell–cell contact likewise remains important for proper migration of more differentiated neuroblasts from the V-SVZ to the OB [194]. Specifically, the chemorepulsive interactions between Robo and Slit ensure proper migration of neuroblasts away from the V-SVZ and toward the OB, but it is less clear if there are differences across subregions [195].

The balance between aNSCs and qNSCs may be considered as another potential layer of lineage heterogeneity in the V-SVZ niche. Transcriptomic analyses of qNSCs have revealed enrichment in transcripts categorized as representing cell–cell adhesion, extracellular-matrix response, and anchorage-dependent niche signals, while aNSCs show enrichment for cell cycle and DNA repair-related gene sets [65]. Specific factors such as Notch1 and Notch3 have been implicated in facilitating this transition, as deletion of *Notch*1 selectively reduces aNSCs [81–85]. Furthermore, Notch3 expression is limited to qNSCs of the lateral and ventral walls, while Notch1 is found more specifically in aNSCs and TAPs in the V-SVZ [86].

## *Epigenetic Regulation of V-SVZ Cell Diversity*

As previously mentioned, heterotopic transplantation studies support a cell-intrinsic component of positional identity [61, 196]. A potential cell-intrinsic mechanism through which NSC heterogeneity is maintained in the V-SVZ is epigenetic regulation. Epigenetic alterations throughout development of the mammalian brain contribute to cell fate and maintenance of cell identity [197]. Additionally, NSCs' distinct epigenetic states are thought to change their sensitivity to external environmental cues over time and even alter the set and amount of transcription factor expression [49]. For example, neuronal precursors exposed to BMPs at E17–18

differentiate into glial cells [198], while at E13–14, they differentiate into neurons [199, 200]. This is a possible result of expressing distinct subtypes of BMP receptors and/or differing abundance of receptors [49, 201, 202]. Although there has been much speculation on the contribution of epigenetics to regional differences throughout the V-SVZ niche, much more is currently known about epigenetic alterations affecting NSC differentiation. Overall, many different epigenetic mechanisms underlie the preservation of NSC features and induction of differentiation in the V-SVZ. One subset of epigenetic alterations involves DNA methylation. While DNA methylation of certain areas of the genome known as CpG islands typically results in inhibition of genes associated with that specific island, de novo methyltransferase 3a (Dnmt3a) methylation of these islands within the V-SVZ results in activation of neural progenitor genes in addition to the inhibition of self-renewal genes to effectively increase differentiation [49, 203, 204].

Histone modifications also include methylation of histone tails. Polycomb group (PcG) and trithorax group (trxG) proteins act through histone methylation in the V-SVZ to silence or activate genes, respectively, and thus influence cell identity [205]. For example, the removal of a specific trxG homolog called *Mll*1 in V-SVZ B1 cells results in NSCs with appropriate markers and the ability to self-renew, but the B1 cells are restricted to the production of glial cells [206]. Other methyltransferases, such as the radial glial PR domain-containing 16 (PRDM16), can regulate the number and position of neurons in the cortex by acting through other epigenetic modifiers [207]. For example, PRDM16 represses transcription of the ubiquitin E3 ligase PDZRN3 in order to increase migration to upper cortical layers [207]. Additional examples of enzymes catalyzing histone modifications that contribute to V-SVZ heterogeneity include BAF, HDACs, Jmjd3, and Il2b (see [208] for review). Furthermore, RNAs that do not directly code for proteins such as microRNAs or lncRNAs can regulate translation by binding and degrading mRNA. For example, homeobox gene Paired box 6 (Pax6) mRNA is expressed along the dorsal–ventral V-SVZ axis, but PAX6 protein expression is restricted to the dorsal subregion due to regional expression of miR-7a, emphasizing additional layers of post-translational regulation of identity [209]. Due to the increased tissue and temporal specificity of lncRNAs compared to microRNAs, lncRNAs could potentially represent a critical mechanism for discrimination between specific V-SVZ subtypes [208, 210, 211]. LncRNAs act through distinct mechanisms to regulate the V-SVZ niche and determine cell fate [211, 212]. One lncRNA, *Pnky*, functions along with PTPB1, a splicing factor, in regulating similar pathways within the V-SVZ to preserve the NSC population [212]. Knockdowns of either *Pnky* or PTPB1 result in increased differentiation and reduced NSCs within the niche [212].

## *Metabolic Differences Within the V-SVZ*

Within the V-SVZ lineage, key metabolic differences exist, including increased protein synthesis in aNSCs as compared to that in qNSCs [95]. More specifically, RNA microarray analysis has demonstrated that qNSCs are enriched for lipid metabolism,

while aNSCs are enriched for DNA/RNA metabolism and proteasome activity [65], similar to transcriptional programs for quiescence and activation in stem cells from other tissue types [213–220]. Additionally, recent emerging work has illustrated that V-SVZ subregions possess differences in basal signaling of growth pathways, adding another layer of V-SVZ regulation that warrants further exploration [221].

### *Heterogeneity in the Aging Niche*

V-SVZ heterogeneity also persists in the aging niche. There is a significant reduction (50–75%) of proliferating cells in aged mice (20–24 months) and a depletion of B1 cells over time via consuming differentiative divisions [21, 52, 222–227]. However, this decline appears to be spatially consistent with OB interneuron subtypes observed at similar ratios over time [228]. This reduction of newborn interneurons in the OB has been shown to interfere with fine olfactory discrimination tasks [223]. As the niche ages, parts of the ventricle wall undergo stenosis and thinning, concentrating neurogenesis to the anterior dorsal subregion [225]. Typically, only 2.5% of young adult VSVZ NSCs are dividing at any particular time [228–230]. However, in aged mice (>1 year), there is an increase in the percentage of total NSCs that are dividing (~16.8% undergoing a mitotic division at any given time) but a reduction in the total number of TAPs [225, 228]. This phenotype was also observed by analysis of the V-SVZ transcriptome—a decline in the division rate of Mash1+ cells up to 18 months of age was noted followed by a "recovery" at 22 months [231]. Further analysis utilizing FACS sorting of V-SVZ populations showed a decline in TAPs and neuroblasts as early as 4 months of age and a progressive increase in G1 phase cell cycle length specifically in aNSCs (lengthening by 3 h at 6 vs. 2 months and then 6.5 h at 12 vs. 2 months) [232]. This increased cell cycle length may explain the increase in the percentage of mitotic NSCs observed in other studies—to compensate for reduced progeny generation due to a longer cell cycle, the V-SVZ may increase the total number of dividing cells. The diversity of V-SVZ NSC abundance and properties throughout aging highlights an additional layer of heterogeneity within a single stem cell niche.

## Future Directions: Uniting Transcript, Signal Transduction, and Function

Since the discovery of stem-like activity in the periventricular niche, research into the mechanical and molecular cues driving neurogenesis has blossomed into a wide-ranging field with applications to regenerative medicine, cancer biology, and evolutionary biology. Although the cytoarchitecture and properties of this fascinating niche are extensively mapped, the advent of additional novel approaches and a renewed appreciation of the many levels at which heterogeneity may exist have

revealed a new generation of questions about neural stem cells. Although recent work has focused heavily on the regulation of NSCs using transcript- or RNA-centered approaches, less is known about the relative activation of key pathways at the protein level, particularly post-translational modifications and protein turnover. Recent work has highlighted the importance of protein processing and turnover of cellular byproducts in the regulation of quiescence [233], but methods to probe these and other features at the per-cell level have not yet been widely adopted. As highly multiplexed cytometry approaches, including mass cytometry, multiplexed imaging, and high-resolution mass spectrometry, continue to advance, the prospect of attaining per-cell data on these additional features emerges. Going forward, a key challenge for researchers interested in spatial or temporal heterogeneity will be the effort to unite information about multiple levels of regulation (e.g., epigenetic, metabolic, and post-translational) within individual cells or tightly regulated populations with functional outcomes—either the normal behavior of neural stem cells across organisms or any subpopulation-specific effects of perturbation of this niche in disease or injury.

**Definitions** Heterogeneity: The quality or state of being diverse in character or content.

Thymidine analog: Common analogs include bromodeoxyuridine (BrdU) and 5-ethynyl-2′-deoxyuridine (EdU). These compounds are synthetic nucleoside analogs of thymidine that are commonly used to detect cell proliferation. They are incorporated into newly synthesized DNA during the S phase of the cell cycle, replacing thymidine during DNA replication.

[$^3$H]-Thymidine: Tritiated thymidine (thymidine labeled with the radioisotope tritium). It is incorporated into dividing cells, and the level of incorporation is considered proportional to the amount of cell proliferation. The levels of [$^3$H]-Thymidine are measured by autoradiography.

Transcription factor: Transcription factors are a range of proteins that are involved in the process of transcribing (converting) DNA to RNA. Transcription factors have DNA-binding domains, and their actions allow for variant gene expression across cell types and throughout developmental time.

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# Chapter 2
# Heterogeneity of Stem Cells in the Hippocampus


**Mehmet Tosun, Fatih Semerci, and Mirjana Maletic-Savatic**

**Abstract** The discovery of neural stem cells in the adult mammalian hippocampus has attracted attention and controversy, which both continue to this day. Hippocampal neural stem cells and their immediate progeny, amplifying neuroprogenitor cells, give rise to neurons and astrocytes in the region. Envisioned as possible key for tissue regeneration, whether mobilized endogenously or transplanted exogenously, neural stem cells have been in the eye of both public and science over the course of the past 20 years. These cells are a heterogeneous population, and here, we review different aspects of their heterogeneity from morphology to metabolism and response to different stimuli.

**Keywords** Adult hippocampal neurogenesis · Neural stem cells · Neuroprogenitors · Metabolism · Aging · Cell cycle · Lunatic fringe · Notch pathway · Wnt pathway · Sonic hedgehog pathway

The unique capacity of the adult hippocampus to produce new neurons throughout life is of great scientific and medical interest [1–3], as adult hippocampal neurogenesis has been associated with a number of processes including learning and memory [4] and mood control [5–9]. Hence, there is considerable interest in modulating

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hippocampal neurogenesis to treat depression, anxiety, addiction, schizophrenia [10–16], and perhaps even age-related disorders [17–22]. However, we have barely begun to untangle the molecular and cellular mechanisms that govern this phenomenon [23].

Adult hippocampal neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus, an intrinsic part of the hippocampal formation and circuitry [24]. A community of various cell types resides there in a spatially restricted environment called the neurogenic niche. This is a very dynamic environment, where turnover of new and old cells continues throughout life. Radial neural stem cells (rNSCs), the primary stem cells of this niche, are the *sine-qua-non* of this space; without them, the niche ceases to exist. Thus, understanding their properties is essential if modulation of adult neurogenesis is to advance into therapies [25–29].

rNSCs, as well as the other parts of the niche, are very sensitive to their environment and changes in both its molecular composition and the electrophysiological activity [30–32]. They react to various stimuli, but, unlike paradigmatic stem cell populations such as hematopoetic stem cells, they behave idiosyncratically. For example, rNSCs are dispensable and not limitlessly renewable: once they enter the cell cycle, they divide several times and then appear to irreversibly transform into astrocytes [33]. They may self-replicate, but these instances are rare [34]. The dramatic decrease in the rNSC population throughout life is considered the most likely reason that neurogenesis diminishes with age [35–38]. Several questions come up: Why do most of rNSCs behave differently than other stem cells, which can enter into a quiescent mode and re-enter the cell cycle when needed? Why do they divide consecutively in a very limited time? Why some of them self-renew and others do not? What makes them irreversibly exit cell cycle—an intrinsic pre-programmed mechanism or signaling that comes from the niche? Why do they seem to have different properties depending on their location—ventral versus dorsal hippocampus? Can we prevent their loss over time or even reverse their terminal astrocytic transformation? The answers to these questions are important not only for elucidation of the mechanisms that exert molecular and cellular control over rNSCs but also for the potential preservation and expansion of this unique source of new neurons in the adult hippocampus, which may have considerable implications for our capacity to treat a variety of disorders.

## Adult Hippocampal Neural Stem and Progenitor Cells and Their Origin

Neurogenesis has traditionally been thought to occur only during embryogenesis and the perinatal stages of the mammalian nervous system development, creating a century-old dogma that “In the adult centers the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated” [39]. However, over the past two decades, research has firmly established that postnatal neurogenesis occurs in rodents as well as primates, including humans, especially, in

the SGZ of the dentate gyrus of the hippocampus [38, 40–43] and the subventricular zone of the lateral ventricles [44–48]. In the SGZ, approximately 9000 newborn neurons are generated daily in the adult rat, replacing about 40% of the dentate gyrus granule cell layer over the life span [49]. In humans, carbon-dating estimates that about 700 newborn neurons are integrated into the adult hippocampal circuitry daily, replacing about 30% of the granule cell layer over the life span [50]. However, recent data on human hippocampal neurogenesis indicate existing controversy about the rate of neurogenesis at different ages [51–53]. Clearly, this phenomenon that attracted so much attention over the past 20 years still needs more work to establish its significance, particularly in humans.

Importantly, based on animal studies, the adult-born neurons integrate into the local hippocampal circuitry and influence both regional physiology and the functional connectivity of the hippocampus with more distant brain regions, such as the prefrontal cortex, amygdala, and other structures within the limbic system [54–59]. Specifically, newborn neurons in the murine dentate gyrus contribute to the encoding of new memories and flexibility [4, 60], memory consolidation [61], pattern separation [60, 62], spatial learning [63–65], and mood regulation [17, 66, 67].

The origin of rNSCs in the SGZ is under debate [68]. rNSCs resemble in morphology the radial glial cells of the embryonic brain. Furthermore, they share common markers, such as nestin, BLBP, GFAP, and Sox2, suggesting that these cells in the adult SGZ might be the remnants of the embryonic brain [69, 70]. However, different reports state different places of origin for the rNSCs. One model proposes that these cells originate from the whole length of the dentate neuroepithelium, which, during the embryonic development, produces granular neurons and postnatally, adult newborn neurons. The other model proposes that adult rNSCs originate from sonic hedgehog (Shh)-responsive cells, which emerge in the ventral hippocampus during late gestation [71, 72]. Shh is critical for tissue patterning and specification during embryonic development and continues to regulate adult rNSCs [73]. According to the second model, the descendants of Shh-responsive cells relocate into the dorsal hippocampus and become the rNSCs postnatally in the adult SGZ [71]. Regardless of origin, adult neurogenesis is considered to be a continuation of the embryonic neurogenesis, rather than an emerging property of the SGZ at some point postnatally.

## SGZ Neurogenic Niche

To grasp the vast complexity of adult neural stem and progenitor cells as well as hippocampal neurogenesis, one needs to first learn about the neurogenic niche and the cascade of events that need to occur in order for a newborn neuron to be generated and eventually incorporated into the local circuitry. The cascade begins with the activation of quiescent rNSCs (type I cells) that reside in a narrow band of tissue between the granule cell layer and the hilus, the so-called “SGZ”. The SGZ represents the microenvironment, or the neurogenic niche, that allows neurogenesis to occur.

The niche comprises a wide variety of cells, including rNSC, neuroprogenitors, neuroblasts, astrocytes [55, 74, 75], and endothelial cells [76], as well as microglia [77]. Thus, the niche provides a unique milieu consisting of cells, extracellular matrix, excreted molecules, and cell-to-cell contacts, which all support and regulate newborn neuron development.

In this niche, upon stimulation, rNSCs divide asymmetrically and produce an amplifying neuroprogenitor (ANP, type II cell), while preserving themselves. ANPs are rapidly dividing progenitors that expand their own population before undergoing either apoptotic cell death or differentiation into neuroblasts [77]. Neuroblasts continue to differentiate into immature neurons and, eventually, mature granule cells that send their axons toward the CA3 pyramidal neurons and incorporate into the local circuitry [78]. Importantly, newborn cells undergo two critical periods of survival: a major early period that occurs within 1–4 days of cell life, when more than 50% of newborn cells die, and a minor late period, which occurs 1–3 weeks after cell birth, when up to 25% of the cells die [77]. These apoptotic periods have great importance in regulation of the output of the neurogenic cascade [79, 80]. Interestingly, the rNSCs in the SGZ produce only neurons and astrocytes and not the oligodendrocytes, at least under physiological conditions [68, 81, 82]. However, they can redirect their fate to oligodendrocytic lineage either through ectopic expression of Ascl1 [82] or following elimination of Drosha-mediated NFIB inhibition [83] in rNSCs. This indicates their pluripotency and hints on possible heterogeneity among rNSCs in pathological conditions that may require production of oligodendrocytes [26, 84].

## Neural Stem and Progenitor Cell Heterogeneity

Stem cells are defined by their capacity to self-renew and their ability to generate specialized progeny. However, stem and progenitor cells are very heterogeneous populations, and distinct stem cells may produce the same lineage depending on a given stimulus [34, 68, 85–88]. Given that these cells are rare, heterogeneous, and exhibit dynamic states, it is very difficult to study their unique properties [23]. Until recently, no single neural stem and progenitor cell could be identified with a single marker (Table 2.1), which highly complicated their isolation for targeted studies [89]. Importantly, a recent discovery of Lunatic Fringe (Lfng), a modifier of the Notch receptor, as a selective marker of rNSC [90] gives hope that more specific studies could be designed to elucidate the properties of these unique cells. In addition, there is a need for new technologies to better probe neural stem and progenitor cell heterogeneity. Recently, single-cell RNA sequencing technology has emerged as a powerful method to describe heterogeneous cell populations and examine cell-to-cell expression variability of thousands of genes [91, 92]. First single-cell RNA sequencing studies have shed light on neural and glial cell heterogeneity [93–97] as well as the cellular and molecular dynamics during development [98–100].

**Table 2.1** Lunatic fringe (Lfng) is the *only* single marker that selectively labels neural stem cells in the SGZ neurogenic niche

| Marker | NSC | ANP | NB | IN | GC | Astrocyte | OPC | Pericyte | Marker | NSC | ANP | NB | IN | GC | Astrocyte | OPC | Pericyte |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nestin | + | + | | | | | + | + | PSA-NCAM | | | + | + | | | | |
| GFAP | + | | | | | + | | | Tuc4 | | | + | + | + | | | |
| Sox2 | + | + | | | | + | | | Neuro-D | | | + | + | + | | | |
| BLBP | + | + | | | | | | | Prox1 | | | + | + | + | | | |
| Lfng | + | | | | | | | | Tuji1 | | | + | + | + | | | |
| Thrsp | (+) | (+) | | | | | | | NeuN | | | | + | + | | | |
| Tbr2 | | (+) | + | | | | | | Calbindin | | | | | + | | | |
| Dcx | | (+) | + | + | | | | | S100β | | | | | | + | | |

Individual cell types are mostly identified by a combination of markers. Most importantly, commonly employed stem cell-related markers such as Nestin or Sox2, label not only radial NSCs, but also their immediate progeny, ANPs, and other cells present in the neurogenic niche. *NSC* radial neural stem cell, *ANP* amplifying neuroprogenitor, *NB* neuroblast, *IN* immature neuron, *GC* granule cell, *OPC* oligoprogenitor cell

Finally, first clonal genetic lineage-tracing studies of individual NSCs have been reported, revealing their heterogeneity [34, 101]. Here, we outline the major differences between subpopulations of rNSCs and their immediate progeny.

## *Morphological Heterogeneity*

Adult hippocampal neurogenic niche is highly complex, not only because of the number of different types of cells it harbors but also because of the heterogeneity of each cell type. Most of the cell types found in the niche form a continuum of heterogeneous lineage with different proliferation potential, lineage plasticity, and lifespans. So, in this context, a combinatorial approach using both cell morphology and immunohistological markers helps us better understand the complexity of different cell types found in the niche.

rNSCs have distinct morphological features that enable us to distinguish them from surrounding cells. Their cell body in the SGZ has a unique triangular shape, which gives rise to a radial process that spans the granule cell layer and ends with fine arborizations in the granule cell layer–molecular layer boundary [102] and endfeet on the vasculature [103]. A recent study has argued that rNSCs can be classified into two different groups: (1) Type α cells, representing three quarters of the rNSC population, which have longer radial processes that modestly branch, and (2) Type β cells, representing a quarter of the rNSC population, which have shorter processes with more elaborate branching pattern (Fig. 2.1) [88]. In addition to these unique morphological features, rNSCs express GFAP, Nestin, BLBP, Vimentin, GLAST, Sox2, Lfng, Hes5, Ascl1, Spot14, and Hopx [89]. However, not all of these markers

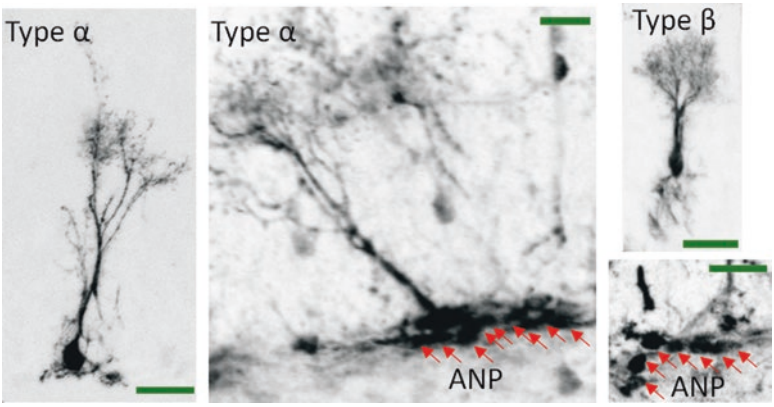


**Fig. 2.1** Radial neural stem cells (rNSCs) may exist as two types by their morphology, Type α and Type β. Both types have radial processes, but their branching differs. In contrast, their progeny, amplifying neuroprogenitors (ANPs), do not have any processes. Scale bar = 20 μm

are expressed in every single rNSC at every single time, raising the possibility of their functional heterogeneity based on the expression of these markers [104]. These observations further support the notion that, although rNSCs mostly share the same morphology, their position in the quiescence/activation/differentiation continuum throughout the mouse life span is heterogenic and that this heterogeneity represents the source of plasticity of the neurogenic niche.

Although morphologically different, as direct descendants of rNSCs, Amplifying NeuroProgenitors (ANPs or Type 2 cells) still share a subset of rNSC markers in early stages, such as Nestin, Sox2, and BLBP. However, further down the proliferation/differentiation route to a neuroblast, they start to express transcription factors and cytoskeletal proteins that restrict their proliferative potential and solidify their fate as neurons. Gradual damping of the Notch signaling pathway results in loss of Sox2 and upregulation Ngn2, NeuroD1, and Tbr2, further strengthening the differentiation [105, 106]. At later stages of ANPs, NeuroD1 expression is upregulated and Prox1, Dcx, Sox4/Sox11 start to be expressed [107–110], resulting in changes not only in the transcriptional landscape of the late progenitors but also in the ANP morphologies, as first neuroblasts start to emerge with extending processes that will become future dendrites.

## *Cell Cycle Heterogeneity*

rNSCs can exist in three functional states with respect to their cell cycling: (1) Quiescence, (2) Self-renewal, and (3) Exhaustion, when they permanently exit the cell cycle.

*Quiescence* is defined as a reversible resting state in which a cell can re-enter the cell cycle when needed and divide to generate progeny [111, 112]. In essence, the cell enters the G0 state and cannot be labeled with any of the commonly used markers of cell cycle, such as Ki67, PH3, BrdU, and its analogs, and others. In fact, to detect quiescent stem cells, one needs to eliminate all proliferating ones; this is mostly achieved by administration of antimitotic agents such as temozolomide. Most rNSCs are relatively quiescent by nature, which makes them different from nonradial and intermediate progenitors, ANPs [44, 113]. To achieve the state of quiescence, the cell needs to express cell cycle inhibitors and to downregulate the cell cycle activators. Quiescent rNSC express different inhibitors of cyclin-dependent kinases such as p57, p27, p16, and p21 [114–118], which were all thought to prevent early exhaustion of these cells. In addition, the chromatin remodeling factor chromodomain-helicase-DNA-binding protein-7 (CHD7), which represses upregulators of cell cycle, as well as Hes-5, is needed for cycling rNSCs to return to quiescence [119]. If these genes are inactivated, rNSCs initially proliferate more but then get depleted, leading to depletion of neurogenesis [114, 119, 120]. Finally, the repressor element 1-silencing transcription (REST) is required to maintain rNSCs in a quiescent and undifferentiated state, at least in part by preventing premature expression of the neuronal differentiation program [121, 122].

Recently, the Notch pathway has also emerged as one of the main regulators of rNSC cell cycle [90, 123–127]. Notch1 is required for self-renewal, and inactivation of the Notch pathway component RBPj results in an initial increase in neurogenesis but eventual depletion of the progenitor cell pool and generation of newborn neurons [126]. Interestingly, Lunatic fringe, a key modifier of Notch receptor, is selectively expressed in rNSCs where it participates in the control of their quiescence [90]. There it helps rNSCs to distinguish between Delta-expressing surrounding neurons and Jagged-expressing progeny, ANPs. Lunatic fringe modifies the Notch receptor by glycosylation so that the modified receptor responds to ligands (Delta and Jagged) differently from the native Notch receptor. Lunatic fringe-modified Notch amplifies Delta-Notch signaling but dampens Jagged-Notch signaling. Therefore, when rNSCs are surrounded by Delta-positive neurons, they are mostly quiescent, in a standby mode, protected from random activation and unnecessary division. On the other hand, when rNSCs are undergoing asymmetric division and are generating ANPs, interaction of the Lunatic fringe-modified Notch receptor with Jagged1 expressed on the daughter ANPs surrounding their mother rNSC eventually slows down Notch signaling in the rNSC, leading to its exit from the cell cycle [90]. The Notch pathway thus finely regulates rNSC cycling to prevent their excessive division and premature exhaustion.

*Self-renewal* refers to the ability of a stem cell to divide symmetrically, generating two stem or progenitor cells, or asymmetrically, generating one stem and one progenitor cell. Self-renewal has been mostly reported for ANPs, and it happens rarely in case of rNSCs. Both Wnt and Notch signaling have been implicated in self-renewal. The canonical Wnt pathway stimulates self-renewal of NSCs [128], while Wnt inhibitors Dickkopf-1 (Dkk1) and secreted frizzled-related protein 3 (sFRP3) promote rNSC quiescence [129, 130]. Long-term rNSC self-renewal is under debate. One model argues that rNSC activation leads to multiple consecutive rounds of proliferation that terminates by transformation of these cells into astrocytes without ever returning to quiescence [33]. On the other hand, clonal analysis showed cycles of rNSC activation, including return to quiescence and re-activation of individual NSCs, with moderate depletion through astrocytic transformation [34]. Further, it appears that there are several different rNSC subpopulations that exhibit varying levels of self-renewal and differentiation capacity [131]. This has particularly become evident when stem cells were labeled using different Cre drivers—different rNSCs in the niche showed discrete proliferation responses to stimulators or inhibitors [104]. For example, social isolation has been shown to favor symmetric division, which leads to self-renewal of rNSCs [30]. However, evidence for molecular substrates of this functional self-renewal heterogeneity is rare; whether it contributes to differences in neurogenesis at the population level remains to be seen. A recent study has shown that depletion of Jag1 from NSC lineage results in an increased number of NSCs [90]. Further, recent single-cell gene expression analysis found only a few genes that were specific to quiescent rNSCs [132]. It is thought that Ascl1 protein half-life is controlled by E3-ubiquitin ligase Huwe1 (HECT, UBA, and WWE domain containing 1) to allow proliferating rNSCs to return back to quiescence [133]. Finally, single rNSCs appear not to be able to self-renew long

term [101, 134], supporting the emerging concept that rNSCs may only persist at a population level.

Unlike rNSCs, ANPs readily self-renew, even though it appears that their capacity to self-renew is also definite [33]. The ANPs may also exhibit cell cycle heterogeneity. One of the possible mediators is the Ascl1/Mash1, mostly expressed in activated ANPs [135] but can also be critically important for activation of the rNSCs [133, 136, 137]. Ascl/Mash1 is under the control of Hes proteins, which oscillate and therefore lead to oscillation in the expression of their targets, including Neurogenin 2 (Neurog2) and Ascl1/Mash1. Ascl1/Mash1 is detected in about one-third of the activated ANPs [136], where its oscillating level promotes proliferation, while its stable levels indicate that the cell is on its differentiation route [138]. In addition, the T-box transcription factor Tbr2 may also be critical for ANP heterogeneity. Namely, ANPs that express Tbr2 mostly exit cell cycle and undergo neuronal differentiation [139], while lack of Tbr2 increased ANP proliferation and prevented the generation of late ANPs and newborn neurons [106, 140]. Some reports indicate the Tbr2-positive ANPs can self-renew, but this finding is still controversial [141, 142].

Finally, *exhaustion* implies a permanent exit of a stem cell from cell cycle and termination of its stemness. Indeed, rNSCs are depleted in different disease models and conditions, and this is particularly striking during aging [35, 143] and in models of epilepsy [32]. Interestingly, the rNSCs do not appear to die by apoptosis or necrotic cell death but get exhausted by transformation into astrocytes [33], while ANPs die by apoptosis in large quantity during the first critical period of the newborn cell survival [77].

## *Epigenetic Heterogeneity*

Epigenetic modifications play an important role in the regulation of rNSC and ANP quiescence and proliferation [144–146]. Active DNA demethylation, mediated by the TET-GADD45 pathway, regulates SGZ rNSC proliferation both cell-autonomously [147] and non-cell-autonomously through modulation of growth factors in mature granule neurons [86]. Loss of Methyl-CpG-binding protein 1 increases NSC proliferation [148], while Methyl-CpG binding protein 2 balances NSC proliferation and differentiation by regulating specific miRNAs, such as miR-137 [149]. Histone modifications also contribute to the epigenetic regulation of SGZ stem and progenitor cells. Enhancer of zeste homolog2 (Ezh2) is expressed in actively dividing rNSCs and is thought to promote rNSC proliferation by suppressing Pten expression and activating the Akt-mTOR pathway [149]. Chromatin proteins such as HMGB2 also play a role in NSC proliferation, during both embryonic and adult neurogenesis [150, 151]. However, it is not known whether different rNSC and ANP subtypes are sensitive to different epigenetic modifications, which would influence their response to diverse stimuli as well as the capacity to produce newborn neurons and/or astrocytes. Given that epigenetic modifications particularly accumulate during aging, it will be interesting to study their effects in the aging populations of rNSCs.

### *Nonautonomous Heterogeneity*

In the SGZ, radial processes extend through the granule cell layer up into the molecular layer of the dentate gyrus where they branch out. The fine end-points of rNSC branches may allow rNSCs to sample and integrate neuronal circuitry activity that occurs there, as impulses arrive to the molecular layer from the entorhinal cortex. Indeed, adult rNSCs are regulated by neurotransmitters released by mature neurons [152]. GABA, the major inhibitory neurotransmitter of the brain, promotes rNSC quiescence by blocking cell cycle progression [153]. SGZ rNSCs can also sense parvalbumin interneuron-released GABA; reducing GABA signaling results in rNSC activation and symmetrical division [154]. Adult rNSCs also communicate with other cells through gap junctions (connexin 43 (Cx43) [155] and direct cell–cell interactions. Loss of Cx30 and Cx43 in rNSCs diminishes rNSC numbers.

### *Metabolic Heterogeneity*

The metabolic state of stem cells differs from the metabolic state of their progeny, as stem cells primarily use anaerobic glycolysis and fatty acid oxidation to derive energy, while postmitotic cells rely on mitochondrial oxidative phosphorylation [156–159]. When a stem or progenitor cell starts to produce a daughter cell, some of its mitochondria become part of the progeny [160]. As there is a demand for increased energy, mitochondrial DNA gradually increases in support of mitochondrial biogenesis: the spherical and cristae-poor mitochondria of primary stem cells are transformed into tubular and cristae-rich structures to guarantee sufficient ATP for the metabolism [161]. Concomitantly, the production of mitochondria-related key enzymes and mitochondrial reactive oxygen species is increased, leading to suppression of glycolytic genes and the production of antioxidants [161–165]. Thus, regulation of energy metabolism is one of the critical components that determines whether a stem cell is in quiescent or proliferative state. Interestingly, neurogenesis is elevated in mice exposed to caloric restriction, while it is diminished in those exposed to diet-induced obesity and diabetes [166, 167].

In the SGZ, rNSCs and ANPs are exposed to low oxygen availability [168], which activates hypoxia-inducible factors (HIFs) and favors anaerobic glycolysis over oxidative phosphorylation [169]. Indeed, lack of HIF1 leads to activation of rNSCs [170], which, by coupling to angiogenesis, leads to increased availability of oxygen for progeny that then switches to oxidative phosphorylation as a source of ATP and starts to differentiate into neuronal lineage. Thus, oxygen participates in regulation of the rNSC maintenance by balancing quiescence and self-renewal of these cells.

In addition, de novo lipogenesis is critical for rNSC cell cycle determination [171, 172]. This is of no surprise, as stem cell needs to double its membrane content

before undergoing mitosis to ensure the structural integrity of the daughter cell. Fatty acid synthase (Fasn) is a key regulator of rNSC and ANP proliferation and is elevated in dividing cells. Indeed, in quiescent rNSC, the thyroid hormone-inducible hepatic protein (THRSP or SPOT14) downregulates Fasn and lipogenesis, preventing active cycling [171]. The accumulation of fatty acids in both rNSCs and ANPs during proliferation [173–175] may allow for their detection in the live brain, through magnetic resonance spectroscopy [3, 176–178].

Finally, recent single-cell transcriptomic data support the switch from glycolytic metabolism to a largely mitochondrial-driven metabolism as quiescent rNSC undergo activation, division, and differentiation of progeny [132, 179]. Altogether, these data indicate that quiescence is associated with a hypometabolic state while proliferation is associated with lipogenesis and hyperactive metabolism necessary to produce energy for the dividing and differentiating cells. Indeed, the hypometabolic state of quiescent rNSC has been proposed to preserve them, by allowing them to withstand metabolic stress, prevent reactive oxygen species from causing cellular damage, and preserve genome integrity throughout the lifespan [24, 170].

## *Heterogenic Response of Neural Stem and Progenitor Cells to Stimuli*

Neural stem and progenitor cells as well as the net outcome of adult neurogenesis are under the influence of various physiological, pathological, and pharmacological stimuli such as exercise, enriched environment, antidepressants, aging, epilepsy, and others [180]. In essence, newborn neurons are generated on demand and not randomly. Most stimuli such as running [31], enriched environment [181], and antidepressants [182] act on the ANPs to increase the number of divisions and production of neuroblasts. Some stimuli such as electroconvulsive shock as well as neuronal hyperactivity as seen in epilepsy, target rNSCs, leading to increased production of ANPs or self-renewal with concomitant transformation into reactive astrocytes, respectively [32].

However, we still do not know the specific target(s) and underlying signaling mechanisms of these physiological and pathological stimuli in rNSCs and ANPs. For example, following the discovery of stimulatory effect of physical exercise on neurogenesis [31], different mechanisms have been proposed to participate: increased proliferation of either rNSCs [113] or only ANPs [183] or both rNSCs and ANPs [81]; increased Notch activity and cell cycle exit of neuroblasts [184]; shortening of the cell cycle length [185]; increased neurite growth and survival [186]; increased blood vessel perimeter in young but not old mice [187]. However, none of these mechanisms are able to explain the observed phenomena by itself [188] but rather point out to the heterogeneity of rNSC population that responds differently to physical exercise. In addition, because of the use of different animal models that

drive expression of fluorescent proteins in diverse cells, it is not always clear whether a particular stimulus affects directly a quiescent rNSC or their immediate progeny. Regardless, the heterogeneity of the responses has been recognized and new tools that precisely target specific neural stem and progenitor sub-populations or different states of the same cell are needed to examine this diversity.

Most controversy exists with respect to biology of rNSCs during aging when a dramatic reduction in the number of proliferating cells and decreased neurogenesis are observed in the dentate gyrus (~90% reduction) [189–191]. Different studies point to different scenarios. Some studies indicate that the rNSC pool is finite, lacks self-renewal capacity, and is depleted during aging [33, 192] either through elimination or division-coupled astrocytosis. Others, however, suggest that rNSCs are maintained, and that they may even increase in number [30, 34, 193–195]. Namely, they can undergo symmetric division, and while their pool declines, it does not decline significantly; however, their capacity to produce newborn neurons diminishes with age, leading to decline in neurogenesis. Most recent studies also indicate that the progeny may revert to rNSC or engage in long-term self-renewal [81, 196], while others indicate that only a subpopulation of rNSC engages in activation and self-renewal at a given time and then disappears through death or differentiation [133]. All these different reports and models ultimately indicate that more precise studies of this unique population of cells are indicated, given their importance for potential improvement and rejuvenation of the hippocampal tissue and cognition.

rNSCs and ANPs are also differentially regulated with age [35, 189]. Different hypothesis have been proposed, from decreased pool of rNSCs, increased quiescence of rNSC population, decline of a quiescent rNSC population that is able to symmetrically divide and replenish the exhausted pool of rNSCs, and others [33, 193, 197]. In addition, reduction in the number of rNSCs in the ventral dentate gyrus is significantly higher than that in the dorsal DG, indicating that the anatomical location also contributes to the heterogeneity of rNSCs decline during aging [198]. It has also been suggested that the aging niche is inhibitory, while the young niche is permissive to neurogenesis [199]. Finally, it has been argued that the decline in rNSC population during aging is cell-intrinsic and may result from the accumulation of DNA damage during aging [200–202]. On the other hand, they may be extrinsic as many inhibitory factors from circulation may affect rNSC and their progeny. For example, during development, the endothelial-derived factor vascular endothelial growth factor (VEGF) promotes stem cell self-renewal, while during adulthood and aging, it promotes quiescence and maintenance of the rNSC pool [203–205]. Blood-borne factors such as glucocorticoids released from the adrenal gland also increase during aging and their increase may affect rNSCs and neurogenesis adversely [206–209]. Ultimately, deep sequencing on a single cell level at different ages as well as environmental and pathological exposures is needed to produce biologically meaningful data applicable to both prevention of neurogenic decline during aging and regeneration of the tissue in case of pathology.

## Conclusion

Overall, hippocampal neural stem and progenitor cells exhibit heterogeneity in many aspects, from their metabolic preference to gene and epigenome differences, which ultimately affect their morphology, cell cycle properties, and response to different stimuli (Fig. 2.2). This heterogeneity is also affected by age and most likely by the physical location of a given cell. It is imperative that we understand the molecular underpinnings of such diversity, as they will give us clues important for both biology of these unique population of cells in the mammalian brain and the targets that might be modified to prevent their depletion in disease and physiological aging.

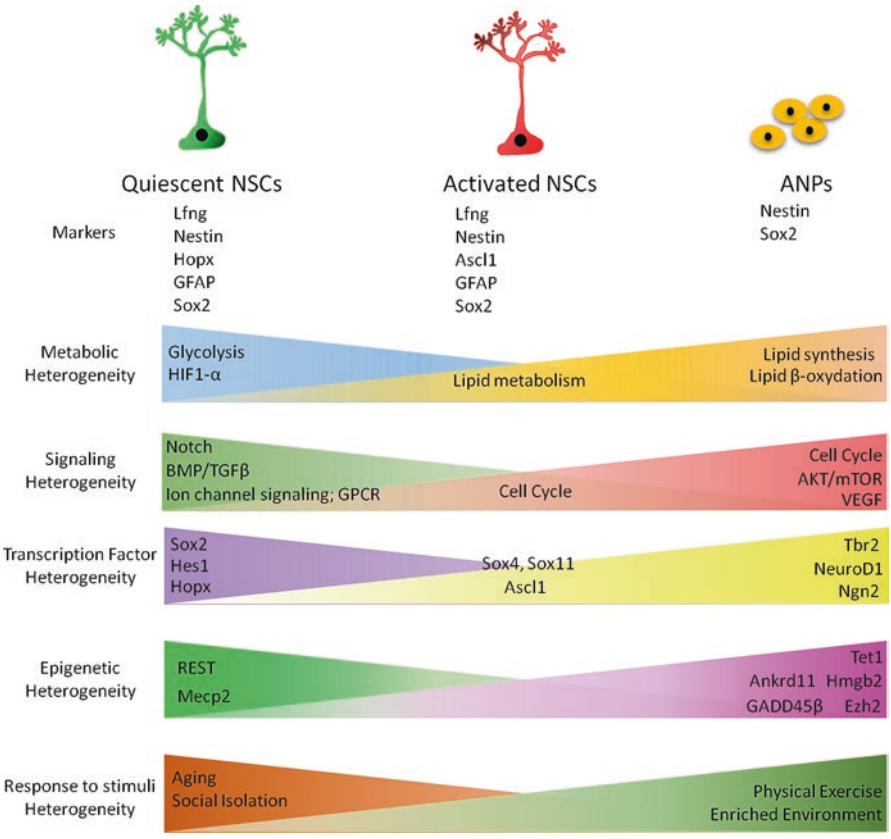


**Fig. 2.2** A schematic outline of the heterogeneity of rNSCs and their immediate progeny, ANPs

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# Chapter 3
# Heterogeneity of Sweat Gland Stem Cells


**Matthias Brandenburger and Charli Kruse**

**Abstract** Sweat glands play an important role in skin physiology and are an integral part of the natural skin barrier. In order to maintain functionality throughout life, sweat glands make use of several types of stem cells. This chapter focuses on the classification of different types of stem cells found in the sweat gland and their physiological roles. First, sweat gland formation during skin maturation is addressed in order to give an overview of sweat gland origin and formation in vivo. Then, different kinds of adult sweat gland stem cells are introduced and classified between different potency levels and corresponding physiological roles. Finally, the importance of these cell sources for future developments, including applications in wound healing and cosmetics research, is discussed.

**Keywords** Adult stem cells · Sweat glands · Wound regeneration · Skin appendages · Nestin · Skin barrier · Glands · Multipotency · Wound healing · Cell differentiation

## Introduction

With densities between 64 and 700 sweat glands per square centimeter [1], sweat glands are an abundant part of the natural skin barrier. Besides the regulation of the body temperature by the secretion of sweat, they were also found to participate in further physiological processes like skin wound healing [2]. Sweat glands can be classified into eccrine, apocrine, and apoeccrine subtypes. Eccrine glands regulate

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body temperature by secretion of sweat onto the skin. They consist of a coiled secretory part located in the dermis and a straight ductal part that transports the sweat to the epithelial surface. In contrast, apocrine sweat glands secrete sweat into the pilary canal of the hair follicle. Apocrine sweat glands secrete pheromones and consist of a larger secretory portion; they are overall larger than eccrine sweat glands. However, a mutation in the ABCC11 gene leads to smaller apocrine glands and lack of body odor in certain Asian subpopulations [3]. Sweat glands, which are related to neither eccrine nor apocrine sweat glands, are described as apoeccrine sweat glands, since they share histological features of both major sweat gland types [4]. In addition, several specialized types of apocrine sweat glands (e.g., ceruminous glands, mammary glands, ciliary glands, etc.) are known. In order to give a structured overview with clear differentiation on other stem cell sources (e.g., the hair follicle), this chapter focuses only on eccrine sweat glands of the skin.

The most important roles of eccrine sweat glands of the skin (abbreviated as sweat glands hereafter) include the support of skin wound healing, regulation of the body temperature by transpiration, and participation in the innate host defense system of the immune system. In order to fulfill these important and complex processes throughout life, sweat glands make use of several types of adult stem cells.

This chapter focuses on the classification of different types of stem cells found in the sweat gland and their physiological roles. First, sweat gland formation during skin maturation is addressed in order to give an overview of sweat gland origin and formation in vivo. Then, different kinds of adult sweat gland stem cells are introduced and classified between different potency levels and corresponding physiological roles. In the final part, the importance of these cell sources for future developments, including applications in wound healing and cosmetics research, is discussed.

## Sweat Gland Formation

Human sweat gland formation begins during weeks 12–20 of embryonic development. At this stage, the placode is formed as an epidermal invagination into the dermis. Lineage tracing experiments have shown that by multipotent epidermal basal cells, which express cytokeratins 5 and 14, predominantly take part in this process [5]. However, multipotency is conserved in basal cells only, whereas luminal and myoepithelial cells exhibit unipotent differentiation potential [5]. During further development, the duct propagates into deeper parts of the dermis. The nascent gland consists of a fully developed ductal part and adjacent myoepithelial and luminal cells, which will form the secretory coil during postnatal development. Various studies have analyzed the activation of different pathways during sweat gland formation. Most importantly, the EDA/EDAR/NF-kB [6, 7], wnt [6, 7], Sonic hedgehog (Shh) [6, 7], and BMP [7] signaling pathways are associated with sweat gland formation.

The process and mechanism of gland formation differ between different types of glands. In contrast to sweat glands, mammary glands undergo major remodeling

processes in adolescence before they reach full functionality. Thus, mammary glands accommodate potent stem cells, which will be activated during pregnancy and take part in further branching and leafing until development of mature mammary glands is completed. In contrast, multipotent stem cells of the adult sweat glands remain in a quiescent state during adulthood and are activated only during regeneration (e.g., regeneration of skin wounds). These different mechanisms of stem cell quiescence and activation might also be reflected by the incidence of different tissue-associated tumors. It is known that adult stem cells can mutate towards cancer cells, thereby initiating cancer [8]. In comparison to sweat gland-associated tumors, mammary cancer shows a high prevalence [9–11]. Like sweat glands, salivary glands or pancreas do not undergo major remodeling after birth and also exhibit stem and progenitor cells, which enable tissue functionality in the adult. However, adult stem cells of the salivary gland and pancreas are supposed to be directly involved in tumor initiation [12, 13].

## Sweat Gland Stem Cells

The skin is one of the largest and most versatile organs of the human body, which is involved in many important physiological processes. The integrity and functionality of the skin are ensured by a number of stem cells located and associated with different skin compartments including skin appendages. In the past, various approaches have demonstrated the existence of sweat gland stem cells. The analysis often included skin areas rich in eccrine sweat glands and lacked other skin appendages (e.g., the hair follicle), enabling clear identification and classification of sweat gland stem cells. Overall, sweat gland progenitors and stem cells can be classified as unipotent and multipotent adult stem cells, which correlate with the physiological function. Unipotent sweat gland stem cells mainly aim for skin homeostasis and closure of minor wounds. In contrast, multipotent stem cells are activated in more complex scenarios, such as regeneration of deeper wounds.

Unipotent stem cells of the sweat gland can be classified into four types, namely epidermal stem cells, basal stem cells of the sweat gland duct, luminal stem cells, and myoepithelial stem cells. These stem cells derive from multipotent stem cells during development and can be further discriminated by their marker expression profile and physiological function. These stem cells are of great importance, especially for homeostasis and minor wound regeneration. Therefore, the introduction of different wounds demonstrated the activation of further tissue-specific progenitors including the epidermis, duct, lumen, or myoepithelium.

Epidermal stem cells are located near the sweat gland duct. These cells highly express cytokeratin 14 and primarily participate in the regeneration of epidermal wounds [5]. In comparison, basal stem cells of the sweat gland duct highly express integrin α6 in addition to cytokeratin 14. In contrast to epidermal stem cells, ductal stem cells mainly regenerate the sweat gland orifice. Luminal cells maintain functionality of the sweat gland by supporting homeostasis. These cells can be discriminated

by the expression of cytokeratins 19, 18, and 15 and CD29 [5]. Lineage tracing experiments have shown that luminal cells emerge from multipotent epidermal stem cells during development but lose their multipotent differentiation potential after sweat gland formation [5]. In this state, luminal progenitors remain unipotent, enabling replacement of luminal cells during homeostasis.

Myoepithelial cells usually exhibit a unipotent differentiation potential in the sweat gland. These cells are characterized by the expression of cytokeratins 14 and 5 [5] and maintain sweat gland functionality. However, when myoepithelial cells are brought into another environment, they also exhibit a multipotent differentiation potential. When transplanted into a mammary gland fat pad, myoepithelial cells were shown to regenerate a functional sweat gland, duct, and epidermis [5]. However, this multipotent differentiation potential is rather of minor importance in vivo.

Multipotent sweat gland-derived stem cells are found in deeper areas of the skin around the secretory coil of sweat glands. These stem cells remain quiescent under physiological conditions and are activated during regeneration of deeper skin wounds or by placing sweat glands in artificial environments. In a mouse model, Ohe et al. demonstrated that reserve stem cells exist around the eccrine unit of the gland. These cells express Bmi1 and usually exhibit a low cycling rate. Upon activation by injury, these cells can differentiate toward rapidly cycling Lgr6-expressing stem cells, which participate in the maintenance of the entire gland and interadnexal epidermis. Thus, these cells serve as a cell reservoir, which enables the regeneration of depleted progenitor cells of the sweat gland. In contrast, Lgr5-expressing cells were shown to participate only in the maintenance of the sweat gland [14].

Another type of multipotent sweat gland stem cell can be found in the sweat gland stroma. Sweat gland stroma-derived stem cells (SGSCs) can robustly be isolated and propagated in vitro, and they highly express nestin [15–17]. Nestin is a type VI intermediate filament and plays a role in cellular remodeling processes of proliferating cells. It is regarded as a marker for neural stem cells and other fetal organs, as well as found in adult stem cells of skin appendages (e.g., the hair follicle). Owing to the closeness to various cell niches for nestin-positive cells (e.g., hair follicle stem cells, neural crest cells, endothelial cells, etc.), the exact origin of SGSCs is still a subject of debate. However, functional characterization demonstrated that SGSCs play an important role in wound healing, including orchestration of important mechanisms, which are usually activated in acute wound healing. The existence of nestin-positive stromal stem cells seems to be a common property of various glands, underlining its physiological importance. Thus, nestin-positive multipotent gland-derived stem cells can be found in various types of glands, including sweat glands [17], salivary glands [18], mammary glands [19], and pancreas [18, 20]. In the past, robust isolation procedures have been developed, which allow for the isolation and propagation of multipotent nestin-positive sweat gland stroma-derived stem cells (SGSCs) [15, 17].

The isolation procedure of SGSCs is based on isolated sweat glands with conserved secretory coil regions, which are subsequently placed on culture dishes. This artificial in vitro wound scenario promotes outgrowth of SGSCs, which is in line with the proposed physiological role of these stem cells in wound healing (Fig. 3.1).

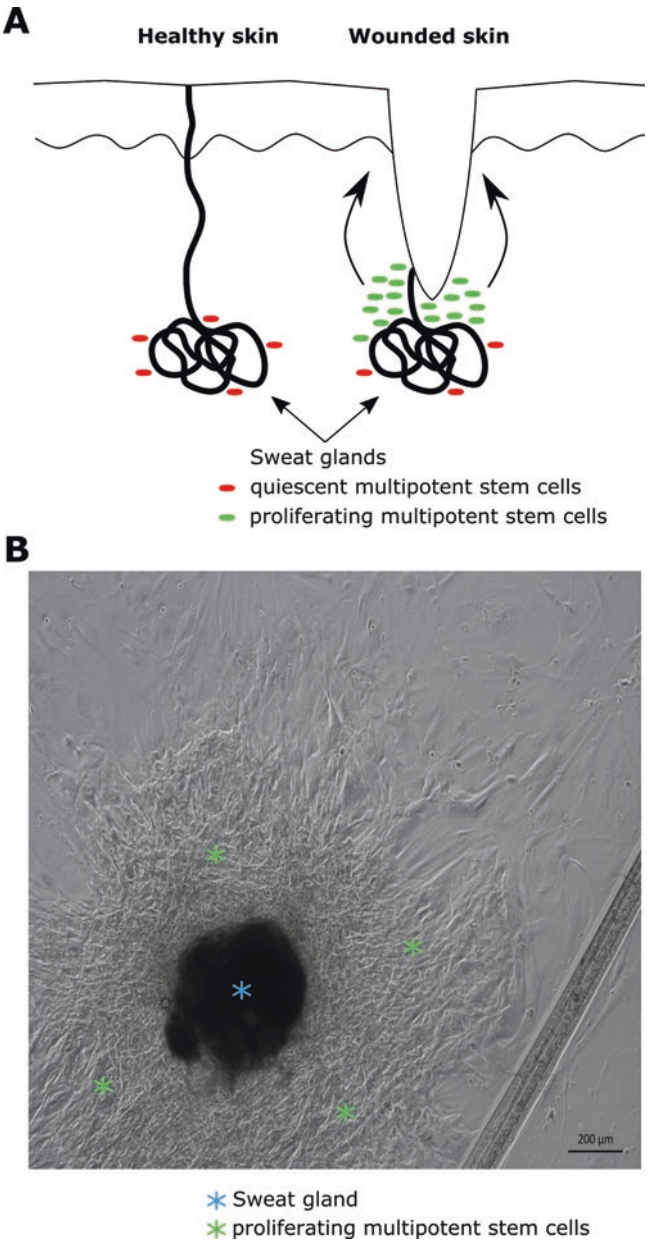


**Fig. 3.1** Activation of multipotent stem cells of the sweat gland. (**a**) Proposed role of multipotent sweat gland stem cells in vivo. Upon injury, quiescent stem cells (red) are activated toward proliferating stem cells (green), which support the regeneration of the sweat gland and skin. (**b**) Multipotent stem cells can be isolated from the sweat gland stroma by generating explant cultures of sweat glands (blue asterisk). This artificial wound scenario promotes the outgrowth of multipotent sweat gland stroma-derived stem cells (SGSCs, green asterisk) in vitro

A recent work by Ma et al. [21] confirmed this approach for the isolation of multipotent stem cells from sweat glands. In this study, sweat gland stem cells were also derived from explant cultures of the secretory portion of the sweat gland. Further characterization revealed closeness to mesenchymal stem cells [21].

The regenerative potential of SGSCs was shown in different in vitro and in vivo assays, which displayed the orchestration of important wound healing mechanisms. Thus, human SGSCs led to an increased re-epithelization of the skin [22] as well as enhanced recellularization of the wound bed with increased sprouting of blood vessels [15] and peripheral nerves [23]. Due to the expression of relevant cytokines, it was hypothesized that the underlying mechanism involves paracrine effects rather than direct differentiation [16]. However, the physiological mechanism of action is still under debate and requires further research.

Another method for the isolation of multipotent glandular stem cell was described by Diao et al. [24]. In this approach, sweat glands from mouse skin were isolated and enzymatically digested. Separated cells were cultivated to form sweat gland organoids (SGOs). The organoids exhibited markers for the sweat gland lumen epithelia (CK18 and CK19), functional markers (AQP5 and aATP), and stem cell markers (SOX9, aSMA). Introduction of SGOs into skin wounds revealed positive effects on wound healing. Furthermore, the potential of sweat gland formation was discussed [24]. These data underline the presence and potency of multipotent stem cells in the sweat gland, which are activated upon transplantation in wounds.

Overall, the exact origin and classification of different multipotent stem cells are still under debate. However, multipotent stem cells share two common properties. The first common property is the activation of these stem cells by a wound scenario. In the case of SGSCs, the introduction of such scenario is generated by explant cultures from adult human sweat glands. In contrast, the approach of Diao et al. introduces the wound scenario by enzymatical separation of sweat glands and subsequent reorganization by organoid formation. Finally, Ohe et al. demonstrated the activation of Bmi1-expressing stem cells upon injury. The second common property is the localization of multipotent sweat gland stem cells near the secretory coil in deeper areas of the skin. Perhaps, this localization was evolutionary advantageous, since it protects these stem cells from UV radiation, which might otherwise lead to degeneration of multipotent stem cells to cancer cells.

Further research is needed to answer the question whether these cells have the same origin. The closeness of the secretory part to blood vessels, peripheral nerves, and fat tissue exhibits several potential stem cell niches, which might be the source of adult multipotent stem cells.

## Future Trends and Directions

Since glandular stem cells play such an important role in homeostasis and wound repair, these cells will be an important target for future developments in various applications including wound healing, cosmetics research, and the development of more complex in vitro model systems.

The easy accessibility of this stem cell source will open opportunities for novel autologous cell therapeutic approaches. The artificial induction of pluripotency marker molecules is unnecessary in these types of cells, which is a huge advantage in terms of safety. Especially for wound healing applications, multipotent glandular stem cells offer a great potential, since they are capable to trigger crucial wound healing mechanisms, which are missing in chronic or burn wounds.

Another interesting aspect is the role of aging for the availability of functional sweat gland stem cells. Besides wound healing, this would be of great interest for cosmetics research, since protection of stem cells involved in tissue homeostasis will likely exhibit benefits in the retardation of skin aging. As a direct effect, stem cells will have increased superficial wound healing capability with reduced scar formation. In addition, sweat glands play an important role in the support of the natural barrier function of the skin. Thus, antimicrobial peptides like dermicidin are secreted by eccrine sweat glands upon activation of the innate host defense of the immune system.

The third part of future directions involves utilization of these distinct stem cells for model system development. In the past, great advances have been made in the development of in vitro model systems of the skin. This led to the availability of tissue-engineered human skin models and provided an alternative to in vivo studies, which led to a reduced demand of laboratory animals. Current reconstituted skin models, however, lack the introduction of skin appendages, which reduces the significance of in vitro models. With the increasing knowledge of different subtypes of sweat gland stem cells and their role during sweat gland development and homeostasis, the basis for more complex in vitro models will be created. With regard to the closeness of sweat glands to mammary glands, the development of model systems for applied research on the treatment on mammary cancer could be of importance. Especially, a deeper insight into the mechanism and regulation of quiescence of multipotent sweat gland stem cells might give more implications for novel treatment options. This would also pave the way to a better understanding of other gland-derived cancers (e.g., salivary glands or pancreas).

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# Chapter 4
# Heterogeneity of Stem Cells in the Human Vocal Fold Mucosa

**Kiminori Sato**

**Abstract**

1. There is growing evidence to suggest that the cells in the maculae flavae are tissue stem cells of the human vocal fold and maculae flavae are a candidate for a stem cell niche.
2. The latest research shows that the cells in the human maculae flavae are involved in the metabolism of extracellular matrices that are essential for viscoelasticity in the human vocal fold mucosa as a vibrating tissue and are considered to be important cells in the growth, development, and aging of the human vocal fold mucosa.
3. Recent evidence has indicated that the cells including vocal fold stellate cells in the maculae flavae of the human vocal fold mucosa are a functionally heterogenous population.
4. The cells in the human maculae flavae possess proteins of all three germ layers, indicating that they are undifferentiated and have the ability of multipotency.
5. The cell division in the human adult maculae flavae is reflective of asymmetric self-renewal, and cultured cells form a colony-forming unit. Therefore, the phenomenon gives rise to the strong possibility that the cells in the human maculae flavae are putative stem cells.
6. Recent research has suggested that the cells in the human maculae flavae arise from the differentiation of bone marrow cells via peripheral circulation.
7. Cultured cell populations in the human maculae flavae are roughly divided into three groups by morphological features: cobblestone-like polygonal cells, vocal fold stellate cell–like cells, and fibroblast-like spindle cells. However, at the present state of our investigation, it is difficult to clarify the stem cell system and hierarchy of stem cells in the human maculae flavae.

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8. Subpopulations of cells in the maculae flavae proliferate extremely slowly and retain stem cell properties.
9. Tension caused by phonation seems to regulate the behavior and heterogeneity of the cells (mechanical regulation) in the maculae flavae of the human vocal fold.
10. The putative stem cells in the maculae flavae appear to differentiate into other kind of cells in the surrounding tissue.

**Keywords** Tissue stem cells · Heterogeneity · Stem cell niche · Macula flava · Vocal fold stellate cells · Vocal fold

## Introduction

Among mammals, only humans can speak and sing songs throughout their lifetime. And only the human adult vocal fold has a vocal ligament, Reinke's space, and a characteristic layered structure [1–5].

The viscoelastic properties of the lamina propria of the human vocal fold mucosa determine its vibratory behavior and depend on extracellular matrices, such as collagen fibers, reticular fibers, elastic fibers, proteoglycan, glycosaminoglycan, and glycoproteins [6]. The three-dimensional structures of these extracellular matrices are indispensable to the viscoelastic properties of the human vocal fold mucosa [6]. The fine structures of the human vocal fold mucosa influence vibrating behavior and voice quality [6].

Adult tissue-specific stem cells (tissue stem cells) have the capacity to self-renew and generate functionally differentiated cells that replenish lost cells throughout an organism's lifetime. Tissue-specific stem cells reside in a niche, whereby a complex microenvironment maintains their multipotency. After birth, adult stem cells, including both germ-line stem cells and tissue stem cells, reside in a specific microenvironment termed a "niche," which varies in nature and location depending on the tissue type [7]. These adult stem cells are an essential component of tissue homeostasis; they support ongoing tissue regeneration, replacing cells lost due to natural cell death (apoptosis) or injury [7].

Human adult maculae flavae are dense masses of cells and extracellular matrices located at the anterior and posterior ends of the membranous portion of the bilateral vocal folds (Fig. 4.1). The histological structure of the maculae flavae in the human adult vocal fold mucosa is unique and not suitable for vibration. Therefore, their roles in the human vocal fold as a vibrating tissue are very interesting. However, their roles in the human vocal fold have not been clarified until recently [8–11].

The latest research shows that the human maculae flavae are involved in the metabolism of extracellular matrices that are essential for the viscoelasticity of the human vocal fold mucosa [11] and are considered to be an important structure in the growth, development, and ageing of the human vocal fold mucosa [12, 13]. In addition, there is growing evidence to suggest that the cells in the maculae flavae are

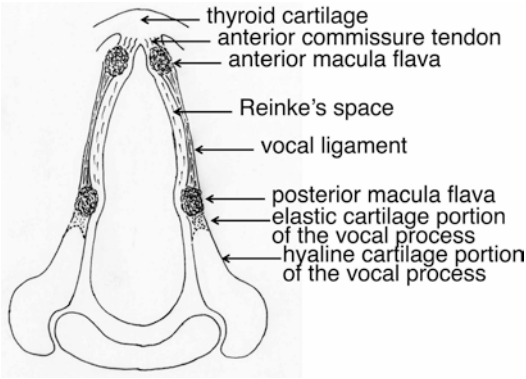


**Fig. 4.1** Human adult vocal fold and maculae flavae

tissue stem cells of the human vocal fold mucosa, and the maculae flavae are a candidate for a stem cell niche [14].

In this chapter, the latest research regarding the heterogeneity and stem cell hierarchies of the cell population in the maculae flavae of the human vocal fold mucosa is summarized.

## Maculae Flavae as a Stem Cell Niche in the Human Vocal Fold Mucosa

### *Maculae Flavae in the Human Adult Vocal Fold Mucosa*

The vibratory portion (membranous portion) of the human vocal fold is connected to the thyroid cartilage anteriorly via the intervening anterior macula flava and anterior commissure tendon. Posteriorly, it is joined to the vocal process of the arytenoid cartilage via the intervening posterior macula flava (Figs. 4.1 and 4.2). The vocal ligament runs between the anterior and posterior maculae flavae.

Human adult maculae flavae are dense masses of cells and extracellular matrices (Fig. 4.3) [11, 15, 16]. The maculae flavae are located at the anterior and posterior ends of the membranous portion of the bilateral vocal folds. They are elliptical in shape, and their size is approximately 1.5 × 1.5 × 1 mm [15]. The border between the maculae flavae and the surrounding soft tissue is clearly delineated (Figs. 4.2 and 4.4) [11].

The extracellular matrices of the human adult maculae flavae are composed of glycoproteins, glycosaminoglycan, and fibrillar proteins such as collagen fibers, reticular fibers, and elastic fibers (Fig. 4.3). These extracellular matrices in the maculae flavae extend to those in the lamina propria (Reinke's space and vocal ligament) of the human vocal fold mucosa [15].

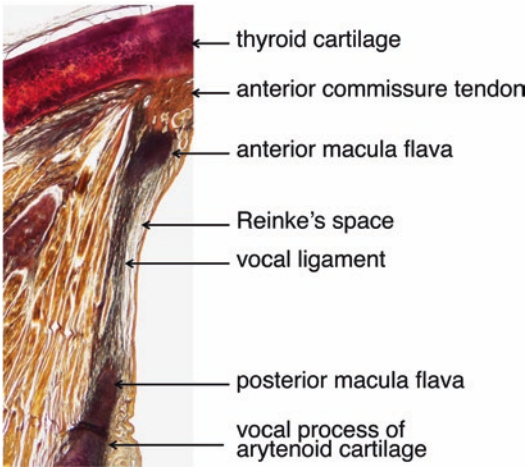


**Fig. 4.2** Transverse section of human adult vocal fold (Elastica van Gieson stain)

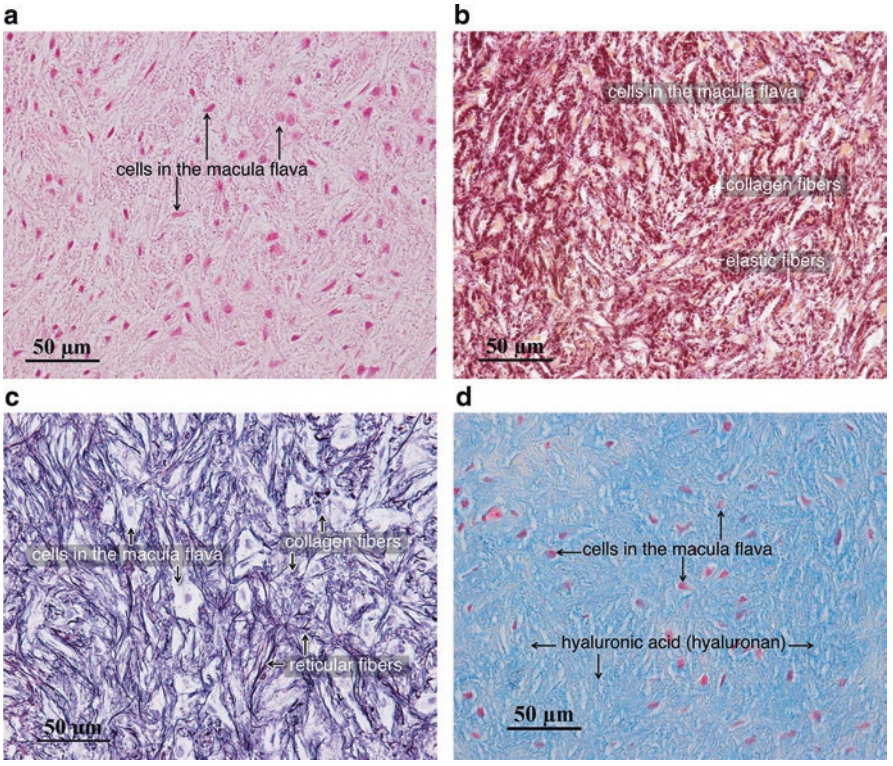


**Fig. 4.3** Macula flava of the human adult vocal fold mucosa. (**a**) Human adult maculae flavae are dense masses of cells (hematoxylin and eosin stain). (**b**) There are many collagen fibers stained red, and elastic fibers stained black around the cells in the human adult maculae flavae (elastic van Gieson stain). (**c**) There are many collagen fibers stained red, and reticular fibers (type III collagen) stained black around cells in the human adult maculae flavae (Silver stain). (**d**) Much glycosaminoglycan (hyaluronan, hyaluronic acid) is situated around the cells in the human adult maculae flavae (Alcian blue stain, pH 2.5). Maculae flavae are strongly stained light blue with Alcian Blue at pH 2.5. Material in the maculae flavae that is strongly stained with Alcian Blue (pH 2.5) is digested by hyaluronidase

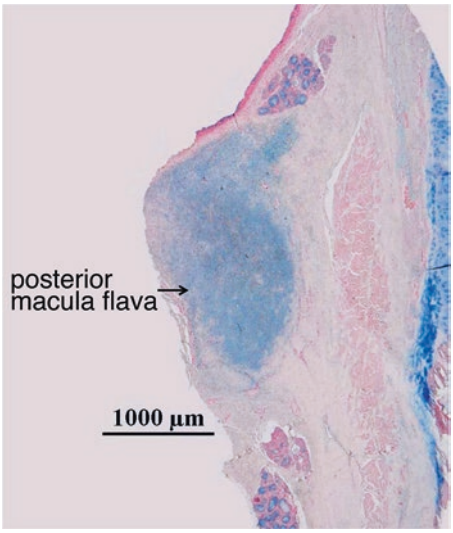


**Fig. 4.4** Coronal section of the posterior macula flava (Alcian blue stain, pH 2.5)

## *Maculae Flavae in the Human Newborn Vocal Fold Mucosa*

Newborns already have maculae flavae at the same sites as in adult vocal folds [17–19]. The newborn maculae flavae are composed of relatively dense masses of cells and are situated at the anterior and posterior ends of the bilateral vocal fold mucosae.

Extracellular matrices composed of collagen fibers, reticular fibers, elastic fibers, and ground substances are not abundant in the newborn maculae flavae. A newborn's maculae flavae are in the process of acquiring a hyaluronan-rich matrix, making it a candidate for a stem cell niche [19].

At birth, the cells have already been supplied, likely from the bone marrow, to the maculae flavae in the newborn vocal fold and are ready to start the growth and development of the human vocal fold mucosa as a vibrating tissue [17].

## *Microenvironment, Hyaluronan-Rich Matrix, of the Maculae Flavae as a Stem Cell Niche in the Human Vocal Fold Mucosa*

The structural and biochemical microenvironment that confers stemness upon cells in multicellular organisms is referred to as the stem cell niche. A stem cell niche is composed of a group of cells in a special tissue location for the maintenance of stem cells [7].

Hyaluronan serves as an important niche component for numerous stem cell populations [20, 21]. After the discovery of hyaluronan, it was assumed that its major functions were in the biophysical and homeostatic properties of tissues.

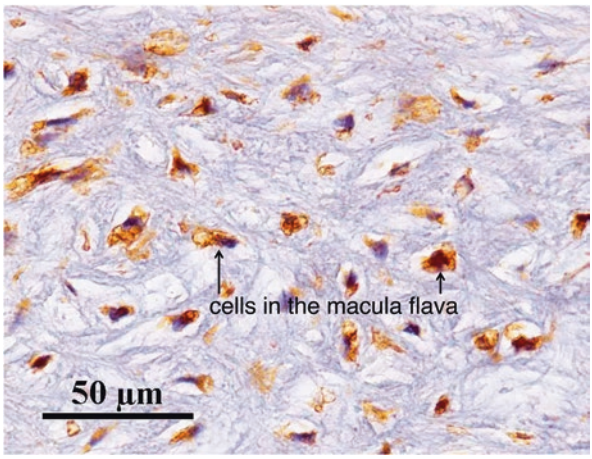


**Fig. 4.5** CD44 in the cytoplasm of cells in the human adult macula flava, shown by immunohistochemical staining

However, current studies have led to the understanding that hyaluronan also plays a crucial role in cell behavior [22]. A hyaluronan-rich matrix, which is composed of the glycosaminoglycan hyaluronan and its transmembrane receptors (cell surface hyaluronan receptors), is able to directly affect the cellular functions of stem cells in a stem cell niche [20, 21].

The maculae flavae in the human adult vocal fold are strongly stained light blue with Alcian Blue at pH 2.5 (Figs. 4.3d and 4.4). The materials in the maculae flavae that are strongly stained with Alcian Blue (pH 2.5) are digested by hyaluronidase. A great deal of glycosaminoglycan (hyaluronan) is situated around the cells in the human adult maculae flavae, and hyaluronan concentration is high. The border between dense masses of hyaluronan (maculae flavae) and the surrounding tissue is clearly delineated (Fig. 4.4). Additionally, most of the cells in the maculae flavae express CD44 (cell surface hyaluronan receptors) (Fig. 4.5). This indicates that the human maculae flavae are a hyaluronan-rich pericellular matrix [23].

Since the cells in the human maculae flavae have cell surface hyaluronan receptors and are surrounded by a high concentration of hyaluronan [14, 23], the maculae flavae are a candidate for a stem cell niche, which is a microenvironment nurturing a pool of putative stem cells [14, 23].

## Heterogeneity of the Cell Population in the Maculae Flavae of the Human Vocal Fold Mucosa

Recent evidence indicates that the cells including vocal fold stellate cells in the maculae flavae of the human vocal fold mucosa are a functionally heterogenous population.

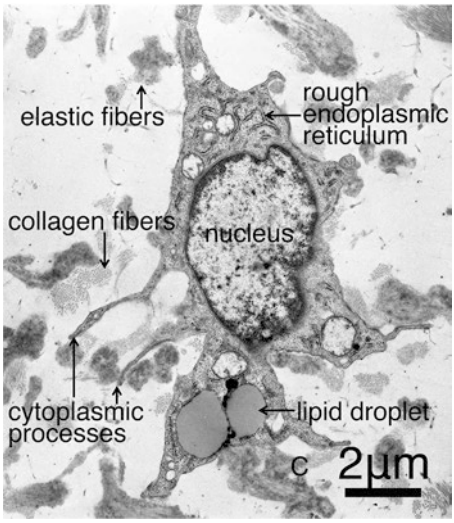


**Fig. 4.6** Transmission electron micrograph of a vocal fold stellate cell in the human macula flava (uranyl acetate and lead citrate stain). Vocal fold stellate cells are stellate in shape and possess slender cytoplasmic processes. They possess vitamin A–storing lipid droplets and intracellular organelles such as the rough endoplasmic reticulum and Golgi apparatus

## *Vocal Fold Stellate Cells in the Human Adult Maculae Flavae*

Interstitial cells with a star-like appearance in the human adult maculae flavae were discovered in our laboratory in 2001 (Fig. 4.6) [24–27]. These cells had no nomenclature and were thus designated "vocal fold stellate cells" in our series of studies. Vocal fold stellate cells are stellate in shape and possess vitamin A–storing lipid droplets [25]. There are a number of morphological differences between vocal fold stellate cells and fibroblasts in the human vocal fold mucosa. Along the surface of the vocal fold stellate cells, a number of vesicles are present, and they constantly synthesize extracellular matrices which are essential for the viscoelastic properties of the human vocal fold mucosa [24].

As a result of this heterogeneity, it is uncertain whether the vocal fold stellate cells derive from the same embryonic source as fibroblasts in the human vocal fold mucosa. The vocal fold stellate cells in the maculae flavae form an independent cell category that is a new category of cells in the human vocal fold mucosa.

## *Intermediate Filaments of the Cell Population in the Human Adult Maculae Flavae*

The expression of proteins in the intermediate filaments of the cytoplasm is specific to cell type and differentiation [28].

Proteins in the intermediate filaments containing cytokeratin, vimentin, glial fibrillary acidic protein (GFAP), and desmin are distributed in the cytoplasm of the cells in the adult maculae flavae [14, 29]. Additionally, cells in the human maculae

flavae express SOX 17, which is an endodermal cell marker [14]. Consequently, the cells in the human adult maculae flavae express proteins of all three germ layers [14]. This suggests that the cells in the maculae flavae are undifferentiated cells and have the ability of multipotency.

## *Radiosensitivity of the Cell Population in the Human Adult Maculae Flavae*

The radiosensitivity of the cells in the maculae flavae is morphologically higher than that of fibroblasts in Reinke's space of the human vocal fold mucosa, indicating that the cells in the maculae flavae are not yet as fully differentiated as fibroblasts [30].

## *Telomerase in the Cell Population in the Human Adult Maculae Flavae*

In multicellular organisms, telomerase resides mainly in the germ cells that give rise to sperms and eggs, and in a few other kinds of proliferating normal cells such as stem cells [31].

Most of the cells in the maculae flavae express telomerase reverse transcriptase, indicating that the special DNA polymerase called telomerase resides in the cells in the maculae flavae [23]. This suggests that the cells in the human maculae flavae are a putative stem cell of the human vocal fold mucosa.

## *Cell Cycle of the Cell Population in the Human Adult Maculae Flavae*

Cells express Ki-67 during proliferation (G1-, S-, G2-, M-phase) in the cell cycle, but cells that are in an arrested state (G0-phase) do not express Ki-67 [32].

The cells in the human maculae flavae do not express Ki-67, indicating that they are resting cells (G0-phase), as are other stem cells [23].

## *Cell Division of the Cell Population in the Human Adult Maculae Flavae*

In vitro culturing of the human maculae flavae yields interesting results. After a few weeks of primary culture in an MF-start primary culture medium (Toyobo, Osaka, Japan), two types of cells, fibroblast-like spindle cells (Group A) and

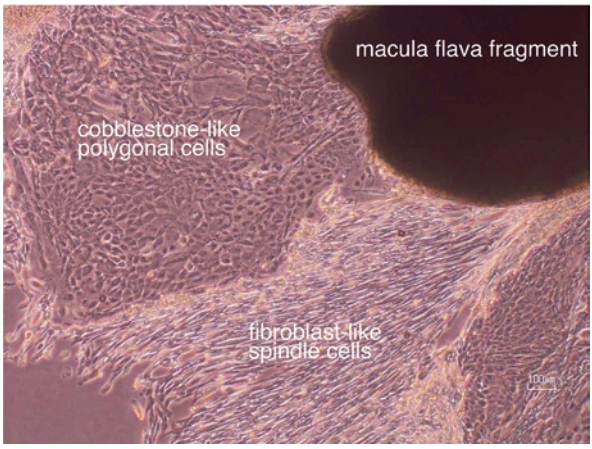


**Fig. 4.7** Primary culture of macula flava with MF-start primary culture medium (Toyobo, Osaka, Japan) (Phase-contrast microscopy). Two types of cells, cobblestone-like polygonal cells and fibroblast-like spindle cells, grow from the macula flava fragments in the primary culture

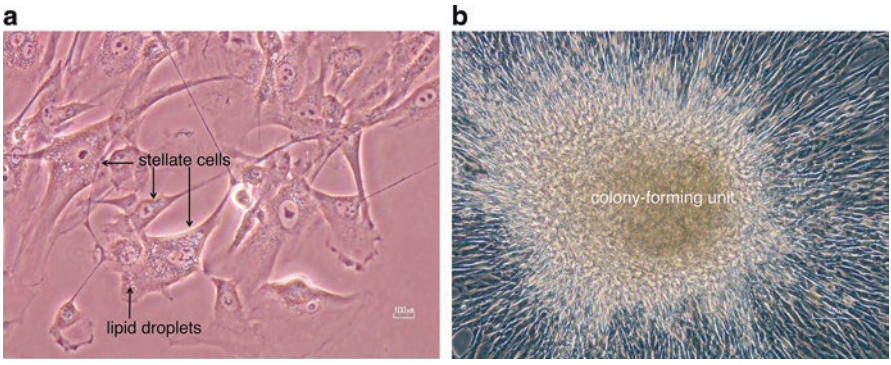


**Fig. 4.8** Individual subculture of each type of cell in an MF-medium (Mesenchymal Stem Cell Growth Medium) (Toyobo, Osaka, Japan) to proliferate the cells (Phase-contrast microscopy). (**a**) Vocal fold stellate cell–like cells. Fibroblast-like cells in the primary culture become stellate in shape and possess slender cytoplasmic processes and have small lipid droplets in the cytoplasm. (**b**) Colony-forming unit. Cobblestone-like polygonal cells in an MF-medium constitute a colony-forming unit

cobblestone-like polygonal cells (Group B), grew from the human macula flava fragments (Fig. 4.7) [33]. After removing the two types of cells by cell scraper, each type of cell was individually subcultured in an MF-medium (Mesenchymal Stem Cell Growth Medium) (Toyobo, Osaka, Japan) to proliferate the cells.

After a week of first subculture, subcultured Group A cells became stellate in shape and possessed slender cytoplasmic processes (Fig. 4.8a). Small lipid droplets were present in the cytoplasm. The nuclei were oval in shape, and their nucleus-cytoplasm ratios were low. These cells were morphologically similar to vocal fold stellate cells.

After a week of second subculture, subcultured Group B cells formed a colony-forming unit (Fig. 4.8b), indicating these cells were mesenchymal stem cells or stromal stem cells in the bone marrow.

Therefore, the colony-forming phenomenon gives rise to the possibility that the cells in the human maculae flavae are stem cells [33].

As mentioned earlier, the cell division in the human adult maculae flavae with mesenchymal stem cell growth medium is reflective of asymmetric self-renewal [33]. Asymmetry in cell division gives rise to the possibility that the maculae flavae in the human adult vocal fold is a stem cell niche containing stem cells [33].

## *Origin of Cell Population in the Human Adult Maculae Flavae*

Bone marrow–derived cells have received a great deal of attention with regard to tissue development and regeneration. Bone marrow–derived cells are considered to contain bone marrow–derived mesenchymal stem cells, which are multipotent cells capable of self-renewal [34, 35], and to be the origin of circulating fibrocytes, which are associated with wound healing and tissue fibrosis [36]. They circulate in the peripheral blood and are distributed to organs under normal conditions. When tissue is injured, they contribute to tissue repair by cell differentiation and migrate into injured tissue as needed [37, 38].

Circulating fibrocytes were first described as blood-born fibroblast-like cells by Bucala et al. [36]. They were found to be unique cells because they co-expressed hematopoietic markers as well as collagen type I and other mesenchymal markers. CD34, CD45, and collagen type I are major markers for circulating fibrocytes derived from bone marrow [39].

The cells in the human maculae flavae express CD34 (hematopoietic stem cell marker), CD45 (leukocyte common antigen), and collagen type I [40]. These proteins are major makers of bone marrow–derived circulating fibrocytes. Consequently, cells in the human maculae flavae quite possibly arise, not from resident interstitial cells of the vocal fold mucosa but from the differentiation of bone marrow cells via peripheral circulation [40].

## *Cell Population in the Human Newborn Maculae Flavae*

The cells in the newborn maculae flavae possess some features of mesenchymal cells [17, 18]. The cells in the human newborn maculae flavae possess proteins of all three germ layers [41]. And, the cells in the human newborn maculae flavae express CD34, CD45, and collagen type I [41]. They are likely undifferentiated cells which arise not from resident interstitial cells but from the differentiation of bone marrow cells [41].

The results of our studies are consistent with the hypothesis that the cells in the maculae flavae are putative stem cells or progenitor cells of the human newborn vocal fold mucosa [19, 41].

## *Hierarchy of Putative Stem Cells in the Human Maculae Flavae*

As mentioned earlier, cultured cell populations in the human maculae flavae are roughly divided into three groups by morphological features: cobblestone-like polygonal cells, vocal fold stellate cell–like cells, and fibroblast-like spindle cells. Thus, such findings raise the question whether all cell populations in the human maculae flavae are equal or if some particular populations retain more stem cell–like ability than others. In addition, another question arises whether the vocal fold stellate cells are putative stem cells or progenitor cells (transit-amplifying cells).

Both colony-forming subcultured cells (cobblestone-like polygonal cells) and non-colony-forming subcultured cells (fibroblast-like spindle cells) (Fig. 4.7) express cytoplasmic cytokeratin, vimentin, GFAP, and desmin [40]. Consequently, both colony-forming cells (cobblestone-like polygonal cells) and non-colony-forming cells (fibroblast-like spindle cells) express ectoderm and mesoderm germ layers. This suggests that they are undifferentiated cells and have the ability of multipotency [40].

The vocal fold stellate cells are possibly transit-amplifying cells, that is, progenitor cells [33]. However, at the present state of our investigation, it is difficult to clarify the stem cell system and hierarchy of stem cells in the human maculae flavae and determine whether the vocal fold stellate cells are putative stem cells or progenitor cells. Individual evaluations of cells in the maculae flavae by clonal analysis are necessary to determine whether all cells in the maculae flavae retain stem cell functions.

## *Slow-Dividing Cell Population in the Maculae Flavae*

Adult tissue stem cells are maintained in a quiescent state and proliferate extremely slowly with stem cell properties. Adult tissue stem cells consistently retain labeled DNA since stem cells divide more infrequently than other cells.

The cells in the maculae flavae of the rat vocal fold retained BrdU labeling, that is, label-retaining cells reside in the maculae flavae [42]. This phenomenon indicates the division cycles of the cells in the maculae flavae are slow and different from other cells in the vocal fold mucosa [42]. Consequently, the results are consistent with the hypothesis that the cells in the maculae flavae are putative stem cells of the vocal fold mucosa [42]. On the other hand, not all of the cells in the maculae flavae are label-retaining cells [42]. These findings imply that subpopulations of cells in the maculae flavae proliferate extremely slowly and retain stem cell properties. Slow-diving cells escape the risk of DNA mutation by repetitive replication and may be at the top of the cell hierarchy.

## Side Population Cells in the Vocal Fold Mucosa

Side population cells are regarded as a cell population enriched with stem cells or progenitor cells and are recognized as a candidate for tissue stem cells.

In one recent study, side population cells were identified in the epithelium and subepithelial tissue including the anterior and posterior maculae flavae [43]. In another recent study, side population cells increased significantly in Reinke's space of an injured vocal fold starting on day 3, with a peak at day 7, followed by a decrease back to baseline values on day 14 [44]. These cells in the maculae flavae participated in the early stages of wound healing [44]. The two investigations cited here suggest that the anterior and posterior maculae flavae contain stem cells or progenitor cells, and these cells have the capacity to play essential roles in tissue regeneration.

## Mechanical Regulation (Cellular Mechanotransduction) of the Cells in the Human Maculae Flavae

Current scientific findings suggest that the magnitude and frequency of tensile strain are particularly important in determining the type of mechanically induced differentiation that stem cells will undergo [45]. The macula flava is the microenvironment where the magnitude and frequency of tensile strain during vocal fold vibration are greatest [46]. The function and fate of the cells in the human maculae flavae are regulated by various microenvironmental factors. In addition to chemical factors, mechanical factors also modulate the behavior and heterogeneity of cells in the human maculae flavae.

We hypothesize that the tensions caused by phonation (vocal fold vibration) after birth stimulate cells in the anterior and posterior maculae flavae to accelerate production of extracellular matrices and form the vocal ligament, Reinke's space, and the layered structure [47]. The results of our studies (morphologic differences are detected between adult vocal fold mucosae that have been phonated and those that have remained unphonated since birth [48–50]) are consistent with this hypothesis.

We also hypothesize that after the layered structure of the adult vocal fold is completed, the tensions caused by phonation (vocal fold vibration) stimulate cells in the anterior and posterior maculae flavae to accelerate production of extracellular matrices and maintain the layered structure of the human adult vocal fold mucosa as a vibrating tissue. The results of our study (morphologic differences are detected between the adult vocal fold mucosae that have remained phonated and those that have been unphonated for a long period [51]) are consistent with this hypothesis.

The bending stresses on the vocal fold associated with phonation (vocal fold vibration) are greatest in the region of the maculae flavae located at both ends of the vocal fold mucosa [46]. Tension caused by phonation seems to regulate the behavior and heterogeneity of the cells (mechanical regulation) in the maculae flavae of

the human vocal fold. It is of interest whether the mechanical forces caused by vocal fold vibration from outside the cells in the maculae flavae influence intracellular signaling cascades through cell to matrix that ultimately alter many cellular behaviors and heterogeneity.

"Mechanotransduction" is the term for the ability of living tissues to sense mechanical stress and respond by tissue remodeling. Cellular mechanotransduction is the mechanism by which cells convert mechanical stimuli into biomechanical responses. More recently, mechanotransduction has expanded to include the sensation of stress, its translation into a biochemical signal, and the sequence of biological responses it produces. Mechanical stress has become increasingly recognized as one of the primary and essential factors controlling biological functions, ultimately affecting the functions of cells, tissue, and organs [52]. It is very likely that the mechanical stress caused by phonation (vocal fold vibration) is one of the primary and essential factors controlling biological functions, ultimately affecting the function and heterogeneity of the cells in the maculae flavae of the human vocal fold mucosa. However, the role of mechanotransduction in the vibrating vocal fold mucosa remains unclear.

It is readily apparent that tensile and compressive strains can have direct effects on cell morphology and structure, including changes in the cell membrane, shape, and volume as well as cytoskeletal structure and organization [45]. These physical changes can be converted into changes in cell signaling and transcriptional activities in the nucleus to cause alterations in cellular differentiation, proliferation, and migration [45].

The function and fate of stem cells are regulated by various microenvironmental factors [45]. In addition to chemical factors, mechanical factors can also modulate stem cell survival, organization, migration, proliferation, and differentiation [45]. Stem cells are potentially one of the main players in the phenotype determination of a tissue in response to mechanical loading [45].

The cells in the human maculae flavae may be sensing mechanical forces, and these tissue-specific mechanical forces (vocal fold vibration) could promote cell differentiation toward the phenotype of the cells residing within the vocal fold tissue. However, little is known about how force affects biological signaling. It is suggested that the combination of multiple mechanical and chemical factors may be involved in more complicated signaling mechanisms, and assessment of the relative importance of each factor needs further investigations.

## Transition Area Between the Human Adult Maculae Flavae and Surrounding Tissue

Examination of the transition area between the maculae flavae and their surrounding tissue is helpful in understanding the heterogeneity of the putative stem cells in the maculae flavae of the human vocal fold mucosa.

The posterior macula flava is attached to the vocal process of the arytenoid cartilage posteriorly (Figs. 4.1 and 4.2). Elastic cartilage located at the tip of the

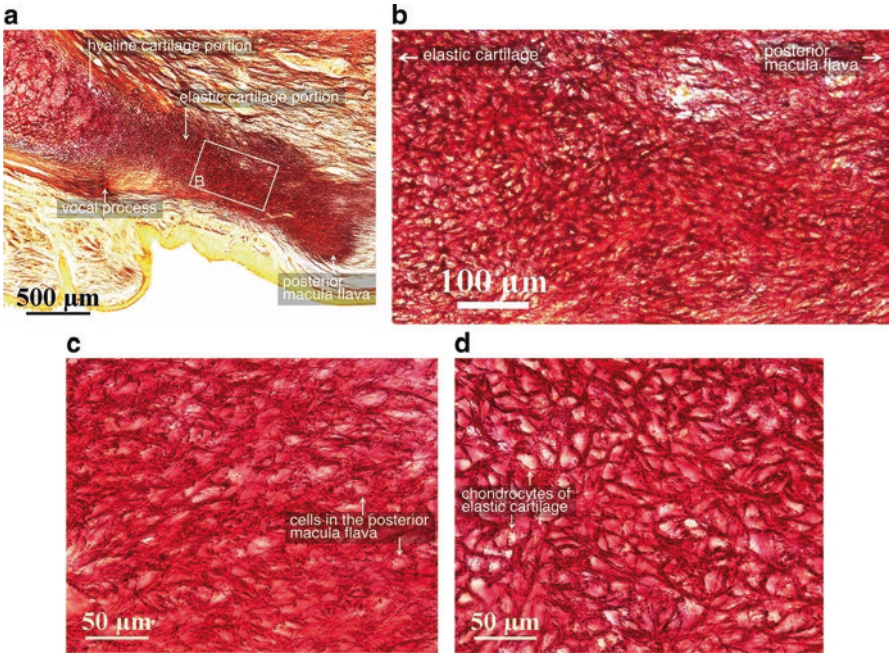


**Fig. 4.9** Transition area between human adult posterior macula flava and tip of vocal process of the arytenoid cartilage. (**a**) Posterior macula flava and tip of vocal process. (**b**) Transition between posterior macula flava and tip of vocal process. (**c**) Posterior macula flava. (**d**) Elastic cartilage portion of vocal process

vocal process facilitates movement of the vocal process during adduction and abduction [53]. The transition of cells and extracellular matrices between the posterior macula flava and the elastic cartilage portion of the vocal process is gradual, and the border between them is not clearly delineated (Fig. 4.9). The cells in the posterior macula flava appear to differentiate into chondrocytes in the tip of the vocal process [14, 23].

The cells in the human maculae flavae express CD44 (mesenchymal stem cell marker). Most of the fibroblasts in the tissue surrounding the maculae flavae do not express CD44. However, CD44-positive fibroblasts are observed at the periphery of the maculae flavae (Fig. 4.10). The cells in the maculae flavae appear to differentiate into fibroblasts in the surrounding tissue [14, 23].

These findings raise the possibility that the putative stem cells in the maculae flavae generate functionally differentiated cells, such as chondrocytes and fibroblasts in the human vocal fold mucosa [14, 23]. Additional investigations are needed to determine whether the putative stem cells in the maculae flavae have the capacity to self-renew and generate functionally differentiated cells (multipotency) that replenish lost cells throughout an organism's lifetime.

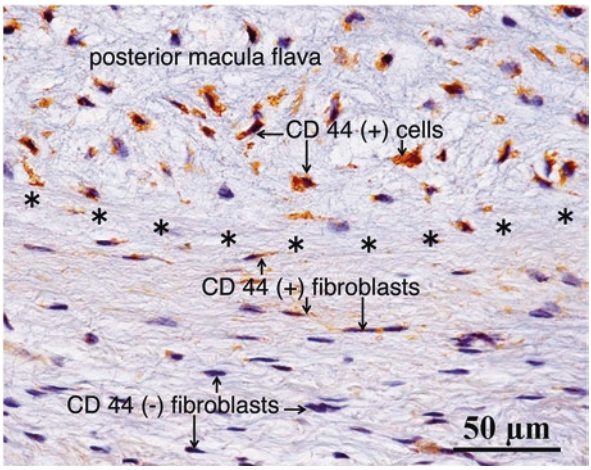


**Fig. 4.10** Border between the human adult posterior macula flava and surrounding tissue (CD44, immunohistochemical staining). The border (asterisks) between the dense mass of the macula flava, containing cells including vocal fold stellate cells, and surrounding tissue is clearly delineated. The CD44-positive fibroblasts are observed at the periphery of the human maculae flavae

## Future Prospects

As a result of the latest research, there is growing evidence to suggest that the cells in the human maculae flavae are adult multipotent stem cells, tissue stem cells, or progenitor cells in the human vocal fold mucosa and that the human maculae flavae are a candidate for a stem cell niche. This chapter has reviewed the recent findings of functionally heterogenous and stem cell–like subpopulations among cells in the maculae flavae of the vocal fold mucosa.

Investigations concerning how to regulate these cells contained in the human maculae flavae are challenging but important in the field of regenerative medicine of the human vocal fold.

The putative stem cells in the maculae flavae are a potential endogenous cell source for vocal fold regeneration and will provide the tools for future therapeutic approaches. The manipulation of not only cells but also their microenvironment is one of the strategies in regenerative medicine. Artificial manipulations of these cells using cutting-edge methods (e.g., via chemical biology) could lead to advanced developments in vocal fold regeneration. Understanding the mechanisms responsible for microenvironmental regulation of the cells in the human maculae flavae will provide the tools needed to manipulate cells through their microenvironment for the development of therapeutic approaches to diseases and tissue injuries of the vocal fold.

Further investigations of stem cell systems including the heterogeneity regarding the putative stem cells in the maculae flavae of the human vocal fold mucosa are needed in the field of regenerative medicine of the vocal fold. Translational medicine focused on how to regulate putative stem cells and extracellular matrices (microenvironments) contained in the maculae flavae of the human vocal folds will contribute to our ability to restore and regenerate human vocal fold tissue.

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# Chapter 5
# Heterogeneity of Stem Cells in the Thyroid


**Giovanni Zito, Antonina Coppola, Giuseppe Pizzolanti, and Carla Giordano**

**Abstract** Identification of thyroid stem cells in the past few years has made important contributions to our understanding of the cellular and molecular mechanisms that induce tissue regeneration and repair. Embryonic stem (ES) cells and induced-pluripotent stem cells have been used to establish reliable protocols to obtain mature thyrocytes and functional follicles for the treatment of thyroid diseases in mice. In addition, the discovery of resident thyroid progenitor cells, along with other sources of stem cells, has defined in detail the mechanisms responsible for tissue repair upon moderate or severe organ injury.

In this chapter, we highlight in detail the current state of research on thyroid stem cells by focusing on (1) the description of the first experiments performed to obtain thyroid follicles from embryonic stem cells, (2) the identification of resident stem cells in the thyroid gland, and (3) the definition of the current translational in vivo and in vitro models used for thyroid tissue repair and regeneration.

**Keywords** Thyroid regeneration · Organ repair · Thyroid resident stem cells · Solid cell nest (SCN) · Side population (SP) · Embryonic stem cells (ES cells) · TSH · Activin-A · Thyrospheres · Thyroid follicles · Parafollicular cells · Thyroglobulin (Tg) · Thyroperoxidase (TPO) · Oct4 · Sca1

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## Introduction

In the past few decades, stem cell biology has contributed to the identification of molecular mechanisms that control tissue regeneration and repair after injury. Stem cells have been identified in several tissues, and their molecular signature has provided important details on (1) tissue development and maintenance and (2) mechanisms underlying diseases and cancer origin and progression [1–6].

Recently, different research groups have begun to identify the putative role of stem cells in the thyroid gland [7–10]. In particular, they started to define their role in tissue homeostasis and in the development of several autoimmune diseases and cancer [11–16]. However, a clear molecular definition of stem cells in the thyroid is still under question. In particular, it is still necessary to define the cellular source of stem cells within the gland, and how they contribute to the regeneration of the organ after injury. The current state of the art maintains the hypothesis that thyroid stem cells derive from different sources within and outside the gland, and they differently contribute to organ homeostasis and tissue repair [17].

In this chapter, we highlight in detail the current state of research on thyroid stem cells by focusing on (1) the description of the first experiments performed to obtain thyroid follicles from embryonic stem (ES) cells, (2) the identification of resident stem cells in the thyroid gland, and (3) the definition of the current translational in vivo and in vitro models used for thyroid tissue repair and regeneration.

Taken together, these data might provide the biological basis to define the next steps that need to be pursued in order to complete our understanding of thyroid regeneration and repair.

## Thyroid Gland Development and Its Molecular Signature

The thyroid gland is mainly composed of thyroid follicular cells (TFCs) and parafollicular C cells. TFCs are organized in spherical structures whose main role is to (1) store thyroglobulin (Tg) and (2) control the release of thyroid hormones [18]. Parafollicular C cells, scattered among the thyroid follicules, are instead responsible for calcitonin production and secretion. While TFCs have endodermal origins and derive from the pharyngeal floor, parafollicular C cells are formed from the ultimobrachial body (UBB) from the fourth pharyngeal pouch [19] (Fig. 5.1).

During development, thyroid gland formation occurs after migration and proliferation of thyroid precursors around E8.5 in mice. In particular, it has been shown that survival and proliferation of thyroid precursors depend on expression of specific transcription factors, including paired box 8 (Pax8), thyroid transcription factor 1 (TTF-1), forkhead box E1 (Foxe1), and hematopoietically expressed homeobox protein 1 (Hhex1). Altogether, their co-expression represents the molecular signature of the developing thyroid gland [20]. However, their functions within the TFCs are distinct: while TTF-1 and Pax8 are required for the survival of TFCs [21, 22],

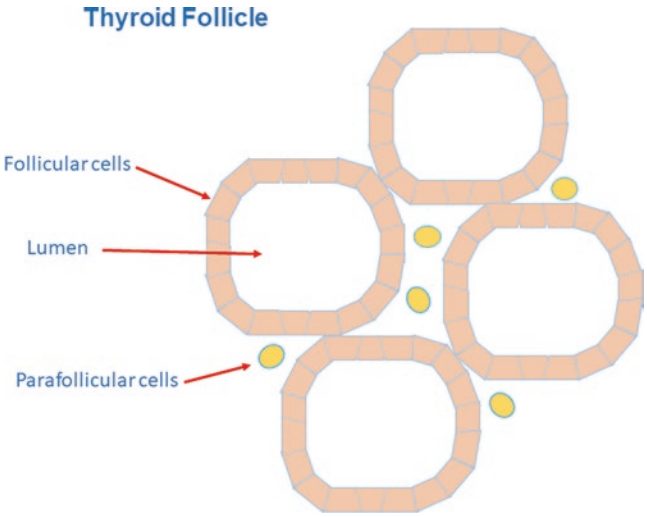


**Fig. 5.1** Cellular structure of functional follicles within the thyroid gland

Foxe1 is essential for migration of the thyroid precursor cells [23], and Hhex, although it still needs to be proved, appears to be important for regulation of the transcription factors mentioned above [24].

Thus, expression of the above markers has been used to monitor differentiation of embryonic stem cells towards the thyroid lineage, as described in detail in the next paragraph.

## Embryonic Stem Cells as a Source of Thyrocyte-Like Cells

Embryonic stem (ES) cells from the inner cell mass retain the ability, under proper culture conditions, to differentiate into different cell types. By these means, they have been used to generate several lineages, including cardiomyocytes, hepatocytes, pancreatic β-cells, neurons, and hematopoietic progenitors [25–30].

Lin et al. [31] established a protocol that allowed formation of mature thyrocytes from murine ES cells. Briefly, they first let ES cells form embryoid bodies (EBs) in the presence of serum. EBs were then treated with thyroid-stimulating hormone (TSH), the main regulator of the mature thyroid gland. After a few days, in these culture conditions, they found that TSH-treated EBs started to upregulate genes that are specifically expressed in mature thyrocytes, including *Pax8*, *$Na^+/I^-$ symporter* (*NIS*), *thyroglobulin* (*Tg*), *thyroperoxidase* (*TPO*), and the *TSH receptor* (*TSHR*). In addition, researchers demonstrated that TSH-treated EBs showed typical thyroid functions, such as cAMP production upon TSH treatment. Despite the big achievements obtained with this first set of experiments, the number of mature thyrocytes was not sufficient for further studies. Thus, new protocols are needed to be employed

to overcome this problem. The same group a few years later engineered a murine ES line by creating a GFP-TSHR fusion protein [10, 32]. In this case too, mature thyrocytes were obtained by using a multistep process, which included treatment of ES cells, in chronological order, with Activin-A, TSH, insulin, and insulin growth factors (IGFs). Gene expression analysis performed on thyroid-like mature cells demonstrated that these cells began to express follicular thyroid markers, including *Pax8*, *NIS*, *TSHR*, and thyroid hormones such as *Tg* and *TPO*. However, while the last set of experiments increased the number of thyroid-like differentiated cells, the limitation of this approach was still related to the difficulty of maintaining the thyrocyte phenotype in the long term. The authors pointed out that after 12 days of differentiation, all the thyroid-specific genes described above were downregulated if not completely switched off.

It has been shown that TSHR knockout mice (TSHR KO) displayed a normal thyroid in the correct anatomical position. Even if the mice showed congenital hypothyroidism, with a reduction of the organ functionality, TSHR KO TFCs were quite normal in their physiological function [33]. Thus, it has been hypothesized that thyroid follicles are partially TSH-independent, and that other factors might need to be used to obtain TFCs. Given the endodermal origin, Ma et al. [34] for the first time used activin-A to obtain thyroid follicles from human W9.5 or TSHR+/− ES cells. Activin-A treatment displayed reduction of the ES markers *Oct-4* and *REX1* and activation of the endodermal markers *GATA-4* and *α-fetoprotein*. Furthermore, after 5 days of culture, the endodermal-induced cells began to express TSHR and NIS protein, along with *Pax8* mRNA. More importantly, these experiments showed that even 21 days after the beginning of treatment, thyroid-like cells maintain their phenotype, with or without addition of TSH or insulin in the culture medium.

All together, these data demonstrated that activin-A induction of a thyroid endoderm is required for long-term maturation of thyrocytes.

The limitation of the studies described above was mainly due to functional application of Embryonic Stem Cells (ESCs)-derived thyrocyte to any thyroid disease. The very low number of differentiated cells obtained with the protocols described made it impossible to envision a hypothetical therapeutic approach to fight thyroid dysfunctions. To overcome this problem, Antonica et al. [35] developed a new protocol to establish functional thyrocytes derived from murine ESCs. Briefly, they transiently overexpressed TTF-1 and Pax8 on ESCs in order to induce thyroid differentiation. While transient overexpression of TTF-1 and Pax8 did not affect ESC pluripotency, the authors demonstrated that activation of these transcription factors induced differentiation of 60% of ESCs into thyrocyte-like cells. In a second step, they treated the differentiated ESC thyrocyte-like cells with TSH in order to obtain in vitro formation of mature thyroid follicles. These follicles not only were nicely formed and molecularly accurate but they were also functionally active, as demonstrated by iodide organification in in vitro experiments. More importantly, ESC-derived thyroid follicles, when grafted into the kidney capsule of thyroid animals, were able to correct the hormone deficiency [35].

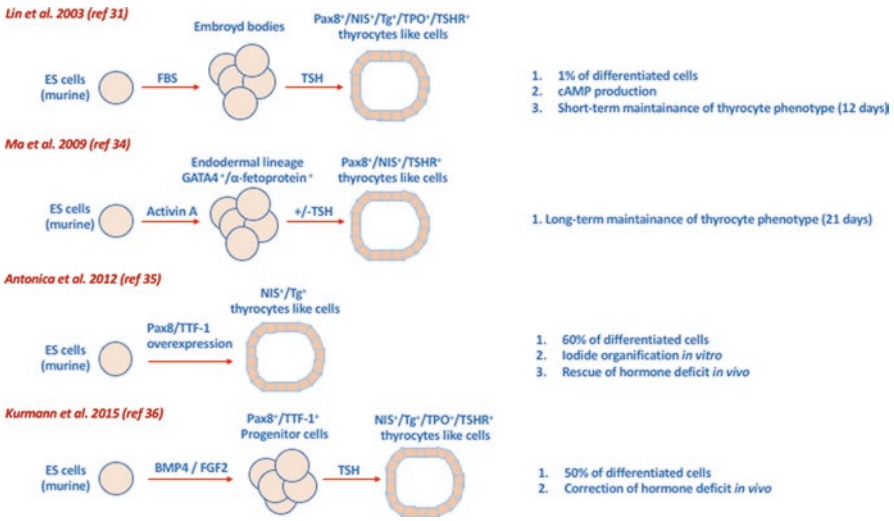


**Fig. 5.2** Schematic of the experiments performed to obtain mature thyrocyte-like cells from ES cells

In 2015, Kurmann et al. [36] further defined new protocols to establish mature and functional thyrocytes from mouse ESCs and induced pluripotent stem cells (iPSCs). They demonstrated that BMP (bone morphogenetic protein) and FGF (fibroblast growth factor) signaling pathways were required for thyroid lineage specification. Similarly Antonica's study, ESC-derived mature thyrocytes were capable in vivo of correcting hormone deficiency in hypothyroid mice. More importantly, by using BMP4 and FGF2, the authors for the first time successfully obtained TTF-1$^+$/Pax8$^+$ human thyroid progenitors in iPSCs from patients with hypothyroidism [36].

Together, these novel findings clearly established new potential protocols to restore thyroid function not only in mice but also in human patients affected by hypothyroidism (Fig. 5.2).

## Adult Thyroid Stem/Progenitor Cells

Several years ago, it was postulated that the thyroid gland is a low proliferating organ, where follicular cells divide occasionally about four to five times during their entire life [37]. However, despite the low rate of turnover, the organ is capable of maintaining its size under physiological conditions. All these observations led Dumont et al. [38] to hypothesize for the first time the possibility that a population of resident stem cells, with self-renewal capability, existed in the thyroid gland. According to these data, the putative number of stem cells in the thyroid is estimated to be around 1 to 1000.

The existence of thyroid stem cells (TSCs) and their isolation and characterization was proven a few years later. In particular, the first attempt to isolate TSCs was performed by Hoshi et al. [7]. They used mouse thyroid to isolate a side population (SP) of cells in contrast to the main cell population (MP).

SP cells were first selected in adult mouse bone marrow from MP because of their ability to efflux the vital dye Hoechst 33342 [39]. By using fluorescence-activating cell sorting (FACS), it was possible to isolate SP cells for their specific characterization. By using this approach, it was demonstrated that SP cells efflux the dye because they expressed ATP-binding cassette-dependent transporter ABCG2 in the cell membrane [40]. In non-hematopoietic tissue, SP cells were shown to have stem cell properties, as they were able to contribute to organ regeneration under physiological conditions [41, 42]. Starting from these observations, it has been hypothesized that SP cells in the thyroid gland could retain stem cell features.

SP isolation and characterization from thyroid gland and thyroid cell lines has been carried out by different groups. In 2007, Hoshi et al. [7] were the first to isolate SP cells from mouse thyroid gland. Specifically, they used some hematopoietic stem cell markers to isolate two different SP populations: $CD45^{-}/c\text{-}KIT^{-}/SCA1^{+}$ (SP1 population) and $CD45^{-}/c\text{-}KIT^{-}/SCA1^{-}$ (SP2 population), along with an MP population. Gene expression analysis performed on SP1, SP2, and MP cells showed that stem cell markers, such as *Oct4*, *nucleostemin*, and *ABCG2*, were highly expressed in SP1 and SP2, while thyroid-specific markers, including *TPO*, *Tg*, *TSHR*, *TTF-1*, and *Pax8*, were mainly upregulated in the MP population, with weak positivity in SP2 [7]. On the basis of this analysis, the authors suggest the hypothesis that SP1 cells were less differentiated with defined characteristics of stem/progenitor cells. In the same year, Mitsutake et al. isolated SP cells from several human thyroid cancer cell lines by using the same approach described above. However, the study demonstrated that SP and non-SP population indistinctly maintain tumorigenic properties in tumorigenesis assay using nude mice [15]. These findings demonstrated that cancer stem cells and SP population are not identical and opened up the possibility that thyroid tumors might arise from a different population of stem cells, not necessarily derived from SP cells.

The isolation of adult stem cells from human thyroid has been performed by different research groups worldwide. In 2006, Derwahl's group identified putative stem cells in human thyroid [8]. In particular, they found the existence of $Oct\text{-}4^{+}$ cells in tissue sections from human goiters, thus suggesting the possibility of a population of resident stem cells within the gland. In addition, they proved that $Oct\text{-}4^{+}$ cells also expressed GATA4 and HFN4α, typical markers of endodermal lineage. Immunohistochemical analysis showed that these cells were largely dispersed within follicles. Finally, the authors clearly demonstrated that $Oct\text{-}4^{+}/GATA4^{+}/HFN4\alpha^{+}$ cells did not express thyroid differentiation markers, while $Oct\text{-}4^{-}/GATA4^{-}/HFN4\alpha^{+}$ cells displayed positivity for Tg and TPO. Altogether, these data represented the first hint for the existence of a few adult progenitor cells in the human thyroid. In 2007, the same research group carried out elegant work by isolating SP cells from cultured human goiters. Gene expression analysis on SP-sorted

cells confirmed the expression of stem cell and endodermal markers *Oct-4*, *ABCG2*, *GATA4*, and *HFN4α*, and showed the absence of *Tg* and *TPO* mRNA. Furthermore, SP cells sorted using Hoechst 33342 were cultured in the presence of stem cell stimuli, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). These culture conditions determined the formation of thyrospheres with an increased percentage of SP cells (from 0.1% to 5%). In addition, serum and TSH treatment induced differentiation of thyrospheres into mature thyrocytes expressing PAX8, Tg, and TPO. These data taken together provide the first evidence of adult stem cells in the thyroid that, under proper stimuli, are able to differentiate into mature and functional thyrocytes [43].

In 2008, Fierabracci et al. [9] isolated TSCs from the normal gland, thyroid adenoma, and Graves' disease for a total of 23 samples. In particular, they obtained thyrospheres by using "spheroid medium" supplemented with EGF and bFGF. The molecular and immunohistochemical characterization of all the lines showed a subset of $CD34^+/CD45^-$ cells, very few lines expressing *Tg* and *TPO*, absence of *TSHR* and *NIS*, and *nestin*, *Oct-4*, and *Nanog* upregulation. More importantly, the author showed that the thyrospheres, under proper culture conditions, were able to differentiate toward the neuronal and the adipogenic phenotype [9]. Thus, the authors, by using different molecular markers and stem cell assays, confirmed the existence of a stem cell population within the thyroid gland.

Taken together, the data just referred to define a stem/progenitor subset of cells in the thyroid gland, with specific expression of stem cell and endodermal markers (*Oct-4*, *ABCG2*, *GATA4*, and *HFN4α*). In addition, the authors clearly showed the ability of TSCs to generate mature follicles when cultured in differentiation media. However, it is still under debate whether these cells are the only ones that contribute to organ regeneration during homeostasis or after injury.

## Stem Cell Heterogeneity in Thyroid Regeneration

As mentioned earlier, the thyroid gland has a very low turnover, along with other organs including the heart, liver, muscle, and brain. In these organs, stem cells play a minor role for their homeostasis. However, when they undergo partial excision, cellular hypertrophy and hyperplasia occur, and in this context, stem cell proliferation and differentiation take place to induce organ regeneration [44]. It has been shown that after partial hepatectomy, the liver is able to regenerate in about 7 days [45]. In a similar manner, previous studies demonstrated that partial thyroidectomy induces hypertrophy and hyperplasia [46, 47]. Thus, these findings could be used to increase our understanding of how the thyroid regenerates and the type of cells that contribute to this biological process.

In the past few decades, partial thyroidectomy was mainly used as a model to understand how the decreased production of thyroid hormone affected liver regeneration, as well as normal brain physiology [48, 49]. Recently, partial thyroidectomy has been used to study the cellular behaviors that occur during thyroid

regeneration. The first work was published in 2012 by Ozaki et al. [11]. In this study, partial thyroidectomy (one entire lobe and 2/5 caudal segment of the other lobe) caused extensive damage, which determined an immediate increase of TSH levels and the development of a goitrogenic condition. However, 2 weeks after partial thyroidectomy, this condition resolved [11]. During the regeneration process, the authors demonstrated the presence of BrdU-positive clear cells in the central area of the gland and in the areas continuous to the cut edge. Importantly, those cells began to express *Foxa2*, a marker of definitive endoderm, thus suggesting that regeneration processes were in place to replenish the lack of follicles due to partial excision. Thus, this study indicates that in the context of massive damage, clear cells contributed to regeneration of the tissue [11]. However, it is still necessary to understand the nature of the clear cells mentioned above, that is whether they were calcitonin-producing C cells or follicular ones.

Ozaki et al. [11] used partial thyroidectomy to study the contribution of SCA1$^+$ stem cells to thyroid regeneration. They used an elegant genetic lineage–tracing approach to follow the fate, and thus the origin of the new generated follicles. In detail, a β-gal reporter mouse was crossed with a mouse where the Cre recombinase was under the control of the *TPO* promoter. Thus, in the progeny *TPO-Cre*, *β-gal*, the reporter gene, was expressed in the TPO$^+$ cells.

Partial thyroidectomy has been performed in order to obtain moderate damage. Specifically, the caudal third of both thyroid lobes was removed. According to the authors, after partial thyroidectomy, SCA1$^+$/BrdU$^+$ cells were found in the non-follicular mesenchymal areas of the tissue. Immunohistochemical analysis showed that SCA1$^+$/BrdU$^+$ cells were negative for TSC markers *Oct-4* and *GATA4*, but started to express the epithelial marker *KRT14*. SCA1$^+$/BrdU$^+$/KRT14$^+$ were found in the thyroid follicles 2 weeks after partial thyroidectomy. It is to be noted that 35 days after thyroidectomy, SCA1$^+$/BrdU$^+$/KRT14$^+$ cells in the newly generated irregular follicles were β-gal-negative, thus indicating that the de novo proliferating cells did not originate from differentiated follicle cells. However, 4 months after thyroidectomy, SCA1$^+$/BrdU$^+$/β-gal$^+$ were found in the new follicles, thus suggesting that the regenerated follicular tissue was functional, as new TPO hormone was produced. Taken together, these important findings demonstrate that after moderate damage to the organ, SCA1$^+$ cells contribute to tissue regeneration, by generating new functional follicle cells. However, the only limit of this work was related to the impossibility of defining the origin of the SCA$^+$ cells. It is still not clearly understood whether they were resident adult TSCs [7] or bone marrow–derived stem cells [39].

Bone marrow stem cells are well known for their pluripotency and for their ability to differentiate into several lineages [50]. In addition, they have been shown to reach the specific tissue and differentiate in response to injury [51]. A recently published study demonstrated that GFP-bone marrow cells derived from C57BL/6 mice were grafted into irradiated C57BL/6 mice to study whether mesenchymal stem cells contribute to thyroid regeneration upon X-ray irradiation. The authors showed that 40 weeks post irradiation, GFP$^+$ cells were found in the thyroid follicles. More importantly, GFP$^+$ cells co-expressed the thyroid hormone Tg, thus specifically demonstrating that bone marrow derives GFP$^+$ stem cells contributed to generation of new functional thyrocytes [52].

The solid cell nest (SCN) is believed to be the embryonic remnant derived from the UBB. This structure is important as it gives rise to calcitonin-producing parafollicular C cells [53]. In the past few years, it has been shown that SCN epithelial basal layer contains a population of $p63^+$ cells, which are thought to be another potential source of stem cells that could (1) contribute to the histogenesis of C cells and follicular cells and (2) participate in repair of thyroid lesions [54–56]. Genetic studies performed on *TTF-1*;*p63*-double null mice showed the existence of another population of cells in the SCN that might be present also in the thyroid of wild-type mice [57]. These cells, called immature cells, present scarce cytoplasm and no clear intracellular organelles or adhesion structures. It is important to mention that these cells do not express *Oct-4* and other TSC markers. Thus, they might be different from the thyroid resident progenitor cells described earlier [57]. Given that immature intrafollicular cells were found sporadically in the newly generated follicles after partial thyroidectomy, it has been proposed that immature stem cells from SCN might contribute to organ regeneration.

## Models for Thyroid Regeneration

Based on what has been discussed so far, we can hypothesize different scenarios for thyroid regeneration, and different stem cell populations contributing to tissue repair according to the size of the injury [17]. According to Kimura's work (Fig. 5.3), Model 1 for thyroid regeneration might occur in a moderate damage, and different stem cell populations have been demonstrated to contribute to tissue repair, including $SCA1^+/BrdU^+$ [7] cells and the immature putative stem cells of the SCN [57].

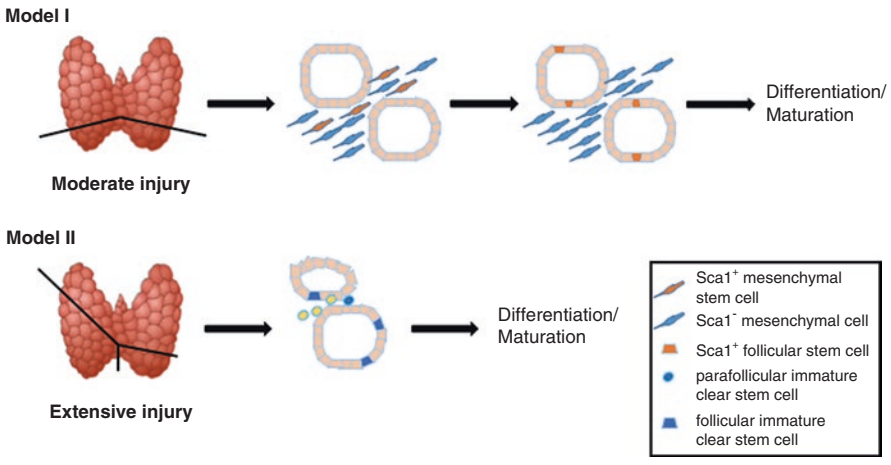


**Fig. 5.3** Proposed models for thyroid regeneration. In a context of moderate injury, $Sca1^+$ stem cells, with mesenchymal or epithelial origin, are involved in regeneration of thyroid follicles. After extensive damage, follicular- and parafollicular-derived immature clear cells, with defined stem cell features, are found to be involved in regeneration of the lesion

It is still under question whether and how these cell populations interact to further sustain tissue repair. However, it is important to mention that SCA1$^+$ cells could have different origins. They can not only be resident thyroid progenitor cells [7] but they could also be bone marrow stem cells [52] that have modified their molecular profile and characteristics after migrating into thyroid tissue.

Model II for thyroid regeneration occurs when the tissue damage is extensive. In this case, the regeneration process needs to be faster in order to maintain organ homeostasis. Thus, stem cell contribution to organ regeneration is unlikely as it requires at least 2–4 weeks to be completed. After semi-total thyroidectomy, calcitonin-producing C cells and follicular cells appear to become immature (clear cells) and start to contribute to thyroid repair.

Interestingly, the two models resemble those that have been hypothesized to be crucial for liver regeneration. In this case, one model describes the contribution of mature liver epithelial cells, while the other one describes the role of liver stem cells [58–60]. Thus, it appears that the mechanisms of tissue regeneration in low-turnover organs are very similar, and involve, according to the extent of the damage, different stem cell populations or epithelial cells, acquiring an immature phenotype [17].

## Concluding Remarks

Thyroid stem cell research is currently a very hot topic, and it has increased our understanding of tissue development and repair. Stem cells and thyroid are two terms that started to be put together just recently, and for different aims. First, the use of ESCs and iPSCs to obtain mature thyrocytes finally leads to the possibility to identify therapeutical strategies that can be used in the treatment of severe hypothyroidism. As discussed earlier, in 15 years, research groups have been able to improve the differentiation protocols in order to obtain acceptable numbers of ESC or iPSC-derived thyrocytes that could restore a thyroid hormone deficit in thyroid mice. This important step is encouraging in the light of the possible application of the same strategies to human patients in the near future. Second, identification of different stem cell population within the thyroid gland led us to better understand the cellular and molecular mechanisms of thyroid regeneration and response to different types of injuries. Definition of the precise steps leading to tissue repair could be extremely useful to define new diagnostic approaches to thyroid diseases.

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# Chapter 6
# Heterogeneity of Pulmonary Stem Cells


**Thomas J. Lynch, Vitaly Ievlev, and Kalpaj R. Parekh**

**Abstract** Epithelial stem cells reside within multiple regions of the lung where they renew various region-specific cells. In addition, there are multiple routes of regeneration after injury through built-in heterogeneity within stem cell populations and through a capacity for cellular plasticity among differentiated cells. These processes are important facets of respiratory tissue resiliency and organism survival. However, this regenerative capacity is not limitless, and repetitive or chronic injuries, environmental stresses, or underlying factors of disease may ultimately lead to or contribute to tissue remodeling and end-stage lung disease. This chapter will review stem cell heterogeneity among pulmonary epithelia in the lower respiratory system, discuss recent findings that may challenge long-held scientific paradigms, and identify several clinically relevant research opportunities for regenerative medicine.

**Keywords** Stem cell · Basal stem cell · Club cell · Myoepithelial cell · Submucosal gland · Obliterative bronchiolitis · Chronic rejection · Ionocyte · Goblet cell · Alveolar type II cell

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## Introduction

The pulmonary epithelium contains multiple distinct functional units with multiple cell types. In the pseudostratified columnar epithelium abundant cell types include: secretory club-like cells, multiciliated cells, and basal cells. Less frequent cell types include: neuroendocrine cells, goblet cells, brush/tuft cells, and the recently discovered ionocytes [1, 2]. Along the cartilaginous tracheobronchial airways, epithelial submucosal glands (SMGs) reside within the airway mesenchyme. SMGs contain secretory mucous and serous cells as well as contractile myoepithelial cells, and glandular ducts contain multiciliated cells as well as basal-like duct cells. The cuboidal epithelium of terminal bronchioles contains secretory club cells, fewer multiciliated cells than those in larger airways, and infrequent airway basal cells. Terminal airways transition into alveoli, which are lined by squamous alveolar type 1 cells and cuboidal alveolar type 2 cells. It is evident that lung epithelial cell types and their fractional distribution in the population change as we move from proximal to distal airways. However, it is also important to acknowledge that stem cells that give rise to these tissues are distinct as well. In this chapter, we discuss the stem cell types within each of these regions and focus on heterogeneity within each population.

Researchers are exploring pulmonary stem cell biology using both human and animal models. Transgenic mouse models allow researchers to study stem cells within their native microenvironment using lineage-labeling approaches. These approaches rely on cell-specific promoter activity to drive expression of Cre recombinase, which provides a spatial selectivity for labeling only specific cell types. Temporal control is accomplished with the use of inducible recombinases such as CreERT2, which is activated with tamoxifen administration. Lineage-tracing experiments have a labeling phase and a fate mapping phase. In the labeling phase, Cre-mediated recombination is usually used to activate the expression of a fluorescent protein. In the fate mapping phase, labeled cells divide and differentiate, and their daughter cells can be followed to establish lineage relationship hierarchies. Lineage labeling experiments can be performed within a normal physiological context to study steady-state turnover or following injury to study regeneration. However, initial labeling specificity is key to interpreting fate mapping results. Cre-drivers used to lineage-label specific cell types must demonstrate specific expression throughout the labeling stage of the experiment. A tamoxifen washout period is often necessary prior to injury to avoid nonspecific labeling of cells that potentially activate the promoter driving Cre as a result of injury. In addition, lineage-labeling cells during developmental windows, when precursor cell identities may still be relatively flexible, could contribute to complexity in interpreting fate mapping results. However, these experiments often provide compelling evidence for the origin of different cell types within a tissue. Overall, despite the constraints of Cre specificity and potential "leakiness" of some Cre-driving promoters, this strategy of stem cell lineage labeling remains to be an accepted standard approach in the field.

In vivo lineage-labeling studies have led to important discoveries of how region-specific stem cell niches maintain quiescence or promote proliferation. For example,

a subtype of Axin2-CreERT2 lineage-labeled alveolar type 2 (AT2) cells is regulated by a single fibroblast cell niche that can secrete different Wnt ligands to promote either a quiescent or a proliferative state [3]. Moreover, fluorescent reporters can enable isolation of lineage-traced cells by fluorescence-activated cell sorting (FACS) for characterization in vitro. For example, in vitro colony formation efficiency and differentiation assays can be useful for experiments designed to test the stemness of various cell types within a highly controlled in vitro context (e.g., in the presence of specified growth factors). Within the mammalian pulmonary system, lineage-labeling approaches have been primarily limited to mouse models, and this may be a limitation for studying biology that is not conserved between mice and humans. However, alternative animal models (like the ferret) are becoming increasingly available for lineage-labeling [4]. Tracking lineage-labeled stem cells under native conditions in vivo or in a controlled environment in vitro can help answer the questions regarding proliferative and differentiation capacity as well as self-renewal capability of the stem cells of interest.

Lineage relationships can also be inferred using high-dimensional single-cell datasets. For example, single-cell mRNA sequencing has been used to study human cell lineage hierarchies [2, 5–9]. Powerful new bioinformatic approaches have even led to the novel discovery of a rare airway cell type—the ionocyte—which amounts to less than 1% of cells in the tracheobronchial surface airway epithelium (SAE), yet accounts for more than half of its CFTR mRNA expression [1, 2]. This discovery has critical implications for cystic fibrosis research considering that mutations in CFTR primarily define the pathology. In addition, single-cell approaches can even delineate complexity within what was previously considered a homogeneous cell population. For example, Montoro et al. [1] discovered that in mice there may be distinct subsets of tuft and goblet cells. However, discoveries using single-cell approaches may be confounded by the fact that cells must be enzymatically isolated from tissue samples and analyzed away from their physiological compartment. Thus, experimental in vivo validation is necessary. To address this challenge, sequencing approaches can also be coupled with in vivo lineage analysis to add temporal and spatial dimension into sequencing experiments. Both lineage labeling and sequencing experiments have accelerated the field of stem cell research, and in the modern age of “Big Data,” combining these technologies has expanded our knowledge even further.

## Basal Cells

Basal cells serve as multipotent stem cells in the SAE of conducting airways (Fig. 6.1). They reside along the airway basal lamina where they contact multiple luminal cell types and play an important role in homeostasis due to their ability to self-renew and differentiate into various luminal cell lineages [10, 15, 16]. Basal cells can also regenerate the airway epithelium following injury [17–19]. However, basal cell regeneration may be insufficient in some pathologic states. For example,

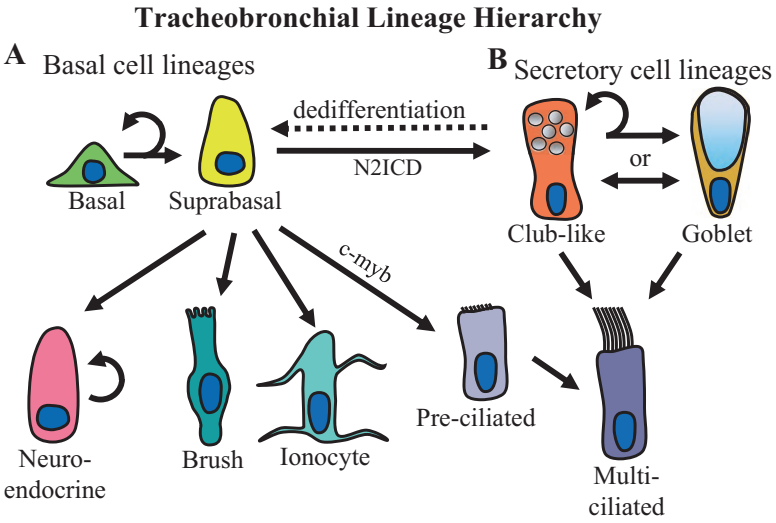


**Fig. 6.1** Lineage relationships of the surface airway epithelium in the trachea and bronchi. (**a**) Basal cells self-renew and give rise to all of the cell types present in the surface airway epithelium. Basal cells differentiate into luminal lineages through an intermediate suprabasal cell state [10]. Basal cells can give rise to self-renewing neuroendocrine cells in addition to brush (or tuft) cells and ionocytes [1, 2]. In addition, a subset of basal cells that display intracellular Notch2 activation (N2ICD) gives rise to secretory cells, whereas basal cells that express low levels of c-myb are able to lineage commit toward multiciliated cells [11]. Recent, single-cell RNA sequencing experiments have suggested that basal cells give rise to multiciliated cells through a differentiation of an intermediate population of pre-ciliated cells [12]. If basal cells are ablated, club-like cells can renew basal cells through a process of dedifferentiation (dashed line) [13]. (**b**) Secretory cells in large airways consist of club-like cells and goblet cells. Club-like cells are similar to bronchiolar club cells in that they express proteins commonly used to identify bronchiolar club cells, such as SCGB1A1 (a.k.a. club cell secretory protein); however, unlike bronchiolar club cells, large airway club-like cells also express mucins such as MUC5B and MUC5AC [14]. Secretory goblet cells have a distinct goblet or cup-like morphology but express many of the same phenotypic markers that club-like cells express; thus, it is unclear if club-like cells and goblet cells are distinct secretory cell fates or if a single population of secretory cells fluctuates between displaying a club-like or goblet cell morphology depending on environmental factors. Multiciliated cell renewal is primarily accomplished by club-like progenitors at steady state [10] and following injury [1, 12]. In addition, it has recently been argued that goblet cells may act as a differentiation intermediate between club-like secretory cells and multiciliated cells [12]

chronic inflammation may eventually overcome basal cell regeneration capacity leading to basal stem cell depletion [20–22]. Thus, we must expand our knowledge of basal cell biology to better understand lung diseases and to refine novel basal cell-targeted regenerative therapies.

At steady state, airway basal cells are most commonly identified by TP63 (p63) and KRT5 expression. Expression of many keratins may help distinguish luminal epithelial cells from basal cells as well as subtypes of basal cells from one another (Table 6.1). For example, Watson et al. [10] discovered that basal cells that express KRT8 are fated to differentiate into luminal cell types. Recent single-cell RNA

**Table 6.1** Keratin expression in airway epithelial cells

| Cell type | Keratins |
|---|---|
| *Basal cells* | |
| Basal | 5[A,B,C], 6A[A,B], 6B[A,B], 6C[A], 14[A,B], 15[A,B,C], 17[A,B] |
| Suprabasal | 4[B], 13[B], 16[B], 23[B] |
| *Luminal cell types* | |
| Secretory | 7[C], 8[B], 18[C], 19[C] |
| Secretory precursor | 4[B], 13[B] |
| Goblet | 7[B], 8[B], 18[B], 19[B] |
| Multiciliated | 8[B], 42P[C], 80[C] |
| Preciliated | 8[B], 10[B] |
| Ionocyte | 7[A], 8[A], 18[A], 80[A] |

A: Plasschaert et al. [2]; B: Ruiz Garcia et al. [12]; C: Zuo et al. [7]

sequencing studies have begun to better highlight the expression of various keratins in order to characterize the heterogeneity of different airway cell types including basal cells (Table 6.1) [2, 7, 12]. For example, several studies have found that KRT4 and KRT13 are differentially expressed in a transitional suprabasal cell population as basal cells differentiate into secretory cells [1, 2, 12]. However, in mice, Krt4/Krt13 may demarcate a heterogeneous population of squamous-like cells found within discrete nonciliated regions of the tracheal epithelium (see the discussion on "Hillocks") [1]. In addition, KRT14 expression is enriched in mitotically active basal cells [23–25]. In the steady state mouse trachea, Krt14 marks a relatively infrequent subset of unipotent and self-renewing basal cells (<20%), but after injury, regenerative Krt14+ basal cells proliferate and become multipotent for all of the major cell lineages of the surface epithelium [26, 27]. KRT14 interacts with KRT5 to establish a structural network of intermediate filaments needed for proliferation [28]. Taken together, composition of keratins can be assessed to distinguish different subpopulations of basal cells, including actively proliferating cells.

A short-term surge in proliferation of basal cells may come at a long-term cost. Evidence supporting this notion in the airway comes from studies of infrequently dividing progenitors. Multiple colony formation efficiency studies have found that infrequently dividing basal cells are highly clonogenic in vitro [27, 29]. Heterogeneity within the stem cell pool to maintain both frequently dividing and infrequently dividing progenitors provides a mechanism of population-level regulation for balancing the capacity to regenerate cells damaged by injury while also safeguarding long-term proliferative potential (Fig. 6.2).

In disease, however, chronic inflammation may lead to proliferative exhaustion of airway basal cells by overstimulating the expansion of KRT14+ basal cells, leading to failed long-term epithelial regeneration and fibrosis. For instance, in the context of chronic rejection following lung transplantation, p63 + KRT5+ basal cells are depleted while KRT14+ basal cells expand in both human patients and in a

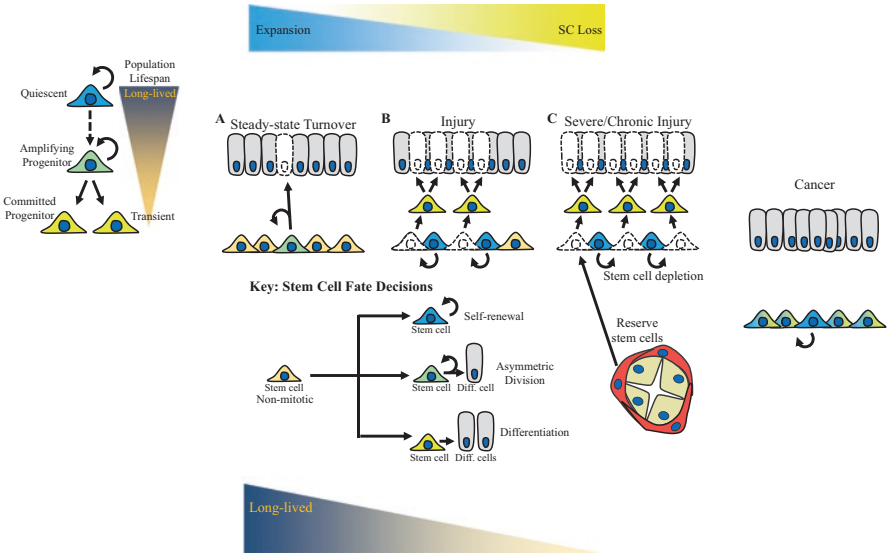


**Fig. 6.2** Proliferative stress requirements of stem cells at steady state and following moderate and severe injury. (**a**) At steady state, the majority of mitotic stem cells undergo asymmetric cell division (green cells), which is capable of compensating for cell loss during a low rate of turnover without depleting the stem cell population. (**b**) Following injury, basal cells may undergo symmetric differentiation (yellow cells) in order to more rapidly compensate for the loss of many cells during a higher rate of turnover. Stem cells that undergo symmetric differentiation are removed from the stem cell pool, but other stem cells may compensate for this loss through symmetric self-renewal (blue cells). (**c**) If the proliferative stress is sustained long enough or the injury is severe enough, the capacity of the stem cell population to self-renew is insufficient to compensate for the loss of stem cells through differentiation. In this case, reserve stem cells from neighboring regenerative stem cell pools may attempt to compensate for stem cell loss. However, in the case of chronic injury, stem cells are ultimately depleted leading to fibrosis and disease

ferret transplantation model. This shifting basal cell phenotype accompanies a decline in clonogenicity and correlates closely with histologic severity of rejection and progression of obliterative bronchiolitis (OB) [22]. Ghosh et al. [21] discovered a similar decline in p63 + KRT5+ basal cells in chronic obstructive pulmonary disease (COPD) and found that an overall decline in basal cells may identify a subset of prediagnostic, non-COPD patients at heightened risk of developing COPD (pending validation by prospective analysis). Meanwhile, others saw that cigarette smoke may induce expansion of KRT14+ basal cells in COPD-affected patients [30, 31]. Interestingly, in idiopathic pulmonary fibrosis (IPF) it appears that airway basal cells migrate from conducting airways to alveolar regions, where they acquire an aberrant differentiation program and may contribute to fibrosis [6, 32]. Factors that influence the flux states of basal cell heterogeneity within the bounds of normal physiology, during relatively mild perturbations of short-term injury, and during pathoprogressive states of disease remain an important research frontier (Fig. 6.2). Establishment of disease progression markers can become a clinically useful early

diagnosis tool of pathologies associated with injury-dependent stem cell depletion, such as OB and COPD.

Researchers are actively investigating the therapeutic potential of engrafting basal cells as a therapy to treat lung diseases. Isolation and cultural expansion of patient-derived basal cells has enormous potential. For example, basal cell therapy has the potential to correct cystic fibrosis (CF) lung disease, which is caused by mutation(s) in the CF transmembrane conductance regulator (CFTR). CF patient basal cells can be harvested and the CFTR mutations in these cells corrected in vitro before the cells are delivered back into the afflicted patients. Recent cell transplantation studies done in mice have shown promising results using this approach and have provided benchmarks for researchers to use toward developing these therapies for humans. For example, based on data extrapolated from mouse studies, research has estimated that a therapeutic dose of 60 million cells is needed for effective treatment [33]. Obtaining such a large number of cells without detriment to cell viability is not trivial, and until recently, it was not clear how well CF cells would grow. However, Hayes Jr. et al. recently demonstrated several landmark findings that promote the feasibility of basal cell therapy for the treatment of CF. For example, both non-CF basal cells as well as CF basal cells can be amplified enough to achieve the estimated therapeutic dose (over 60 million cells), and these culturally expanded basal cells retain the capacity to differentiate into secretory and ciliated cell types [34]. Still, many critical questions remain. For example, barriers to cell engraftment must be identified and overcome. In mice, for example, researchers found that it was critically important to injure the airway epithelium before transplanted cells could efficiently engraft [33]. However, Hayes Jr. et al. [34] propose an interesting suggestion to administer basal cells to lung transplant recipients to prevent primary graft dysfunction, which causes profound epithelial sloughing in 25–29% of lung allografts within 3–14 days following transplantation [35]. However, reaching the estimated therapeutic dose of 60 million cells within 3–14 days is currently pushing beyond technological capabilities. It is feasible, however, for clinicians to store culturally expanded cells obtained from patients by brush biopsy well prior to the lung transplantation procedure; thus, it may be necessary to interrogate how cryo-storage affects basal cell viability and performance. In aggregate, the therapeutic potential of basal cell therapy to treat human disease is poised for clinical usage within the coming years. However, basic questions regarding human airway basal cell biology still linger.

Many aspects of basal cell biology have been clarified using mouse models. However, there are species-specific differences in the biology and distribution of basal cells between mice and larger mammals, including humans. The importance of some of these differences remains unclear. For example, in the mouse lung, the distribution of Krt5 + p63+ basal cells is largely limited to the trachea and bronchi, whereas in human lungs, these cells are distributed throughout conducting airways down to the terminal bronchioles [17, 18]. It might be due to the size of the lung: the bigger it is, the more basal stem cells the organism would need to maintain it. It is still unclear if and how basal cells differ across airway levels in terms of various properties such as proliferative rate or differentiation capacity. However, Okuda

et al. [14] demonstrated that large airway epithelial (LAE) cells and small airway epithelial (SAE) cells held an architecture and protein expression phenotype that were indicative of their site of origin upon establishing well-differentiated air–liquid interface (ALI) cultures. This suggests that basal cells retain a site-specific imprint on their multipotency in vitro. Therefore, animal models that are similar to humans in their basal cell distribution throughout the respiratory tree may better recapitulate critically important mechanisms that control stem cell dynamics than rodent models would. For example, ferret airway basal cell distribution resembles human airways, and even the distal airways in the ferret have basal cells similar to that in human lungs [22].

## Submucosal Gland Progenitors

Submucosal glands (SMGs) are specialized epithelial invaginations of the superficial epithelium located throughout human cartilaginous airways. SMGs secrete fluids containing hundreds of proteins involved in maintaining sterility of the airway including multiple gel-forming mucins, antimicrobials, surfactants, immune regulators, and many other enzymes [36]. Structurally, submucosal glands are made of compound tubuloacinar epithelia containing four domains: ciliated ducts, collecting ducts, mucous tubules, and serous acini [37]. Ciliated ducts are contiguous with the SAE and contain similar cell types. For example, both multiciliated cells and secretory cells are present in ciliated ducts, and duct cells are similar to SAE basal cells. Although several studies have suggested that glandular duct cells are distinct from basal cells that reside in the superficial epithelium [38–40], duct cell-specific lineage analysis is needed to further support the hypothesis that ductal cells are truly distinct from basal cells. Collecting ducts contain a simple columnar epithelium that is poorly defined and may vary in different airways and between different species. Collecting ducts also tend to be larger in proximal airways than in distal airways and are absent in mice [36]. Branching tubules of mucous cells terminate with bulbous acini of serous cells that comprise the most distal secretory components of the glands [41]. In addition, contractile myoepithelial cells line the mesenchymal surfaces of glands except for the ducts [42, 43], and the glandular epithelium is maintained by myoepithelial stem/progenitor cells (Fig. 6.3).

Several studies have shown that SMGs are a niche for slowly cycling stem cells that can long-term retain pulsed nucleotide analogs and/or tetracycline-inducible H2B-GFP [19, 27, 29, 47, 48]. Compared to nearby SAE basal cells, glandular progenitors exhibit a greater proliferative capacity in colony formation efficiency assays grown at the air–liquid interface and in denuded tracheal xenografts [27]. Furthermore, in vitro clonal analysis has revealed that glandular progenitors are able to differentiate into both SAE and SMG cell types [27, 39], suggesting that glandular stem cells may contribute to the renewal of both the surface epithelium and SMGs. Moreover, lineage-labeling of myoepithelial cells in mice has revealed that myoepithelial cells are multipotent stem cells that can self-renew and give rise

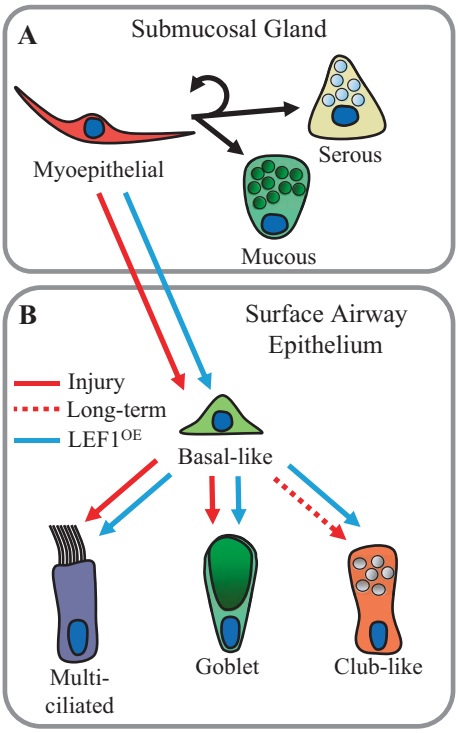


**Fig. 6.3** Submucosal gland myoepithelial cell lineages at steady state and following injury. The tracheobronchial airways possess epithelial submucosal glands that secrete mucous and serous fluids that help regulate mucociliary clearance on the airway surface. (**a**) Myoepithelial cells within the glands are self-renewing stem cells. At steady state, myoepithelial cells can differentiate into glandular serous and mucous cells [44–46]. (**b**) Following injury to the airway surface epithelium by naphthalene or $SO_2$ (red arrows), myoepithelial cells activate a Lef1 transcriptional program that promotes their migration to the airway surface where they are capable of establishing long-lived basal cell progenitors [45, 46]. In addition, ectopic overexpression of LEF1 (blue arrows) is sufficient to initiate this process without injury. Initially, following injury, myoepithelial-derived basal-like cells are less likely to renew into SCGB1A1+ secretory club-like cells and have a lineage bias toward multiciliated and secretory goblet cells (mucus-positive cells). However, with increasing time after injury and distance away from the submucosal glands, myoepithelial-derived basal-like cells become increasingly able to differentiate into SCGB1A1+ secretory club-like cells (dashed red line). In addition, ectopic overexpression of LEF1 accelerates this process [45]

to glandular mucous cells, serous cells, and duct cells during development and at homeostasis [44] (Fig. 6.3). Recently, it was shown that following severe injury to the SAE of both mice and pigs, myoepithelial cells act as reserve stem cells capable of repopulating surface basal cells and other SAE cell types by extension [45, 46]. Myoepithelial cells can repopulate surface basal cells with lasting regenerative capacity. However, at least in mice, myoepithelial-derived basal cells are less likely to generate surface secretory cells that express Scgb1a1 or Scgb3a2 than basal cells

that normally reside in the surface epithelium. On the other hand, myoepithelial-derived basal cells were likely to give rise to multiciliated cells and Muc5B secretory cells [45]. Interestingly, although MUC5B is expressed in both glandular mucous cells and SAE secretory cell types, SCGB1A1 is not expressed in SMGs [14]. This may reflect a propensity to retain a gland-like lineage bias and may help provide mechanistic insight into epithelial remodeling that occurs with recurrent epithelial injury. However, it is just as likely that glandular stem cells may play a more significant role in human airways since SMGs are much more abundant in human airways than in mouse ones. The evidence outlined above makes a compelling case that myoepithelial cells (MEC) can act as reserve multipotent stem cells that can contribute to repair of surface airway epithelium (SAE); however, the regenerative contribution of MECs in the SAE might be skewed toward multiciliated cells and Muc5B+ secretory cells.

Glands in mice are confined to the proximal trachea extending from the cricoid cartilage to no further than the first few cartilage rings of the proximal trachea [19], although age, gender, and mouse strain may contribute to variations in gland size and abundance [49–51]. On the other hand, ferrets, like humans, have SMGs throughout their cartilaginous airways [52]. Recently, it has been shown that SMGs are destroyed in transplanted lungs in both human and ferret allografts as they develop a form of chronic lung allograft dysfunction (CLAD) known as obliterative bronchiolitis (OB). Depletion of the SMG stem cell niche occurs, as allografts progressively lose clonogenic surface basal cells in both distal and proximal airways [22]. The existing paradigm regarding pathoprogression of OB has suggested that disease pathology is limited to distal airways. For example, some researchers have suggested that bronchiolar club cells are selectively affected in OB [53], and indeed, there is evidence of bronchiolar club cell loss in OB lungs [22, 53, 54]. However, recent findings in human and ferret allografts have suggested that the depletion of stem cells occurs more globally in both large and small airway basal cell populations as well as in reserve stem cell niches of SMGs [22]. This may indicate that epithelial remodeling occurs as regenerative stem cell populations are depleted through a process of proliferative exhaustion. Moreover, murine myoepithelial-derived reserve basal cells may not generate SCGB1A1-expressing club cells as readily as do surface-resident basal cells [45], and if this biology is conserved in ferrets and humans, it may suggest a possible mechanism for club cell loss with OB. Depletion of surface epithelial basal cells and reserve stem cells from SMGs might be a root cause of OB. Thus, the ferret is an excellent transplant model to study this pathology because the distribution of airway basal cells and submucosal glands in ferrets is strikingly similar to that of humans, and the progressive loss of these stem cell niches is also similar.

## Secretory Cells

Historically, airway secretory cells have been segregated into four distinct cell types based on their morphology and ultrastructure: club cells, goblet cells, serous cells, and pulmonary neuroendocrine cells (PNECs) [55, 56]. However, since these early morphometry studies, PNECs have largely been disassociated from other secretory cell types, and we will briefly discuss their contribution to pulmonary stem cell biology in a later section. In humans, serous cells are mainly present in fetal airways and in adult submucosal glands, although they have been occasionally observed in adult bronchioles by transmission electron microscopy [57]. Their lineage relationships with other cell types have not been explored in recent years. In this section, we will discuss characteristics and controversies surrounding club cells and goblet cells as secretory cell types placing an emphasis on population heterogeneity and their contribution to different epithelial lineages.

Club cells have been identified as a major progenitor cell type of the airway epithelium (Figs. 6.1 and 6.4) [10, 68]. Club cells are commonly identified by their expression of SCGB1A1 (aka club cell secretory protein or CCSP), but classically, they must also be dome-shaped columnar cells lacking periodic acid-Schiff (PAS) staining, and by this definition, Boers et al. found that CCs are largely restricted to terminal and respiratory bronchioles in healthy human airways [69]. By contrast, goblet cells are mucus-producing cells prototypically identified by their "flask-shaped" morphology and prominent PAS-reactive cytoplasmic vacuoles, and by this histologic definition, Boers et al. [69] found that goblet cells are more abundant in proximal airways and are largely absent from terminal and respiratory bronchioles. However, these narrow morphological definitions of secretory club cells and goblet cells do not completely describe the diversity of secretory cell types nor can club cells be specifically identified by SCGB1A1 expression alone.

Indeed, club-like secretory cells that do not fit the classical club cell description but do express SCGB1A1 can be found throughout the human conducting airways (Fig. 6.5). For example, Boers et al. [69] described "indeterminate" SCGB1A1-expressing, PAS-positive cells present in human bronchi and to a lesser extent in distal nonterminal and terminal bronchioles. In normal/healthy human airways, SCGB1A1 marks club-like cells of the superficial epithelium from the trachea to the terminal bronchioles, and SCGB1A1 expression is absent only from submucosal glands. Notably, most club-like cells also express the mucins MUC5B and/or MUC5AC at all levels of the respiratory tree except for in terminal bronchioles where mucins are not expressed. In addition, cells with typical goblet cell morphology were rarely observed in healthy human lungs that were selected using rigorous inclusion criteria, which included only nonsmoking donor lungs with minimal exposure to mechanical ventilation [14]. However, various aeroallergens and toxins, viral infections, and even mechanical stresses such as hyperventilation can induce inflammatory mediators that promote goblet cell metaplasia and hyperplasia [70–73]. Considering that the airway surface epithelium is constantly exposed to the external environment, it is unclear to what extent goblet cell abundance and/or

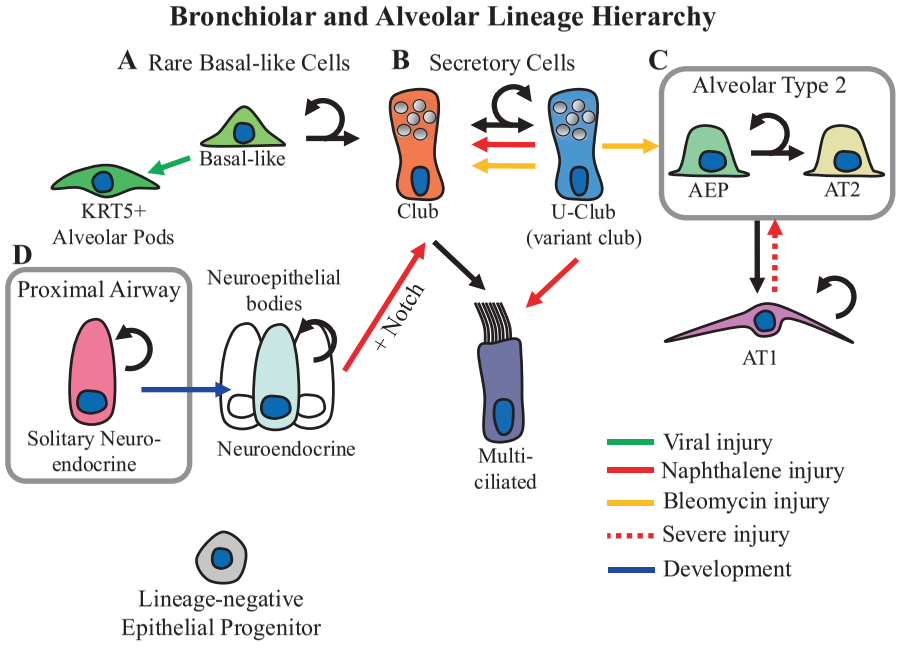


**Fig. 6.4** Small airway bronchiolar and alveolar lineages. (**a**) Basal-like progenitors are rare in small airways, yet they play an important role in restoring the structural integrity of alveolar epithelium after catastrophic viral injury (green arrow) by generating KRT5+ alveolar pods [58–60]. Recent evidence also suggests that small airway basal-like cells give rise to bronchiolar club cells [7]. (**b**) A rare subtype of club cells expressing Uroplakin3a (U-Club cells) is capable of renewing the larger club cell population at steady state. Following naphthalene injury (red arrow), U-club cells can also give rise to multiciliated cells, and following bleomycin injury (yellow arrow), U-Club cells renew alveolar type 2 (AT2) cells [61]. (**c**) The alveolar epithelium consists of AT2 cells and alveolar type 1 (AT1) cells, and AT2 cells are self-renewing and can lineage commit to AT1 cells [62]. Recently, a population of highly clonogenic stem cells called alveolar epithelial progenitors (AEPs) has been identified within the AT2 cell population [63]. In addition, AT1 cells are able to dedifferentiate following severe injury (dashed red arrow) into AT2 cells [64, 65]. (**d**) In addition, small airways harbor neuroepithelial bodies, which consist of clusters of self-renewing neuroendocrine cells. Forced induction of notch signaling in the context of injury induces neuroendocrine to club-like cell transdifferentiation [66]. During development (blue arrow), solitary neuroendocrine cells residing in proximal airways migrate to small airway branch points where they organize into neuroepithelial bodies [67]

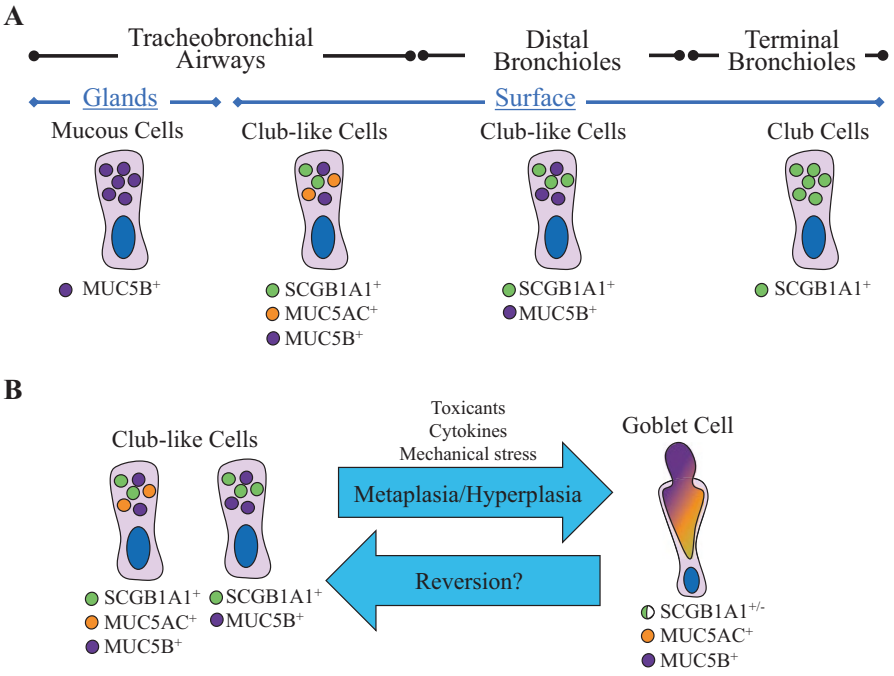


**Fig. 6.5** Nuances among pulmonary secretory cells. Recent studies have provided compelling data for anatomical specificity of secretory cell types in the human lung. (**a**) Based on the expression of MUC5B, MUC5AC, and SCGB1A1 (CCSP), there are at least four distinct secretory cell types. Submucosal glands possess MUC5B-expressing secretory mucous cells. In proximal large airways, secretory club-like cells express MUC5B, MUC5AC, and SCGB1A1. In small bronchial airways, secretory club-like cells express MUC5B and SCGB1A1 but not MUC5AC, and in terminal bronchioles, secretory club-like cells express only SCGB1A1 [14]. (**b**) Given that club-like cells express many of the phenotypic characteristic of goblet cells, it may be necessary to experimentally challenge the paradigm that goblet cells are indeed a divergent cell type rather than simply being a hypersecretory state of club-like cells. For example, if goblet cell metaplasia/hyperplasia is readily reversible back to a club-like cell phenotype, this may suggest that goblet cells and club-like cells are the same population of secretory cells

morphology can fluctuate within the bounds of normal physiology. For example, this may suggest that the classically defined goblet cell morphology may in fact reflect an elastic response to various forms of harmful environmental stimuli. On the other hand, changes in goblet cell abundance are a hallmark of many chronic respiratory diseases, and goblet cell hyperplasia is a shared feature of asthma [74] and COPD [75], whereas surface goblet cells are hypertrophic in cystic fibrosis (CF) [76]. Taken together, these data may suggest either that goblet cells are a distinct cell fate or that club-like cells may fluctuate in (and perhaps out of) a goblet cell state. Club-like cell plasticity has been observed in dedifferentiating club-like cells that regenerate airway basal cells after basal cell ablation [13]. A better understanding of the lineage relationship between club-like cells and goblet cells may lead to

important discoveries regarding disease progression while also advancing basic scientific knowledge regarding the continuum between flexible cell states and committed cell fates. For example, are goblet cells simply stress-induced hyperactive club-like cells or are they a terminally committed cell lineage that is distinct from club-like cells?

Furthermore, it is unclear to what extent bronchiolar club cells differ from club-like cells from other regions of the lung. Bronchial club-like cells and bronchiolar club cells may share a similar origin. KRT5+ basal cells have been identified as progenitors for both club-like cells and club cells based on bioinformatic analyses of single-cell transcriptomes of human bronchial epithelial cells (HBECs) and small airway epithelial cells (biopsied from the 10th- to 12th-generation airways) [2, 7]. Interestingly, basal cells isolated from either large airways or small airways generated well-differentiated ALI cultures that contained secretory cells that phenotypically mirrored the in vivo region-specific cell types [14]. Taken together, this suggests that classically defined club cells in human terminal bronchioles and club-like cells throughout the rest of the tracheobronchial airways share many similar characteristics but may in fact be distinct subtypes of airway secretory cells.

Support for the notion that club cells and club-like cells retain lineage-specific programs has also been demonstrated in mice, and it was hypothesized that this region-specific programming ensures that cellular composition is restored to maintain healthy functionality [77]. How finely are airway progenitors defined by region specificity, and what are the molecular mechanisms responsible for this, remains to be discovered. Perhaps, most importantly, more research is needed to investigate whether this semi-predetermined state of stem cells can be overcome in the interest of developing efficient cell-based therapies.

Airway secretory cell heterogeneity has been reported in many studies of human and other species. For example, ultrastructural studies have reported the presence of club cells within the upper airways of many mammalian species [78–81]. However, some groups have argued that club cells cannot be reliably identified based solely on their ultrastructure, as club cells share no definitive features between different species, and there may be a considerable variability within a single animal subject [82]. This heterogeneity may be critically important for maintaining normal physiology. The larger club cell population contains subsets of cells with variable expression of different detoxifying enzymes such as members of the cytochrome P450 family. Cytochrome P450 enzymes oxidize multiple small-molecule substrates and therefore can produce cytotoxic intermediates, which is the primary reason for injury after naphthalene exposure. Variant club cells that do not express Cyp2f2—a member of the cytochrome P450 family—are resistant to naphthalene and can regenerate the bronchiolar epithelia after injury [83–85]. Recently, a subset of variant club cells that express Upk3a (U-club cells) was reported to contribute to homeostatic renewal of airway epithelial cells and to be able to regenerate alveolar type 1 (AT1) and alveolar type 2 (AT2) cells following bleomycin injury in mice [61]. It is thus evident that heterogeneity of airway secretory cells contributes to the differential ability of these cells to repair various types of injury. Research that helps to delineate the molecular mechanisms that expand progenitor cell potency and

differentiation potential may help improve the likelihood that regenerative cell-based therapies can become a mainstream treatment option for acute or chronic airway injuries.

## Pulmonary Neuroendocrine Cells (PNECs)

PNECs can exist as solitary cells within proximal airways but in distal airways they are mostly found within clusters called neuroepithelial bodies (NEBs). NEBs may be further specified by their anatomical organization. Nodal NEBs are located at airway branching points, whereas internodal NEBS are located between branching points [67]. Mature NEBs are basally innervated by vagal nerve afferents and serve as an important interface between the central nervous system and the conducting epithelium to regulate breathing [86]. PNECs may have many functions effecting regeneration, including an important role in regulating U-club cells organized around NEBs, and after naphthalene injury, epithelial regeneration by U-club cells and possibly other variant club cells occurs preferentially around nodal NEBs [61, 85, 87]. However, PNECs may also directly participate in regeneration following naphthalene injury coupled with ectopic activation of Notch signaling by transdifferentiating into club cells (Fig. 6.4) [66]. This plasticity may suggest that PNECs have the capacity to serve as potential reserve stem cells for small airways in a similar way that glandular myoepithelial cells do for the superficial epithelia in larger airways. Additionally, small cell lung cancers are thought to arise from PNECs [88–90]. However, it may be difficult to appreciate heterogeneity within the PNEC population given their relative rarity among other cell types. However, one conspicuous question that remains is how solitary PNECs in proximal airways differ from those found within small airway NEBs. However, it has already been discovered that during development, solitary PNECs in proximal airways migrate toward distal airways where they establish static nodal NEBs but not internodal NEBs [67, 91]. This divergent developmental ontogeny between nodal and internodal NEBs may point toward a provocative difference in the physiological function and regenerative capacity of PNECs throughout the conducting airway tree. Overall, PNECs remain relatively understudied in the context of pulmonary stem cell biology, although there is some evidence that PNECs may function as a reserve stem cell population.

## Alveolar Progenitors

Lung alveolar epithelium is composed of squamous alveolar type 1 (AT1) cells and cuboidal alveolar type 2 (AT2) cells. AT2 cells are the predominant stem cell for alveoli, as they can both self-renew and generate AT1 cells (Fig. 6.4) [62, 92, 93]. However, AT1 cells may also have a limited capacity to proliferate after injury [64,

65]. AT2 cells are typically identified by their expression of SFTPC, ABCA3, and/or LAMP3, while AT1 cells can be marked with PDPN, AGER, AQP5, and HOPX [94]. Both AT1 and AT2 cells are capable of at least some regenerative proliferation, but the bulk of this function is executed by AT2 cuboidal alveolar cells.

Recently, a subset of AT2 cells (SFTPC+/HOPX-) that express AXIN2 at steady state has been identified as a highly regenerative and clonogenic alveolar stem cell population. These airway epithelial progenitors (AEP) are divergent from other AT2 cells, possessing a distinct chromatin structure and transcriptomic profile, and AEPs can be isolated from human lungs by their expression of the surface marker TM4SF1 [63]. Unlike SOX2-derived TP63+ KRT5+ airway basal-like progenitors that primarily restore epithelial barrier function by forming "KRT5+ alveolar pods" following influenza injury [58], AEPs restore the alveolar epithelium by regenerating both AT1 and AT2 cells (Fig. 6.4) [63]. At steady state single fibroblasts directly neighboring AEPs maintain their "stemness" with paracrine Wnt signals that act to prevent AEPs from transdifferentiating into AT1 cells. However, after injury, AEPs exit their stromal niche to engage in regeneration [3]. Noncell autonomous signals that steer lineage commitment of AEPs into AT1 or AT2 cells may be elucidated in forthcoming studies. Further understanding of processes that regulate this biology will bring an efficient cell-based therapy to repair alveolar damage within reach. Likewise, given that AEPs have only recently been discovered, it is still unknown how AEPs are affected by disease or contribute to disease such as cancer. Interestingly, in a previous murine adenocarcinoma study that induced KRAS mutations in AT2 cells, only a rare subset of AT2 cells initiated tumors [92]. Whether or not these tumor-initiating AT2 cells are the newly appreciated AEPs remains to be seen. Taken together, this emerging body of research on AEPs suggests that this specific subset of AXIN2-positive AT2 cells might be the driver of "stemness" in this population.

In addition to AT2 cells, AT1 cells may also have a limited capacity to proliferate under the right conditions. Lineage-labeled Hopx+ AT1 cells were able to proliferate and generate AT2 cells following partial pneumonectomy and also established colonies in 3D culture [64]. Additionally, HOPX expression was dynamically downregulated both in a mouse model of pulmonary fibrosis induced by bleomycin injury and in human IPF lungs. Therefore, HOPX expression may promote progenitor cell quiescence at homeostasis and work to restrain excessive proliferation following injury [65].

## Hillocks

Morphologically, hillocks were recently described in the steady-state mouse trachea as distinct structures with a stratified epithelial architecture lacking multiciliated cells and demarcated by Krt4 and Krt13 expression [1, 2]. Hillocks contain specific basal (Trp63 + Krt13+) and club (Scgb1a1 + Krt13+) cell types, which have a transcription profile that is distinct from those of other tracheal cells [1]. As to their

function within the mouse trachea, Montoro et al. [1] offered evidence that hillock cells have heightened turnover and increased expression of genes involved in squamous barrier function and immunomodulation. Published in a separate manuscript at the same time, Plasschaert et al. corroborated the existence of a distinct population of Krt4+/Krt13+ cells in mouse tracheal epithelia and found that KRT4/KRT13 expression defined a major axis of heterogeneity within the basal cell population of culturally expanded and differentiated human bronchial epithelial cells. However, Plasschaert et al. [2] interpreted this population of KRT4+/KRT13+ cells to be an intermediate population between basal stem cells and differentiated luminal secretory cells. To ascertain if hillocks are metaplastic zones in flux or represent a distinct niche, further work is needed to better understand their origin and purpose. In addition, the question of how hillocks relate to human airway epithelial physiology at steady state cannot yet be clearly answered because this structure has not been described outside of the mouse trachea.

## Conclusions

In this chapter, we discussed how pulmonary stem cell types differ depending on their anatomical compartment of origin, how heterogeneity within each population may preferentially give rise to various differentiated cell types, and how the type of injury and its severity may affect the regenerative process. Different compartments within the lung have their own stem cell niches. The superficial epithelium of conducting airways is maintained by basal stem cells and to some extent is also maintained by club-like cells in proximal airways but in terminal bronchioles, it is maintained by club cells. Submucosal glands are maintained by myoepithelial cells that can also act as reserve stem cells for the superficial airway epithelium in the context of severe injury. Hillocks of KRT4+/KRT13+ cells are a novel structure found in the mouse trachea and may potentially represent yet another distinct stem cell niche maintained by hillock-specific progenitors. A subset of PNECs organized within nodal NEBs at airway bifurcations may also contribute to epithelial renewal as a reserve stem cell niche for distal airways. Finally, the alveolar epithelium is maintained by a population of cuboidal AT2 cells that contains a subpopulation of quiescent AEP stem cells.

Stem cells renew differentiated cells that are normally present within their respective compartments at steady state, and they may also expand more rapidly following an injury to compensate for increased cell death. Additionally, in case of severe or chronic injury reserve stem cells, such as myoepithelial cells in submucosal glands, they can migrate outside of their niche and aid in the repair of other tissues such as the surface airway epithelium. However, the reparative contribution of reserve stem cells from alternative compartments may skew the epithelial population composition toward that of the compartment where the reserve stem cell originated. This is likely due to epigenetic programming that is partially retained within reserve stem cells. However, with time reserve myoepithelial stem cells become

increasingly similar to native surface basal cells as they migrate away from their glandular origin, and this process can be accelerated with overexpression of LEF1 in myoepithelial cells. From a therapeutic standpoint, determination and possible modification of these epigenetic marks might be an important step toward development of stem cell supplementation therapies.

Another important aspect of stem cell biology to consider is their long-term ability to self-renew. Impaired self-renewal of stem cells is a hallmark of OB and COPD; therefore, research on what drives this stem cell niche depletion/exhaustion is highly clinically relevant considering that most lung transplantation patients develop OB within 5 years of the surgery [95]. If regeneration is accomplished not by the region-specific stem cell but rather by a reserve stem cell population from an alternative compartment, then the function of the newly repaired epithelia may by impaired. Epithelium that is repaired by a reserve stem cell may have a slightly different composition that is skewed toward the compartment where reserve stem cells originated. For example, regeneration of small airway epithelium by reserve stem cells that migrated from large airways may lead to an overabundance of mucous-secreting cells in the small airways, which could lead to obstruction of airflow or impaired mucociliary clearance. Overall heterogeneity of lung stem cells should be an important consideration in designing cell- and drug-based therapies. As the research in this field advances, we expect to see an increasing number of clinical applications of stem cells and pharmaceuticals that target stem cells.

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# Chapter 7
# Heterogeneity of Mammary Stem Cells


**Rhiannon French and Giusy Tornillo**

**Abstract** Adult female mammals are endowed with the unique ability to produce milk for nourishing their newborn offspring. Milk is secreted on demand by the mammary gland, an organ which develops during puberty, further matures during pregnancy and lactation, but reverts to a resting state after weaning. The glandular tissue (re)generated through this series of structural and functional changes is finely sourced by resident stem cells under the control of systemic hormones and local stimuli.

Over the past decades a plethora of studies have been carried out in order to identify and characterize mammary stem cells, primarily in mice and humans. Intriguingly, it is now emerging that multiple mammary stem cell pools (co)exist and are characterized by distinctive molecular markers and context-dependent functions.

This chapter reviews the heterogeneity of the mammary stem cell compartment with emphasis on the key properties and molecular regulators of distinct stem cell populations in both the mouse and human glands.

**Keywords** Stem cell · Cell heterogeneity · Mammary epithelium · Breast · Cell differentiation · Cell self-renewal · Mammary gland development · Stem cell markers

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## Murine Mammary Stem Cells

### *Mouse Mammary Gland Development at a Glance*

Mammary gland development begins during embryogenesis once epithelial cells from the ventral ectoderm invade the underlying fatty stroma and form a rudimentary arboreal structure [1, 2]. After birth, growth of the mammary rudiment is allometric until puberty when ducts rapidly elongate and branch throughout the fat pad, in a process known as branching morphogenesis. In adults the mammary gland undergoes periodic remodelling at each oestrous cycle with recurring formation of short-lived branches and alveolar buds. During pregnancy the mammary tree further expands and fully matures into an organ competent for lactation by developing alveoli capable of synthetizing and secreting milk. However, weaning triggers 'involution' of the gland, leading to the collapse of the secretory alveoli and the restoration of a simple resting ductal tree with the potential to expand and regress again across multiple rounds of pregnancies [2]. Thus, in the adult mammary gland—unlike in many other organs—the stem cell reservoir supplies not only the homeostatic cell turnover but also the high and rapid demand of specialized tissue throughout postnatal development (Fig. 7.1).

The functional component of the mammary gland is its epithelium which consists of two cell layers: luminal cells lining ducts and alveoli, surrounded by basal cells, mostly contractile myoepithelial cells, adjacent to the basement membrane and responsible for milk propulsion through the ducts. Despite being characterized by the widespread expression of distinctive cytokeratins, each of these epithelial layers exhibits a heterogeneous cellular composition, including cell types unique to specific developmental stages [2–6].

### *Professional Mouse Mammary Stem Cells from Embryonic to Maternal Life*

The identity of mouse mammary stem cells (MaSCs) differs depending on developmental stage. In late gestation the basal and luminal lineages are not yet fully resolved within the mouse embryo mammary epithelium (Fig. 7.1) and cells are mostly proliferating [7], co-express basal and luminal markers and the stem cell–specific isoform of SHIP (s-SHIP) [7–12], but lack oestrogen receptor expression (ER) [12]. Tracing of cells labelled by using either a basal or a luminal cell promoter revealed the identity of the earliest mouse MaSC population [9, 13]. Embryonic mouse MaSCs are multipotent stem cells that are able to generate all the epithelial cell types in the adult gland [9, 13], but a proportion of them persist as quiescent postnatally and may contribute to subsequent phases of development [7, 14]. Interestingly, amongst the many signalling mediators found to be expressed in the mouse mammary primordial epithelium [12], the canonical Wnt-beta catenin

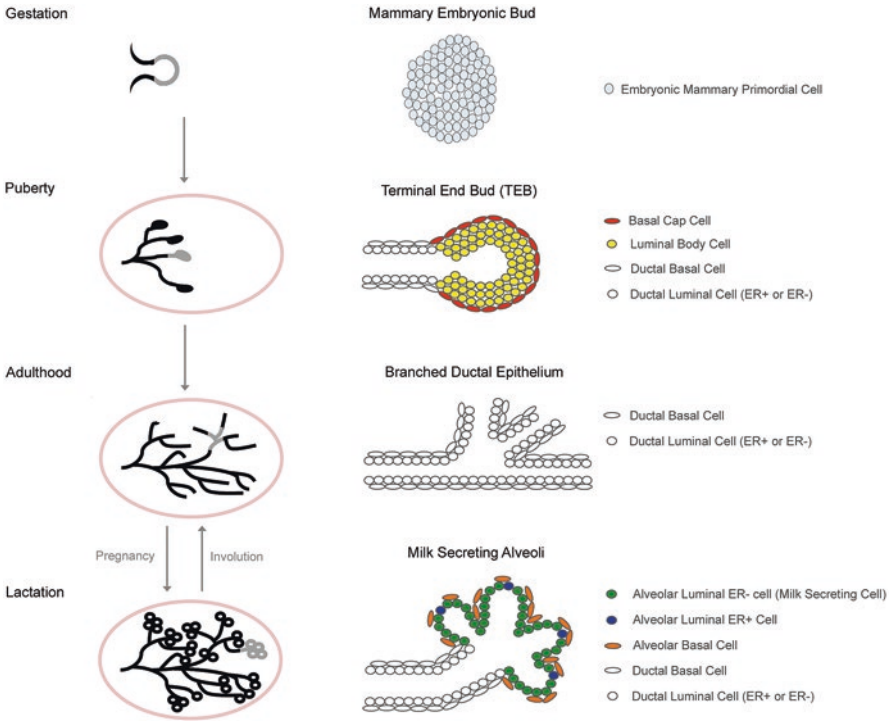


**Fig. 7.1** Schematic of mouse mammary gland development. The mammary epithelium is depicted in black within fat pads indicated in pink (left panels). Portions of the epithelium highlighted in grey correspond to images on the right

pathway does not seem to be active in embryonic multipotent MaSCs but rather marks precursors of the luminal lineage alone [15].

The mammary epithelium hugely expands during puberty via elongation and bifurcation of the ducts from their distal ends, known as terminal end buds (TEBs), throughout the mammary fat pad [16]. The TEB is composed of a single outer layer of cap cells with underlying multi-layered body cells, expressing basal and luminal cell markers, respectively, and held together through a highly specialized adhesion complex [17] (Fig. 7.1). TEBs function as a transient stem cell niche at the growing tips of the ducts, where extensive proliferation provides the subtending regions with newly generated cells [2, 16, 18, 19]. As a proportion of cells migrate from the outer layer into the inner body, cap cells have been long hypothesized to be a pool of bipotent stem cells capable to give rise to both basal and luminal cells [16]. Expression of the stem cell-associated gene s-SHIP is restricted to the outer cap cell layer of TEBs in puberty but cannot be detected either in the primitive ducts before puberty or in the mature ducts after puberty, indicating that cap cells may be MaSCs specifically activated for branching morphogenesis [18]. Furthermore, cap cells possess high canonical Wnt signalling activity [20] and the progeny of cells

responsive to Wnt-beta catenin during puberty was found in both basal and luminal cells of the mature ductal epithelium [15].

However, more recent studies indicate that cap cells may not be multipotent MaSCs but are rather restricted to the basal lineage. In fact, cap cells migrating into the body cell layers seem to stop proliferating and undergo apoptosis without contributing substantially to the luminal lineage [21]. According to a more intricate model, pubertal morphogenesis relies upon luminal and basal lineage-restricted heterogenous MaSC pools, including short-term stem cells, located at the border of the TEBs, biased towards differentiation and contributing to the growth of the adjacent duct, along with stem cells primed for self-renewal at the tip of the TEB. Random cell segregation at each TEB bifurcation allows stem cell pools to be rearranged and positional biases to be re-assigned thus preserving a balanced MaSC repertoire [19].

At the end of puberty the TEBs disappear and the development of the branched mammary skeleton upon which alveoli-like buds and alveoli form during every oestrus cycle and pregnancy, respectively, is complete [2].

Alveologenesis is sustained by multiple lineage-restricted precursors—other than those recruited for ductal formation—which cooperate to generate the distinct cell types present within the mature secretory alveoli [4–6, 9, 22–25] (Fig. 7.1). Amongst whey acidic protein (WAP)-expressing cells, mostly abundant in late pregnancy and lactation, a population of cells has been identified as capable of surviving the involution process and act as self-renewing alveolar precursors in subsequent pregnancies [26]. Known as parity-induced mammary epithelial cells (PI-MECs) [27], these cells live confined to the luminal epithelium at the extremities of ducts in the mammary gland of parous mice and, when recruited during pregnancy, they contribute to the generation of luminal—but not of myoepithelial—cells within alveoli [22].

In addition, at the beginning of pregnancy, the stem cell gene s-SHIP is exclusively expressed in proliferating cells in the basal layer of the alveolar buds [18], but whether these cells represent the ancestors of alveolar myoepithelial cells remains to be determined.

## *Mouse Mammary Stem Cells Across the Basal and Luminal Mammary Epithelium*

In the late 1950s pioneering experiments showed that portions of normal mammary epithelium from donor mice could regenerate an entire functional mammary epithelial tree when transplanted into recipient fat pads devoid of their endogenous epithelium, thus demonstrating the existence of cells with stem cell behaviour [28].

Since then numerous studies have aimed to identify MaSCs by testing the reconstitution ability of different epithelial cell populations, isolated by fluorescence-activated cell sorting (FACS), upon injection into cleared mouse mammary fat pads [4–6, 29]. Single cells within the basal epithelial fraction were found be able to

repopulate cleared mammary fat pads with the highest efficiency, indicating that a proportion of basal cells might be multipotent stem cells [6, 30].

However, cells able to reconstitute the glandular epithelium during the non-physiological process of transplantation, better known as Mammary Repopulating Units (MRUs), might not be the stem cells contributing to the production of the distinct mammary epithelial cell lineages during normal development and homeostasis. One major concern with using mammary transplantation experiments to infer cell stemness is that cells to be tested are removed from their original microenvironment after tissue disaggregation which could induce alterations in behaviour and differentiation potential [13, 24, 31, 32].

On the other hand, lineage-tracing approaches in mice overcome this caveat by enabling fate mapping of cells in situ without perturbation and have so far greatly helped to uncover the complexity and heterogeneity of the stem cell compartment in the mouse mammary gland. Lineage tracing has in fact provided evidence in support of the existence of both multipotent (contributing to both the luminal and the basal lineages) and unipotent (luminal lineage-restricted and basal lineage-restricted) adult MaSCs that are long-lived and can survive multiple rounds of pregnancy/involution [9, 13, 15, 19, 22, 23, 25, 33, 34] (Fig. 7.2). Yet, the latest approaches, including lineage-saturation labelling [35] and stochastic single-cell genetic tracing [36], combined with high-resolution three-dimensional microscopy, have shown that in the adult mammary gland, both the basal and the luminal lineages are self-sustained, even though the presence of non-targeted or rare MaSCs with multi-lineage differentiation potential has not been fully ruled out [37].

For instance, a recently identified Wnt3a target, the protein C receptor (Procr), was found to mark a rare population of multipotent MaSCs located in the basal mammary epithelium. Compared to the rest of basal cells, Procr+ cells express lower levels of basal keratins, exhibit traits of epithelial-mesenchymal transition (EMT) and appear to be actively cycling stem cells, which can give rise to both basal and luminal cells via asymmetric division. Despite being individually dispersed along the ducts rather than accumulated at their distal ends, Procr+ cells

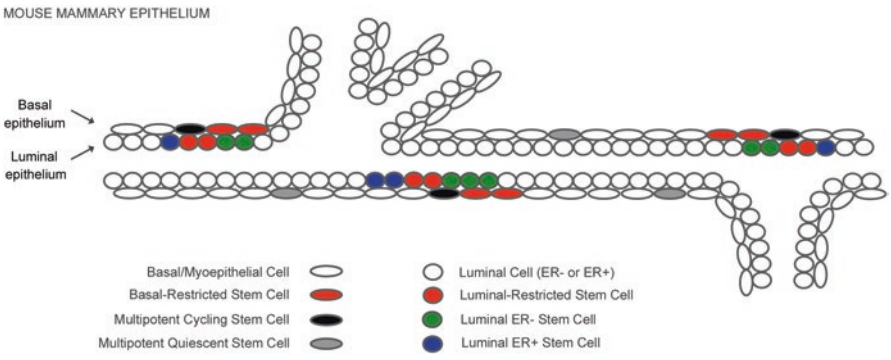


**Fig. 7.2** Overview of stem cell heterogeneity in the mouse mammary epithelium. See main text for details regarding distinct mouse mammary stem cell populations

contribute to ductal morphogenesis during puberty as well as alveolar formation during pregnancy [34].

In contrast to basal cells, no evidence has been so far reported in support of the presence of multipotent MaSCs within the luminal cell compartment, which, however, encompasses a number of long-lived luminal-restricted stem cells differing in their ability to generate distinct specific luminal cell types.

During postnatal mammary development and homeostasis, Prominin+ cells specifically maintain ER+ luminal cells, whereas SOX9+ luminal cells serve as source for ER−, but not ER+, luminal cells [25].

In particular, Notch1-expressing cells are luminal ER− cells which are able to self-renew and generate exclusively luminal ER− cells, both in ducts and alveoli [9]. Given that SOX9 is a key downstream target of Notch1 [38], Notch1+ cells are likely to overlap to a great extent with SOX9+ luminal precursors.

In vivo genetic labelling and tracing of cells expressing another member of the Notch receptor family, Notch3, instead revealed that these are rare transiently quiescent luminal cells with self-renewal ability and a broader differentiation potential. Indeed, the progeny of Notch3+ cells comprises both ER+ and ER− ductal luminal cells as well as luminal alveolar cells. Furthermore, Notch3 activation itself negatively regulates the expansion of this luminal precursor subset by maintaining a resting cell state [23].

## *Quiescent Stem Cells in the Mouse Mammary Gland*

The presence of long label-retaining cells (LRCs) in the mammary epithelium has pointed to the existence of MaSCs, which divide infrequently but can still retain the ability to expand and fulfil the demand for mature functional cells, whilst preserving the long-term regenerative potential of the mammary gland [39]. Although significant heterogeneity is also emerging within the slow-dividing/quiescent MaSCs, several studies indicate that these cells preferentially reside in the basal compartment of the mammary epithelium [7, 14, 30, 40–42] (Fig. 7.2).

In this regard, in vivo labelling of basal cells with an inducible nuclear fluorescent protein (H2B-GFP) led to the identification of a small population of dormant cells in virgin mice, further reduced after pregnancy and located at the tips of the ductal tree. Importantly, this cell population is characterized by the expression of genes related to G-protein-coupled receptors and Wnt/beta catenin signalling and can be prospectively isolated by using the cell surface protein CD1 together with basal cell markers [42].

Another marker for quiescent MaSCs is the transcription factor Bcl11b, which has been demonstrated to be functionally required for cell cycle–arrest induction and for the overall long-term maintenance of the mammary gland. Only a minority of basal cells that express the cytokeratin 17 and are sparsely scattered at the interface of basal and luminal layers of the ducts exhibit high expression of Bcl11b (Bcl11b$^{High}$). Whereas Bcll11b and Procr mark two distinct cell populations, basal

CD1+ cells are enriched for Bcl11b expression, suggesting that the CD1+ and Bcl11b$^{High}$ cell pools may overlap at least in part [41].

Whilst the Wnt target gene Lgr5 is unequivocally a marker for actively dividing stem cells in a number of epithelial tissues [43, 44], the precise role of Lgr5+ cells in the mammary gland has long been controversial. In the mammary epithelium, Lgr5 has been associated with the expression of genes implicated in embryonic development and negative regulation of non-canonical Wnt signalling [14]. Moreover, according to several lineage-tracing studies, Lgr5 marks a very small population of basal cells, which are not highly proliferative but act as MaSCs, whose potency, either multi-lineage or basal-restricted, is, however, still a matter of debate [13, 33, 45, 46]. Lately, based on the expression of Lgr5 and the Tetraspannin family member Tspan8, three distinct quiescent MaSC subsets (Lgr5+Tspan$^{High}$, Lgr5+Tspan- and Lgr5-Tspan$^{High}$) have been defined along with their characteristic spatial localization, transcriptional and epigenetic profiles [14]. Notably, neither CD1 nor Bcl11b mRNAs are enriched in the recently identified quiescent basal stem cell populations defined by Lgr5 and Tspan8, implying that these might represent additional separate MaSC pools. Restricted to the proximal portion of the gland in the nipple area, the deeply quiescent LGR5+ Tspan8$^{High}$ cell subset appear to originate in the embryonic mammary primordium and switch to a dormant state after birth to serve as stem cell reservoir that can be activated by hormonal stimuli [14]. Therefore, LGR5+ Tspan8$^{High}$ cells are highly reminiscent of mammary embryonic cells, previously identified as embryonic long-label-retaining cells (eLLRCs) [7], which become quiescent early in mammary morphogenesis and are deposited near the origin of the ducts in the adult gland but can re-enter the cell cycle during puberty and pregnancy.

Finally, quiescent MaSCs, identified as CD1+ or Bcl111b$^{High}$ or LGR5+ Tspan8$^{High}$ cells, all show extraordinary reconstituting potential after isolation and transplantation into cleared mammary fat pads [41, 42, 47], implying that intrinsic mechanisms might link the quiescent state to their superior regenerative capability.

## Human Mammary Stem Cells

### *Structure of the Human Mammary Gland*

Some notable differences in the structure and development of the mouse and human glands prevent the direct translation of findings from murine studies to human biology [48]. The human mammary gland is generally more complex, having branched structures ending in terminal ductal lobular units (TDLUs). These are not present in the mouse mammary gland which instead forms alveolar buds at each oestrous cycle [29]. In addition, keratin expression is less strict in the human gland where cytokeratins K5 and K14 can also mark the luminal as well as the basal lineage [49]. Stromal composition also differs in terms of fibroblast and adipocyte content [50].

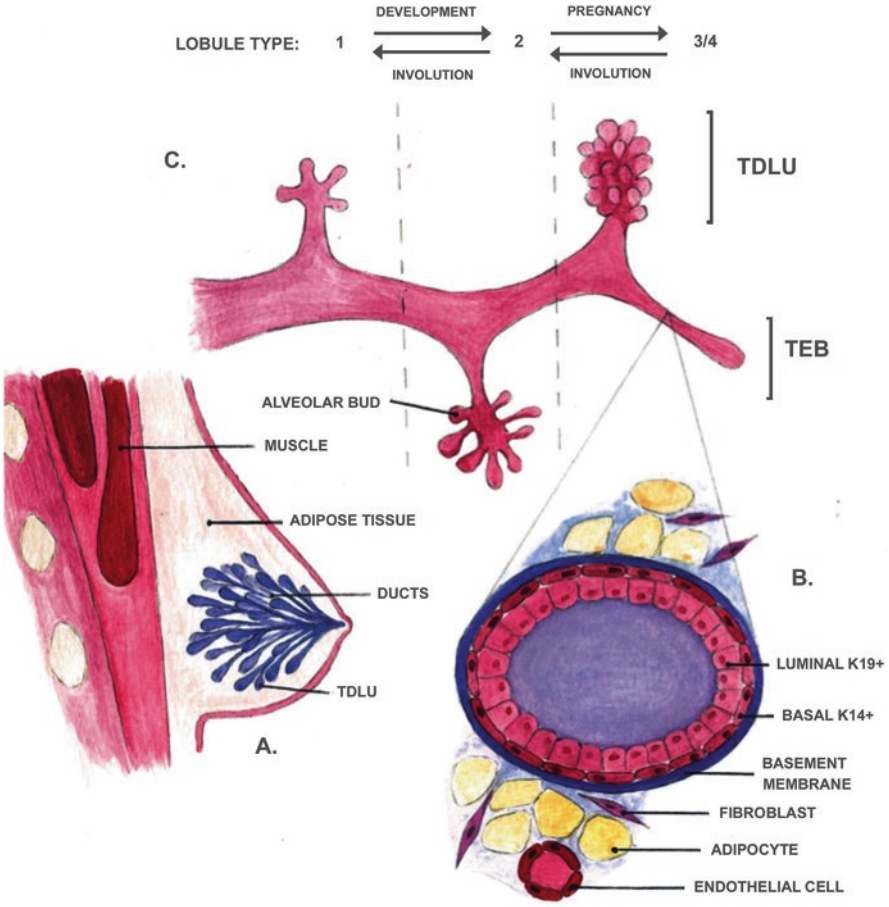


**Fig. 7.3** Structure of the human mammary gland. The human mammary gland consists of a network of branching ducts ending in terminal ductal lobular units (TDLU) (**a**). Transverse section of a duct; luminal and basal cells are indicated (**b**). Lobular development showing four lobule types (**c**). See main text for details

Therefore, an understanding of the cellular organization of the human mammary gland is necessary in order to address the question of the human mammary stem cell (hMaSC).

The human mammary gland is classified as a compound tubuloalveolar exocrine gland, this consisting of branching secretory ducts terminating in alveolar or acinar structures (Fig. 7.3a). The parenchymal tissue of the gland is a bilayered epithelium structured as a network of 11–58 ducts which radiate out from the nipple and end in terminal ductal lobular units (TDLUs), the structure and terminology of which varies according to developmental stage [51]. The stroma includes extracellular matrix, fibroblasts, immune cells and adipose tissue, which provides support for parenchyma and contains the lipid store which can be turned into milk. During lactation,

luminally positioned epithelial cells secrete milk into ducts, aided by the contractile and basally positioned myoepithelial cells (Fig. 7.3b). Cell lineages present within the human gland can be distinguished by the expression of cell type–specific proteins: cytokeratins K14 and K19 mark the basal and luminal cells, respectively; however, some luminal cells in large ducts can also stain positive for K14, and double-positive cells are also present [49]. Luminal cells can also be identified by cytokeratins K18 or K19, EpCAM (or ESA), MUC1 or CD24. As basal cells are myoepithelial, they can also be identified by alpha-smooth muscle actin (α-SMA) or myosin, and also CD49f, CD10 (CALLA), CD44v6 and p63. Basal cells also stain positive for some mesenchymal markers such as vimentin [52, 53] (Fig. 7.3b).

Human mammary gland developmental stage is characterized by the number of alveolar buds, and lobule structure has been divided into four types [51]. Lobule types 1 and 2, containing approximately 11 and 47 alveolar buds, respectively are characteristic of non-parous women. Pregnancy induces further production of alveolar buds to approximately 80 per lobule (type 3). Type 4 lobules are those present during lactation when alveolar buds are termed acini (Fig. 7.3c). The accumulation of milk after weaning inhibits its further production and induces involution of the gland. This remodelling process occurs via cell autolysis and causes lobule structures to revert back from type 4 to 3. Further involution also occurs at menopause when lobule structures revert back from lobule types 3 to 2 to 1 with increasing age. However, type 1 lobules produced by involution differ from those of non-parous females, which are less differentiated and more proliferative [54].

## *Evidence for Human Mammary Stem Cells*

X-linked chromosome inactivation patterns observed in the human mammary gland suggested that it is derived from a single cell during development [55]. However, as adult or somatic stem cells are defined by their capacity to generate all the cell lineages of the tissue in which they reside, the identification of adult mammary stem cells requires approaches capable of measuring bipotent potential (Fig. 7.4). The first evidence for functional bipotency in the human mammary gland was measured by the ability of single human mammary epithelial cells (HMECs) taken from

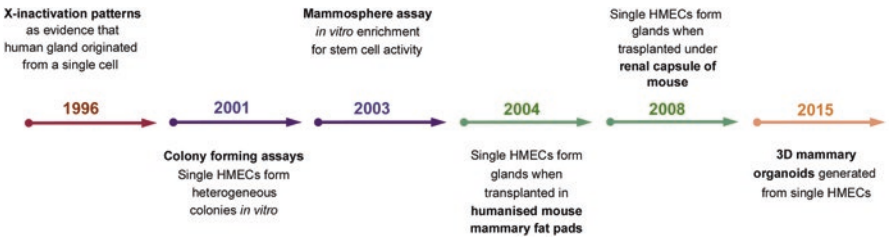


**Fig. 7.4** Timeline of technological advances facilitating the identification and characterization of hMaSCs. See main text for references

reduction mammoplasties to generate heterogeneous colonies in 2D collagen gels. Such colonies had both luminal and basal characteristics, suggesting that the parent cell must have bipotent potential [56, 57]. The development of the mammosphere assay later enabled the identification of HMECs with the stem cell–like properties of anoikis resistance and self-renewal. Passaged spheres are enriched for bipotent cells and can also be quantified as a surrogate readout for stem cell number [58].

Further advances enabled the identification of HMECs with regenerative ability. Analysis of prospective human MaSCs in vivo was first made possible by the 'humanization' of the mouse mammary fat pad, by injection of human fibroblasts into the cleared mouse gland [59]. This was followed by an alternative technique of transplantation of HMECs together with fibroblasts in collagen gels under the mouse renal capsule [60, 61]. Using these methods it was possible to reconstitute the human gland (i.e., containing both lineages) from a single cell. Stem-like cells defined by these methods are termed mammary-repopulating units (MRUs). The more recent development of mammary organoid cultures has also enabled testing for the regenerative ability of HMECs in vitro. Using a 3D collagen matrix it has been possible to generate branching structures of ducts with alveolar buds from single cells, again evidence for bipotent potential [62].

## *Characterization of Putative Human MaSCs*

Many studies have attempted to identify a cell surface marker profile that defines hMaSCs. Fluorescence-activated cell-sorting (FACS) enables the separation of cells based on surface epitopes, thus allowing a prospective analysis of the bipotent potential of marker-defined populations. The first characterization of hMaSCs was performed by separation of HMECs based on the expression of MUC1, CD10, and EpCAM, which were then assessed for colony formation in 2D collagen gels. The population defined by MUC-1$^{lo/med}$, CD10$^{med/hi}$ and EpCAM$^{hi}$ was enriched for cells with the ability to generate heterogeneous colonies [56]. A MUC-1$^{lo/med}$ EpCAM$^{hi}$ population was also shown to produce branched structures within a reconstituted basement membrane [63]. Improvement in single-cell plating efficiencies using fibroblast feeder layers in the colony-formation assay refined the bipotent population to within the CD49f$^{hi}$EpCAM$^{med}$ subset. This population produced heterogeneous colonies, whereas CD49f$^{hi}$EpCAM$^{lo}$ cells produced only myoepithelial colonies and CD49f$^{lo}$EpCAM$^{hi}$ only luminal colonies [57]. Later, studies found that MRUs reside within the CD49f$^{hi}$EpCAM$^{lo}$ subset of HMECs [61, 64]. Specifically, the CD10-positive subset of the CD49f$^{hi}$EpCAM$^{lo}$ population has also been shown to produce mammary organoids in vivo [62]. The predominance of basal-specific proteins and the low EpCAM expression in these putative stem marker profiles suggest that MaSCs may be part of the basal lineage. In support of this, the basal-specific profile CD44$^{hi}$CD24$^{lo}$ has also been shown to enrich for mesenchymal-like cells with mammosphere-forming potential [65]. Furthermore, stem-like CD44$^{hi}$CD24$^{lo}$ cells could be generated from non-stem populations by inducing an

epithelial-to-mesenchymal transition (EMT), suggesting that cells need to lose epithelial features in order to gain stem cell properties [66]. This would correlate with the proposed location of MaSCs at the leading edge of developing organoids, as mesenchymal characteristics may be required to invade the surrounding matrix [67]. However, some of these studies have used immortalized or established cell lines, which have been known to change phenotype in culture, gaining more mesenchymal characteristics over time [66, 68, 69].

In contrast to these findings, the luminal-like $EpCAM^{hi}CD49f^{+}$ population has been shown to form TDLU-like structures in matrigel. This subset was also positive for both K19 and K14 and could give rise to all other cell types [70]. Sarrio et al. also found that it was the $EpCAM^{hi}$ cells of normal breast cell lines that exhibited bipotency: although $CD44^{hi}CD24^{lo}$ cells could spontaneously generate from $EpCAM^{hi}$ cells via an EMT-like process, the reverse was not possible, that is, $CD44^{hi}CD24^{lo}$ cells were lineage-restricted. This led the authors to propose that EMT in mammary cells may reflect an aberrant differentiation to the myoepithelial state as opposed to de-differentiation [69].

Conflicting observations have been made using aldehyde dehydrogenase (ALDH) as a putative MaSC marker. Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme which oxidizes aldehydes such as retinol. ALDH activity can be measured using Aldefluor, a fluorescent substrate activated by ALDH. Using Aldefluor, it was possible to mark a population of approximately 8% of HMECs that generated heterogeneous colonies and were enriched for mammosphere-formation and mammary-repopulating potential in vivo [71]. This suggested that ALDH marked a MaSC population and was supported by a later study, showing that inhibition of the ALDH1A1 isoform reduced sphere formation and branching morphogenesis in vitro [72]. However, in direct contrast to these findings, Eirew et al. showed that mammary-repopulating cells had low Aldefluor activity, also overlapping with the $CD49f^{hi}EpCAM^{hi}CD10^{lo}$ luminal progenitor fraction. The authors suggested that Aldefluor-positivity was not a stem cell marker but was instead acquired by MaSCs at the point of differentiation to the luminal lineage, as in the mouse gland [3, 4, 73]. The discrepancies between the ALDH studies may be due to differences in the in vivo techniques employed: Ginestier et al. used humanization of the mouse mammary fat pad to generate a supportive environment for HMECs to repopulate, whereas Eirew et al. implanted cells in collagen gels under the mouse renal capsule. However, the Aldefluor assay does not discriminate between the isoforms of ALDH and detects active ALDH only. The active ALDH isoform has been shown to differ between samples of HMECs: Eirew et al. found active ALDH to be ALDH1A3, whereas Ginestier et al. and Honeth et al. observed active ALDH1A1 [72, 73]. This could account for the discrepancies between these studies and suggest that only ALDH1A1 may define a MaSC population, whereas ALDH1A3 marks luminal progenitors [4, 52, 72, 73].

Although technical limitations are likely to be the cause of some discrepancies between studies, the observed differences in putative MaSC marker profiles could also be the evidence of heterogeneity within the stem compartment, with some findings providing evidence for at least two separate MaSC populations. The Wicha

group have identified two hMaSC populations defined by $CD44^{hi}CD24^{lo}$ and $ALDH^{pos}$, with mesenchymal and epithelial attributes respectively, and possessing differing stem-like potential. MaSCs could transition between these states, and an overlapping population was also identified, which was enriched for mammosphere formation and expressed both epithelial and mesenchymal markers [65, 74]. Importantly, the $CD44^{hi}CD24^{lo}$ cells were contained with the $CD49f^{hi}EpCam^{lo}$ population, whereas the $ALDH^{pos}$ cells were from the $EpCAm^{hi}CD49f^{hi}$ fraction. Isacke and colleagues [69] have also suggested the existence of two MaSC populations with different stem-like properties; $EpCam^{lo}CD44^{hi}CD24^{lo}$ MaSCs being sphere-forming and invasive, whereas $EpCam^{hi}CD44^{hi}$ $ALDH^{pos}$ MaSCs having bipotent potential.

One study alone has identified a cell population within the adult human mammary gland with pluripotent potential. By using an epigenetic mark to identify cells with repression of $p16^{INK4a}$/CDKN2A, it was possible to pull out cells with extensive lineage plasticity. These cells could be defined by the surface marker profile $CD73^{hi}CD90^{lo}$. Under appropriate conditions, this population was able to differentiate into pancreatic cells (endoderm), adipocytes or cardiomyocytes (mesoderm), and milk-secreting mammary cells (ectoderm). $CD73^{hi}CD90^{lo}$ cells also expressed the pluripotency genes NANOG, OCT3/4 and SOX2, and were also $EpCAM^{hi}$. It was suggested that the $CD73^{hi}CD90^{lo}$ population was active under certain conditions such as wound healing. Its mortality in culture suggested that it provides a short-term contribution to the gland as opposed to previously described populations which may function to maintain homeostasis [75].

Although many findings have been replicated across research groups, the extent of variation between studies currently prevents a firm resolution of one or more stem cell populations defined by surface markers. A summary of stem cell properties attributed to different marker profiles is shown in Table 7.1.

**Table 7.1** Stem-like properties of human mammary cells with different marker profiles

| Marker Profile → / Stem Cell Property ↓ | $MUC\text{-}1^{lo/med}$ $EpCAM^{hi}$ | $CD49f^{hi}$ $EpCam^{lo}$ ($CD10^{hi}$) | $CD44^{hi}$ $CD24^{lo}$ | $ALDH1A1^{pos}$ | $ALDH^{pos}$ $CD44^{hi}$ $CD24^{lo}$ | $CD73^{hi}$ $CD90^{lo}$ |
|---|---|---|---|---|---|---|
| Heterogeneous colonies | + | + | | + | | |
| Sphere formation | | + | + | + | + | |
| MRU (in vivo) | | + | | + | | + |
| organoid formation | + | + | | + | | |
| Pluripotency | | | | | | + |
| References | [56, 63] | [57, 61, 62, 64, 65] | [65] | [52, 71, 72] | [65] | [75] |

The existence of hMaSCs would imply a hierarchical relationship between cell types of the human mammary gland, and some studies have attempted to resolve all cellular compartments by surface marker profiling. The markers CD49f and EpCAM have been used to define subpopulations corresponding to cells of both lineages. In addition to enriching for cells with bipotent potential, the CD49f$^{hi}$EpCam$^{lo}$ profile was also thought to mark basal progenitors, whereas CD49f$^{hi}$EpCam$^{hi}$ marked luminal progenitors [64]. Differential expression of Aldefluor and ERBB3 has been used to divide the CD49f$^{hi}$EpCam$^{hi}$ luminal progenitor compartment into three subpopulations, the proportions of which varied between individuals. In this study, a novel ERBB3-negative luminal progenitor was identified that was present in only 25% of patient samples. The ERBB3-negative profile was thought to mark the most primitive form of luminal progenitor as these cells also had low MUC1 and increased expression of basal-specific genes. Aldefluor-positive progenitors had increased expression of Elf5, suggesting that they were primed for milk production, perhaps analogous to the ER-negative population in mice [4]. Committed luminal populations have also been characterized as ductal (CD49f$^{hi}$EpCam$^{hi}$) and lobular (CD49f$^{lo}$EpCam$^{hi}$) [70].

A potential hierarchical tree taking into account the heterogeneity of both stem and progenitor compartments is proposed in Fig. 7.5.

## *Molecular Heterogeneity of hMaSCs*

A number of pathways have been implicated in the regulation of human MaSCs, notably Wnt, Notch and PI3K [76, 77]. However, as MaSC heterogeneity is a relatively recent observation, few studies have addressed differences in signalling between stem populations. Observations so far would suggest that MaSC populations can be distinguished on the basis of epithelial and mesenchymal markers, which would imply that each population is also different in terms of signalling pathway activation. Single-cell RNA-sequencing of human MaSC populations separated on the basis of ALDH$^{+}$ vs. CD44$^{+}$CD24$^{-}$ marker profiles has identified differences at the transcriptional level: ALDH-positive cells had higher expression of WNT2, insulin-like growth factor 1 (IGF1) and the notch ligand DLL1, whereas CD44$^{+}$/CD24$^{-}$ cells expressed higher levels of the Wnt pathway proteins DKK3, CCND2, PRICKLE2 and DRAXIN [74]. Differences in marker profiles used to separate human MaSCs are also suggestive of distinct signalling mechanisms existing within each population. For example, CD44 is a receptor for hyaluronin that can activate downstream MMP signalling, whereas ALDH is associated with Notch signalling in cancer [78, 79]. These observations suggest potential differences between human MaSC populations in the aforementioned core signalling pathways (Wnt, Notch and PI3K); however, further investigation including analysis at the protein level is required.

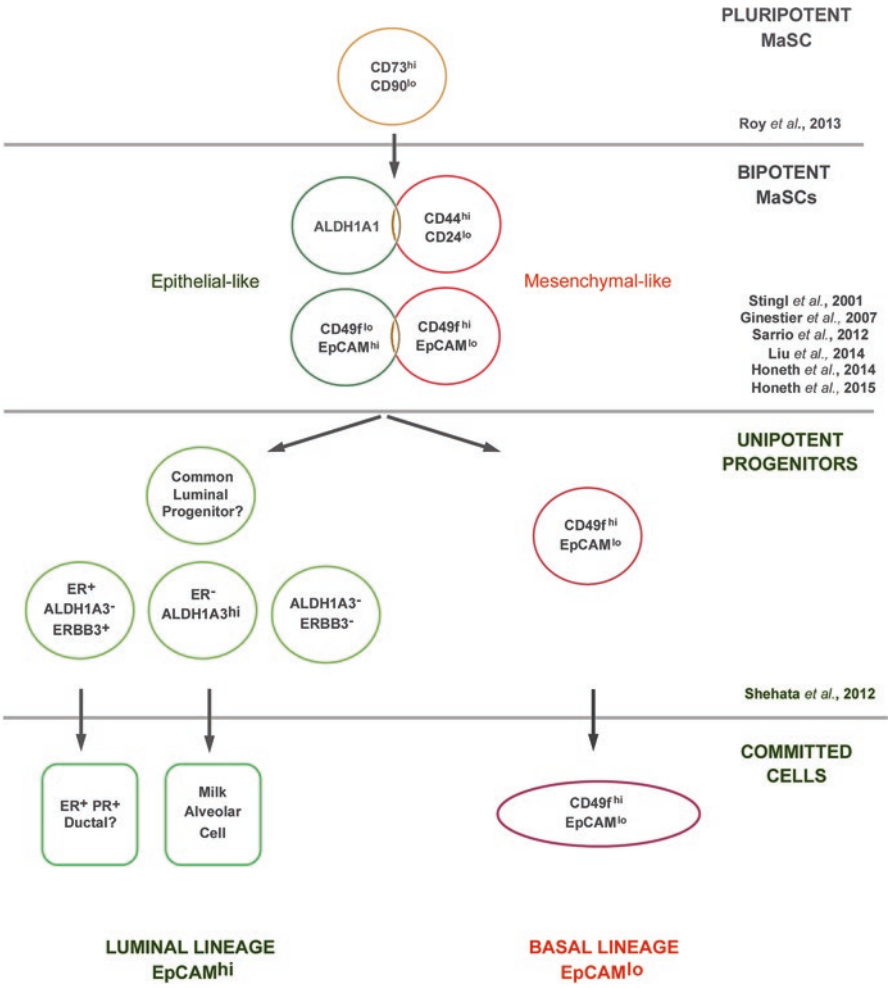


**Fig. 7.5** Proposed cellular hierarchy of human mammary epithelium

## *Location of Mammary Stem Cells and Their Niche*

Some studies have attempted to identify and characterize hMaSCs by location. Development of organoid culture has enabled the modelling of human mammary gland regeneration in vitro and also allows for the possibility of lineage tracing, which gives information such as the extent and location of clonal progeny. Sokol et al. cultured organoids starting from clusters of HMECs which self-organized into basal and luminal lineages and produced complex ducts and lobules in a 3D hydrogel matrix. Using this method in combination with lineage tracing, they observed mass cell migration of clonal progeny, which were dispersed throughout the 'gland.' Time-lapse microscopy of developing organoids identified cells protruding from the leading edge of elongating ducts which were polarized along the direction of travel.

During organoid development, these leader cells extended into the ECM which was followed by ductal elongation determined by the direction of leader cells, a process that has not been observed in mouse cells [80]. The stem cell markers Slug and Sox9 marked cells in the TDLUs of organoids, mostly those in contact with the ECM, including the leader cells, but positive cells were rare in the core and ducts. These findings suggested that MaSCs reside at the leading edge of the developing gland [67].

Honeth et al. used a different approach to come to a similar conclusion. This group attempted to locate MaSCs by generating 1D and 3D computer models of human mammary development. Fate decisions of MaSCs modelled comprised (1) asymmetric or symmetric self-renewal, (2) high or low rate of entering the cell cycle from quiescence and (3) proximal or distal orientation of progenitors relative to the parent cell. Models were generated based on different combinations of fate choices and were evaluated by observation of markers for proliferation and differentiation in sections from human mammoplasties. The model which most accurately reflected distribution of cells in vivo resulted from the MaSC fate choices of asymmetric self-renewal, high rate of cell division and distal orientation of the most undifferentiated progeny. This model predicted MaSCs to be located at the leading edge of the developing lobule and also at branching points. A 3D model also predicted MaSCs to occur at branching points and the ends of ductules. To correlate known markers with the predicted location of MaSCs, sections from mammoplasties were stained with a range of putative MaSC markers by immunofluorescence. The marker profile ALDH1A1$^{+}$ SSEA4$^{+}$ K14$^{+}$ K19$^{+}$ localized to the predicted MaSC regions at the ends of ductules. These areas were less organized and had low expression of MCM2 and Ki67, suggesting quiescence. This profile did not overlap with either ALDH1A3$^{+}$ or CD49f$^{hi}$EpCam$^{lo}$ cells, which were located in the extralobular ducts [52].

In contrast to these findings, a previous study identified ducts as the location of hMaSCs, as ductal-derived cells formed larger colonies and mammospheres than those from lobules. As with the above study, the CD49f$^{hi}$EpCam$^{lo}$ profile was present in the ducts, but ducts also stained positive for co-expression of lineage markers K14 and K19, and also had lower Ki67 expression, indicating quiescence. This study suggested that MaSCs reside in quiescent ducts whereas the lobules are actively dividing [70]. These discrepancies may again be evidence of two separate pools of MaSCs, one present in established ducts and the other at the leading edge of developing TEBs.

The computer models generated by Honeth et al. also predicted type 1 lobules to be enriched for MaSCs, which concurs with previous findings suggesting that they are a more immature lobule type [54]. ALDH1A1 expression was also increased in smaller lobules and in nulliparous women [52]. A separate study analysed the cellular composition of lobules microdissected from mammoplasties based on lobule type [47]. Type 1 lobules had increased K14 expression, lower expression of luminal markers and increased co-expression of lineage markers. Type 1 lobules also had less differentiated cells and increased progenitors. Type 2 and 3 lobules had increased K8 expression, reduced K14 and were lineage-restricted and more epithelial. These findings suggested that stem and progenitor cells were enriched in

immature lobules whereas more mature lobules had increased hierarchical complexity. However, staining patterns were highly variable even between the lobules of the same patient, with some quiescent and some expanding. Much heterogeneity was also observed across patient samples [47].

The development of organoid culture models has also allowed for the possibility of studying the influence of the gland environment on MaSCs. The possible location of MaSCs at the leading edge of ducts during development, and their observed interaction with the ECM, suggests that ECM may be involved in their regulation [67]. Using collagen gels, it has now been possible to develop organoids from single cells; however, it was found that the type of collagen matrix used greatly influenced organoid morphology. In floating gels which allowed for contraction of organoids, HMECs formed branched structures with alveoli and long thin ducts. In attached gels without the possibility of contraction, HMECs formed structures with more branches, but no alveologenesis occurred. Detachment of these gels allowing for contraction induced alveologenesis in forming organoids, whereas attachment of gels inhibited the polarization of p63 and GATA expression, indicative of the basal and luminal lineages, respectively. These findings suggest that the elasticity of ECM has significant influence on MaSC and progenitor differentiation [62].

## Conclusions and Future Directions

Current evidence suggests that both the human and mouse mammary stem cell compartment is heterogeneous, with two or more populations of MaSCs existing in distinct locations and functioning in different situations. However, further efforts are required to reach a consensus on the marker profiles which identify these populations.

Some conflicting observations may be due to technical limitations arising in the study of stem cell biology. The improvement of lineage-tracing approaches in vivo in mice, thanks to cutting-edge techniques for labelling and visualization of individual cells, has the potential to provide further information to the field. However, a functional analysis of MaSC potential sometimes necessitates observations outside of the context of the natural environment, and it is highly likely that cell isolation and maintenance within experimentally defined conditions will induce phenotypic changes that alter the number and properties of MaSCs in a population. Indeed, the recent development of organoid models has demonstrated the importance of the extracellular environment in affecting human MaSC behaviour [67]. A stem cell phenotype will therefore be influenced by the choice of assay used to define it, and so observations may not accurately reflect cellular properties in the native gland. For example, transplantation in vivo may induce a capacity to regenerate in cells which did not possess this attribute previously. Future developments in organoid culture models may improve upon in vivo techniques by allowing for the possibility of manipulating cellular context, and thus producing a more accurate model of the human gland. This technology may help to reach a consensus as to the marker

profiles defining MaSC populations, and also determine how MaSCs are regulated by their niche. Furthermore, high sample variability has also been observed in some studies using human cells derived from reduction mammoplasties [4, 47]. It is likely that MaSC numbers will vary between individuals, as type 1 lobules characteristic of non-parous women have been shown to be more undifferentiated [51, 52]. This variation may be an additional cause of conflicting results and highlights the need for large or pooled sample datasets correlated with age and parity.

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# Chapter 8
# Heterogeneity of Adult Cardiac Stem Cells


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**Abstract** Cardiac biology and heart regeneration have been intensively investigated and debated in the last 15 years. Nowadays, the well-established and old dogma that the adult heart lacks of any myocyte-regenerative capacity has been firmly overturned by the evidence of cardiomyocyte renewal throughout the mammalian life as part of normal organ cell homeostasis, which is increased in response to injury. Concurrently, reproducible evidences from independent laboratories have convincingly shown that the adult heart possesses a pool of multipotent cardiac stem/progenitor cells (CSCs or CPCs) capable of sustaining cardiomyocyte and vascular tissue refreshment after injury. CSC transplantation in animal models displays an effective regenerative potential and may be helpful to treat chronic heart failure (CHF), obviating at the poor/modest results using non-cardiac cells in clinical trials. Nevertheless, the degree/significance of cardiomyocyte turnover in the adult heart, which is insufficient to regenerate extensive damage from ischemic and non-ischemic origin, remains strongly disputed. Concurrently, different methodologies used to detect CSCs in situ have created the paradox of the adult heart harboring more than seven different cardiac progenitor populations. The latter was likely secondary to the intrinsic heterogeneity of any regenerative cell agent in an adult tissue but also to the confusion created by the heterogeneity of the cell population identified by a single cell marker used to detect the CSCs in situ. On the other hand, some recent studies using genetic fate mapping strategies claimed that CSCs are an irrelevant endogenous source of new cardiomyocytes in the adult. On the basis of these contradictory findings, here we critically reviewed the available data on adult CSC biology and their role in myocardial cell homeostasis and repair.

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## Introduction: Need for an Effective and Widely Available Therapy for Cardiac Repair/Regeneration

A combination of clinical and basic cardiovascular (CV) research in the past few decades has significantly reduced most fatal acute cardiovascular syndromes in Western countries [1]. This success has resulted in an increase in chronic heart failure (CHF), which has reached epidemic levels and is the number one killer among CV diseases [2]. This is so because in the majority of these patients irreversible myocardial damage has already occurred at the time of acute life-saving interventions. CHF once developed remains resistant to most available therapies with an average survival of only 3–5 years after onset, a prognosis that is poorer than that for most cancers [1, 2]. This negative outlook is worsened when put in a societal context. There are ~38 million patients with this diagnosis worldwide, with ≤one million hospital admissions due to heart failure in the USA alone—and a similar number in the EU—with an estimated annual cost of several billions of dollars to the US healthcare systems. All heart failure treatments currently in use—with the exception of heart transplant with its insurmountable medical and logistic limitations—are palliative at best. They are aimed at improving symptoms and preserving/enhancing the function of the surviving contractile cells, the myocytes, while none is directed toward replacing the lost myocytes, which is the primary cause of CHF [1].

The main proximate cause of CHF is ischemic cardiac disease, in particular myocardial infarction [1, 2]. Even when CHF is of non-ischemic origin, as is the case of structural cardiomyopathies, the key issue is the failure of the myocardium to undergo a robust cardiomyocyte replacement. It is not surprising, therefore, that regenerative biology/medicine has been at the forefront in the search to find an effective and broadly applicable cellular replacement for the contractile cells lost and/or damaged by the primary insult [3]. Unfortunately, disagreements about the intrinsic regenerative capacity of the adult mammalian myocardium, in general and the human in particular, together with a lack of consensus on its underlying biology (see below), have spawned often contradictory approaches to accomplish myocardial repair/regeneration.

It stands to reason that unless the controversy about basic myocardial biology is settled through evidence based on robust and reproducible scientific data, any clinical repair/regenerative protocols, unless spectacularly positive, will likely be either un-interpretable or will fall short of providing conclusive answers about their utility.

## Biology of the Adult Heart: The Old Paradigm

A long-standing and widely accepted concept about the adult mammalian heart was grounded on the total lack of regenerative capacity of its main parenchymal cells, the cardiomyocytes (CMs) [4]. In adult mammals, CMs are terminally differentiated cells permanently withdrawn from the cell cycle and with no capacity to replicate [5]. Thus, adult CMs are *elementi perenni*, similar to neurons and believed to last a lifetime. Accordingly, the CM response to increased workload or loss of contractile power in the adult is an increase in size to accommodate a larger number of its contractile units, the sarcomeres, which results in physiological or pathological CM hypertrophy but not in adult CM hyperplasia. By necessity, this view assumed that the CM cohort is the same from cradle to grave. This paradigm also required that CM death be an extraordinarily rare event to preserve enough contractile mass needed to generate cardiac output throughout life, including very old age. Thus, neither CM death nor new CM formation played any role after the early postnatal life.

The search to reactivate the mitotic competence of mature CM was and remains stimulated by the prospect that such reactivation would result in CM regeneration in the adult [6]. However, all direct attempts to reactivate mitotic CM's competence have resulted in an increased polyploidy and death by apoptosis, both in vitro and in vivo [6, 7]. Additionally, for still unknown reasons, the heart is a privileged organ with an extremely low incidence of neoplastic transformation [8]. Any manipulations affecting the tight control of cell cycle regulation in this tissue runs a high risk of also breaking the protection of the heart to neoplastic development. It would be a Faustian bargain to trade a dubious CM cell cycle re-entrance for a potential higher risk of cardiac neoplasias.

While the regenerative potential of the adult heart was considered to be nil and its response to increased workload limited to CM hypertrophy, the probability of developing any effective protocol for myocardial regeneration was negligible. This scenario has been the linchpin for most cardiovascular therapeutic research till date.

## Biology of the Adult Heart: The New Paradigm

Mammalian CM terminal differentiation and permanent withdrawal from the cell cycle, based on solid observational and experimental data [4–7, 9], became complicated early on with isolated publications of sporadic new CM formation in the normal and pathological adult heart [10, 11]. As these observations were rare, not mechanistically explained, and did not fit neatly within the prevalent paradigm, they became a curiosity, which was mostly ignored.

Recently, more sensitive biochemical and genetic tracking techniques have indisputably documented that new CMs are continuously born in the post neonatal mammalian heart, including the human, in response to either physiological wear and tear

or myocardial injury [12–15]. The rate of this adult CM turnover seems to be specific for each species and increases significantly after injury. Its precise quantification, however, remains more passionately argued than the existence of the phenomenon itself [16]. Reports based on radioactive isotope decay have calculated an annual CM turnover rate of ~0.5% in adult healthy humans [12, 17]. These data need to be taken with a grain of salt because of methodological issues [16]. In small mammals, where measurements can be more precise, the results are even more problematic, ranging from 0.001% to 4% annually, which highlights that the methodology used is not reliable.

Because replacement of CMs lost by wear and tear and/or injury will underpin most regeneration protocols, it is important to quantify accurately CM turnover in health and disease and at different ages. Despite the lack of consisting and reproducible scientific data about turnover rates, and the significance of the myocardial regenerative response, there is consensus that this response by itself is not sufficient to counteract the CM loss and dysfunction post-MI and in CHF. Nonetheless, the existence of an intrinsic regenerative response in the adult myocardium suggests that identifying its nature and mastering its underlying mechanism(s) might provide an opportunity for the development of clinically meaningful protocols of myocardial protection, repair, and/or regeneration [1].

During physiological mammalian growth, approximately 40% of all adult CMs are generated in neonatal life. This regenerative response is observed in the first days of the neonatal mouse heart, and it is lost by 7 days of age [16]. After this period and during early adulthood, cardiac growth is characterized by a transition from a hyperplastic to a hypertrophic phase, with formation of binucleated CMs that permanently withdraw from the cell cycle, becoming terminally differentiated cells [18, 19]. Thus, the adult mammalian heart has been considered a static organ constituted by CMs unable to re-enter the cell cycle to duplicate [18, 19].

For a long time, the cardiac mass growth that occurs postnatally has been explained by CM hypertrophy, which, in time, becomes enlarged and aged, like the age of the organism. However, this static notion of the adult heart has been followed by the evidence that the organism and organ age do not proceed at the same rate [5] and that the adult mammalian heart maintains a cell turnover during the organismal life essential to generate new cardiac cells after injury through activation of an endogenous pool of stem cells [7–15, 20].

Endogenous cardiac stem/progenitor cells (CSCs/CPCs) were first identified in 2003 by Beltrami et al. [20]. Because heart, blood vessels, and blood cells of the circulatory system share the same primary germ layer origin, to identify the adult stem/progenitor cells in the cardiac tissue, we have used several typical stemness markers such as c-kit [20] and Sca-1 [21]. These markers were first discovered and then used to isolate hematopoietic stem cells (HSC) that could differentiate in several cell lines under certain conditions [20, 22, 23]. Using this strategy, they identified a population of cardiac cells, negative for common hematopoietic lineage markers (such as CD45, CD34, CD3, CD14, CD16, CD19, CD20, and CD56), and able to self-renewal and to differentiate in cardiac cell lineage [19]. In rodent heart (as well as human) cardiomyocyte-depleted freshly isolated cell preparations, only

a small fraction (~10%) of the total c-kit positive (c-kit$^{pos}$) cells possess tissue-specific stem/progenitor characteristics and properties. Indeed, ~90% of c-kit$^{pos}$ cells co-express blood/endothelial markers such as CD45 and CD31 (Lineage positive, Lin$^{pos}$) [24]. A negative sorting (Lineage negative, Lin$^{neg}$) for CD45 and CD31 is necessary to eliminate from the c-kit cardiac cells the vast majority of lineage-committed cells. Half of the Lin$^{neg}$ c-kit$^{pos}$ cardiac cells express also Sca-1 [24] (Fig. 8.1).

Thus, unlike HSCs, for which surface markers have been extensively characterized, resident CPCs show a mixed and overlapping expression of several stem cell markers and an apparent multiplicity and heterogeneity of CPC sub-population. Different CPC populations have been reported in the developing and adult heart: c-kit$^{pos}$ CPCs [20, 24–28]; cardiosphere-derived cells (CDCs) [29, 30]; epicardium-derived cells (EPDCs) [31, 32]; cardiac side population cells (SP) [22, 33, 34]; Sca-1$^{pos}$ CPCs (stem cell antigen-1) [35–37]; Islet-1$^{pos}$ expressing CPCs [38, 39], and PDGFRα$^{pos}$ expressing CPCs (platelet-derived growth factor receptor-alpha) [40]. These cardiac cell populations are clonogenic, self-renewing, and multipotent both in vitro and in vivo and express specific transcription factors (Isl-1, Nkx2.5, MEF2C, and GATA4) in the embryonic and adult heart. Moreover, these populations express several markers of stemness (Oct3/4, Bmi-1, and Nanog) and show significant regenerative potential in vivo (Table 8.1).

Based on these data, a variety of studies have established that the heart contains a reservoir of stem and progenitor cells. Indeed, CSCs have been isolated from different animal models by selection based on c-kit, Sca-1, and/or Abcg2 (MDR-1) expression. Because the "stemness" of a cell is not linked to a single specific biological marker, many reporting groups have independently described a "unique" CSC or progenitor cell that has demonstrated to be different from those previously reported, showing a combination of different stem cell–associated cell surface markers. With the exception of the Islet-1 cells, which decrease dramatically in number into adulthood [39] and seem to be remnants from the cardiac primordia [41], the identification of different cardiac stem progenitor cells by expression of precise membrane markers suggests that these phenotypically different cells are likely to be phenotypic variations of a unique cell type. It is highly unlikely that a tissue, which, until recently was believed to lack any self-renewal capability, is indeed populated by several different types of tissue-specific stem cells.

## C-kit$^{pos}$ Cardiac Stem/Progenitor Cells

Most mammalian adult tissues harbor a subpopulation of stem and progenitor cells (hereafter referred as stem cells) that differentiate into some or all of the parenchymal cells of their tissue of origin [42]. In 2003, for the first time, the identification and characterization of small niches of endogenous cardiac stem cells in the adult mammalian heart has been associated with the expression of type III receptor tyrosine kinase c-kit (CD117 or SCFR-stem cell factor receptor) [20]. The expression of

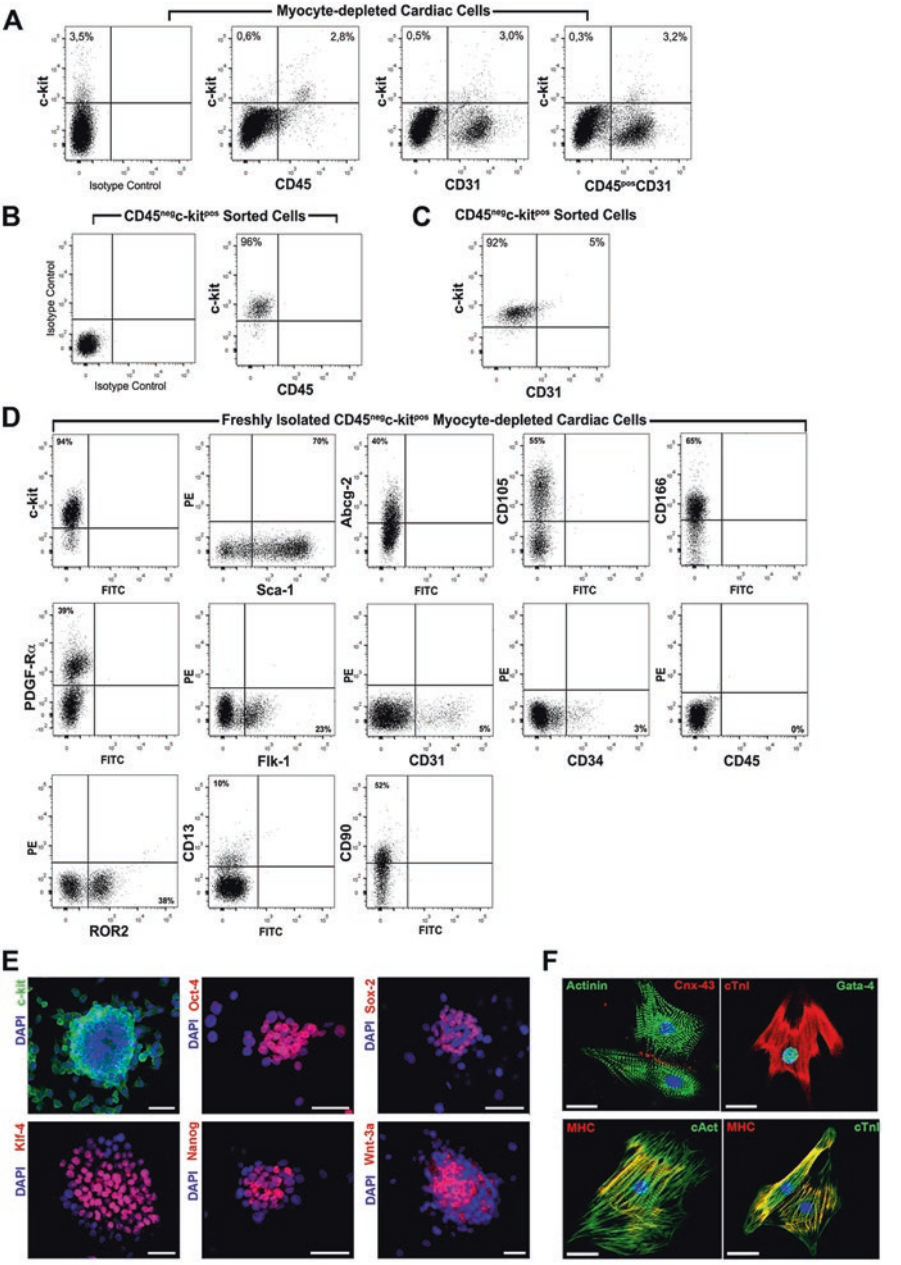


**Fig. 8.1** Phenotype- and tissue-specific stem/progenitor potential of freshly isolated myocyte-depleted c-kit$^{pos}$ cardiac cells. (**a**) Flow cytometry dot plots (representative of $n = 6$) show the percentage of total c-kit$^{pos}$ cells, c-kit$^{pos}$/CD45$^{pos}$, and c-kit$^{pos}$/CD31$^{pos}$ within the myocyte-depleted cardiac cells of adult mouse hearts. The right panel shows that the majority of c-kit$^{pos}$ cardiac cells (~90%) are either CD45- or CD31-positive, while only a minority (~10%) are CD45- and CD31-negative. (**b**) Flow cytometry dot plot shows that after sequential CD45-negative and c-kit-positive

**Table 8.1** Summary of CSC populations

| Phenotype | Markers | Source |
|---|---|---|
| c-kit$^{pos}$ eCSCs | CD34$^{neg}$, CD45$^{neg}$, Sca-1$^{pos}$, Abcg2$^{pos}$, CD105$^{pos}$, CD166$^{pos}$, GATA4$^{pos}$, NKX2–5$^{pos/neg\ or\ low}$, MEF2C$^{pos}$ | Mouse, rat, dog, pig, human |
| c-kit$^{pos}$ eCSCs | CD34$^{neg}$, CD45$^{neg}$, FLK1$^{neg}$, c-kit$^{pos/neg\ or\ low}$, GATA4$^{pos}$, NKX2–5$^{pos/neg\ or\ low}$, MEF2C$^{pos}$ | Mouse, human |
| Side population cells | CD34$^{pos}$, CD45$^{pos}$, Abcg2$^{pos}$, Sca-1$^{pos}$, c-kit$^{pos}$, NKX2–5$^{neg}$, GATA4$^{neg}$ | Mouse |
| Cardiosphere-derived cells | CD105$^{pos}$, CD34$^{pos}$, CD45$^{pos}$, Abcg2$^{pos}$, Sca1$^{pos}$, c-Kit$^{low}$ | Mouse, rat, dog, pig, human |
| Colony-forming unit fibroblasts (CFUFs) | Sca-1$^{pos}$, PDGFR-α$^{pos}$, CD31$^{neg}$, c-Kit$^{low}$, CD45$^{neg}$, FLK1$^{neg}$, CD44$^{pos}$, CD90$^{pos}$, CD29$^{pos}$ and CD105$^{pos}$ | Mouse |
| Cardiac mesangioblasts | CD31$^{pos}$, CD34$^{pos}$, CD44$^{pos}$, CD45$^{neg}$, Sca-1$^{pos}$, c-kit$^{pos}$ | Mouse, human |
| Isl1$^{pos}$ CPCs | CD31$^{neg}$, Sca-1$^{neg}$, c-kit$^{neg}$, GATA4$^{pos}$, NKX2–5$^{pos}$ | Mouse, rat, human |

*CSCs* cardiac stem cells, *CPCs* cardiac progenitor cells (adapted from Smith et al. [28])

c-kit has been shown to be involved in important cellular processes underlying progenitor maintenance, differentiation, proliferation, and migration in hematopoietic, germ, melanocytes, dendritic and mast cells, and other lineages [43–48]. c-kit$^{pos}$ endogenous cardiac stem cells (eCSCs) have been identified and characterized in the rat [20], mouse [30, 49], dog [50], pig [26], and human [25, 30, 51, 52] heart. These cells are present at a similar density in all species (~1 eCSC per 1000 cardiomyocytes or 45,000 human eCSCs per gram of tissue) [53]. Similar to the rodent heart, the distribution of c-kit$^{pos}$ eCSCs in pig and human heart varies in different cardiac chambers. c-kit$^{pos}$ cardiac resident stem cells in embryonic, neonatal, and adult mammalian heart have been identified by using different membrane markers such as Sca-1, Abcg-2, Flk-1, and PDGFR-α and transcription factors (Isl-1, Nkx2.5, GATA4, and Wt-1) [32, 53–56]. It is likely that a number of these identified cell populations represent different developmental and/or physiological stages of a unique resident stem cell [57].

**Fig. 8.1** (continued) sorting, sorted cardiac cells are uniformly CD45-negative and homogeneously c-kit-positive. (**c**) Flow cytometry dot plot shows that CD45$^{neg}$c-kit$^{pos}$-sorted cardiac cells contain a miniscule fraction of CD31-positive cells. (**d**) Flow cytometry dot plots (representative of $n = 5$) for membrane CD markers of freshly isolated and MACS-sorted CD45$^{neg}$c-kit$^{pos}$ cardiac cells from normal adult mouse hearts (for each FACS analysis, a minimum of six digested hearts were pooled together before MACS sorting). (**e**) c-kit$^{pos}$ (green) CSC cardiospheres express multipotent stemness markers (c-kit, Oct-4, Sox-2, Klf-4, and Nanog) and Wnt3a (red). Bar = 50 μm (**f**) CSC-derived contracting CMs express contractile proteins (Actinin, cTnI, MHC, and cardiac Actin) with co-expression of cardiac transcription factor (Gata-4). The CSC-derived CMs exhibit well-defined sarcomeric structures (*z* lines and dots) as well as gap junction formation (Cnx-43) between cells. DAPI stains nuclei in blue. Scale bar = 20 μm

In the adult myocardium, the lone identification of a c-kit$^{pos}$ cardiac cell population cannot be used to distinguish a specific CSC among all the other c-kit$^{pos}$ cardiac cells. Indeed, the adult heart contains a heterogeneous mixture of c-kit$^{pos}$ cells, mainly composed of mast and endothelial/progenitor cells. This heterogeneity of cardiac c-kit$^{pos}$ cells has generated confusion and controversy about the existence and role of CSCs in the adult heart. Most cardiac c-kit$^{pos}$ cells in myocyte-depleted cell preparations co-express blood/endothelial cell lineage commitment markers (Lin$^{pos}$) such as CD45 and CD31. CD45 and CD31 are expressed in the majority of cardiac c-kit$^{pos}$ cells (which also includes cells expressing CD34), while only ~10% is negative for blood/endothelial lineage markers (Lin$^{neg}$) (Fig. 8.1). In a cardiac cell population, a CD45-negative selection followed by a c-kit-positive sorting enriches for a population of cells that exhibit stem/progenitor characteristics. This strategy removes almost all the CD31- and CD34-positive cells from the total c-kit$^{pos}$ cardiac cells and allows the identification of a small population of CD45$^{neg}$ c-kit$^{pos}$ cells that are also positive, at different percentages, for Sca-1, Abcg2, CD105, CD166, PDGFRα, CD90, Flk-1, ROR2, and CD13. CD45$^{neg}$c-kit$^{pos}$ cardiac cells express Tert and Bmi-1, which are regulatory genes of stem cell proliferation and self-renewal, as well as the transcription factors, Gata-4 and Nkx2.5, which predict cardiac differentiation potential, and the genes involved in stem cell renewal and cardiac development, Oct-4, Nanog, Klf-4, and Sox-2 [24] (Figs. 8.1 and 8.2). This double selection still yields a progenitor cell population with a heterogeneous phenotype and developmental potential, comprising a mixture of primitive cells and more committed progenitors. CD45$^{neg}$c-kit$^{pos}$ cardiac cells can be propagated over long-term culture and maintained in an undifferentiated, self-renewing, and stable state, without showing evidence of senescence or abnormal karyotype [58]. eCSCs are clonogenic in vitro and grown in suspension, they form spheres of hundreds of cells similar to the pseudo-embryoid bodies formed by the neural stem cells (neurospheres), which, by analogy, we named cardiospheres, and they represent a distinctive feature of multipotent cells [20]. Indeed, CSCs grown in differentiation media for endothelial (EC), smooth muscle (SMC), and cardiomyocyte (CM) lineages acquire phenotypic characteristics of these different cell types (Fig. 8.1). In contrast, Lin$^{pos}$c-kit$^{pos}$ cardiac cells (i.e., CD45$^{pos}$CD31$^{pos}$c-kit$^{pos}$, ~90% of total myocardial c-kit$^{pos}$ cells) are not able to form spheres or show clonal expansion, but they become vWF-positive in EC differentiation conditions. Similarly, c-kit$^{neg}$ cardiac cells neither clone nor generate CSs. Lin$^{pos}$c-kit$^{pos}$ cardiac cells plated in the cardiomyogenic medium negligibly become cTnI-positive, while in the SMC medium, only a small number acquires smooth muscle actin (SMA) expression [24] (Fig. 8.1).

During cardiac development, well-orchestrated signaling pathways involving several morphogen families regulate various interactions between transcriptional and growth factors necessary for cardiac tissue specification. These extracellular

**Fig. 8.2** (continued) express both Sca-1 and PDGF-Rα. This negative/positive multiple-marker expression of freshly isolated cells is similarly shown by multipotent single cell-derived CSC clones propagated in vitro, and it represents the minimal or "essential phenotype for the identification and isolation of mammalian adult endogenous CSCs"

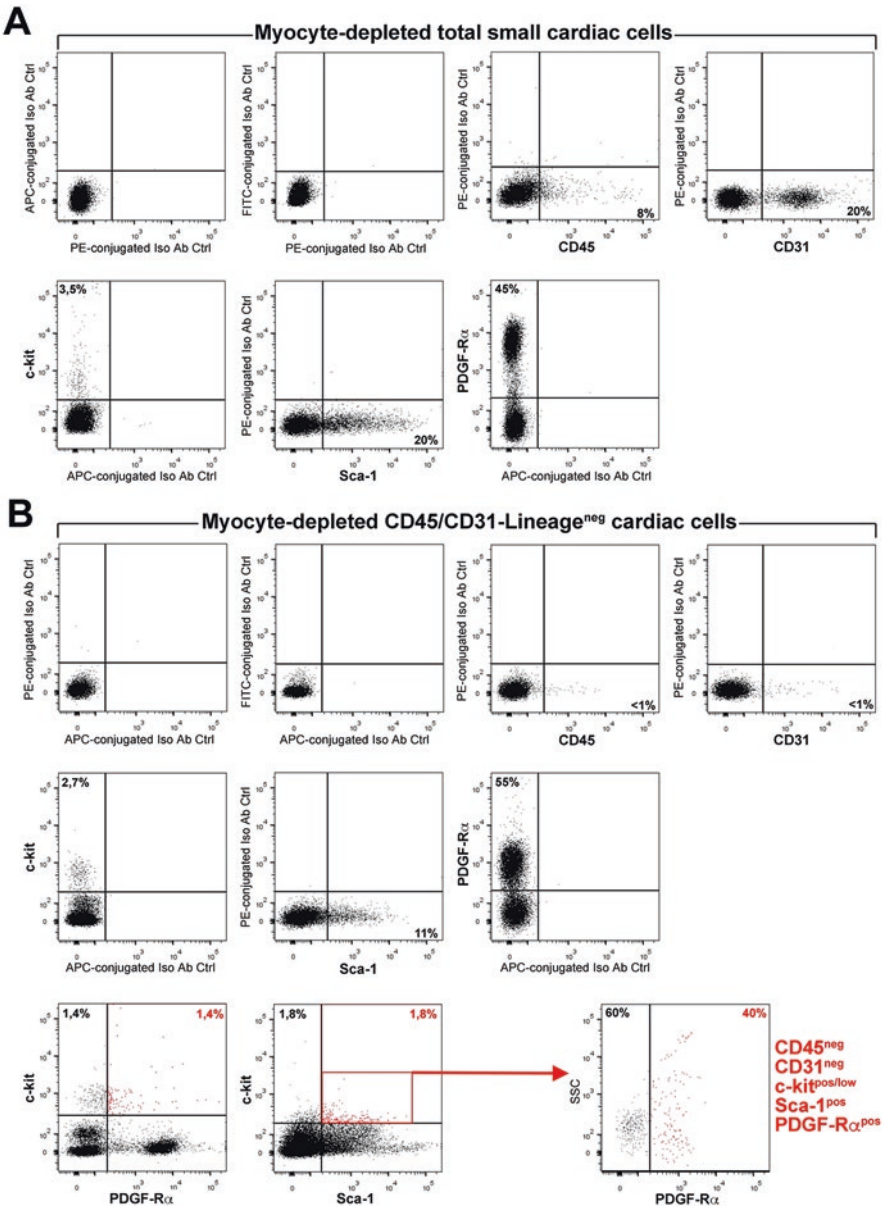


**Fig. 8.2** (**a**) Flow cytometry dot plots (representative of $n = 3$) show expression of CD45, CD31, c-kit, Sca-1, and PDGF-Rα in the myocyte-depleted total cardiac cells obtained through enzymatic digestion of a mouse heart by retrograde perfusion. (**b**) After CD45 and CD31 negative sorting, the flow cytometry analysis shows the efficiency of CD45 and CD31 removal from the cell preparation. The CD45/CD31 lineage-negative cardiac cells still express c-kit, Sca-1, and PDGF-Rα. Importantly, half of the $CD45^{neg}/CD31^{neg}$ $c\text{-}kit^{pos}$ cardiac cells (that are enriched for CSCs) express Sca-1 or PDGF-Rα. More importantly, nearly 20% of the $CD45^{neg}/CD31^{neg}$ $c\text{-}kit^{pos}$ cardiac cells

instructive morphogens or cardiopoietic growth factors (cGFs) [59, 60] finally regulate proliferation and differentiation of embryonic stem cells, promoting the development of the heart through progenitor cells committed [61]. Cardiac progenitor cell proliferation in mesoderm is regulated by the canonical Wnt, FGF, and Hedgehog pathways. On the contrary, Notch and non-canonical Wnt signaling pathways regulate the differentiation events during heart formation [62–65]. As expected from a true cardiac-specific stem/progenitor cell population, cloned $Lin^{neg}$c-$kit^{pos}$ CSCs respond in vitro to the Wnt/β-catenin and TGF-β/SMAD signaling pathways. The cardiomyocyte differentiation program in c-$kit^{pos}$ CSCs follows a step-by-step finely regulated molecular cascade that is closely reminiscent of the known molecular program at the basis of the cardiac development from primary heart tube to the fetal/neonatal heart. In vitro administration of cardiac morphogens, through gain- and loss-of-function experiments, allows us to modulate the self-renewal potential and cardiomyogenic specification of CSCs to generate fully differentiated contracting CMs [24, 66–69]. CSCs express cell surface receptor of the Wnt/β-catenin canonical pathway, Frizzled, as well as its co-receptor, low-density lipoprotein receptor-related protein 6. Wnt-3a, Wnt-3a-conditioned medium, and bromoindirubin-3′-oxime (BIO) stimulate CSC expansion and clonogenicity, while canonical Wnt inhibition decreases CSC proliferation and clonogenicity. In contrast, Dickkopf-1 (Dkk-1) increases CSC myocyte specification, even though its effect is not sufficient to produce a fully differentiated beating phenotype. Cultured CSCs express the cell surface receptor for TGF-β/SMAD signaling, TGF-β-R1. The TGFβ family, also comprising BMPs and Activin A, plays critical and specific roles in cardiac development and CM commitment [61, 70]. In CM differentiation medium, BMP-2, BMP-4, TGF-β1, and Activin-A, increase significantly the expression of myogenic lineage markers and the number of cTnI$^{pos}$ myocyte-committed cells. These evidences confirm that CSCs respond to known cardiac morphogens. Wnt canonical pathway inhibition and TGF-β family activation, each independently, promote cardiomyogenic commitment. Nevertheless, individual modulation of each of these cGFs is insufficient to generate fully differentiated contracting CMs. Transcriptome comparison of RNA-seq data from CSCs, CSCs-derived CMs, neonatal CMs, and adult CMs showed high similarity between CSCs-derived CMs and neonatal CMs. Accordingly, the in vitro myogenic specification of clonogenic adult CSCs produces bona fide cardiomyocytes whose structural, molecular, and functional maturity is nearly indistinguishable from that of neonatal mammalian cardiomyocytes [24].

The regenerative capacity of adult endogenous $CD45^{neg}$c-$kit^{pos}$ cardiac stem cells has been evaluated using different rodent models of diffuse myocardial damage inducing acute heart failure [71, 72]. It has been shown that after transplantation of adult endogenous $CD45^{neg}$c-$kit^{pos}$ cardiac stem cells, the myocardium is able to the regenerate cardiomyocytes and microvasculature [20, 73–76]. Moreover, administration of a cell progeny derived from a single $CD45^{neg}$c-$kit^{pos}$ clonogenic CSC, genetically marked with GFP (GFP$^{pos}$-CSC), in syngeneic rats has been reported. After experimental acute myocardial infarction (AMI), robust histological and functional myocardial regeneration occurs. Indeed, 28 days after AMI and, GFP$^{pos}$-CSC

transplantation, histological analysis of rat hearts revealed a high cell engraftment rate in the border/infarct zone, yielding myocardial regeneration with formation of new cardiomyocytes, capillaries, and arterioles (Fig. 8.3). Furthermore, GFP$^{pos}$-CSC transplantation reduced myocyte apoptosis and hypertrophy, significantly decreased scar size and left ventricle dilation, and improved fractional shortening and ejection fraction. On the contrary, the administration of a population of total GFP$^{pos}$-c-kit$^{pos}$ cardiac cells after AMI showed no protective effect on the cardiac function and a slightest formation of new cardiomyocytes only has been detected. Most of the c-kit$^{pos}$ total cardiac cells acquired endothelial lineage specification (Fig. 8.3).

Overall, only true clonogenic and multipotent CSCs (~1–2% of the total cardiac c-kit$^{pos}$ cells) have a robust cardiomyogenic potential and the capacity to functionally regenerate the infarcted myocardium. The large majority of c-kit$^{pos}$ cardiac cells are not and should not be considered CSCs. The expression of the stem cell receptor c-kit is an essential marker of the mammalian CSCs, but alone, it is not sufficient to identify the true cardiac stem cells [24].

## Sca-1-Positive Cardiac Cells

Sca-1, or lymphocyte activation protein-6A (Ly-6A), belonging to the Ly6 gene family, is a glycosyl phosphatidylinositol-anchored cell surface protein (GPI-AP) first reported as a cell surface marker of hematopoietic stem cells (HSCs) in combination with c-kit. Sca-1 expression in a cardiac cell population has been documented and described for the first time in 2003. Oh et al. [35], using Sca-1 as the principal surface marker, identified a population of cardiac cells in the murine adult myocardium with telomerase activity analogous to that observed in the newborn heart. Indeed, the majority of heart-resident TERT-positive cells could be identified through Sca-1 expression [77]. Although transcripts for cardiac structural genes were absent, these Sca-1$^{pos}$ cardiac cells expressed transcriptional regulators indicative of cardiac commitment such as GATA-4, Mef2c, and Tef1 [35, 78, 79] and a fraction of them exhibited adult tissue-specific stem cell properties [55, 78]. It has been reported that the level of Sca-1 expression in a cardiac cell population may actually play a role in their differentiation potential with a high Sca-1 expression having a broader differentiation potential than low Sca-1 expression in CPCs [80].

Sca-1$^{pos}$ cardiac cells are considered distinct from HSCs due to the lack of CD45, CD34, Lmo2, GATA-2, and Tal1/Scl protein expression. Sca-1$^{pos}$ cardiac cells are also distinct from endothelial progenitor/precursor cells since the lack of expression of CD34, Flk-1, or Flt-1. In 6- to 12-week-old mice, isolated Sca-1$^{pos}$ CPCs are capable of cardiomyogenic differentiation in vitro [35, 55, 78, 80, 81] upon induction with a cocktail of factors such as the cytosine analog 5-azacytadine, TGF-β1, DKK-1, DMSO, BMP2, FGF4, and FGF8 and vitamin C [81], which turn on the expression of Nkx2.5, connexin 43, α-sarcomeric actinin, cardiac troponin, and α-MHC [35, 79]. Furthermore, Sca-1$^{pos}$ CPCs are also able to differentiate in vitro

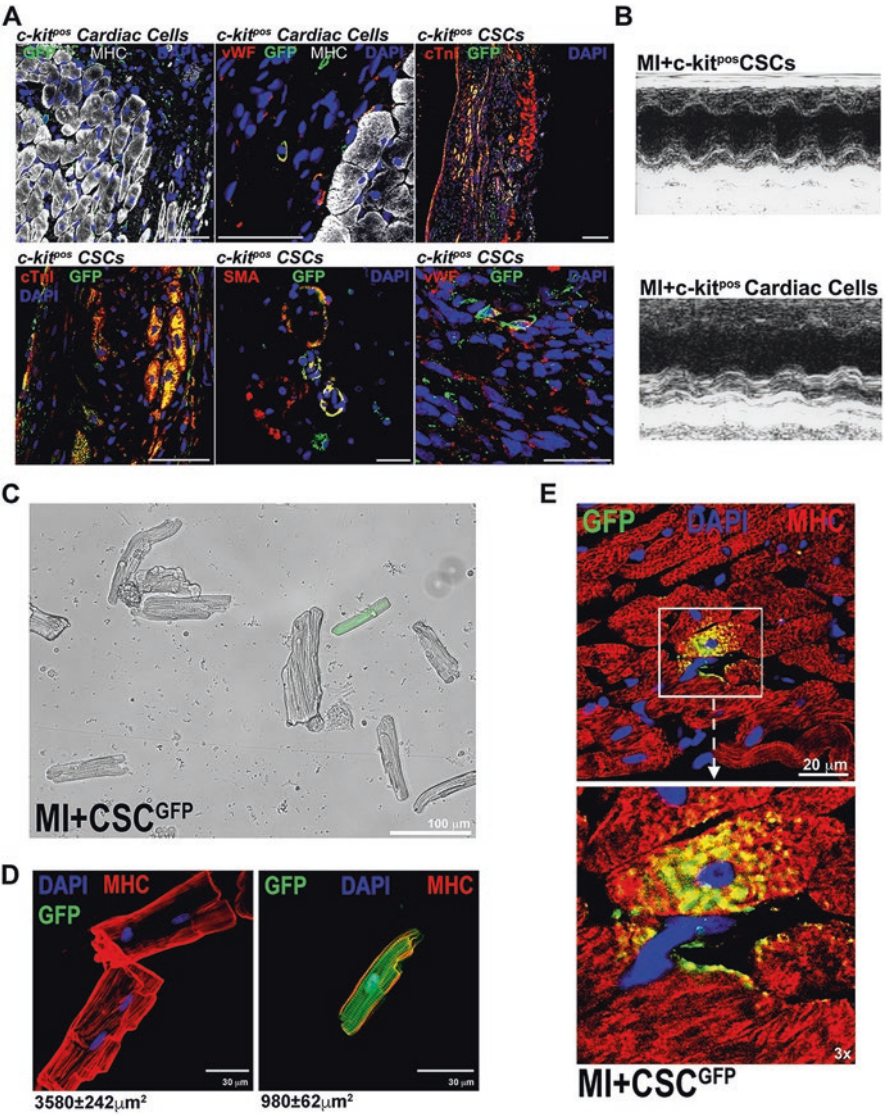


**Fig. 8.3** c-kit$^{pos}$ CSCs not c-kit$^{pos}$ cardiac cells are multipotent in vivo. (**a**) Representative confocal images show GFP-positive cell engraftment and differentiation 28 days after MI in rats treated with GFP-transduced total c-kit$^{pos}$ cardiac cells or c-kit$^{pos}$ CSCs. Most of the GFP$^{pos}$ c-kit$^{pos}$ cardiac cells acquired endothelial lineage specification (vWF, red, upper mid panel). On the contrary, GFP$^{pos}$ c-kit$^{pos}$ CSCs differentiate into CMs (cTnI, red), arterioles (SMA, red), and capillaries (vWF, red). DAPI stains nuclei in blue. Bar = 50 μm except for upper right panel = 200 μm and bottom right panel = 25 μm. (**b**) Representative M-mode echocardiography images for rats treated with GFP$^{pos}$ total c-kit$^{pos}$ cardiac cells or GFP$^{pos}$ c-kit$^{pos}$ CSCs, 28 days after MI. (**c**) Light microscopy image of freshly isolated adult cardiomyocytes from a dissociated heart 28 days after myocardial infarction (MI) and CSC$^{GFP}$ injection (MI + CSC$^{GFP}$) shows a CSC-derived GFP-positive cardiomyocyte. (**d**) Confocal microscopy images show host-derived pre-existing GFP$^{neg}$ cardio

into both endothelial and smooth muscle lineages expressing, respectively, CD31, vWF, and Flk-1, and SMA [79]. When administered intravenously after ischemic/reperfusion injury, Sca-1$^{pos}$ CPCs exhibit in vivo regenerative potential [35]. These cells were able to home to the injured myocardium and to form new CMs [35], attenuating adverse structural remodeling and increasing LV ejection fraction [81]. Moreover, Sca-1$^{pos}$ cells also had the ability to differentiate into endothelial cells in vivo [80].

Despite the fact that a human ortholog of the Sca-1 protein has not been identified so far, Goumans [82] have reported the successful isolation of a cardiac progenitor cell population in the human fetal and adult heart based on an antibody directed against the mouse Sca-1 epitope. These human Sca-1 cardiomyocyte progenitor cells (CMPCs) showed a capability for self-renewal and multipotency by differentiating toward cardiac myocytes and/or vascular tube-like endothelial cells positive for PECAM-1. Tested in immunodeficient mice for their regenerative capacity, fetal human Sca-1 CMPCs improved cardiac function following infarction and showed in vivo differentiation toward a cardiomyocyte-like phenotype based on the presence of troponin I [36]. Human Sca-1$^{pos}$-like cells express early cardiac transcription factors (GATA-4, Mef2c, Isl-1, and Nkx-2.5) and differentiate into contractile CMs [36].

To better understand the Sca-1 gene function and its contribution to the heart, different animal models, modified to track the Sca-1 gene or the fate of Sca-1$^{pos}$ cells, have been used. Genetic deletion of Sca-1 (knockout, KO) causes primary cardiac defects in myocardial contractility [83]. Sca-1-KO mice display age-related cardiac hypertrophy, fibrosis, and dysfunction after pressure overload in a myocardial infarction (MI) model evolving in myocardial and cardiac progenitor cell function impairment [83]. As result, mice display defects in cardiac repair consistent with impairment of resident cardiac progenitor cell proliferative capacity associated with altered canonical Wnt signaling [83].

Mapping the fate of Sca-1$^{pos}$ cells, several reports confirm that Sca-1 expression is not detected in adult CMs [84], in healthy or injured heart, where the descendants of Sca-1$^{pos}$ cells contribute to CM formation with an evident and continuous replacement of Sca-1-derived CMs during the lifespan and after MI [84]. With the same experimental approaches, it seems that only a few number of Sca-1-derived CMs are detectable before 2 months of age [84]. Thus, nowadays, it is believed in the existence of two distinct CM progenitor cells, one that generates most CMs during fetal life and another that generates the small number of new CMs detected in adult heart [84]. Another intriguing option is referred to the possibility that a few CM progenitors may acquire Sca-1 expression after birth.

**Fig. 8.3** (continued) myocytes as compared to CSC-derived GFP$^{pos}$ cardiomyocyte isolated from MI + CSC$^{GFP}$ rat hearts at 28 days after MI. Note that GFP$^{pos}$ cardiomyocytes are of smaller size and mononucleated than surviving binucleated GFP$^{neg}$ cardiomyocytes of the host. Average area profile values (in $\mu m^2$) are reported below each image. Data are presented as mean ± SD ($p < 0.05$). $N = 3$ rats. (**e**) Representative confocal images show, at high magnification, a CSC-derived newly formed GFP$^{pos}$ cardiomyocyte in the infarct-border zone 28 days after MI treated with CSC$^{GFP}$

In the past few years, a biological hierarchy and correlation, still unclear, between cardiac side population (SP) cells and Sca-1$^{pos}$ cells have been identified, where a high percentage (~80%) of adult cardiac SP cells express Sca-1 and a small percentage (1 ~ 3.5%) of cardiac Sca-1$^{pos}$ cells are included in SP fraction [33, 35]. Pfister et al. [33] have demonstrated that a subpopulation of Sca-1$^{pos}$/CD31$^{neg}$ cells within cardiac SP cells was enriched for cardiomyogenic potential. These reports suggest and confirm that there is a substantial heterogeneity in each of the primary isolated cardiac stem/progenitor cells including SP and Sca-1$^{pos}$ cells. The purification of cardiac SP and Sca-1$^{pos}$ cells in combination with several markers may be useful to examine the more precise dynamics of proliferation and differentiation of cardiac stem cells. Using Sca-1 (Ly6a) gene-targeted mice containing either a constitutive or inducible Cre recombinase to perform genetic lineage tracing of Sca-1$^{pos}$ cells in vivo, Molkentin's group claimed that cardiac-resident Sca-1$^{pos}$ cells are not significant contributors to cardiomyocyte renewal in vivo [85]. However, this genetic tracing system only negligibly marked the Sca-1$^{pos}$ CD3$^{neg}$ cardiac cell population, the Sca-1$^{pos}$ cardiac fraction with myogenic potential in vitro and in vivo [33]. Thus, the data by Vagnozzi et al. traced only the endothelial differentiation potential of the heterogeneous Sca-1$^{pos}$ cardiac cell population, while their data on cardiomyocyte renewal are significantly flawed.

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 SP and c-kit$^{pos}$ endogenous CSCs. Therefore, what now needs to be determined is whether these distinct cardiac cell populations represent different cell types or the same one at different developmental/differentiation stages.

## Side Population Cells

Side population cells (SPCs) were first discovered in the murine bone marrow allowing an enrichment of the hematopoietic stem cell population [86]. SPCs were identified for their capacity to partially extrude the DNA-binding dye Hoechst 33342 through the ATP-binding cassette transporter ABCG2 (also referred to as MDR-1) [22, 87], a transmembrane protein already known to confer resistance to the cells by mediating the efflux of drug out of the cytoplasm [88].

Since their first identification, SPCs have been isolated from several tissues, identifying progenitor and stem cells throughout the body [86, 89]. In 2002, Hierlihy et al. [90] first reported the presence of an endogenous cardiac side population (cSP) with stem cell-like activity in the adult myocardium. They found that this population was ~1% of the total cell number in the adult murine heart [90]. In the last decade, several protocols have been used to assess the differentiation potential of these cells. cSP cells differentiate into cardiomyocyte-like cells expressing cardiac markers when co-cultured with neonatal or adult rat ventricular cardiomyocytes [33, 91] or treated with oxytocin or trichostatin A [92]. These cells successfully

generate CMs that are functionally and structurally comparable to adult cardiomyocytes, having well-organized sarcomeres and beating spontaneously. FACS analysis on isolated cSPCs revealed that they do not express hematopoietic markers such as CD45 and CD34 but that they are positive for the cellular adhesion markers CD31. Moreover, they are positive for the stem cell marker Sca-1 but negative for c-kit [33]. These data were confirmed by microarray-based transcriptional profile carried from Dey et al. [93].

Remarkably, among the Sca-1$^{pos}$ cSPCs, it is possible to distinguish two distinct subpopulations on the basis of the expression of CD31. In fact, CD31$^{pos}$/Sca1$^{pos}$ constitutes the majority of total cSPCs (~75%), while CD31$^{neg}$/Sca1$^{pos}$ cells represent only 10%. The analysis of these two subpopulations showed that the higher cardiomyogenic potential is associated with the CD31$^{neg}$/Sca1$^{pos}$ cells. Furthermore, phenotypic analysis of CD31$^{neg}$/Sca1$^{pos}$ CSPs revealed that these cells also expressed cardiac-specific proteins such as Nkx2.5, GATA4, SMA, and desmin at baseline and that, when co-cultured with freshly isolated adult cardiomyocytes, they acquire a more mature phenotype expressing α-actinin, troponin I, and connexin-43 and undergo spontaneous contraction [33].

Notably, the activation of cSPCs in response to cardiac injury has been reproducibly demonstrated, both in mice [94, 95] and in humans [96, 97]. The therapeutic potential of cSPCs was first tested injecting CD31$^{neg}$/Sca1$^{pos}$ cells in MI mouse models where cell administration was able to improve LV ejection fraction (LVEF) and promote myocardial neo-angiogenesis [81]. Subsequently, Oyama et al. [92] found that GFP-labeled cSPCs, isolated from neonatal rats, were able to home to the site of injury and to form new cardiomyocytes, fibroblasts, and endothelial and smooth muscle cells in cryoinjured hearts [92]. Similar data were reported in mice by Noseda et al. [40] using clonally expanded cSPCs from adult mice. Twelve weeks after coronary artery ligation and cell injection into the border infarct zone, transplanted cells were able to engraft and to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells. Consequently, it was possible to observe in cSPC-injected mice an improved ejection fraction and a reduced scar size when compared to those of the uninjected control mice [40].

## Cardiosphere-Derived Cells

Cardiosphere-derived cells (CDC) constitute a candidate pool of cardiac stem/progenitor cells. They represent a heterogeneous population of cardiac cells with clonogenic and self-renewal potential [98], ability to differentiate into cardiomyocytes and endothelial and smooth muscle cells [20, 99]. CDCs are undifferentiated, yet heterogeneous cells grow in vitro as self-adherent “clusters” and express stemness markers such as c-kit and Sca-1 and also the endothelial markers CD34 and CD31. Spheres have been considered a feature of stemness as described for neural stem cells [100]. CDCs have been isolated successfully from various species (rodent, porcine, canine, primate, and human) by several groups [30, 98, 99, 101–103].

Messina et al. [30] for the first time reported that cardiac progenitor cells, clonally expanded from murine and human myocardial biopsy, were able to form three-dimensional spherical structures in vitro. An unresolved issue is whether CDCs are endogenous to the heart or whether they home to the myocardium from an extracardiac origin, perhaps the bone marrow [13, 20]. To this aim, White et al. [104] have demonstrated, by three independent molecular methods, that CDCs originate within the heart, with no evident extracardiac contribution.

In the last years, different cardiosphere isolation protocols have been tested and used, giving results sometimes in contrast to each other [105], but overall, every isolated CDC population, in vivo tested into a myocardial infarction model, has demonstrated to be a promising resource for regenerative therapies [106]. Indeed, it has been proposed that CDCs mimic several features of cardiac stem cell niches, including the presence of both primitive and differentiating cells and expression of endothelial cells, which are associated with enhanced in vivo cell survival and cardioprotection after MI [107]. Furthermore, it has been demonstrated that CDCs reduce scar formation after MI, increase viable myocardium, and boost cardiac function in preclinical animal models [30, 98, 107, 108]. Recently, different progenitor cell types, including CDCs, were tested in vitro and in vivo to assess their cell potency and their functional myocardium repair. In particular, Li et al. have shown that human CDCs display the greatest myogenic differentiation potency in vitro when compared with human BM-derived MSCs, adipose tissue-derived MSCs, and BM-derived mononuclear cells. Furthermore, a series of preclinical studies in patients with left ventricular dysfunction have been achieved by intracoronary delivery of CDCs, which resulted in the formation of new cardiac tissue, reduction of the infarct size, and improvement of hemodynamic parameters [109].

Li et al. [110] showed that in vivo injection of CDCs into infarcted mouse hearts results in a significant improvement of cardiac function. Moreover, they showed that 3 weeks after treatment, there is a high engraftment of these cells in the heart and a high myogenic differentiation rates, with a lower number of apoptotic cells, when compared with the regenerative potential of all the above cited stem cell types [107].

Finally, these cells have already entered a phase I clinical trial (CADUCEUS) [111]. Results from the trial, after a 1-year follow-up, displayed an increased viability of cardiac muscle without clear benefits on cardiac function [112].

## Mesenchymal Cardiac Stem Cells

The presence of mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells or fibroblast colony-forming units (CFU-F), was first demonstrated in the bone marrow in the 1970s [113] and then reported also from other mature tissues such as umbilical cord, adipose tissue, endometrial polyps, heart, liver, etc. MSCs are non hematopoietic cells able to replicate extensively in vitro and to form clones. Furthermore, they have angiogenic properties and immunoregulative activity [114]. For their reported ability to differentiate into the three different lineages, namely,

mesoderm, ectoderm, and endoderm, they have attracted enormous attention owing to their broad therapeutic potential. In 2006, the International Society for Cellular Therapy established the three minimal characters to define MSCs: (1) plastic adherence, when cultured in standard conditions; (2) expression of CD105, CD73, and CD90 and negativity for CD45, CD34, CD11b or CD14, CD19 or CD79, and for HLA-II molecules; and (3) ability to differentiate in vitro in adipocytes, chondroblasts, and osteoblasts [115].

In particular, the presence of the MSCs in the adult heart has been recently considered as an alternative resource of cells to maintain myocardial homeostasis and to stimulate cardiac regeneration, giving rise to cardiomyocytes (cardio-mesenchymal stem cells—CMSCs) [116–118]. Interestingly, it was demonstrated that the MSCs can be isolated from adult human heart and expanded in vitro above 40 population doublings, reflecting their ability to self-renew. Moreover, their expression profile, telomerase activity, immunophenotype, and expression of growth factors and cytokines are identical to those of bone marrow- and liver-derived MSCs [119].

MSCs isolated from the adult heart express the PDGFRα and SCA-1 [116]. Clonal mesenchymal colonies also express some cardiac developmental transcription factors, including GATA4, TBX5, HAND1, and MEF2C, suggesting a cardiac identity and/or lineage-committed state [120].

Recently, a new population of MSCs have been identified on the basis of expression of the W8B2 antigen. These cells can differentiate in vitro into cardiac-like cells when treated with specific cardiogenic factors [121]. Thanks to its cardiac specificity, this subpopulation is characterized by a specific paracrine activity, with protective and proangiogenic effects, representing a new attractive resource of cells in the treatment of heart diseases. In fact, transplantation of MCSs in an infarcted heart shows a cardiac protection effect possibly due to the direct action of these cells in the tissue or to the secretion of MSC-derived factors acting on the adult resident cardiac stem cells.

In short, adult cardiac MSCs are likely to represent a cardiac lineage progenitor; able to maintain the stromal, matrix, and vascular compartments of the heart during homeostasis; and to contribute to the heart repair following an injury. They also act with a paracrine function, mediating dialogs between the other cardiac resident cells, including cardiomyocytes and immune cells. However, the MSC biology has not yet been fully understood, indeed, although the proliferative capacity of cardiac MSCs in vitro reflects their self-renewal and progenitor ability; in vivo further analysis is needed to demonstrate their exact cardiomyogenic potential in the adult heart.

## Isl-1$^{pos}$ Cardiac Progenitor Cells

A completely separate population of progenitor cells resident in the heart has been identified by the expression of the LIM homeodomain transcription factor Islet-1 (Isl-1) [39]. These cells, originally also identified in rodent and human postnatal myocardium [39], are a major source of embryonic cardiac progenitors that

primarily contribute to the atria, outflow tract and right ventricle, and second heart field (SHF)-derived structures [41] through a process dependent on Wnt/β-catenin signaling [122].

Isl-1 is expressed in other cell lineages during embryogenesis [123, 124]. Homozygous mutant mice for Isl-1 exhibit growth retardation and death during embryonic life because of cardiac malformation due to single ventricular chamber and severe reduction in atrial tissue [125, 126]. These observations demonstrated that Isl-1 marks undifferentiated cardiac progenitors that contribute substantially to the embryonic heart with a functional role of Isl-1 more critical in the second myocardial lineage than in the first.

Despite the key role of Isl-1, this transcription factor is not considered an unequivocal marker of this subpopulation of SHF progenitor cells. Isl-1$^{pos}$ cells are also involved in the development of the proepicardium and endocardium [127, 128]. The neural crest also contributes to an Isl-1-expressing cardiac cell population during development [129]. Expression of Isl-1 is involved in the direction of primitive cardiac progenitors to more specific lineages-restricted progenitor cells [130]. Indeed, using two independent transgenic and gene-targeting approaches in human embryonic stem cell lines (ESC), Isl-1$^{pos}$ progenitors are capable of self-renewal and expansion before differentiation into the three major cell types of the heart [38]. The definite cardiac origin of these cells, their contribution to cardiac development and their multipotency, has been clearly defined, by genetic fate mapping results [131]. Successful derivation of Isl-1-positive multipotent cells has been achieved from ESCs [38, 130] or from neonatal cardiac tissue [39], but there is far less evidence of Isl-1-expressing cells playing a significant role in adult life. The scarcity of Isl-1$^{pos}$ cells after embryonic development, with very few identified throughout the heart in the 1-day-old neonatal rat [39], or 2- and 6-day neonatal human tissue [132], 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 the adult cardiac tissue. Recently, Genead et al. [133] demonstrated the contemporary expression of c-kit and Isl1 markers in a cell population of rat adult hearts in normal, pregnant, and infarcted animals. Moreover, few Isl-1$^{pos}$ cells were detected in the adult (11–13 weeks) rat heart, although these were also all cardiac troponin-I positive, indicating their cardiomyogenic differentiation [134]. These cells have also been studied in non-physiological situations, where Isl-1$^{pos}$ cells have been identified in the periphery of an infarct in the mouse heart following pretreatment with thymosin-β-4 [135]. In addition, Isl-1$^{pos}$ cells have been obtained in vitro from cardiosphere-derived cells (CDCs), which were in turn obtained from cells activated in vivo following myocardial infarction in 9-month-old mice [79]. Other authors using heterozygous Isl-1- LacZ mice [136] have supported the hypothesis that, in the adult heart, different cell populations derive from Isl-1$^{pos}$ embryonic precursors, such as smooth muscle cells, parasympathetic neurons, and sinoatrial node (SAN) cells. However, these results did not provide evidence for Islet-1$^{pos}$ cells to serve as myogenic progenitors in the adult heart [136].

## Epicardium-Derived Cells (EPDCs)

The outermost layer of the adult mammalian myocardium, named epicardium, is constituted by a layer of epithelial cells that has been shown to contribute to the formation of the coronary vasculature during embryogenesis [137]. The epicardium consists of a quiescent single-cell layer; despite the structure of the epicardium not being completely known, several epicardial cell-specific proteins have been identified, including WT1 [138], Tbx18 [139], Tcf21 [140], Gata5 [141], and cytokeratin [142]. As observed in Bollini et al. [31], they express also the mesenchymal markers endoglin (CD105), the hyaluronan receptor CD44, the major T-cell antigen 1 (Thy-1 or CD90), and the platelet-derived growth factor receptor (PDGFR)-β.

During heart development, the proepicardial-derived cells cover the myocardium with a multicellular epithelium coating the ventricles [143]. A portion of epicardial cells is subjected to epithelial-to-mesenchymal transition (EMT), during which epicardial cells, first, lose the epithelial signature (defined by apical-basal polarity, and cell–cell contacts) by reducing the expression of the transmembrane adhesion proteins E-cadherin and zonula occludens-1 (ZO-1). Subsequently, the epicardial cells acquire typical mesenchymal cell characteristics displaying a spindle shape morphology and upregulating the expression of fibronectin, N-cadherin, and matrix metalloproteases (MMPs). The mesenchymal epicardium-derived cells (EPDCs), activating a migratory process, are able to move into the myocardial interstitium in which they can differentiate into several cell types and contribute to the development and maturation of the myocardium [143, 144]. The EPDCs can differentiate into cardiac cell types (mainly to non myocyte supporting cells relevant to cardiomyocytes) such as interstitial fibroblasts producing the cardiac extracellular matrix, smooth muscle cells (SMCs), and adventitial fibroblasts sustaining the coronary vasculature.

EMT and migratory process of the EPDCs are finely regulated by PDGF-β and PDGFRβ [145], Tbx5 [146], thymosin β4 [32], and Ets transcription factors [143]. Among these factors, the peptide thymosin β4 was identified by Bock-Marquette et al. [147] to enhance survival and repair of adult cardiomyocytes and can induce the adult epicardium to contribute coronary endothelial and smooth muscle cells and initiate vascular repair [148].

Many studies have demonstrated cardioprotective effects of Tβ4 and its role in regeneration of the ischemic heart by promoting cardiomyocyte survival, by modulating the inflammatory environment, and by promoting neovascularization [149]. Moreover, in vivo experiments on murine models indicate that the adult heart can respond to injury with a modest increase in $Wt1^{pos}$ EPDCs but without initiating a cardiogenic program. Tβ4 enhances this response through a significant reactivation of Wt1 (Wilm's tumor 1) expression, a key embryonic epicardial gene, ultimately resulting in cardiomyocyte refreshment [32]. Furthermore, Tβ4 treatment in mouse epicardial explant cultures showed extensive outgrowth of cells that differentiated in both endothelial and smooth muscle cells. These data suggested that the adult

heart contains a resident epicardial-derived stem/progenitor cell population, which has the potential to contribute bona fide terminally differentiated cardiomyocytes after myocardial infarction [32].

Limana et al. [137] reported that human and mouse epicardial/subepicardial compartments include a population of cells expressing stem cell antigens c-kit and CD34. In particular, these cells are localized in the mesothelial layer, the main constituent of murine epicardium, as well as in the subepicardial compartment of the human epicardium, characterized by the presence of adipose tissue. Epicardial c-kit cells also express MDR1. In the mouse, after myocardial infarction (MI), epicardial c-kit cells have been shown to participate to the reparative process by proliferating and differentiating into myocardial and vascular cells [137].

The experiments performed on human cells demonstrated that EPDCs treated with transforming growth factor-1 (TGF-1) or bone morphogenetic protein-2 (BMP2) have the ability to differentiate into smooth muscle cells but not into endothelial cells, thus recapitulating, at least in part, the differentiation potential of their embryonic counterpart [150]. Furthermore, transplantation of hEPDCs into infarcted mouse hearts preserved left ventricular function and attenuated pathological remodeling [151].

On the basis of these findings, epicardial cells and EPDCs show multipotent progenitor cell behavior in the embryo proper and they might be potentially of interest for AMI treatment and to understand cardiac disease-related mechanisms.

## Pericytes

Pericytes or perivascular mesenchymal cells are mural cells that surround blood vessels, adjacent to endothelial cells (ECs). Due to their presence in different tissues, pericytes have been termed according to their function and morphology, such as hepatic stellate cells in the liver and glomerular mesangial cells in the kidney [152, 153]. Their morphology can appear stellate or spindle-like, with finger-like projections surrounding the vessels [154]. They play critical roles in maturation and maintenance of vascular branching morphogenesis [155]. In addition, pericytes have also been shown to play a crucial role in niche maintenance for hematopoietic stem cells in the bone marrow [156].

The characterization of pericyte molecular phenotype, based on their localization, has been a challenge in the last two decades. The 3G5 antigen, originally suggested to be expressed by pericytes of the retina and adipose tissue, was later recognized as a ubiquitous pericyte marker [157, 158]. Other markers have been described such as the melanoma-associated antigen, Thy1.1; the ephrin receptor and its ligands, neuropilin-1 and -2; and the Notch receptor and its ligands, Jagged-1 and Jagged-2. Vimentin and desmin have been shown to be expressed in most chick pericytes as well as smooth muscle cells [159]. The identification and the isolation

of pericytes from adult human skeletal muscle has been conducted by using alkaline phosphatase (ALP) [160, 161], and its presence, indeed, was subsequently confirmed on pericytes from different tissues [162]. Crisan et al. [163] have demonstrated that CD146, NG2, and PDGFRβ can be used to purify human pericytes from fetal and adult human tissues. Although several markers of pericytes have been identified, these are not uniquely found on pericytes and are often dynamically expressed [163–165]. Some of these pericyte markers are also expressed on other cell types, most particularly endothelial and smooth muscle cells [164]. αSMA, in addition to pericytes, for example, may also be robustly expressed in both skeletal muscle and heart myofibroblasts, which may reside in a perivascular distribution, particularly after injury [166–168]. Recent evidences suggest that pericytes in human skeletal muscle are able to proliferate/mobilize in response to exercise-induced angiogenesis with a significant increase in pericyte density, thickness, and endothelial coverage [169]. Furthermore, skeletal muscle pericytes are also considered to be myogenic precursors distinct from satellite cells, the primary source of postnatal myoblasts [170]. During cardiac development, cells from epicardium undergo epithelial-to-mesenchymal transition (EMT) generating mesenchymal cells that subsequently invade the developing myocardium and give rise to cardiac fibroblasts, pericytes, and coronary vascular smooth muscle cells [171]. Recent insights into the relationship between cardiac pericytes and cardiac progenitor cells have begun to shed lights on a close resemblance between the two cell types [172]. In particular, a common epicardial origin with pericytes has been described for a subset of Sca1-positive cells [40], cardiac MSsC [173], and, possibly, human cardiac progenitors [172]. Moreover, both cardiac MSCs [173] and human cardiac progenitors [174] co-express some pericyte markers (e.g., NG2 and PDGFRβ) and share the expression of pluripotency genes with fetal cardiac pericytes [175]. Based on these considerations, these differences could reflect the existence of distinct cell types in vivo that are probably recruited to a perivascular localization. Considering the crucial role of pericytes in the mechanisms of preservation of a normal cardiac structure and function (e.g., angiogenesis, blood flow, vessel stability, maturation, and vascular permeability, as well as production of trophic factors), Katare et al. [176] have recently assessed their possible use for the treatment of acute myocardial infarction. They found that these cells are potent inducers of reparative vascularization and cardiac healing processes involving reciprocal interactions between donor cells and the ischemic environment [176]. Furthermore, O'Farrell et al. [177], using a mouse model with myocardial ischemia/reperfusion, revealed that pericytes contribute to the no-reflow phenomenon post-ischemia in the heart [178]. Overall, these data show that pericytes are abundant in the human body and are also present within the myocardial tissue. They play an active role in angiogenesis, vessel stabilization, and blood flow regulation and possess the capacity to differentiate into multiple cells of the mesenchymal lineage in vitro and in vivo. Therefore, this evidence indicates that pericytes represent a promising therapeutic candidate for myocardial regeneration.

## Controversy over the Role and Myogenic Properties of the eCSCs

In the last decade, the existence and the potential of tissue-specific endogenous CSCs as regenerative agents have been well documented [73, 74, 78, 92, 98]. Despite some controversy on the actual myogenic potential of transplanted CSCs [179–183], the discussion has moved from the identity to the nature and extent of CSC role in myocardial homeostasis and repair [184].

"Genetic fate mapping" technology uses a class of molecules known as "site-specific recombinases" that, through the capacity to produce precise DNA excisions, are capable of transforming in a specific cell/tissue a silenced reporter transgene into a constitutively expressed one. Fate map strategy system is therefore an extremely powerful tool for biologists, establishing the correspondence between individual cells at one stage of development or adult life and their progeny at later stages of development or life. Thus, genetic fate map systems, mainly Cre/Lox technology, have been used to analyze the contribution of c-kit expressing CSCs in the heart homeostasis/repair.

Using a lentiviral construct carrying the Cre recombinase driven by the c-kit promoter [73] to prospectively and specifically tag c-kit-expressing cells in the adult myocardium of mice with a floxed reporter gene [73], we rigorously documented that true c-kit$^{pos}$ CSCs efficiently differentiate into bona fide cardiomyocytes in vitro and in vivo [73]. These and additional results led to our conclusion that endogenous CSCs are necessary and sufficient for cardiomyocyte regeneration/replenishment after injury [73, 74].

Despite the reproducibility of the published data showing the proper identity and myogenic potential of CSCs, considerable confusion has arisen recently about the physiological role and regenerative capacity of what has come to be called the "c-kit$^{pos}$ cardiac cells" [74, 179–183, 185–188]. This controversy was initiated by reports that c-kit-expressing cardiac cells possess a robust cardiomyogenic potential in the neonatal period, but it becomes significantly reduced in the adult [188, 189], a change that coincides with an increase in myocardial c-kit$^{pos}$ mast cells, which lowers the relative abundance of true CSCs among the "c-kit$^{pos}$ cardiac cells." More problematically, three publications, which did not attempt to replicate our published work, have challenged the conclusion that the CSCs are responsible for the replacement of CMs lost by wear and tear and after injury [181–183]. Van Berlo et al. [183], Sultana et al. [182], and Liu et al. [181] showed that the tagged c-kit$^{pos}$ cells generated on the short and long term a small/minimal number of CMs. Therefore, they concluded that there is adult cardiomyogenesis, which undoubtedly is generated by the (some?) so-called "c-kit$^{pos}$ CSCs." However, this differentiation is negligible at best. Whether their results were due to a low myogenic potential of all the "c-kit$^{pos}$ CSCs" or to a few tagged "c-kit$^{pos}$ cells" with a high myogenic potential was nevertheless not addressed. Despite the significant negative impact that these papers have had on the field of myocardial biology and repair/regeneration, they

have some critical shortcomings that should have been addressed but were not before their publication [190, 191]. Indeed, using a fate mapping strategy, it is important to establish the extent to which Cre expression matches that of the endogenous gene promoter. If Cre levels trigger recombination in only a subset of those cells in which the specific promoter is normally active, the resultant fate map will underestimate the descendant population. Thus, it is critical to determine whether all or only part of an initial cell cohort identified by expression of the cell type-specific gene used to drive Cre is being fate mapped. This is a significant issue if the Cre-driving gene exhibits a heterogeneous level of expression in different cell types of the cohort. It is possible, indeed likely, that the cells with lowest expression of Cre in this heterogeneous population might fail to have their fate tracked because their level of Cre is below the threshold needed to trigger site-specific recombination and ensuing reporter expression [190]. It is then fundamental to check for all these potential pitfalls to avoid false-negative results [190].

Importantly, van Berlo et al. [183], Sultana et al. [182], and Liu et al. [181] have knocked-in (KI) Cre into exon 1 of the c-kit mouse locus (c-kit$^{Cre}$ allele). Using these mice, the three publications have concluded that endogenous c-kit$^{pos}$ cells mainly differentiate into endothelial cells and minimally, if at all, form new cardiomyocytes [181–183]. Some even state that the "cardiac c-kit$^{pos}$ cells" are not CSCs at all but endothelial cells and their precursors. Instead of cardiomyogenic potential, these papers report that "the cardiac c-kit$^{pos}$ cells" have a largely vasculogenic and adventitial cell lineage predisposition. This result was not unexpected considering that >90% of c-kit$^{pos}$ cells in the adult heart are CD45 positive and CD31 positive [24]. Moreover, the fraction of c-kit$^{pos}$ cells that become genetically tagged in these mouse strains resembles the bone marrow-derived c-kit$^{pos}$/Sca-1$^{pos}$/Flk-1$^{pos}$ cells identified by Fazel et al. [192]. These cells, in response to injury, home to the heart and contribute to the revascularization of the damaged area.

In order for a cell to be defined a stem cell, it must exhibit "stem cell" properties: clonogenicity, self-renewal, and multipotency in vitro and in vivo. Identifying, tracing, and characterizing stem/progenitor cells according to the expression of a single surface receptor such as c-kit [181–183, 188, 189] is not sufficient to identify the CSCs. As shown above, the majority of the total CSCs (~90%) are mast cells and endothelial (progenitor) cells, while only ~1% are demonstrably multipotent clonogenic CSCs, but the mentioned publications assumed that all, or most, c-kit$^{pos}$ cells in the heart are CSCs. Thus, relying on genetic tagging to determine the prospective fate or regenerative potential of c-kit$^{pos}$ cells within any tissue, including the heart, and for any quantification is a major biological and practical pitfall. At a minimum, the authors should have determined first, by single cell testing, what fraction of the "c-kit$^{pos}$ CSCs" is true CSC and what fraction of these cells recombines the marker gene after induction of Cre. The publications have challenged the role and/or existence of the CSCs, all using a cell-specific genetic cell-fate mapping strategy, whereby Cre (constitutive or TAM-inducible) was knocked-in the Exon1 of the c-kit locus [181–183]. Their faulty rational was that Cre/lox KIs are fool proof to

track the fate of the myocardial "c-kit$^{pos}$ cells" and to identify and quantify their myogenic contribution [187]. However, all the Cre KIs in the c-kit locus reported so far, including those under discussion [181–183], have rendered the targeted allele a null mutant, resulting in hemizygous expression and a c-kit protein deficiency [191, 193–195]. These c-kit hypomorphs exhibit growth and differentiation defects in many stem and somatic cell types [191, 193–195]. More critically, the Cre-dependent recombination efficiency is directly proportional to the level of Cre expression from the mutated c-kit allele [190, 191, 196]. Therefore, because of the low level of c-kit expression in most stem cell types [197, 198], and particularly in the c-kit$^{pos}$ CSCs [198], and the low abundance of CSCs among the "c-kit$^{pos}$ cells," it was highly questionable whether the hemizygous c-kit$^{Cre}$-KI strategy could recombine a meaningful fraction of the c-kit$^{pos}$ CSCs to track their fate [191] among the noise generated by the easier to recombine mast and endothelial progenitor cells (EPCs).

Our recent data show that c-kit is expressed in CSCs at a significantly lower level than that in the mast cells and EPCs [198]. Inexplicably, the efficiency of Cre-recombination of the CSCs, a critical assumption of their experiment, was not determined. We have therefore addressed this issue and characterized the effect of the c-kit$^{Cre}$-KI-insertion on CSC biology and cardiomyogenic potential [198]. Our data show that c-kit expression level in the CSCs is too low to produce enough Cre (when knocked-in in the first c-kit exon) to effectively recombine the floxed marker and tag the CSCs and their progeny [198]. The c-kit$^{Cre}$-KI model [182, 183] only minimally if not negligibly, tags and fate maps resident CSCs. Furthermore, Cre-KI into the first intron of the c-kit locus in all cases has produced a null c-kit allele, which is responsible for the W phenotype of these mice; the c-kit$^{Cre}$ null-allele fatally impairs in vitro and in vivo CSC growth, self-renewal, myogenicity, and regenerative potential, properties which are rescued by BAC-mediated single-copy c-kit transgenesis [198]. These fate map strategies investigate neither the identity nor the fate of CSCs because the protocol used fails to tag the vast majority, if not all, of them. The low number of c-kit$^{pos}$ progenitor-generated cardiomyocytes detected in the c-kit$^{Cre}$-KI mice, simply reflects the absence of efficient recombination in the CSCs to track their progeny in fetal and adult life together with the defective myogenesis consequence of the mutated c-kit allele in the CSC [198]. Furthermore, proper c-kit expression is necessary for cardiomyogenesis, a conclusion in agreement with our recent finding that a gain-of-function mutation in the c-kit kinase domain increases CSC's myogenic and angiogenic potential in vitro and in vivo [24, 199].

Taken together, the results shown here reinforce our previous conclusion [198] that the CSCs are necessary and sufficient for robust cardiomyogenesis and to support myocardial regeneration/repair in response to diverse types of damage. Confirmation of these conclusions should clear the way for the potential development of CSC-based myocardial regenerative protocols.

## Cardiac Stem and Progenitor Cells: The Same or Different Cells?

For a long time, the heart has been considered a terminally differentiated organ without any regenerative potential. The latter has been classically based on two lines of evidences: first, the cardiomyocytes, the main cell type of the adult heart, are terminally differentiated cells unable to divide under any physiological or pathological stimuli, and second, the absence of a pool of resident tissue-specific stem cells. This view has been radically changed by the discovery of resident cardiac stem and progenitor cells throughout the atria and ventricles of the adult mammalian heart. However, as described above, at minimum, apparently different cell types with tissue-specific characteristics of stem and/or progenitor cells have been described in the adult heart so far. Thus, we have changed from a view of the heart as a static tissue to one of an organ with the highest number of tissue-specific stem and progenitor cell populations. As the latter is improbable to be proved correct, aside from Isl-1$^{pos}$ cardiac progenitor cells, it is likely that the different putative adult cardiac stem and progenitor cells reported so far do not represent different cell types but, instead, different developmental and/or physiological stages of a unique resident adult cardiac stem cell.

One of the main reasons for the apparent confusion surrounding the myocardial stem and progenitor cells is that it is not yet known, at least for the majority of them, the origin of these cells, that is, whether they are intrinsic cells present in the myocardium from embryonic and fetal life or cells of extra-cardiac origin, which have colonized the myocardium in postnatal life, where they acquire tissue-specific properties.

Three papers have described a population of cells resident in the embryonic heart that give rise to all three cardiac lineages, suggesting a developmental origin of a common ancestor for the different cardiac progenitor cells [200]. Although pertinent, the phenotype of the multipotent cardiac progenitor cells (Isl$^{pos}$/Flk1$^{pos}$) described by Moretti et al. [131] and Kattman et al. [66] does not include c-kit. Also, both studies describe the location and in vitro differentiation of these cells, but they have not yet shown the existence of similar multipotent cells in the adult heart or their ability to reconstitute functional myocardium upon injury. Interestingly, Wu et al. [201] described c-kit$^{pos}$/Nkx2.5$^{pos}$ bi-potential myogenic precursor cells in the developing mouse embryo which more closely relates to adult c-kit$^{pos}$ CSCs and therefore supports a developmental origin of CSCs. c-kit$^{pos}$/Nkx2.5$^{pos}$ bipotent progenitor cells underwent in vitro differentiation into both myocardial and smooth muscle cells and demonstrated engraftment and differentiation when transplanted into the chick embryo [201]. It is highly tempting to speculate that these cells might represent different developmental stages of the same cell population, which acquire different phenotypes and express a particular array of epitopes in response to local cues throughout development and in different regions of the heart. However, this remains to be demonstrated.

In this regard, the many varied phenotypes of cardiac progenitor/stem cells identified in the adult mammalian myocardium bring into question whether they are all exclusively different or actually of the same population of cell yet selected and identified at different physiological states.

Recent work from our lab favors a transitional developmental sequence, which involves changes in expression of different receptors and transcription factors before differentiation into one of the three cardiac lineages [24]. Under this view, it would be fair to argue the existence of one CSC and would predict the existence of a "true" stem cell in the adult heart, which exhibits more primitive characteristics than all the previously described adult "cardiac stem/progenitor cells." Indeed, we and others have found a small population of Oct-4$^{pos}$/c-kit$^{neg}$ cells in the myocardium of adult Oct-4/EGFP transgenic mice [24]. Interestingly, the number of Oct4$^{pos}$ cells decreases with age (unpublished observations). A fraction of the Oct-4$^{pos}$ cells was also positive for c-kit suggesting a developmental response of the stem cell as it goes from being perhaps the "early" quiescent stem cell to an amplifying progenitor. Oct-4$^{pos}$ cells were also positive for other embryonic pluripotent markers, that is., Nanog, Sox-2, and the stage-specific embryonic antigen SSEA-1 [24].

On the other hand, our data show that adult CSC biology and regenerative potential in vitro and in vivo requires and is dependent upon a diploid level of c-kit expression [198]. However, the main findings emanating from the use of c-kit$^{Cre}$ mice raise an intriguing and unexpected aspect of c-kit in cardiomyogenesis. Indeed, the available evidence seems to portrait a dichotomy on c-kit role during heart embryonic development as opposed to adult cardiac regeneration. c-kit deletion, as it occurs in homozygous W-mutated mice, [198] is incompatible with life and mice die prematurely in the very last fetal days or very early in the neonatal life. However, those mice appear to have a heart anatomically and macroscopically normally developed [183]. Yet, c-kit-defective adult hearts and adult CSCs (from heterozygous c-kit$^{Cre}$-KI mice) have a significant defect in their regeneration potential in vivo [198]. The latter is unpredicted when considering all the attempts currently undergoing to decipher the pathways of developmental cardiac generation and neonatal heart regeneration to instruct effective protocols of adult cardiac regeneration. Indeed, these data lead to the hypothesis that physiologically and clinically useful protocols for adult myocardial repair/regeneration will not necessarily be based or depend on the genetic regulatory pathways which regulate embryonic and fetal cardiogenesis. In other words, cellular and molecular basis of adult myocardial regeneration may not resemble cardiac development.

## Conclusions and Future Perspective

On the basis of data accumulated over the past decade, it is evident that the vast majority of adult mammalian cardiomyocytes are terminally differentiated cells, which are not a source of cardiomyocyte renewal in the adult mammalian heart. Indeed, even though it is possible to identify rare cardiomyocytes undergoing

mitotic division, they seem to be recently born and still immature myocytes, which have not yet reached terminal differentiation. Furthermore, it is a fact that all the experimental attempts to induce cell cycle in adult cardiomyocytes have resulted in myocardial disarray and overt dysfunction. On the other hand, the identification of cardiac stem cells (CSCs) in the adult myocardium provided a satisfactory explanation for myocardial cell homeostasis throughout the lifespan of the individual and raised the expectation that myocardial regeneration might be accomplishable in the future. Unfortunately, recent uncertainty about the intrinsic regenerative capacity of the adult mammalian myocardium, including the human, raised by the faulty data generated by the c-kit$^{cre}$KI mouse lines, together with a lack of consensus about the underlying biology of neo-myogenesis in the adult has jeopardized progress in the development of clinically applicable myocardial repair/regeneration protocols. It stands to reason that unless this biological unknown of basic myocardial biology is settled through definitive experimental evidence based on robust and reproducible scientific data, any clinical test of different repair/regenerative protocols, unless spectacularly positive, will be un-interpretable at best and will always fall short of providing a convincing and conclusive answers about their clinical potential. In this review article, we have critically discussed the available evidence in support of the c-kit$^{pos}$CSCs as the main, or only, source of new myocytes in the adult myocardium and have highlighted the shortcomings which have muddled this field, which have mainly arose from a poorly controlled and superficial interpretation of Cre/Lox-based genetic cell fate mapping. Nonetheless, we strongly believe that better designed and properly-and-closely controlled new cell tracking methods are what is needed to fully detect, measure, and decipher the regulatory mechanisms responsible for myocardial cell homeostasis and how to manipulate them in order to foster physiologically meaningful endogenous cardiac regeneration.

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# Chapter 9
# Skeletal Muscle Progenitor Cell Heterogeneity


**Dong Seong Cho and Jason D. Doles**

**Abstract** Tissue-specific stem cells contribute to adult tissue maintenance, repair, and regeneration. In skeletal muscle, many different mononuclear cell types are capable of giving rise to differentiated muscle. Of these tissue stem-like cells, satellite cells (SCs) are the most studied muscle stem cell population and are widely considered the main cellular source driving muscle repair and regeneration in adult tissue. Within the satellite cell pool, many distinct subpopulations exist, each exhibiting differential abilities to exit quiescence, expand, differentiate, and self-renew. In this chapter, we discuss the different stem cell types that can give rise to skeletal muscle tissue and then focus on satellite cell heterogeneity during the process of myogenesis/muscle regeneration. Finally, we highlight emerging opportunities to better characterize muscle stem cell heterogeneity, which will ultimately deepen our appreciation of stem cells in muscle development, repair/regeneration, aging, and disease.

**Keywords** Satellite cell · Muscle regeneration · Stem cell · Myogenesis · Differentiation · Fibroblast · Mesenchymal stem cell · Pericyte · Cellular heterogeneity · Cell cycle · Asymmetric division

## Skeletal Muscle Regeneration

Skeletal muscle is one of the most abundant tissues in the human body. It accounts for about 30–50% of total body mass and is necessary for locomotion, respiration, temperature regulation, and organismal metabolism. In addition to meeting daily demands of normal tissue wear-and-tear, skeletal muscle has a remarkable capacity to regenerate following injury [1, 2]. Skeletal muscle repair and regeneration has many similarities to skeletal muscle development, and perhaps not surprisingly, many of the same mechanisms driving muscle development also contribute to muscle regeneration. One common feature shared between muscle development and

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regeneration is the muscle stem cell, or satellite cell, a mononuclear progenitor cell that serves as the building block of skeletal muscle tissue. Accordingly, the underlying mechanisms of satellite cell function have long been of interest to musculoskeletal researchers.

Satellite cells were first discovered by electron microscopy in 1961 by Alexander Mauro on the periphery of skeletal muscle myofibers [3]. These mononucleated cells are located between the basal lamina and plasma membrane of muscle myofibers [3]. Based on his initial observations, Mauro made hypotheses for possible functions of these newly identified satellite cells and proposed that these cells might contribute to skeletal muscle myofiber regeneration upon injury [3]. Subsequent research revealed that satellite cells can proliferate, differentiate, and fuse into multinucleated muscle myofibers in response to injuries [4, 5]. In addition, satellite cells were shown to be mitotically quiescent in resting skeletal muscle and that they enter the cell cycle following injury [6]. Perhaps the best evidence for the functional importance of satellite cells came from a series of genetic ablation studies showing that depletion of satellite cells can disrupt tissue regeneration [7], recovery following burn trauma [8], and hypertrophy dynamics [9]. These findings provide definitive evidence that satellite cells are a major contributor to skeletal muscle homeostasis.

During muscle regeneration, satellite cells progress through several distinct cell states (Fig. 9.1). As mentioned earlier, satellite cells predominantly exist in a mitotically quiescent state in resting muscle. Upon activation from injury or normal tissue

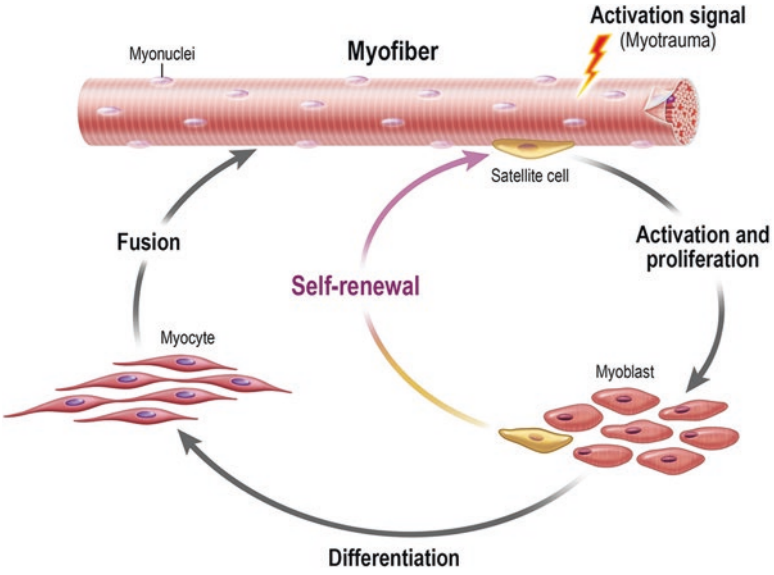


**Fig. 9.1.** Illustration of myogenic progression. Shown is the ordered progression of how a normally quiescent satellite cell, upon receipt of an activation signal, undergoes activation, proliferation, differentiation, and fusion to promote muscle repair. Importantly, a subset of activated satellite cells undergoes self-renewal, a critical stem cell property that permits lifelong maintenance of the progenitor cell pool

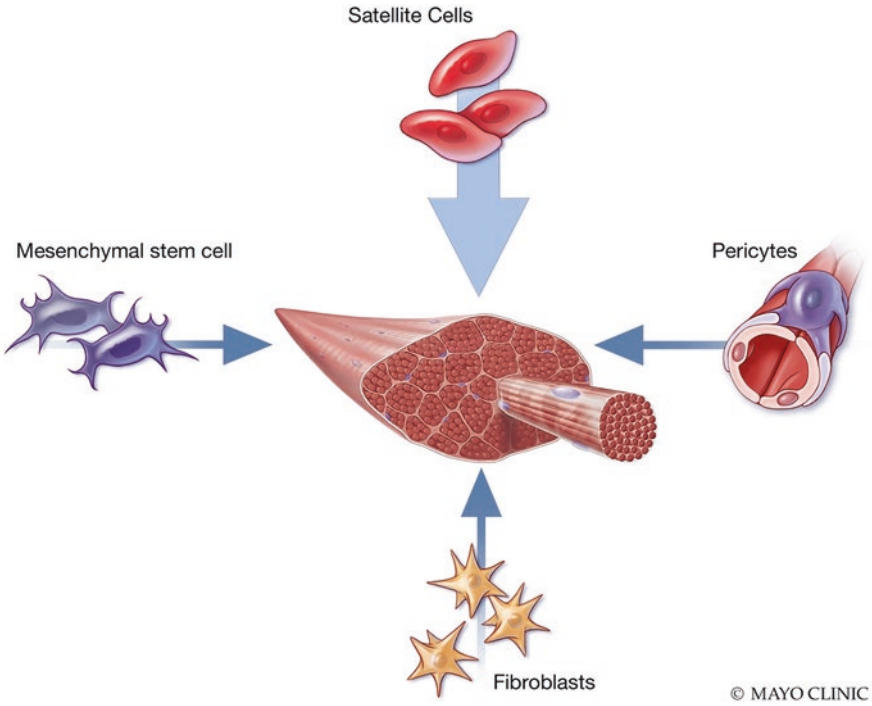


**Fig. 9.2** Illustration of the various muscle-resident cell types that contribute to skeletal muscle repair and regeneration. Shown are satellite cells (the main adult stem cell giving rise to new skeletal muscle tissue) and non-satellite cells that also participate in repair/regeneration, including pericytes, mesenchymal progenitors, fibro/adipogenic progenitors, and fibroblasts

turnover, satellite cells enter the cell cycle and transition to a proliferative myoblast state [6]. Then, myoblasts can further differentiate into myocytes and fuse into regenerating myofibers, or return to a quiescent state, thus replenishing the satellite cell pool [10, 11]. Satellite cells along this myogenic trajectory have distinct gene expression profiles, cell cycle status, and metabolic activities. Of note, in addition to satellite cells, skeletal muscle contains many other cell types capable of contributing to skeletal muscle repair and regeneration, such as pericytes, fibroblasts, mesenchymal progenitors, and fibro/adipogenic progenitors (FAPs) (Fig. 9.2). In this chapter, skeletal muscle heterogeneity will be discussed, with a focus on satellite cells during the process of myogenesis.

## Heterogeneity in Skeletal Muscle-Resident Stem Cells

In addition to satellite cells, adult skeletal muscle contains other stem-like cell types including pericytes, fibroblasts, mesenchymal progenitors, and FAPs—all of which contribute to muscle repair and regeneration to varying degrees. Pericytes resident

in skeletal muscle are located beneath the basal lamina of small vessels adjacent to muscle fibers, and they have differentiation capacity into skeletal muscle cells [12–15]. Moreover, skeletal muscle–resident pericytes are able to generate satellite cells and directly contribute to muscle regeneration [13]. Skeletal muscle–resident pericytes can be subdivided into two subgroups: type-1 (Nestin-GFP$^-$/NG2-DsRed$^+$) and type-2 (Nestin-GFP$^+$/NG2-DsRed$^+$), and these two subtypes have functionally distinct properties [16–19]. Specifically, type-1 pericytes contribute to fat accumulation and to fibrous tissue deposition in aged skeletal muscle, whereas type-2 pericytes contribute to muscle regeneration and angiogenesis [16, 18, 19]. Muscle-resident fibroblasts also coordinate muscle regeneration [7, 20] largely via their ability to influence satellite cell function. Indeed, ablation of fibroblasts in skeletal muscle resulted in premature differentiation of satellite cells and depletion of satellite cell pool, leading to impairment of healthy muscle regeneration [7]. In addition, fibroblasts can stimulate proliferation, differentiation, and fusion of myogenic precursor cells [20]. Mesenchymal progenitors and muscle-resident FAPs are CD45$^-$/CD31$^-$/PDGFRα$^+$ [21, 22] and CD45$^-$/CD31$^-$/integrin-α7$^-$/Sca1$^+$/CD34$^+$ populations, respectively, and they share similar properties. Both mesenchymal progenitors and FAPs are capable of differentiation into fibroblasts and adipocytes, and they generally do not directly contribute to new muscle myofibers [21–23]. In addition, 85% of CD45$^-$/CD31$^-$/PDGFRα$^+$ cells co-express FAP markers (CD45$^-$/CD31$^-$/integrin-α7$^-$/Sca1$^+$/CD34$^+$) [23], indicating that mesenchymal progenitors and FAPs represent overlapping populations. Despite little or no ability of muscle myofiber formation directly from these cells, they support muscle regeneration by promoting differentiation of muscle progenitors [23]. These studies collectively show that interplay between these supporting cell types and satellite cells are important for muscle regeneration, and underscore the role of satellite cells as the major cell type responsible for providing new cellular material during skeletal muscle regeneration.

## Heterogeneity in Satellite Cell Gene Expression

Satellite cells in different cell states exhibit unique gene expression signatures. All undifferentiated quiescent satellite cells express Pax7, a member of the paired-box transcription factor family [24]. In addition to its expression in adult satellite cells, Pax7 is known to have regulatory roles during embryonic muscle development [25]. In vitro and in vivo analyses of skeletal muscle from *Pax7*$^{-/-}$ mice revealed severe muscle defects and lack of a defined satellite cell population, highlighting the essential role of Pax7 in satellite cell specification [24]. In contrast to Pax7, quiescent satellite cells lack expression of myoblast determination protein 1 (MyoD) and myogenin (Myog) [26–28]. MyoD and Myog are transcription factors involved in muscle development and are typically associated with activated and/or committed myoblasts/myocytes. MyoD is required for skeletal muscle formation and myoblast formation [29], and Myog is essential for late muscle development [30, 31]. When

satellite cells are activated, MyoD is co-expressed with Pax7 [11, 27]. In some proliferating cells, Pax7 downregulation and Myog upregulation signify a commitment to terminal differentiation, whereas maintenance of Pax7 expression, downregulation of MyoD and lack of Myog upregulation signify a return to quiescence [11]. Following commitment to terminal differentiation, myocytes fuse into muscle fibers, and Myog is silenced [32, 33]. Hence, Pax7, MyoD, and Myog are key markers characterizing the progressive differentiation of satellite cells.

Comparison of quiescent and activated satellite cells revealed additional markers besides the aforementioned transcription factors. Microarray analysis identified several potential quiescent satellite cell markers, and it was confirmed by qRT-PCR that quiescent satellite cells highly express *Rgs2*, *Pmp22*, *p57*, *Spry1*, *Bmp4*, *Bmp6*, *Msc*, *Heyl*, *VE-cadherin*, *Vcam1*, *Icam1*, *Cldn5*, *Esam*, *Pcdhb9*, and *Calcr*, which are not expressed in activated satellite cells [34]. Of these, *Pmp22* and *p57* are negative regulators of cell cycle [35, 36]. *Bmp4*, *Bmp6*, *Msc*, and *Heyl* are known to be myogenic inhibitors [37–40], possibly involved in maintenance of quiescence of satellite cells. *VE-cadherin*, *Vcam1*, *Icam1*, *Cldn5*, *Esam*, *Pcdhb9*, and *Calcr* are surface markers that can be potentially used to isolate quiescent satellite cells. By contrast, activated satellite cells highly express genes involved in progression of cell cycle and mitochondria activity, reflecting their proliferative state [34].

As discussed, each satellite cell state can be defined by distinct sets of genes associated with their phenotypes. Interestingly, however, heterogeneous expression of some of these established markers has been observed within each cell state. For example, while expression of CD34, M-cadherin, and *Myf5* (transcript) are widely used markers to define quiescent satellite cells, several groups have reported rare populations of quiescent satellite cells that lack expression of CD34, M-cadherin, and *Myf5* (transcript) [41–43]. In a second example of heterogeneous transcript expression, MyoD and *Myf5* are generally shown to be co-expressed in activated satellite cells [27, 32, 44]. Detailed studies of MyoD and *Myf5,* however, have shown that *Myf5* expression is heterogeneous in MyoD$^+$-activated satellite cells [27, 44]. Furthermore, in *Myf5*$^{nlacZ/+}$ mice, *Myf5*$^+$ cells also had heterogeneous expression of MyoD [45]. Finally, detailed time-course studies revealed that expression of MyoD and Myf5 did not always coincide with each other during myogenic progression in vitro and in vivo [32, 46]. Third, heterogeneity in gene expression of MyoD$^+$-activated satellite cells was also seen in single-cell analysis on fluorescence-activated cell sorting (FACS)-sorted satellite cells [47]. The authors used a CD34$^+$/integrin-α7$^+$/CD45$^-$/CD11b$^-$/Sca1$^-$/CD31$^-$ identification strategy to isolate satellite cells and perform transcript analyses. All of the isolated cells expressed *Pax7* and *Myf5*, and 25% of these cells expressed *MyoD*, indicating that they were activated. However, these *MyoD*$^+$ cells had varied expression of *Pax3*, another commonly used satellite cell marker. Heterogeneous expression of *Pax3* was also found in a subset of *Pax7*$^+$/*MyoD*$^-$/*Myf5*$^+$ cells, which are usually classified as quiescent satellite cells. As Pax3 is known to regulate expression of MyoD [25, 48], Pax3 expression in quiescent satellite cells may stratify satellite cells with differing activation potential. Fourth, MyoD$^+$ human satellite cells contain subset of Dlk1$^+$ and Dlk1$^-$ populations [49]. Finally, a single-cell RNA-sequencing analysis of 21 individual Pax7-positive

satellite cells revealed extensive heterogeneity based on global transcriptome expression profiles [50]. Moreover, each cell in this study had a few hundred genes that were expressed uniquely by that cell. Altogether, these studies provide strong evidence that satellite cells have heterogeneous gene expression patterns not only between cell states but also likely along the entire continuum of myogenic progression.

Is heterogeneous gene expression indicative of functional differences within the satellite cell pool? Rocheteau et al. [51] compared functional behaviors of undifferentiated Pax7-expressing cells using transgenic *Tg:Pax7-nGFP* mice to label and stratify $Pax7^+$ satellite cells based on nGFP expression. Compared to Pax7-$nGFP^{Lo}$ cells, Pax7-$nGFP^{Hi}$ cells displayed functional properties of less committed satellite cells, such as lower metabolic activities, delayed first cell division upon activation, and lower gene expression level of *Myog*. In a second study using *Myf5-Cre/ROSA-YFP* mice, behaviors of $Pax7^+/YFP^+$ and $Pax7^+/YFP^-$ cells were compared by transplanting YFP subpopulations into *Pax7*$^{-/-}$ mice which lack satellite cells [52]. Transplantation of $Pax7^+/YFP^+$ cells gave rise to significantly greater number of myofibers than transplantation of $Pax7^+/YFP^-$ cells, whereas transplanted $Pax7^+/YFP^-$ cells extensively contributed to the satellite cell pool. These results indicate that $Pax7^+/YFP^+$ satellite cells are more committed than $Pax7^+/YFP^-$ satellite cells. In a third study, MyoD protein expression was observed only in the satellite cells that already had expression of *Myf5* transcript [45]. These data suggest that *Myf5*-expressing cells within a quiescent satellite cell population may perhaps be more committed or poised to be activated/enter cell cycle. Taken together, satellite cell gene expression is clearly heterogeneous and may underlie functional heterogeneity of the satellite cell pool. Thus, the current definition of satellite cell differentiation states (quiescent, activated, committed) may need to be re-evaluated as new or "hybrid" satellite cell subpopulations are identified.

## Satellite Cell Heterogeneity in Cell Cycle Status

Quiescent satellite cells exist in a reversible $G_0$ state. Upon activation, these quiescent satellite cells enter the $G_1$ phase of the cell cycle and proliferate. A small subset of activated satellite cells then returns to $G_0$ to replenish the satellite cell pool [10, 11]. As mentioned in the previous section, quiescent satellite cells highly express negative regulators of cell cycle, including *Pmp22* and *p57* [34], reflecting their mitotically dormant state. In addition to these cell cycle genes, *Notch3* and the Notch effector gene, *Heyl*, are also upregulated in quiescent satellite cells [34]. Notch signaling has been identified as a key regulator of satellite cell quiescence [53, 54]. Disruption of Notch signaling induced spontaneous differentiation of satellite cells without transition into S phase [54]. Moreover, Notch signaling has been shown to be required for maintenance of satellite cell quiescence via regulation of self-renewal and differentiation [53].

Once activated, quiescent satellite cells transit to $G_1$ phase and enter the cell cycle [46]. In activated satellite cells, expression of MyoD and Myf5 dynamically changes depending on their phase in cell cycle [46]. Expression of MyoD is high in $G_1$, low during the transition to S phase, and high in $G_2$ and M phases [46]. In contrast, Myf5, which is low in $G_1$, is upregulated during the transition from $G_1$ to S phase, and its expression is maintained until M phase [46]. Genes associated with progression of cell cycle, including *Cdc2a*, *Cdc20*, *Cdc25c*, *Ccnb1*, and *Ccna2*, were upregulated in activated satellite cells compared to quiescent satellite cells [34], reflecting their state of cell cycle entry. On a signaling level, several key regulators of cell cycle progression include Fgf2-mediated p38α/β MAPK signaling and IGF-I signaling [55–59]. Addition of FGF2 in ex vivo cell/myofiber cultures promotes proliferation and antagonizes satellite cell differentiation [57, 59]. Inhibition of p38α/β MAPK signaling induced satellite cells to return to quiescence, thus impairing cell cycle entry. These studies demonstrate the essential role of Fgf2-mediated p38α/β MAPK signaling during the $G_1$-S phase transition in satellite cells [56, 57]. Similarly, like FGF2, IGF-I also promotes proliferation of satellite cells in vitro, as well as muscle hypertrophy in aged rats [55]. Furthermore, IGF-I can inactivate the Forkhead transcription factor, FoxO1, to downregulate the activity of the promoter of cell cyclin inhibitor, $p27^{Kip1}$, resulting in cell cycle entry and proliferation of satellite cells [58].

In a recent report, Rodgers et al. [60] have shown that satellite cells exist in at least two distinct cell cycle states prior to cell cycle entry. The authors termed this cell cycle state "$G_{Alert}$" because the cells at this state have an intermediate cell cycle phenotype that appears poised between quiescence ($G_0$) and full activation ($G_1$). The authors suggest that $G_{Alert}$ is engaged when non-activated satellite cells "sense" an activation stimulus but do not themselves fully activate and enter the cell cycle. In a single hindlimb injury model, they show that satellite cells isolated from the uninjured contralateral limb exhibit greater cell size, mitochondrial activity, mtDNA, and intracellular ATP than quiescent satellite cells isolated from naïve (injured) mice. Moreover, these contralateral satellite cells are more responsive to proliferate than quiescent satellite cells, and their transcriptome profile reflects a mix of transcripts associated with both activation and quiescence.

Several studies have shown that activated satellite cells exit the cell cycle to reversibly return to a quiescent state [10, 11]. For example, $Pax7^+/MyoD^-$ quiescent satellite cells can arise from $Pax7^+/MyoD^+$ cells, indicating that activated satellite cells are capable of returning to quiescent satellite cells [11]. In addition, when a proliferation marker (BrdU) was administered to mice just after injury, 98% of quiescent satellite cells upon resolution of the injury were BrdU-positive, showing that the vast majority of quiescent satellite cell pool was replenished by proliferating satellite cells [10]. Furthermore, the authors found that *Spry1*, a receptor tyrosine kinase signaling inhibitor, was highly expressed in quiescent satellite cells and satellite cells returning to quiescent state, but not in proliferating satellite cells. Using satellite cell–specific *Spry1* mutant mice, the number of quiescent satellite cells was significantly reduced in *Spry1* mutant after injury, whereas the number of proliferat-

ing satellite cells was same as the control, showing that *Spry1* plays a key role on satellite cells to exit cell cycle and return to quiescent state [10].

Proliferating satellite cells can exit the cell cycle not only to return to a quiescent state but also to terminally differentiate. Cell cycle control is essential for differentiation of myogenic cells, as shown with the requirement of cell cycle inhibitors, p21 and p57, for skeletal muscle differentiation [61]. Expression of cell cycle inhibitors including retinoblastoma (Rb), p18, p19, and p27 (as well as p21 and p57) are upregulated during later stages of myogenesis in vitro and in vivo [62–70], reflecting cell cycle exit upon terminal differentiation. Intriguingly, it has been previously shown that MyoD can regulate cell cycle–associated genes [65, 66, 68, 71, 72]. For example, MyoD can directly lead to the upregulation of Rb, p21, and cyclin D3 mRNA, and protein [65, 66, 68, 71, 72]. Clearly, myogenic cell fate decisions and cell cycle status are intimately intertwined, with alterations in either process impacting the other.

As described earlier, satellite cells readily transit from quiescent $G_0/G_{Alert}$ to mitotically active cell cycle ($G_1$-S-$G_2$-M) phases, and back to quiescence via terminal differentiation or self-renewal. Together with highly variable gene expression patterns (discussed in the previous section), satellite cells are indeed a heterogeneous cell population. Although the key regulators of cell cycle transitions in satellite cells, such as Notch signaling, p38α/β MAPK signaling, *Spry1*, and cell cycle regulators, are known, it will be important to understand how these different signaling pathways interact to coordinate myogenesis and maintain the satellite cell pool during tissue aging.

## Asymmetric Division Contributes to Satellite Cell Heterogeneity

During muscle regeneration, satellite cells enter the cell cycle and proliferate. Each round of cell division gives rise to two daughter cells with similar (symmetric division) or divergent fates (asymmetric division) [52, 73–76]. In symmetric division, activated satellite cells give rise to two identical satellite cells that are not lineage committed, contributing to the maintenance of satellite stem cell pool [52]. By contrast, satellite cells can undergo asymmetric division, generating one satellite stem cell and one myogenic progenitor cell which expresses MyoD or Myf5 [52, 75]. In addition, one proliferating satellite cell and one differentiating cell can be generated from one satellite cell through asymmetric division [77]. In this section, we will discuss asymmetric satellite cell division, and how this process contributes to satellite cell heterogeneity.

One example of cell division giving rise to transcriptionally divergent satellite cells is the asymmetric cell division of $Pax7^+/Myf5^-$ satellite cells, which can give rise to both $Pax7^+/Myf5^-$ and $Pax7^+/Myf5^+$ cells [52]. The authors found that most of the cell divisions of $Pax7^+/Myf5^-$ satellite cells were planar with respect to the orientation of the daughter cells on muscle myofibers. The daughter cells in 92% of these planar divisions were identical to each other ($Pax7^+/Myf5^-$). On the other

hand, $Pax7^+/Myf5^-$ cell divisions in the apical-basal direction predominately gave rise to one $Pax7^+/Myf5^-$ cell and one $Pax7^+/Myf5^+$ cell. Moreover, the majority of the daughter cells in the apical side were $Pax7^+/Myf5^+$ while basally positioned cells were predominantly $Pax7^+/Myf5^-$. The $Pax7^+/Myf5^+$ daughter cells were functionally more responsive to differentiation, whereas the $Pax7^+/Myf5^-$ daughter cells were more prone to contribute to the satellite stem cell compartment. Similar to asymmetric segregation of Myf5, MyoD can also be asymmetrically distributed from $Pax7^+/MyoD^+$ satellite cells into two daughter cells: $Pax7^+/MyoD^+$ and $Pax7^+/MyoD^-$ cells [75]. In contrast to the aforementioned asymmetric divisions which gave rise to one quiescent satellite cell and one proliferating satellite cell, the set of daughter cells with one proliferating cell and one differentiating cell can also be generated via asymmetric division [75, 77]. For example, $Pax7^+/MyoD^+$ cells can give rise to two sister cells of $Pax7^+/MyoD^+$ and $Pax7^-/MyoD^+$ cells [75], and $Myog^+$ daughter cells can be generated with asymmetric segregation of Myog into the two daughter cells [77].

Along with asymmetric segregation of daughter cells based on myogenic transcription factor expression, asymmetric DNA distribution into daughter cells during cell division has been observed in satellite cells [51, 76, 78]. The first observation of asymmetric division of satellite cells was demonstrated with asymmetric distribution of Numb protein and template DNA during satellite cell division [78]. Biased (non-random) DNA segregation during satellite cell divisions appear to be correlated with asymmetric cell fate decisions. For example, Pax7-$nGFP^{Hi}$ cells in $Pax7^+$ populations undergo asymmetric DNA segregation more frequently than Pax7-$nGFP^{Lo}$ cells, giving rise to one satellite stem cell and one committed daughter cell [51]. In another study, asymmetric DNA segregation was correlated with asymmetric cell division of $Pax7^+$ cells into $Pax7^+/Myog^-$ and $Pax7^+/Myog^+$ cells [77].

These asymmetric cell fate decisions during cell division are regulated by differential signaling activity in the two daughter cells [52, 76]. In the asymmetric cell divisions of satellite cells into $Pax7^+/Myf5^+$ and $Pax7^+/Myf5^-$ cells discussed earlier, Notch signaling-associated genes were differentially expressed in the two daughter cells [52]. As Notch signaling is essential for maintenance of quiescence of satellite cells [53, 54], the differential expression of Notch signaling-associated genes likely regulates the cell-fate decision of the daughter cells. In addition to Notch, the Par complex was identified as asymmetrically localized in a subset of the daughter cells during satellite cell division [76]. This led to the asymmetric activation of p38α/β MAPK signaling in the two daughter cells, thus giving rise to progeny destined to proliferate or self-renew.

## Future Perspectives and Concluding Remarks

Satellite cells are rare, stem-like cells that reside in adult skeletal muscle [79, 80], and are highly heterogeneous on many levels, including gene expression and cell cycle status. Additionally, satellite cell subpopulations are functionally

heterogeneous, even though they are often regarded as a functionally homogeneous population [47, 51, 81]. Although there have been many studies revealing satellite cell heterogeneity, current knowledge is still limited for the following reasons. First, the studies discussed earlier [27, 44, 45, 47, 51, 52, 75, 77, 81] have so far investigated heterogeneity of satellite cell populations with characterization of only a few well-known markers of satellite cells, such as Pax7, Pax3, Myf5, MyoD, and Myog. These are all transcription factors that have a number of downstream target genes with important roles on myogenic regulation [79, 80, 82]. Hence, the heterogeneous expression of these master regulators should contribute to a much more complex gene expression landscape than is currently known. As shown in a recent transcriptome-wide RNA-seq analysis of 21 single satellite cells, each cell expressed unique sets of signature genes that are absent in all other cells [50]. The full extent of transcriptional diversity in individual satellite cells will be important to determine in future studies. Second, while there have been single-cell analysis to compare gene expression of satellite cells on a single-cell level [27, 47, 50], the number of analyzed cells was extremely limited. This makes any definitive assessment of overall heterogeneity very difficult. Despite all of the data supporting the claim that satellite cells are a heterogeneous cell population, several important questions remain. Are there additional satellite cell subpopulations that have not yet been identified? How distinct are their cell cycle states and metabolic activities? These and other questions are primed for investigation as new profiling technologies evolve.

Lineage tracing models, such as Pax7, Pax3, and Myf5 transgenic mice [51, 52, 83–86], are useful tools to track satellite cell fate. Most of the analyses performed in these models are accompanied by immunofluorescence staining and/or flow cytometry (FACS) analysis. These analyses limit the number of parameters that can be measured simultaneously in a same cell, and often, less than four markers are characterized. To faithfully capture true population heterogeneity, it will be necessary to characterize many markers on a single-cell level from a large number of cells. Recent progress along these lines include a recently developed single-cell technique—single-cell mass cytometry—that allows the measurement of more than 40 protein marker-based parameters in individual cells [87]. Application of this technique to satellite cells should provide a better understanding of population heterogeneity. Indeed, mass cytometry-based analysis of individual satellite cells uncovered a new signature of activated satellite cells ($CD44^+/CD98^+/MyoD^+$), as well as novel cell surface markers [88]. Single-cell proteomics by mass spectrometry (SCoPE-MS), another single-cell analysis to quantify protein expression in single-cell level [89], may also be used as a useful tool to reveal satellite cell heterogeneity. In addition to these protein quantification techniques, single-cell RNA-sequencing with a droplet-based platform to prepare libraries (i.e., DROP-seq) enables transcriptome analyses of thousands of cells on a single-cell level [90]. This technique should help to overcome the previous limitation of the low number of characterized satellite cells while still permitting evaluation of the entire satellite cell transcriptome. Overall, these and other emerging technologies to robustly profile single cells will likely lead the way in advancing our understanding of satellite cell/muscle stem cell heterogeneity.

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# Chapter 10
# Hematopoietic Stem Cell Heterogeneity


**Roland Jurecic**

**Abstract** Hematopoietic stem cells (HSCs) maintain lifelong production of mature blood cells and regenerate the hematopoietic system after cytotoxic injury. Use of expanding cell surface marker panels and advanced functional analyses have revealed the presence of several immunophenotypically different HSC subsets with distinct self-renewal and repopulating capacity and bias toward selective lineage differentiation. This chapter summarizes current understanding of the phenotypic and functional heterogeneity within the HSC pool, with emphasis on the immunophenotypes and functional features of several known HSC subsets, and their roles in steady-state and emergency hematopoiesis, and in aging. The chapter also highlights some of the future research directions to elucidate further the biology and function of different HSC subsets in health and disease states.

**Keywords** Hematopoietic stem cells · Hematopoiesis · HSC subsets · Functional heterogeneity · Differentiation bias

## Introduction

Hematopoietic stem cells (HSCs) are defined by their ability to sustain life long production of mature blood cells of all lineages, and to repopulate all hematopoietic lineages after radiation or chemically induced cytotoxic insult. Healthy HSCs are capable of (a) long-term multilineage reconstitution and in situ recovery of the hematopoietic system (e.g., after massive cytotoxic injury induced by radiation or chemotherapy) and (b) long-term engraftment and multilineage repopulation after adoptive transplant into preconditioned recipients.

Identification, enrichment, and functional characterization of mouse adult HSCs in 1988 [1] paved the way for more precise isolation and characterization of HSCs.

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Subsequent studies have catalyzed continuous advancements in phenotypic profiling and enrichment of murine HSCs, as well as functional characterization of HSCs [2–9].

Important progress in identifying human HSCs was accomplished in 1992 through isolation of a candidate human HSC population from the fetal bone marrow [10]. Succeeding studies reported phenotypic and functional characterization of human HSCs residing in the cord blood, adult bone marrow (BM), the peripheral blood of patients after mobilization with granulocyte/macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF), and in the peripheral blood of cancer patients undergoing chemotherapy [11–13]. These studies fueled identification of new human HSC markers and more advanced phenotypic and functional characterization of human HSCs residing in the bone marrow, fetal liver, cord blood, and peripheral blood [14–17].

Remarkably, early on it was apparent that the population of HSCs is heterogeneous [18]. Numerous studies since then have defined phenotypic and functional heterogeneity within HSC pool and have revealed the coexistence of several HSC subsets with distinct proliferation, self-renewal, and differentiation potentials [19–29]. Cumulatively, these findings transformed our perception of HSCs as a functionally uniform pool to that of a heterogeneous pool consisting of different HSC subsets.

Recent review brought up important questions about the origins of HSC heterogeneity [30]. The origins of HSC heterogeneity and intrinsic and extrinsic factors that may shape functional diversification of HSCs are not well understood. These factors may include: (a) differential genetic and epigenetic reprogramming during early development and cell maturation, (b) differential localization in BM niches, and (c) genetic and epigenetic reprogramming brought on by responses to different molecular and cellular stimuli [14, 30].

## Phenotypic and Functional Distinction of Short-Term and Long-Term Repopulating Hematopoietic Stem Cells

Transplantation of different BM cell populations phenotypically defined by differential expression of cell surface markers revealed that the HSC compartment is heterogeneous in regard to prototypical HSC functional features: self-renewal capacity and lifespan of individual HSC clones, multilineage repopulation of hematopoiesis in lethally irradiated mice, and the duration of repopulation after the transplant. Multiple studies used different sets of cell surface markers, retroviral cell marking, single cell transplant approaches, cellular barcoding, and serial transplantation to establish the existence and define functional properties of short-term repopulating HSCs (STR-HSC) and long-term repopulating HSCs (LTR-HSC) in mice and humans. In mice, STR-HSCs have a limited self-renewal capacity and upon transplantation are able to support hematopoiesis in lethally irradiated

recipients for up to several months. In contrast, LTR-HSCs display an extensive self-renewal capacity and can support hematopoiesis for ≥6 months and longer. Serial transplantation remains a standard for in vivo assessment of long-term self-renewal and multilineage repopulating potential of HSCs. In that regard, murine LTR-HSCs can be distinguished from STR-HSC by their capacity to reconstitute hematopoietic systems of lethally irradiated recipients in a successive manner through serial transplantation [27, 31–45] (Fig. 10.1a).

Human LTR-HSCs can maintain human hematopoiesis in serially transplanted recipient immunodeficient mice. Matsuoka et al. have reported that human cord blood CD34+/− cells that do not express thrombopoietin (TPO) receptor myeloproliferative leukemia protein (MPL) maintain long-term human hematopoiesis in primary, secondary, and tertiary recipient immunodeficient mice and represent LTR-HSCs. The population of CD34+ MPL+ cells represents intermediate-term HSCs, whereas CD34- MPL+ cells are short-term repopulating HSCs [46].

Further studies revealed differential CD34 expression on murine LTR-HSCs during developmental stages. During fetal and neonatal period, LTR-HSCs express CD34, whereas LTR-HSCs from adult mice are $CD34^{neg}$. Notably, the expression of CD34 marker on adult mouse LTR-HSCs is reversible. During steady-state hematopoiesis in adult mice, the majority of LTR-HSCs are $CD34^{neg}$. However, after cytotoxic injury, LTR-HSCs exist in two "states": $CD34^{neg}$ and $CD34^{pos}$ states. More importantly, when hematopoiesis achieves steady state after the injury, the $CD34^{pos}$ HSCs revert to $CD34^{neg}$ state [47–49] (Fig. 10.1b).

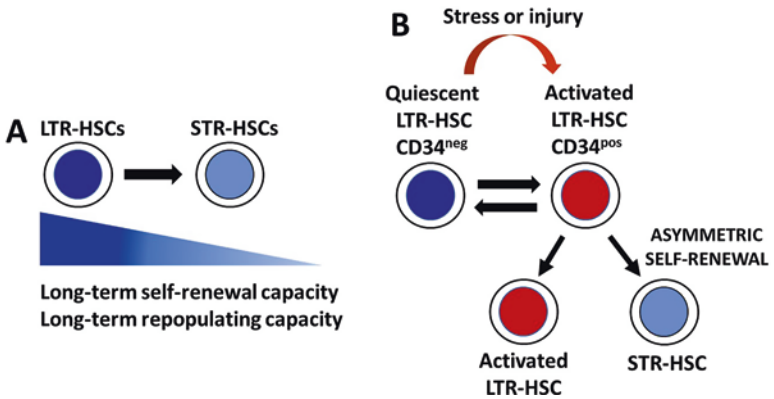


**Fig. 10.1** Functional features of short-term repopulating HSCs (STR-HSC) and long-term repopulating HSCs (LTR-HSC). (**a**) LTR-HSCs display long-term self-renewal and repopulating capacity, whereas STR-HSCs exhibit short-term self-renewal and repopulating capacity. (**b**) During homeostasis, the majority of LTR-HSCs are $CD34^{neg}$, and are quiescent and dividing infrequently. In response to stress and injury, dormant HSCs are activated and start to express CD34, and cycle more frequently. Activated HSCs are maintaining themselves and are generating STR-HSCs through asymmetric self-renewal divisions. After restoring homeostasis of the hematopoietic system, activated HSCs return to a quiescent state

Notably, studies of adult human HSCs have also documented reversible in vivo expression of CD34, and revealed that both $CD34^{neg}$ and $CD34^{pos}$ HSC subsets exhibit long-term in vivo multilineage engraftment capacity [50, 51].

Expression of CD34 on mouse LTR-HSCs is also a marker of HSC dormancy and activation. During homeostasis, the majority of infrequently dividing and dormant HSCs are in the $CD34^{neg}$ subset, whereas $CD34^{pos}$ subset contains active self-renewing HSCs. In response to stress and injury (e.g., infections, acute blood cell loss, chemotherapy, or irradiation-induced cytotoxicity), dormant $CD34^{neg}$ HSCs switch to actively cell cycling $CD34^{pos}$ state. Importantly, once the blood system is regenerated and the homeostasis is re-established, activated HSCs return to dormancy [52–55]. These observations indicate that HSCs can reversibly switch from dormant to activated state in response to hematopoietic stress and injury. Moreover, these studies have revealed that reversible CD34 expression demarcates activation states of HSCs during homeostatic and pathological conditions.

In response to stress and injury (e.g., infections, acute blood cell loss, chemotherapy, or irradiation-induced cytotoxicity), HSCs exit dormancy to restore the homeostasis of the hematopoiesis, and then return to a quiescent state (Fig. 10.1b). Return of HSCs to a quiescent state minimizes replicative stress and DNA damage accumulation in HSCs and is extremely important for maintaining the fitness of individual HSCs and entire HSC pool, thus preventing HSC exhaustion and possible bone marrow failure [56, 57].

## Lineage-Biased Hematopoietic Stem Cell Subsets

Initial prevailing concept was that LTR-HSCs are uniform in their self-renewal, long-term repopulating, and multilineage differentiation capacities. However, transplantation of single HSCs and in vivo tracking of their progeny and lineage differentiation potential in a serial transplant setting have revealed quite a functional diversity and consequential heterogeneity among LTR-HSCs. These studies have defined several HSC subsets that exhibit lineage differentiation bias and differential propensity in vivo to generate myeloid and lymphoid lineages. While these lineage-biased HSCs can generate all hematopoietic lineages, the ratios of lymphoid and myeloid cells they produce differ [19, 23, 26, 58].

Lineage-biased HSC subsets that exhibit distinct lineage output profiles in vivo and are present in young and old mice were isolated and characterized based on differential levels of expression of signaling lymphocytic activation molecule 1 (SLAMf1) or CD150. CD150 is one of the SLAM family markers used to distinguish HSCs from multipotent progenitors (MPPs) and downstream oligopotent progenitors [27, 33].

The $CD150^{hi}$ subset of LTR-HSCs, named Myeloid-biased HSCs (My-HSCs), predominantly generated myeloid lineages and has stably maintained the same differentiation pattern and self-renewal potential throughout serial transplantation. In addition, the lymphoid progeny of My-HSCs exhibited reduced response to IL-7, a

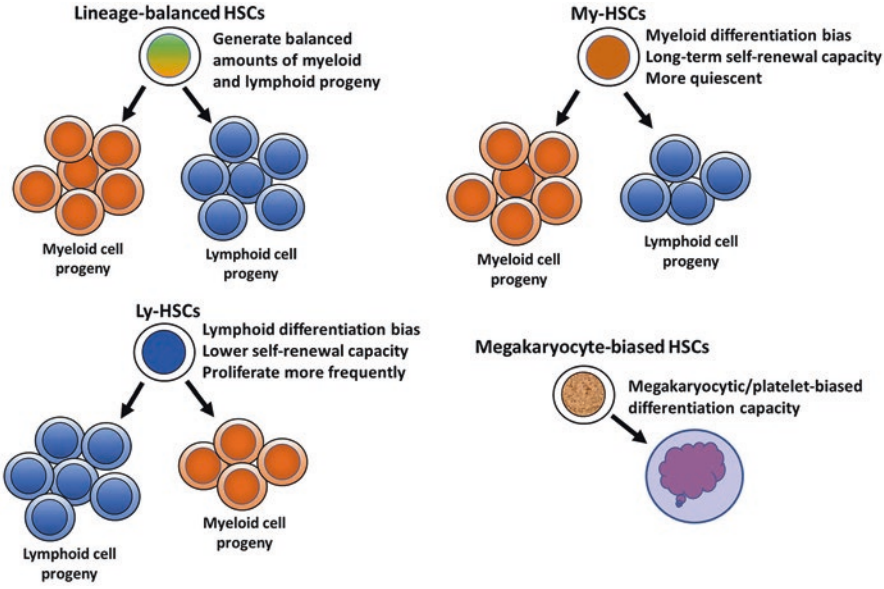


**Fig. 10.2** Lineage-biased HSC subsets exhibit in vivo lineage differentiation bias. Lineage-balanced HSCs produce balanced amounts of myeloid and lymphoid progeny. Myeloid-biased HSCs exhibit long-term self-renewal capacity and myeloid differentiation bias. Lymphoid-biased HSCs display lymphoid differentiation bias and lower self-renewal capacity and proliferate more frequently. Megakaryocyte-biased HSCs display megakaryocytic/platelet-biased differentiation capacity

cytokine with an important role in differentiation and proliferation of lymphoid cells. The $CD150^{low}$ HSC subset, named Lymphoid-biased HSCs (Ly-HSCs), largely generated lymphoid lineages (Fig. 10.2). The third $CD150^{med}$ HSC subset produces balanced amounts of myeloid and lymphoid progeny and represents lineage-balanced HSC subset [19, 23, 26, 58, 59] (Fig. 10.2).

In addition to differential lineage outputs, lineage-biased HSC subsets exhibit different self-renewal and LTR capacity, and differentiation kinetics. Among lineage-biased HSCs My-HSCs are more quiescent and exhibit the highest long-term self-renewal and LTR capacity. In contrast, Ly-HSCs divide more frequently and exhibit lesser and shorter lasting self-renewal potential, as observed after serial transplantation of clonally derived Ly-HSCs [19, 23, 26, 58, 60]. Serial transplantation experiments have also revealed the stability of lineage bias among lineage-biased HSC subsets, indicating that the biased differentiation predisposition is a stable functional feature of these HSC subsets.

Expression of CD41 (platelet integrin alpha chain 2b) was reported to also distinguish myeloid-biased and lymphoid-biased HSC subsets [61]. $CD41^{pos}$ HSC subset is more quiescent and selectively expresses key transcription factors driving myelo-erythroid and megakaryocyte differentiation. $CD41^{neg}$ HSC subset on the other hand divides more frequently and exhibits lymphoid lineage gene priming.

In serial transplantation experiments, CD41$^{pos}$ HSCs exhibited robust long-term repopulation capacity and marked bias toward myeloid differentiation, whereas CD41$^{neg}$ HSCs exhibited attenuated long-term repopulation capacity and marked bias toward generating lymphoid progeny. Analysis of CD41 KO mice revealed significant decreases in platelet, erythrocyte, and all leukocyte lineages. Furthermore, loss of CD41 resulted in perturbed hematopoiesis and decreased survival and quiescence of HSCs [61].

In addition to myeloid and lymphoid-biased HSC subsets, several studies have detected the presence of HSC subsets that display megakaryocytic/platelet-biased differentiation capacity [59, 62–65] (Fig. 10.2).

Platelets are generated in the bone marrow through megakaryopoiesis, and together with red blood cells comprise >90% of the cells produced daily. In addition to their role in thrombosis and wound healing, activated platelets modulate innate and adaptive immune responses to infections [66, 67]. Rapid consumption of platelets due to excessive bleeding, injury, and infection can lead to transient thrombocytopenia, which must be resolved by rapid platelet replenishment.

Analysis of Vwf-eGFP BAC transgenic mice with eGFP reporter driven by the megakaryocyte-associated von Willebrand factor (VWF) identified vWF− and vWF+ HSC subsets. Functional analysis of lineage differentiation bias of vWF+ and vWF− HSCs at the clonal level revealed that vWF+ HSCs displayed long-term repopulating capacity with platelet/myeloid-biased repopulation of recipients, while vWF− HSCs showed long-term repopulating capacity with clear lymphoid-biased repopulation of recipients [59]. Single-cell transplantation and single-cell RNASeq expression analysis detected a subset of HSCs expressing high levels of von Willebrand factor (VWF), which could represent a population of megakaryocyte-biased HSCs [64].

During steady-state hematopoiesis, there is a log-fold range in the level of cell surface expression of stem cell factor (SCF) receptor c-kit on HSCs. Since c-kit expression is the obligatory marker for isolation of HSCs, and SCF and c-kit have an important role in HSC function and maintenance, Shin et al. investigated the functional and genetic properties of HSCs expressing low and high levels of c-kit. Functional and transcriptome analyses in that study have revealed that HSCs expressing the same level of CD150 marker, but different level of c-kit, are functionally diverse subsets [63]. HSCs with low level of surface c-kit expression exhibited enhanced self-renewal and long-term repopulating potential. In addition, c-kit$^{low}$ HSCs are more quiescent and display delayed multilineage differentiation. In contrast, HSCs with high level of surface c-kit expression displayed increased cell cycling and rapid multilineage differentiation kinetics, but impaired long-term repopulation capacity and self-renewal after serial transplant. In vitro and in vivo studies of the differentiation potential of c-kit$^{low}$ and c-kit$^{hi}$ HSCs have also shown that c-kit$^{hi}$ HSC subset possess much higher megakaryocyte differentiation capacity and gives rise more rapidly to megakaryocytes than c-kit$^{low}$ HSCs, and thus exhibits a megakaryocytic lineage bias [63].

In human hematopoiesis, study by Matsuoka et al. demonstrated that human cord blood CD34− and CD34+ HSCs represent myeloid-biased and lymphoid-biased

HSC subsets with long-term repopulating capacities [68]. A recent study has confirmed that the CD34− subset of cord blood HSCs represents the most primitive human LTR-HSCs, which also display megakaryocyte/erythrocyte lineage priming and much higher megakaryocyte/erythrocyte differentiation potential in vivo than the CD34+ HSC subset [69].

It is evident that the LTR-HSC pool is functionally and immunophenotypically diverse, encompassing HSC subsets with different self-renewal and long-term repopulating capacity, proliferation and differentiation kinetics, and lineage differentiation bias.

Expanding cell surface marker panels and advancing technologies for purification and genetic and functional analysis of a small population of cells will undoubtedly lead to more definitive immunophenotypic and functional classification of known lineage-biased HSC subsets in homeostasis, and perhaps the discovery of new types of lineage-biased HSCs.

It will be interesting and important to determine whether lineage-biased HSC subsets are developmentally independent, or if there is a developmental hierarchy involved, such as the one described for murine $CD41^{pos}$ and $CD41^{neg}$ HSC subsets [61], and how are they maintained and regulated [70–72].

There is still much to be learned about: (a) the origins of HSC heterogeneity and developmental emergence of different HSC subsets [30] and (b) functional properties (self-renewal and LTR capacity, differentiation kinetics, and profile) and cell surface immunophenotypes of different subsets of HSCs under pathophysiological conditions (e.g., inflammatory and oxidative stress, infections) and during and after cytotoxic injury (radiation, chemotherapy).

## Hematopoietic Stem Cells and Emergency Hematopoiesis

In the last decade, it became apparent that hematopoiesis is a very adaptable and responsive process that can quickly react to external stimuli such as infections and change to meet the need for the specific type of blood cells. Viral and bacterial infections and associated inflammation mobilize present innate immune effector cells (granulocytes, monocytes) to contain or eradicate pathogens. During infection, innate immune effector cells are rapidly used and need to be continuously replenished. Continuous need for newly generated innate immune effector cells causes a shift from steady-state hematopoiesis to emergency hematopoiesis (EH), which ensures increased output of innate immune effector cells and fast innate immune responses. Both EH and subsequent adaptive immune response are necessary to control and clear viral and bacterial infections.

A number of studies have established that EH involves and starts with a response of HSCs to infections. HSCs express pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs). TLR engagement by bacterial or viral PAMPs activates the NF-κB and

interferon regulatory factor (IRF) pathways, which mediate inflammation and antibacterial and antiviral defense. HSCs are also responsive to inflammatory cytokines and chemokines that are being produced in response to pathogens. Thus, HSCs can "detect" infection via PAMPs and inflammatory cytokines and chemokines and initiate rapid generation of innate immune cells. During EH, inflammatory cytokines (e.g., Type I and Type II IFNs and IL-6) and chemokines activate HSCs, which results in increased proliferation and temporary expansion of HSCs pool, myeloid lineage-biased differentiation and mobilization of HSCs into peripheral blood, and migration to spleen (extramedullary hematopoiesis) [73–83].

Several studies have reported that exposure to recurring infections and chronic inflammation are eroding self-renewal and repopulating capacity of LTR-HSCs, leading to reduction of HSC pool and sometimes even to their depletion [74, 76, 77, 84–87].

Notably, in an animal model of Lymphocytic choriomeningitis virus (LCMV) infection, IFNγ was found to promote differentiation of myeloid-biased HSCs but not lymphoid-biased HSCs during an innate immune response to infection [88]. Other studies reported that *Ehrlichia muris* infection causes activation of LTR-HSCs and myeloid differentiation bias, and that TLR-mediated stimulation of HSCs promotes myeloid differentiation and activates myeloid-biased HSCs [71, 77, 79]. Thus, it appears that pathogens are preferentially inducing differentiation of My-HSC subset.

As mentioned earlier, the platelets play an important role in innate and adaptive immune responses to infections [66, 67]. Infections can increase platelet turnover, which can lead to transient thrombocytopenia. In that situation, the hematopoietic system must quickly ramp up the production of platelets.

In an effort to understand how the hematopoietic system counteracts infection-induced thrombocytopenia, Haas et al. discovered a new stem-like megakaryocyte-committed progenitor cell population (SL-MkPs) in the HSC compartment. Although SL-MkPs share some of the features with multipotent HSCs, this cell subset is unipotent and produces only megakaryocyte lineage [89]. In the steady-state hematopoiesis, SL-MkPs are in a quiescent state, and do not participate much in a steady-state megakaryopoiesis. However, infection-related inflammation induces emergency megakaryopoiesis, activates this cell subset, and promotes rapid differentiation into megakaryocytes and generation of platelets [89]. These findings further illustrate how the entire HSC compartment and different HSC subsets are equipped to respond to and rapidly deal with diverse emergency conditions. It will be interesting to study further the features and function of SL-MkPs subset in real infection scenarios.

Overall, further studies are necessary to determine the effects of acute and chronic bacterial and viral infections on the function of different lineage-biased HSCs in young, middle-aged, and old hosts.

## Aging of Hematopoietic Stem Cell Subsets

Aging brings on wide-ranging adverse changes in the function of the hematopoietic system and causes profound and distinct functional and quantitative changes among HSC subsets which are at the core of altered and dysregulated hematopoiesis and immunity. The hallmarks of the aged hematopoietic system are enhanced myelopoiesis and increased production of myeloid cells, impaired lymphopoiesis and decreased output of lymphoid cells, declining function of the immune system, and anemia. Functional impairments of the adaptive immune system and dysregulation of innate immunity are contributing to (a) increased susceptibility to and occurrence of serious infections, (b) impaired would healing, and (c) increased incidence of inflammatory and autoimmune diseases, and hematological and other malignancies. All these adverse effects of the hematopoietic system aging are significantly increasing morbidity and mortality among the elderly [90–94].

In young and middle-aged mice, the stable balance is maintained between myeloid-biased, lymphoid-biased, and balanced HSC subsets [23, 58–60] (Fig. 10.3a). In older mice, the pool of myeloid-biased HSCs is expanding, and that subset becomes a predominant type of HSCs in very old mice. More importantly, old myeloid-biased HSCs display increased cycling, declining long-term self-renewal and repopulating capacity, and reduced generation of mature blood cells in comparison to young myeloid-biased HSCs [19, 91, 92, 95–97] (Fig. 10.3a).

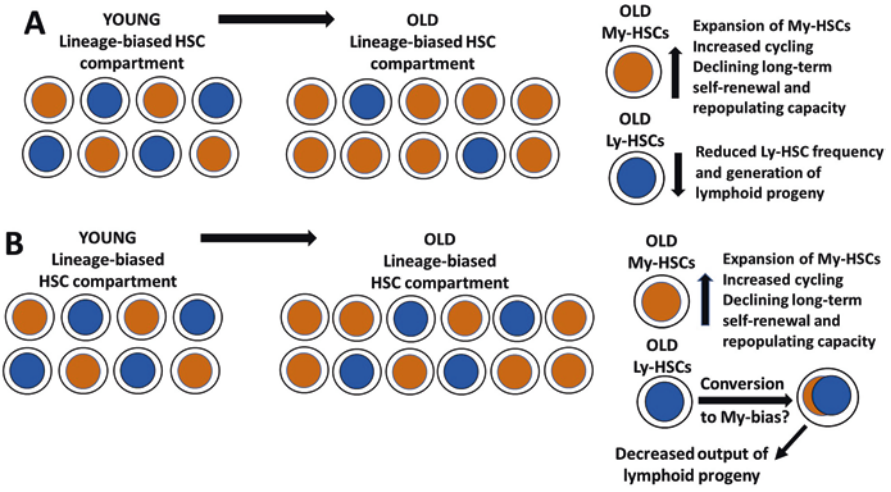


**Fig. 10.3** Age-induced changes in lineage-biased HSC subsets. (**a**) In a current model of HSC aging, the subset of My-HSCs expands, but their long-term self-renewal and repopulating capacity are declining. In contrast, the pool of Ly-HSCs is reduced, contributing to reduced lymphopoiesis and decreased output of lymphoid progeny. (**b**) In an alternative newly proposed model, the numbers of old Ly-HSCs do not change, but their lineage differentiation bias could be switching to myelopoiesis, resulting in decreased output of lymphoid progeny

Functional and transcriptome analysis of murine young and old HSCs at the single-cell level identified upregulated platelet-lineage gene expression and functional platelet bias at the single-cell level among old HSCs. Moreover, the pool of old HSCs contains a high proportion of previously unknown platelet-restricted HSCs that almost exclusively produce platelets [98]. Observation that inactivation of FOG-1 transcription factor (involved in normal development of megakaryocytes and erythrocytes in mice and humans) increases lymphoid lineage output, suggested that platelet-biased priming of HSCs may contribute to reduced production of lymphoid cell lineages [98].

Significantly reduced and altered lymphopoiesis and decreased output of T and B cell lineages in old age are thought to be caused in part by reduction of the pool of lymphoid-biased HSCs and their age-related functional decline [91, 99, 100] (Fig. 10.3a). The most recent paper reported that phenotypic and functional changes in the aged immune system are largely due to functional and epigenetic changes in old HSCs, which are defining the transcriptional profile and impaired function of their T and B cell progeny [101]. Regrettably, the LTR-HSCs analyzed in that study were not separated into lymphoid-biased and myeloid-biased HSC subsets.

Previous studies have reported reduced frequency of lymphoid-biased HSCs in old mice, leading to conclusion that age-related impaired lymphopoiesis is due to loss of lymphoid-biased HSCs.

Dorshkind group [102] and our group as well (unpublished data) have observed that when the total numbers of HSCs and lymphoid-biased HSCs are determined instead of their frequency, the number of lymphoid-biased HSCs is not statistically different between young and old mice [102] (Fig. 10.3b). More importantly, the transcriptome profiles of old My-HSCs and Ly-HSCs were found to be similar, suggesting that old Ly-HSCs acquire myeloid-biased characteristics. Based on these findings, Kong et al. are suggesting a revised model of Ly-HSC aging, wherein the switch to myeloid-biased transcriptional profile rather than reduction of the pool of old Ly-HSCs contributes to diminished lymphopoiesis in old age [102] (Fig. 10.3b). Further studies of old Ly-HSCs are needed to elucidate further genetic and functional changes undergoing in that HSC subset and to understand better how these age-associated changes impact the lymphopoiesis and function of immune system in old age.

Notably, the age-induced functional and quantitative changes of HSCs subsets in old mice and elderly humans are quite similar. The number of HSCs in elderly humans is expanded as well, and old HSCs are predominantly myeloid biased and are less quiescent, but with reduced self-renewal and repopulating capacity. More importantly, aged human HSCs display transcriptional upregulation of genes associated with cell cycle, myeloid lineage specification, and myeloid malignancies. Moreover, the pool of aged myeloid-biased HSCs contains HSCs that carry genetic and epigenetic changes, which are increasing the risk of developing age-associated hematopoietic diseases such as myelodysplastic syndromes (MDS), myeloproliferative disorders (MPDs), bone marrow failure disorders, and myeloid leukemias [90–94, 103].

A plethora of cell-intrinsic factors and changes (increased myeloid priming and differentiation signaling pathways, downregulated lymphoid priming and lymphoid differentiation signaling pathways, impaired response to genotoxic stress, epigenetic changes, etc.) and cell-extrinsic factors (aged microenvironment, aged HSC niches, inflammation, oxidative stress) contribute to aging-related functional changes of lineage-biased HSC subsets [104–108].

Continued functional and molecular profiling of murine and human HSC subsets during aging are necessary for development of therapeutic approaches that would: (a) slow down or attenuate HSC aging and (b) modify bias of old HSC subsets toward selective lineage differentiation with the goal to improve the function of old HSCs and hematopoietic and immune systems in elderly.

## Concluding Remarks and Future Directions

Ongoing progress in HSC research revealed increasing cellular and functional heterogeneity and complexity of the HSC compartment. Current understanding indicates that the HSC pool is composed of functionally diverse and yet synergistic array of HSC subsets. Combined, these HSC subsets can cover most if not all "bases" to maintain optimal functioning of the hematopoietic system in steady state and during emergency conditions (e.g., inflammatory and oxidative stress), and to rapidly regenerate the hematopoietic system after cytotoxic injury and restore its homeostasis.

It is very important and clinically highly relevant to amplify our understanding of the function of all HSC subsets in a steady state and especially under pathophysiological and stress conditions. Below are some of the future research directions with a clinical relevance, whose outcomes could pave the way for targeted modification of genetic and epigenetic makeup and functional properties (e.g., lineage differentiation bias) of different HSC subsets to improve function of the hematopoietic and immune systems in aging and disease:

- Analysis of clonal succession and dominance among different HSC subsets from young to old age, and in homeostatic versus pathophysiological conditions
- Analysis of the function of different HSC subsets during acute and chronic infections (acute and chronic emergency hematopoiesis) in young, middle-aged, and old hosts
- Analysis of the function of different HSC subsets during acute and chronic inflammation
- Characterization of the effects of pre-therapy cancer progression on the function of all HSC subsets (Cancer Hematopoiesis)
- Characterization of the acute effects of cancer and therapy (radiation and chemotherapy) on the function of all HSC subsets in tumor-bearing mouse models and in cancer patients

- Characterization of the long-lasting effects of cancer and therapy (radiation and chemotherapy) on the function of all HSC subsets in murine cancer survivor models and in cancer survivors.

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# Chapter 11
# Heterogeneity of Stem Cells in the Ovary


**Deepa Bhartiya, Hiren Patel, and Diksha Sharma**

**Abstract** Every organ in the body is thought to harbor two populations of stem cells, including the quiescent and the actively dividing, that leads to heterogeneity among them. It is generally believed that the ovary harbors a fixed number of follicles at birth that differentiate during fetal development from the primordial germ cells. The numbers of follicles decrease by age, leading to menopause. However, in 2004, it was suggested that ovary may harbor stem cells that are possibly involved in the formation of new follicles throughout reproductive life. Research over little more than a decade shows that ovarian stem cells include a quiescent population of very small embryonic-like stem cells (VSELs) and slightly bigger, actively dividing ovarian stem cells (OSCs). This heterogeneity among ovarian stem cells is similar to the presence of VSELs along with spermatogonial stem cells (SSCs) in the testis or hematopoietic stem cells (HSCs) in the hematopoietic system. VSELs express embryonic markers, including nuclear OCT-4, and are lodged in the ovary surface epithelium (OSE). Ovarian VSELs undergo asymmetric cell division to self-renew and give rise to OSCs that in turn undergo symmetric cell divisions and clonal expansion (germ cell nest) followed by meiosis to form an oocyte that gets assembled as a primordial follicle. Both VSELs and OSCs also express receptors for follicle-stimulating hormone (FSHR) and are directly activated by FSH to undergo neo-oogenesis and primordial follicle assembly. Whether stimulation of ovaries by FSH in Infertility Clinics activates the stem cells leading to the formation of multiple follicles needs further investigation. Epithelial cells lining the surface of ovary provide a niche to the stem cells under normal circumstances and undergo epithelial-mesenchymal transition (EMT) to form granulosa cells for primordial follicle assembly. Compromised function of the epithelial cells with age possibly leads to inability of stem cells to form follicles, leading to menopause. More than 90% of ovarian cancers arise in the OSE, possibly due to excessive self-renewal of VSELs. Altered biology of the OSE cells results in the formation of myofibroblasts by EMT and may

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provide a cancerous niche that supports excessive expansion of the stem cells lodged in the OSE, leading to ovarian cancer. Ovarian cancer cells express markers like OCT-4 and FSHR, which are also expressed by the VSELs lodged in the OSE, whereas the epithelial cells are distinctly negative for the same. Lot more research is required in the field to gain further understanding of ovarian stem cell biology.

**Keywords** Ovary · Stem cells · Very small embryonic-like stem cells · Ovary surface epithelium · OCT-4 · Asymmetric cell division · Germ cell nest · Primordial follicle · Ovarian cancer · FSH

## Introduction

Heterogeneity among stem cells basically implies presence of different types of stem cells in any tissue. In 2018, attempts are being made by leaders in the field to relook at the definition of adult stem cells [1]. The classical and best-studied stem cells in the body are the hematopoietic and testicular stem cells. However, the presence of stem cells with similar properties in other adult tissues remains vague at present. Earlier, it was suggested that adult tissues harbor two types of stem cells, including the quiescent and the actively dividing [2, 3]. But now, questions are being raised on the very existence of stem cells in certain adult organs. Organs like liver, pancreas, and lungs are thought not to harbor stem cells, although they possess huge potential to regenerate after surgical ablation. Rather than stem cells playing a role, it has been suggested that the adult cell types dedifferentiate and/or expand to bring about regeneration, for example, after hepatectomy, liver hepatocytes rapidly divide to regenerate the liver. Whereas in other adult organs like testis, gut, and skin epithelium with huge turnover, scientists are yet to discover the quiescent stem cells that undergo asymmetric cell divisions. Quiescent stem cells are reported as satellite stem cells in muscles, and a subpopulation of stem cells exist among the hematopoietic stem cells that are termed long term hematopoietic stem cells (LTSCs)/short term hematopoietic stem cells (STSCs). Interestingly, Clevers and Watt [1] in their review on adult stem cells do not even acknowledge presence of stem cells in the adult ovaries. In 2015, Clevers [4] discussed what is an adult stem cell? Due to lack of clarity at present, it was proposed that rather than defining stem cells as an entity, it may be better to define them based on their function [1].

Let us first understand what is an adult stem cell? Stem cells are expected to be quiescent in nature, to be able to self-renew and give rise to tissue-specific progenitors by undergoing asymmetrical cell division (ACD), and to have ability to differentiate into various cell types. It is indeed intriguing that even HSCs ability to remain quiescent and undergo ACD remains questionable. HSCs have the ability to differentiate into various blood cell types, but their ability to differentiate into various lineages to accomplish regeneration does not exist, as evident from failed global efforts. Thus, it is important to have more clarity on how to define adult stem cells.

We recently compiled the work done over the years in our lab to show that there exists a subpopulation of pluripotent stem cells in various adult tissues that undergo ACD to self-renew and give rise to tissue-specific progenitors [5]. Results challenge existing views of lack of stem cells in tissues like pancreas and liver. We showed that adult pancreas harbors stem cells that regenerate pancreas after partial pancreatectomy [6] and that these stem cells undergo ACD. ACD among very small embryonic-like stem cells (VSELs) was first reported in mice testis [7], and it was later confirmed by the differential expression of NUMB in the mouse bone marrow [8], ovary [9], and in the uterus [10]. Results challenge existing views of dedifferentiation/reprogramming of adult cells to bring about regeneration.

These pluripotent stem cells are small in size and have been termed very small embryonic-like stem cells (VSELs) for the first time by Ratajczak's group from University of Louisville, Kentucky, USA. They were reviewed extensively by various groups [11, 12, 13, 14]. VSELs are pluripotent stem cells proposed to be equivalent to primordial germ cells (PGCs), which, rather than migrating only to the developing gonadal ridge during development, travel to all developing organs and survive throughout life as VSELs [15, 16]. Both human and mouse VSELs have been shown to differentiate into three germ layers [17, 18, 19, 20], and recent efforts have been successful to also expand them in vitro [16, 18]. But being small in size and scarce, these stem cells have been unknowingly discarded over decades. VSELs and are still struggling to be acknowledged widely by the scientific community [21].

Data have also piled up to show that the VSELs are relatively quiescent in nature and survive chemo- as well as radiotherapies. Ratajczak et al. [22] showed that VSELs, being quiescent in nature, survive total body radiotherapy in the mouse bone marrow, whereas the HSCs are destroyed (since they are actively dividing). VSELs in fact get activated and undergo proliferation (increased take up of BrdU) in an attempt to regenerate the damaged marrow. Later, our group has shown that VSELs survive chemotherapy in mouse testis [17, 23, 24] and ovary [25]. These stem cells have also been reported in azoospermic human testes of cancer survivors [26, 27] as well as in ovaries, despite complete absence of follicles [28]. We have also observed that these pluripotent stem cells survive in atrophied mouse uterus after bilateral ovariectomy by the presence of embryonic markers including Oct-4A by RT-PCR and also by OCT-4 immunoexpression [10, 29].

Thus VSELs could possibly be the true stem cells in various adult tissues as they are quiescent by nature, undergo ACD, and have the required plasticity to bring about regeneration. An urgent need is felt to accept presence of VSELs in adult tissues, relook at the definitions of stem cells and progenitors, and arrive at a consensus. This was discussed earlier by our group [30, 31]. After the hematopoietic compartment, testis is considered to be the best-studied tissue for spermatogonial stem cells (SSCs). However, scientists are still struggling to identify the subpopulation that undergoes ACD in the testis. SSCs invariably undergo symmetric cell divisions, and our group, for the first time, reported that VSELs in the testis undergo ACD and give rise to SSCs which in turn undergo symmetrical cell divisions (SCD) and clonal expansion [7]. Thus, heterogeneity does exist in the stem cells compartment in various adult tissues including hematopoietic system as well as in the testes.

They harbor different populations of stem cells, including similar population of VSELs and tissue-specific progenitors such as HSCs in the hematopoietic system and SSCs in the testis. In the present chapter, we will discuss in depth the available data, suggesting heterogeneity among ovarian stem cells.

## Main Text

### *Ovarian Stem Cells*

Jonathan Tilly's group for the first time reported stem cells in adult mouse ovary [32]. Their findings contradicted age-long belief that ovaries have a fixed numbers of follicles that get depleted with age, resulting in menopause later on in life. By simple logic of daily counting numbers of atretic follicles, they argued that the ovary should get depleted of follicles within a month, but the ovaries remain functional for more than a year, thereby suggesting active renewal of follicles from the stem cells, that is, the ovarian stem cells (OSCs). The group reported actively dividing cells co-expressing mouse vasa homolog (MVH) and bromodeoxyuridine (BrdU) in mouse ovary surface epithelium (OSE). In 2016, the group had described detailed protocols to isolate OSCs form the ovary by flow cytometry [33]. Recently, Tilly's group could also detect VSELs by flow cytometry in mouse ovaries and showed that germ cell marker DDX-4 is not expressed by the VSELs [34] but questioned the link between VSELs and OSCs. The developmental link between VSELs and OSCs/SSCs is not hypothetical but rather based on scientific evidence. VSELs in various adult tissues express nuclear OCT-4 (pluripotent marker) whereas OSCs/SSCs express cytoplasmic OCT-4. It is common knowledge that pluripotent embryonic stem cells express nuclear OCT-4A and the cultures have to be terminated if they start expressing cytoplasmic OCT-4B (suggestive of their differentiation). Thus, as reported earlier, VSELs give rise to the OSCs by undergoing ACD [5, 35] and are the stem cells whereas OSCs are indeed ovary specific progenitors.

Later on, Irma Virant-Klun's group [28] reported pluripotent stem cells in the human OSE for the first time. These stem cells were very small in size (3–5 μm), and VSELs that expressed pluripotent markers on culture resulted in the differentiation of these stem cells into >90 μm oocyte-like structures with a well-defined zona pellucida. Virant-Klun [36] recently showed that the oocyte-like structures obtained by differentiation of VSELs lodged in the OSE are capable of undergoing fertilization and cortical reaction when incubated with sperm. The group has also shown that the VSELs exist in large numbers in the ovarian tissue collected from women with borderline ovarian cancer and high-grade serous ovarian carcinoma, thus indicating their potential involvement in ovarian cancer [37]. These findings have been confirmed by another group as well [38, 39]. Role of VSELs in cancers was recently reviewed [40].

Our group studied OSE cells isolated from rabbit, marmoset, sheep, and human ovaries and showed two distinct populations of stem cells (Figs. 11.1, 11.2 and 11.3) based on their size with the small VSELs expressing nuclear OCT-4 and the bigger

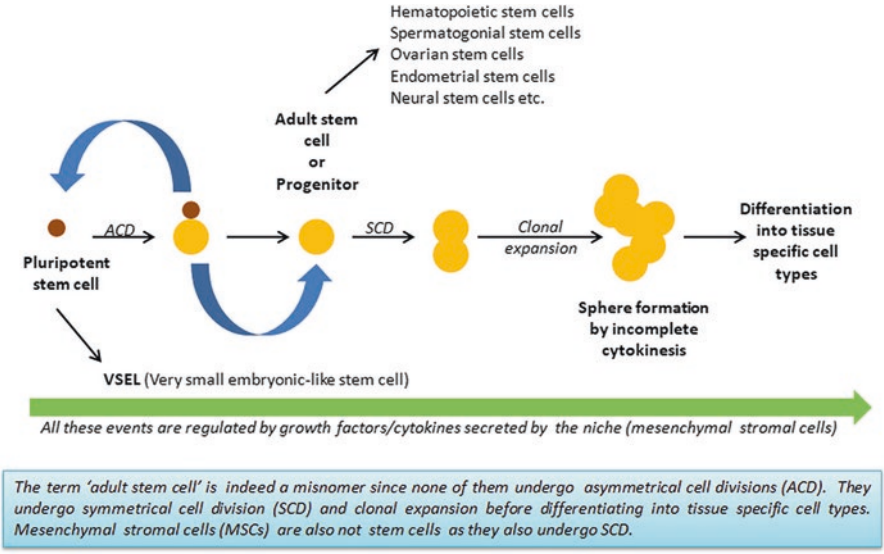


**Fig. 11.1** Basic stem cells biology in adult tissues to explain stem cells heterogeneity. Small, spherical VSELs exist in all adult tissues, undergo asymmetric cell divisions to give rise to tissue-committed progenitors which undergo symmetric cell divisions and clonal expansion (rapid proliferation with incomplete cytokinesis) before differentiating into tissue-specific cell types

OSCs expressing cytoplasmic OCT-4 [41, 42]. Based on our experience with human embryonic stem cells [43] and testicular stem cells [12], it was evident that VSELs are the pluripotent stem cells and OSCs are ovary-specific progenitors, and both are lodged in the OSE, similar to VSELs and SSCs in the testis and VSELs and HSCs in the hematopoietic system. Parte et al. [41, 42] further reported that the stem cells on culture resulted in the formation of oocyte-like structures, providing support to earlier findings of Virant-Klun's group [28]. The stem cells differentiated into oocyte-like structures whereas the epithelial cells provided trophic support to the differentiating stem cells. Recently, Cafforio's group from Italy has confirmed presence of two populations of stem cells in human ovary [44]. We discussed their work [45], and hopefully, more groups will confirm these findings and will result in a paradigm shift in the field of ovarian biology (Figs. 11.1, 11.2, 11.3, and 11.4).

Lei and Spradling [46] reported germ cell nests (GCNs) in fetal ovaries but did not find any such structures in the adult ovary and thus concluded that there are no stem cells in the adult ovary [47]. These GCNs or cysts are formed by rapid proliferation and have cytoplasmic continuity just prior to entering meiosis. They are considered as functional units for oogenesis, which eventually breakdown, transform into individual oocytes, and assemble as primordial follicle. Oocytes differentiate by receiving organelles and cytoplasm from sister germ cells and build a Balbiani body to become oocytes, whereas nurse-like germ cells die [46]. Our group had earlier reported similar GCN in adult ovary [45] and argued that absence of evidence is not evidence for absence [48]. We explained why Lei and Spradling [47] failed to observe the GCN in adult ovary.

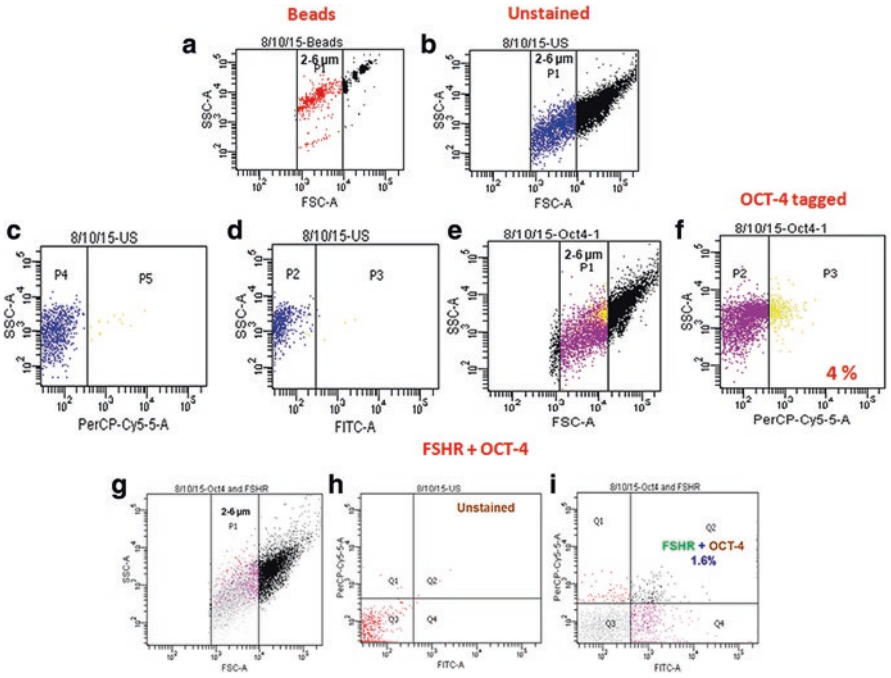


**Fig. 11.2** Immunophenotyping studies on stem cells in sheep ovarian surface epithelial (OSE) cells. (**a**) Immunophenotyping analysis for OCT-4 was carried out on sheep OSE cells (**b**) in the size range of 2–6 µm, representing P1 population (**c**) gated based on calibration size beads. Immunophenotyping analysis shows that 4.4% OCT-4 positive stem cells in the size range of 2–6 µm (**d**) expressed OCT-4 using indirect method of labeling (**e**) which (**f**) represent percentage of cell population negative for OCT-4 using Alexafluor 488 without primary antibody. These data are reprinted with permissions from Patel and Bhartiya [21]

## *What Are These Germ Cell Nests Described in Fetal Ovaries?*

Cysts arise by clonal expansion of a single cell by undergoing incomplete cytokinesis to form a cluster of cells connected by cytoplasmic bridges. Cyst formation is followed by meiosis that results in the formation of eggs and sperms [49, 50]. However, such spheres are the characteristic property of stem cells [51], are formed in various normal and neoplastic tissues, and are described on the basis of tissue of origin, like mammospheres, neurospheres, cardiospheres, pancreatospheres, and so on. Sphere formation is the ability of stem cells to undergo self-renewal and is used to study stem cells in cancer tissues. Only a self-renewing stem cell can form a sphere whereby they can divide while maintaining the undifferentiated state. Sphere formation may not necessarily identify the quiescent, adult stem cells which reside in the G0 state, which likely prevents their depletion in vivo, and the possibility of the introduction of mutations during replication.

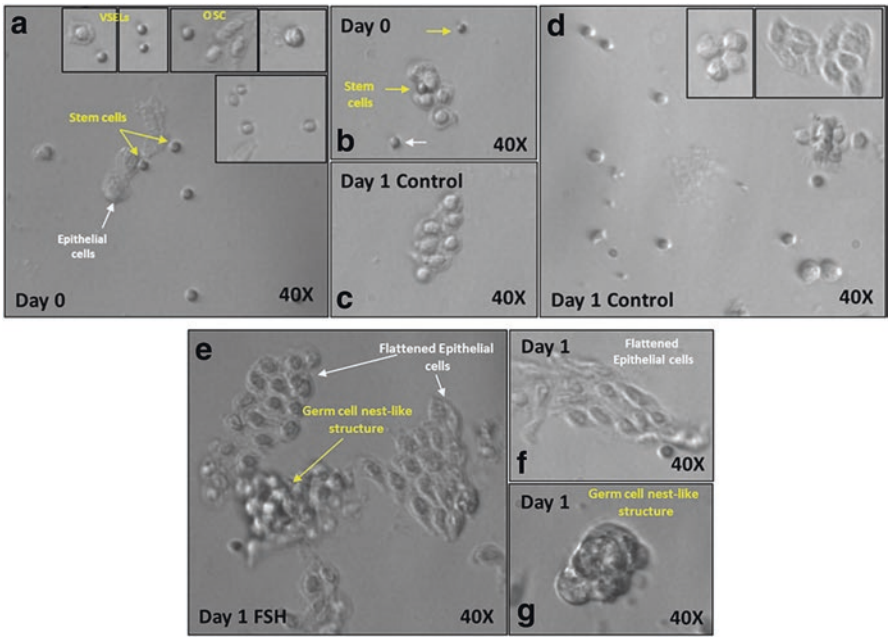


**Fig. 11.3** In vitro culture of manually scraped sheep ovary surface epithelial (OSE) cells in the presence of FSH. (**a** and **b**) OSE cells in initial culture show presence of different cell types including large epithelial cells (white arrow), small spherical VSELs, and slightly larger OSCs (yellow arrow). Stem cells are observed in close association with large epithelial cells. (**c** and **d**) Untreated (without FSH) OSE cells after 24 h of culture show minimal effect on stem cells (**e** and **g**) FSH treatment for 24 h resulted in distinct changes in OSE culture. Spherical stem cells appeared to increase in numbers and formed small spheres in close association with epithelial cells bed. (**f**) Epithelial cells became flat and were attached to bottom of the culture dish. (**g**) Cluster of stem cells formed by incomplete cytokinesis (clonal expansion) resembled germ cell nest-like structures. These data are reprinted with permissions from Patel and Bhartiya [21]

Our model clearly shows that under normal homeostatic conditions, rather than the stem cells, it is the tissue-specific progenitors that undergo symmetric cell divisions and clonal expansion to form spheres [5]. However, in ovarian cancers, the VSELs, rather than forming OCSs by ACD, undergo excessive self-renewal to initiate cancer since the cancer cells express several embryonic markers. Patel et al. [9, 35] showed that sheep ovarian VSELs in the OSE undergo ACD to give rise to the OSCs, which in turn form spheres. This study also provides first evidence showing presence of a germ cell nest in adult sheep ovarian cortex. Culture of sheep OSE cells shows formation of GCN, which express OCT-4 and FSHR [52] (Figs 11.3 and 11.4). This stem cells biology occurs in a subtle nature throughout adult life, leading to the assembly of primordial follicles [53]. Loss of function of these stem cells with age due to a compromised stem cells niche possibly results in menopause [54].

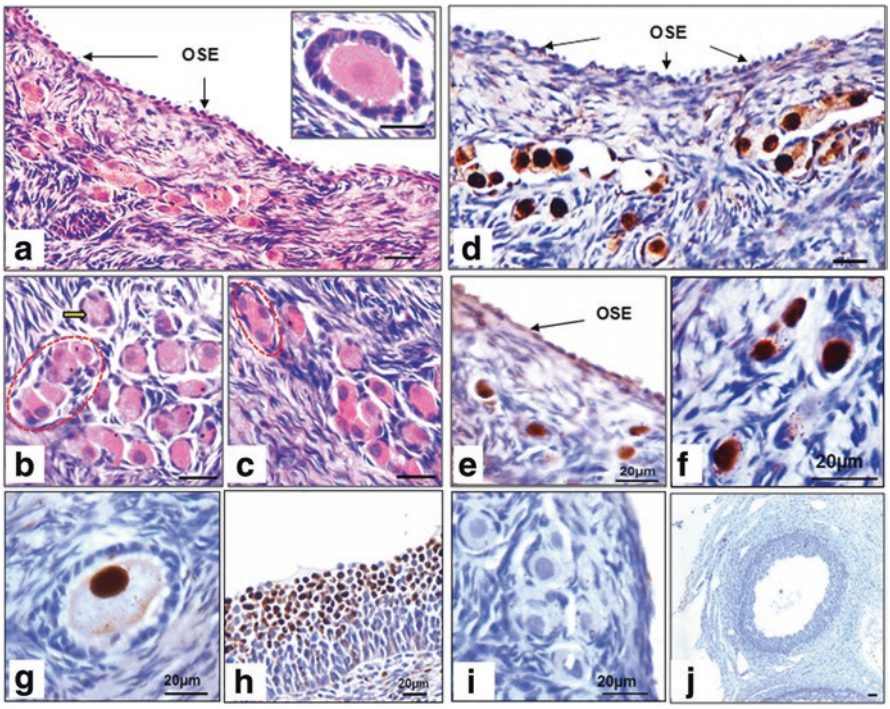


**Fig. 11.4** Proliferating cell nuclear antigen (PCNA) immunolocalization on sheep ovarian sections. H&E staining of ovarian sections (**a**) shows a distinct layer of OSE cells (**b** and **c**) cohort of cytoplasmically connected oocytes, that is, a germ cell nest surrounded by few pregranulosa cells. These structures are referred to as ovigerous cords in literature (Smith et al. 2014). Few primordial and primary follicles were located in the cortical region, whereas large oocytes were present in medulla region. (**d**–**h**) Immunolocalization with PCNA showed few OSE cells positive for PCNA, cluster of oocytes/germ cell cyst, individual primordial, and primary follicles located in cortical region of ovary showed strong nuclear PCNA. Interestingly, surrounding granulosa cells and stromal cells were completely negative for PCNA. Large Graffian follicle in medulla region showed weak PCNA expression in both nucleus and ooplasm of oocytes; however, (**h**) surrounding granulosa cells were strongly positive for PCNA. (**i** and **j**) Negative control. These data are reprinted with permissions from Patel and Bhartiya [21]

## *Conclusions and Directions for Future Research*

Heterogeneity exists among stem cells lodged in the ovary surface epithelium. Stem cells include VSELs and OSCs, of which VSELs are capable of undergoing ACD while OSCs undergo symmetric cell divisions and clonal expansion to form germ cell nests. Rather than defining as heterogeneity among the stem cells, we need to label VSELs as stem cells and OSCs as tissue-specific progenitors. VSELs express nuclear OCT-4 and undergo ACD to self-renew and give rise to "tissue-specific

progenitors" OSCs with cytoplasmic OCT-4. The OSCs further form GCNs by rapid proliferation and incomplete cytokinesis. Similar heterogeneity exists in the hematopoietic system (VSELs and HSCs) and testes (VSELs and SSCs). It is the stem cells lodged in the OSE (and not the surface epithelial cells) that possibly initiate ovarian cancer.

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# Chapter 12
# Heterogeneity of Spermatogonial Stem Cells


**Hiroshi Kubota**

**Abstract** Germ cells transfer genetic materials from one generation to the next, which ensures the continuation of the species. Spermatogenesis, the process of male germ cell production, is one of the most productive systems in adult tissues. This high productivity depends on the well-coordinated differentiation cascade in spermatogonia, occurring via their synchronized cell division and proliferation. Spermatogonial stem cells (SSCs) are responsible for maintaining the spermatogonial population via self-renewal and the continuous generation of committed progenitor cells that differentiate into spermatozoa. Like other stem cells in the body, SSCs are defined by their self-renewal and differentiation abilities. A functional transplantation assay, in which these biological properties of SSCs can be quantitatively evaluated, was developed using mice, and the cell surface characteristics and intracellular marker gene expression of murine SSCs were successfully determined. Another approach to elucidate SSC identity is a cell lineage-tracing experiment using transgenic mice, which can track the SSC behavior in the testes. Recent studies using both these experimental approaches have revealed that the SSC identity changed depending upon the developmental, homeostatic, and regenerative circumstances. In addition, single-cell transcriptomic analyses have further indicated the instability of marker gene expression in SSCs. More studies are needed to unify the results of the determination of SSC identity based on the functional properties and accumulating transcriptomic data of SSCs, to elucidate the functional interaction between SSC behavior and gene products and illustrate the conserved features of SSCs amidst their heterogeneity. Furthermore, the deterministic roles of distinct SSC niches under different physiological conditions in the SSC heterogeneity and its causal regulators must also be clarified in future studies.

**Keywords** Spermatogonial stem cells · Germline stem cells · Functional assay · Stem cell transplantation · Cell lineage-tracing · Genetic-labeling · Stem cell

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identity · Stem cell markers · Stem cell niche · Self-renewal · Differentiation · Regeneration · Spermatogonia · Spermatogenesis · Testis

## Introduction

Spermatogenesis occurs in the seminiferous tubules in the testes, and it is presumed to be one of the most productive cell-renewing systems in the adult tissues. In males, millions of spermatozoa are produced daily since the onset of puberty until old age [1]. The high productivity of spermatogenesis is conserved among most animals; therefore, this process is advantageous for the continuation of the species. This high productivity depends on the well-coordinated differentiation cascade in spermatogonia, which occurs via their synchronized cell division and proliferation. Because one spermatocyte produces only four spermatids by reductive cell division, a continuous supply of large numbers of spermatocytes requires at least a quarter of the population of spermatogonia, which are the precursors of spermatocytes and represent mitotic cell populations in spermatogenesis [2].

In the testes, the basic structure of seminiferous tubules is formed by the epithelia of Sertoli cells and the surrounding peritubular myoid cells (Fig. 12.1). The tight junctions of Sertoli cells form the blood-testis barrier and separate the basal and adluminal compartments of the seminiferous tubules. The basal compartment contains spermatogonia, which are located on the basement membrane, and preleptotene spermatocytes. The adluminal compartment contains pachytene spermatocytes and subsequent haploid germ cells, including spermatids and spermatozoa, which are never exposed to the blood and lymph constituents. This is important to keep them separate from the immune system in order to avoid unwanted immune reactions against the haploid germ cells after meiotic recombination [3].

Spermatogonial stem cells (SSCs) are responsible for maintaining the spermatogonial population during reproductive life via self-renewal and the generation of daughter cells that commit to differentiation [4, 5]. Among various mammalian species, mouse spermatogenesis is the most intensely investigated, and thus, mice are the most established model for studying spermatogenesis in several mammals [6]. Although spermatogenesis in non-primate mammals is similar to mouse spermatogenesis, primate spermatogenesis, including that in humans, displays notable differences in the classification of spermatogonial cell populations and their differentiation process, compared to those in non-primates [7]. The biology of human SSCs is tremendously important in order to understand the unique human reproductive system and to develop new therapies for male infertility; however, the identity of human SSCs has not yet been unequivocally determined and still raises marked controversy among researchers [8]. Currently, our knowledge about mammalian SSCs has stemmed mainly from mouse studies. In addition, several critical experimental methods and approaches to elucidate stem cell behavior are only available in mouse-based systems. Therefore, this review focuses on mouse SSCs and discusses their heterogeneity.

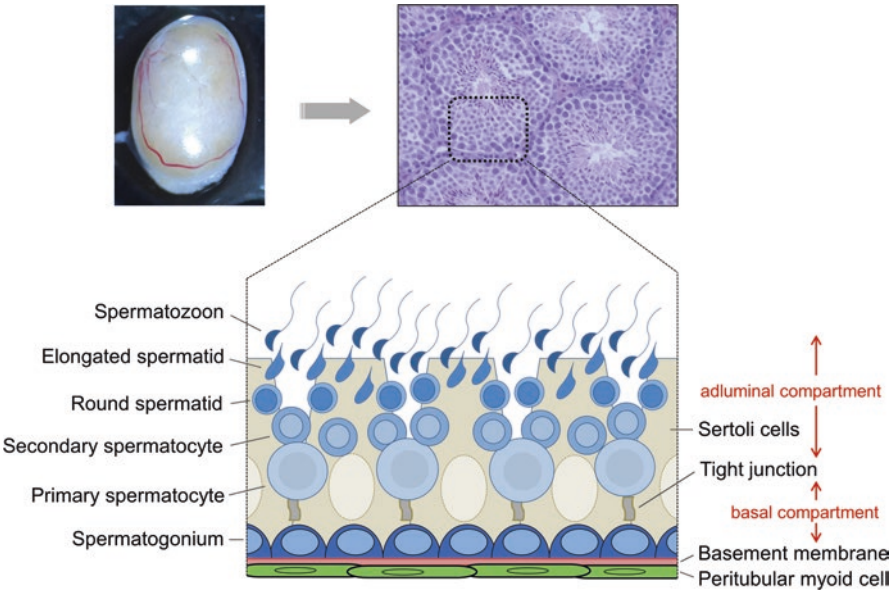


**Fig. 12.1** Schematic representation of mouse seminiferous tubules. (Upper left) Adult mouse testis. (Upper right) Cross-section of adult mouse testis stained with hematoxylin–eosin. (Bottom) Schematic magnified view of the indicated square region of a seminiferous tubule in the histological section. The seminiferous tubules consist of the epithelia of Sertoli cells, the surrounding peritubular myoid cells, and the germ cells at various stages of their development. The tight junctions between Sertoli cells form the blood-testis barrier separate the basal and adluminal compartments of the seminiferous tubules. The basal compartment contains spermatogonia, which are located on the basement membrane, and early primary spermatocytes (preleptotene spermatocytes), which are not shown in this diagram. The adluminal compartment contains late primary spermatocytes (pachytene spermatocytes–), secondary spermatocytes, spermatids, and spermatozoa

## Classical Spermatogonial Stem Cell Definition Based on Morphology

Spermatogonia are located in the basal compartment and on the basement membrane in the periphery of the seminiferous tubules (Fig. 12.1). They are classified into several subpopulations. At first, they were subdivided into type A and type B. Type A spermatogonia present no heterochromatin in the nuclei, whereas type B spermatogonia display heterochromatin. In mice, subsequently, spermatogonia of an intermediate (In) type were found; the nuclei of In spermatogonia contain a moderate amount of heterochromatin [2]. Type A spermatogonia form the initial population of cells that undergo spermatogenesis, followed by In and type B spermatogonia, which give rise to spermatocytes (Fig. 12.2). Type A spermatogonia are further subdivided into undifferentiated, A1, A2, A3, and A4 spermatogonia (Fig. 12.2). The six types of spermatogonia from A1 to type B are generated by one cell division

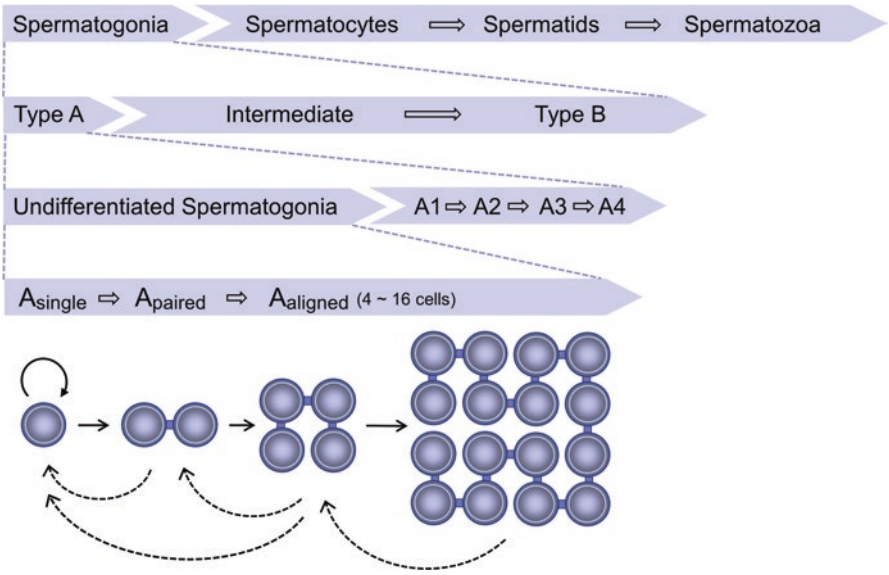


**Fig. 12.2** Scheme of male germ cell lineage in mice. Murine spermatogonia are classified into type A, intermediate type (In), and type B spermatogonia. Type A spermatogonia are further subdivided into undifferentiated, A1, A2, A3, and A4 spermatogonia. Undifferentiated spermatogonia, in turn, are further subdivided to $A_s$, $A_{pr}$, and $A_{al}$. $A_{al}$ have $2^n$ ($n$ = 2–4, rarely 5) cells due to their incomplete cytokinesis. The $A_s$ model assumes that only the $A_s$ undifferentiated spermatogonia are SSCs that can self-renew (circle arrow). The dotted arrows indicate fragmentation from longer $A_{al}$ to shorter $A_{al}$, $A_{pr}$, or $A_s$. This suggests that all undifferentiated spermatogonia have stem-cell potential. Refer to the text for details

each [9], and a single type B spermatogonium divides into two preleptotene spermatocytes. Thus, one A1 spermatogonium can generate 64 preleptotene spermatocytes, which eventually produce 256 spermatozoa. In each cell division, the two daughter cells do not separate completely and are connected by an intercellular bridge [10]. Because subsequent cell divisions are synchronized, their differentiation process proceeds in a well-coordinated manner.

The most immature type A spermatogonia are named undifferentiated spermatogonia, which are further subdivided into $A_{single}$ ($A_s$), $A_{paired}$ ($A_{pr}$), and $A_{aligned}$ ($A_{al}$), based on their morphological characteristics (Fig. 12.2). An $A_s$ cell represents a single or an isolated undifferentiated spermatogonium, while $A_{pr}$ cells are two interconnected undifferentiated spermatogonia with an intercellular bridge. $A_{al\text{-}4}$, $A_{al\text{-}8}$, $A_{al\text{-}16}$, and the rare $A_{al\text{-}32}$ comprise 4, 8, 16, and 32 undifferentiated spermatogonia, respectively, with intercellular bridges. These $A_{al}$ spermatogonia differentiate into A1 spermatogonia without cell division; thus, theoretically, a single $A_s$ spermatogonium can generate a maximum of 4096 or 8192 spermatozoa. However, because a significant number of germ cells undergo apoptosis during differentiation [11], this high yield of spermatozoa does not occur naturally.

The morphological analyses, in combination with cell kinetic studies via radioisotope labeling, whole mount analysis, and differentiation-arrest models, including models fed with vitamin A-deficient diets and those with cryptorchid testes, support the hypothesis that $A_s$ spermatogonia are SSCs ($A_s$ model) [4, 9]. When $A_s$ spermatogonia divide, they have two options: one cell can either generate two $A_s$ spermatogonia or two $A_{pr}$ spermatogonia. The former represents cell division for self-renewal, and the latter represents differentiation, which results in the generation of $A_{al\text{-}4}$, $A_{al\text{-}8}$, $A_{al\text{-}16}$, and $A_{al\text{-}32}$ following subsequent synchronized cell divisions. In adult testes, the number of SSCs is consistent; therefore, after a self-renewal cell division, only one $A_s$ is maintained as an $A_s$ spermatogonium, whereas the other undergoes differentiation and generates $A_{pr}$ by the subsequent cell division. In contrast, during the developmental phase after birth or regeneration phases after injury such as irradiation or chemotherapy, $A_s$ spermatogonia in the testes must increase in number and repeat self-renewal cell divisions. In the $A_s$ model, the generation of $A_{pr}$ is the first step of differentiation. Furthermore, the model suggests that $A_s$ cells are assumed to only be derived from self-renewing cell divisions of $A_s$ cells; therefore, $A_s$ cells are the most primitive spermatogonia and have been exclusively considered as SSCs in the testes [12, 13]. It is not clear, however, whether all $A_s$ have the ability to maintain and regenerate long-term spermatogenesis and whether non-$A_s$ subpopulations such as $A_{pr}$, $A_{al}$, or other spermatogonia have such a potential or occasionally behave as SSCs. Although the $A_s$ model assumes that both $A_{pr}$ and $A_{al}$ are irreversibly committed for differentiation, recent reports have observed fragmentation from long $A_{al}$ to short $A_{al}$, $A_{pr}$, and $A_s$, which accompanied dedifferentiation [14, 15]. The differentiation order of mouse spermatogonia is indicated in Fig. 12.2.

## Spermatogonial Stem Cell Definition Based on Functional Transplantation

Stem cells are defined by their biological activity [16]; however, until 1994, a method to assess the stem cell activity was only available for murine hematopoietic stem cells. When testicular germ cells were introduced into germ cell-depleted seminiferous tubules, a particular cell population migrated and colonized the basement membrane and regenerated, resulting in donor-derived spermatogenesis [17] (Fig. 12.3). The reconstituted spermatogenesis continued throughout the lifespan of the host and successfully generated a progeny upon mating with females [18]. One spermatogenic colony in the recipient testes could be generated from a single colonized cell [19, 20]. These results demonstrated that the spermatogenic colony-forming cells had the ability to self-renew and differentiate into functional gametes, and the transplantation system served as a quantitative functional assay for SSCs [8]. This SSC transplantation system, in which both the self-renewal and differentiation activities of SSCs can be evaluated, is the second functional assay for stem cells, the first transplantation assay being bone marrow transplantation into

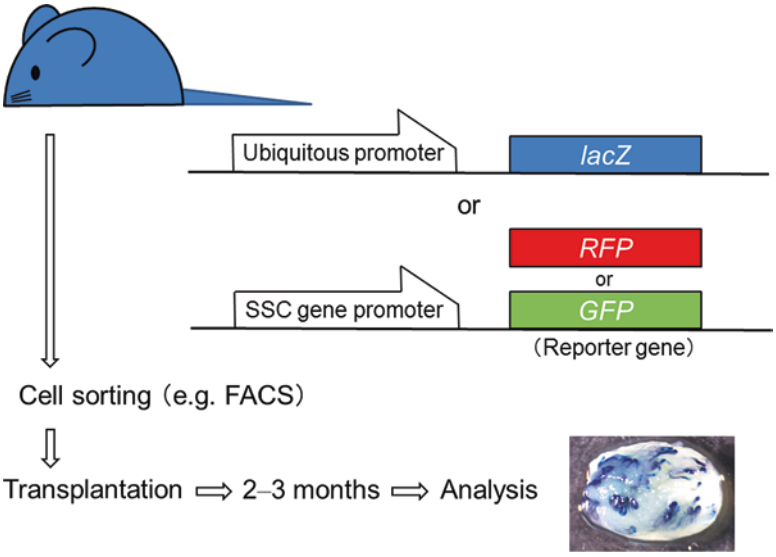


**Fig. 12.3** Schematic representation of the transplantation assay for SSCs to determine the SSC identity. To explore the antigenic profile of SSCs, donor cells are prepared from transgenic mice that constitutively express a reporter gene (e.g., *lacZ* encoding β-galactosidase) under the control of a ubiquitous promoter (e.g., *Rosa26* locus), isolated into their candidate fractions by FACS or MACS, and transplanted into the seminiferous tubules of infertile recipient mice. About 2–3 months after transplantation, the recipient testes were analyzed to identify donor-derived spermatogenic colonies (β-galactosidase-expressing colonies can be identified as blue colonies by staining with X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). To explore the intracellular molecules expressed in SSCs, donor cells are prepared from transgenic mice that express a fluorescent reporter gene (e.g., GFP or RFP) under the control of an endogenous gene promoter, which possibly drives SSC-specific gene expression (SSC gene promoter). The fluorescent signal-positive cells are isolated by FACS, followed by the transplantation assay. About 2–3 months after transplantation, the recipient testes are analyzed to identify donor-derived fluorescent spermatogenic colonies (not shown in this figure)

recipients with hematopoietic destruction, for hematopoietic stem cells, which was established in 1961 [21].

Using the transplantation assay for SSCs in combination with fluorescence-activated cell sorting (FACS), the antigenic profile of SSCs, which is a phenotypic identity of SSCs, can be determined (Fig. 12.3). A series of studies using antibodies against various cell surface molecules have identified the ITGA6, ITGB1, THY1, EPCAM, MCAM, CD9, CD24, CDH1, and GFRA1 expressed on SSCs [22–28]. Because these molecules are also expressed on non-stem spermatogonia, no SSC-specific surface molecules have been identified. During the course of studies, to determine the surface phenotype of SSCs, negative results (no expression) have been found to be important. For example, SSC activity was concentrated in the major histocompatibility complex-1 (MHC-1)$^{-}$- and KIT$^{-}$-cell fractions [25]. MHC-1 is expressed in most somatic cells, whereas KIT is expressed in spermatogonia, differentiating from A1 to type B [29]. A combination of the positive and

aforementioned negative markers has facilitated the identification of a specific subpopulation of testicular cells enriched for SSCs. In diploid testicular cells, MHC-1$^-$ KIT$^-$ cells represent undifferentiated spermatogonia, in which ITGA6, CD9, CDH1, MCAM1, and THY1 are expressed. THY1 was strongly expressed in a few somatic cells, including fibroblasts and T lymphocytes; however, its expression in a germ cell fraction was detected at a low level [25, 30]. A THY1$^+$ ITGA6$^+$ KIT$^-$ MHC-1$^-$ subpopulation comprises SSC activity-containing undifferentiated spermatogonia and is one of the most SSC-enriched subpopulations, in which 1 in 15–30 cells is assumed to be an SSC [25]. THY1 appeared to be heterogeneously expressed in ITGA6$^+$ spermatogonia [30]. High SSC activity was detected in THY1$^+$, whereas low SSC activity was detected in THY1$^-$ spermatogonia, indicating the heterogeneity of the cell surface phenotype of SSCs. In addition, THY1 expression on SSCs decreased gradually from neonates to adults, suggesting that the cell-surface phenotype of SSCs in infants and adults or in a proliferation phase and stable phase is different [30]. The regulatory mechanism of THY1 expression in undifferentiated spermatogonia needs to be determined.

The transplantation assay, in conjunction with FACS, can be utilized for investigating intracellular molecules expressed in transplantable SSCs (Fig. 12.3). To identify the SSCs via intracellular molecules, transgenic mice expressing noninvasive reporter genes, such as GFP, YFP, or RFP, under the regulatory elements of the genes of interest, are required. In most cases, a reporter gene is knocked-in to the original genomic locus or inserted into a large genomic fragment or bacterial artificial chromosome (BAC) clone containing regulatory sequences of the gene. Based on the reporter gene expression, subpopulations of spermatogonia can be isolated by FACS, and the SSC activity of these spermatogonia can be determined via the transplantation assay. Using this approach, it has been reported that *Pou5f1 (Oct3/4)*, *Sox2*, *Tert*, and *Id4* are expressed in SSCs [31–34]. *Pou5f1* and *Sox2* are transcription factors essential for pluripotency, and *Tert* is telomerase reverse transcriptase, which is expressed in self-renewing cells to ensure unlimited proliferation. *Id4*, a transcriptional repressor, was identified in SSC-enriched THY1$^+$ undifferentiated spermatogonia from juvenile males [35]. The biological function of ID4 in the SSCs has not been elucidated. Nonetheless, determination of the cell surface phenotype of transplantable SSCs has made it possible to obtain SSC-enriched populations by FACS or magnetic-activated cell sorting (MACS), which could be used for the identification of genes expressed in SSCs, such as *Id4*, by transcriptomic analyses. The transplantation assay for SSCs using reporter mice is a valuable approach to validate the functionality of the gene products with regard to the self-renewal and differentiation potential of SSCs.

These reporter gene-expressing transgenic mice can be used to assess the topographical distribution and cell identity of the reporter gene$^+$ cells in seminiferous tubules. Histological analyses of adult *Id4-GFP* transgenic mice injected with an *Id4*-BAC clone revealed that the GFP signal in the testes was detected in a small subset of *Zbtb16* (*Plzf*)$^+$ undifferentiated spermatogonia (~2%), which were primarily $A_s$ and a few $A_{pr}$, and pachytene spermatocytes, indicating that *Id4* could be an $A_s$ marker [34]. *Zbtb16* is a transcription repressor that is required for the maintenance

of continuous spermatogenesis in adult males and is expressed in all undifferentiated spermatogonia [36–38]. Intriguingly, the histological and flow cytometric analyses of adult testes of *Id4-GFP* knock-in mice indicated a more widespread GFP expression, including that from ~30% of *Zbtb16*$^+$ spermatogonia, spermatocytes, and spermatids [38, 39]. Although these results of the *Id4-GFP* knock-in mice were contrasting to those obtained for the *Id4*-BAC clone transgenic mice, which claimed that the major *Id4-GFP*$^+$ cells in the testes were $A_s$, the expression of *Id4-GFP* in a minor subset of undifferentiated spermatogonia was commonly observed in both the *Id4-GFP* transgenic mouse lines. However, it should be noted that flow cytometric analysis of the *Id4*-BAC clone transgenic mice was performed in the developing testes (8 days postpartum; 8 dpp), which did not contain differentiated and haploid germ cells [40]; therefore, it is important to compare the flow cytometric data of the *Id4-GFP*$^+$ cells in the adult testes of the *Id4*-BAC clone transgenic mice with those of the *Id4-GFP*$^+$ cells in the adult testes of the *Id4-GFP* knock-in mice, to clarify the proportion of *Id4*$^+$ cells in mouse testes.

Because it has been reported that SSCs and gonocytes, the precursor cells of SSCs, in postnatal testes could transform the pluripotent stem cells in culture [41–44], the expression of *Pou5f1* and *Sox2*, which are critical transcription factors for pluripotency, in SSCs, is of great interest. Although several *Pou5f1* transgenic mice have been developed, two *Pou5f1-GFP* transgenic mouse lines developed by introducing an 18-kb genomic fragment containing the minimal promoter and proximal and distal enhancers (GOF18) or an 18-kb genomic fragment lacking the proximal enhancer sequences (GOF18ΔPE) were used for the SSC transplantation assay [31, 45]. Either construct is sufficient for reproducing the endogenous gene expression pattern in the germline [46]. Almost all gonocytes in the neonatal testes of the two *Pou5f1-GFP* transgenic mice were GFP$^+$; however, the GFP expression of the undifferentiated spermatogonia in juvenile testes (5–8 dpp) was heterogeneous [31, 38, 47]. While the *Pou5f1-GFP*$^+$ cells from the developing testes contained transplantable SSCs, *Pou5f1-GFP*$^-$ cells also generated spermatogenic colonies, albeit at low numbers [31, 47]. Intriguingly, *Pou5f1-GFP*$^+$ cells in the adult testes (GOF18ΔPE) comprised $A_{al}$ undifferentiated spermatogonia, KIT$^+$ spermatogonia, and spermatids [38]. $A_s$ and $A_{pr}$ undifferentiated spermatogonia in the mice were *Pou5f1-GFP*$^-$ cells. As expected, the *Pou5f1-GFP*$^-$ cells, which were basically *Gfra1*$^+$, generated spermatogenic colonies following transplantation; however, the *Pou5f1-GFP*$^+$ cells in adult testes, which had no $A_s$ spermatogonia, also generated spermatogenic colonies [38]. The SSC activity in the *Pou5f1-GFP*$^-$ undifferentiated spermatogonia of the adult testes was threefold higher than that of the *Pou5f1-GFP*$^+$ cells. Collectively, the expression of *Pou5f1* in SSCs changes developmentally and does not associate faithfully with SSC activity.

To investigate *Sox2* expression in SSCs, *Sox2-GFP* knock-in mice were generated [32]. In the adult testes of the *Sox2-GFP* mice, rare GFP$^+$ cells were detected on the basement membrane of the seminiferous tubules of adult testes. However, the flow cytometric analysis of testicular cells from young *Sox2-GFP* mice (2-week-old) revealed twice as many KIT$^+$ *Sox2-GFP*$^+$ cells as KIT$^-$ Sox2-GFP$^+$ cells, indicating that the differentiating spermatogonia expressed *Sox2*. In the transplantation

assay, the KIT$^-$ *Sox2-GFP*$^+$ cells isolated by FACS displayed SSC activity; however, the KIT$^+$ *Sox2-GFP*$^+$ cells did not generate any colonies, indicating that no transplantable SSCs existed in the KIT$^+$ *Sox2-GFP*$^+$ spermatogonia. The discrepancy in the results of the flow cytometric analysis and immunohistochemistry of GFP expression in the *Sox2-GFP* mouse testes remains unexplained; however, *Sox2*, a crucial transcription factor for pluripotency, is expressed in transplantable SSCs. Furthermore, these studies indicate that the expression patterns of *Pou5f1* and *Sox2* in transplantable SSCs are not correlated with each other.

Telomere maintenance is critical for self-renewal in stem cells [48]. In testicular germ cells of adult *Tert-RFP* mice, the *Tert*$^{high}$ population comprised undifferentiated spermatogonia and KIT$^+$-differentiating spermatogonia, whereas the *Tert*$^{low}$ comprised KIT$^+$ differentiated spermatogonia [33, 49]. Transplantation assay demonstrated that SSC activity was detected only in the *Tert*$^{high}$ KIT$^-$ cell population. Although the *Tert*$^{high}$ KIT$^-$ cells expressed homogenous ZBTB16, GFRA1 was heterogeneously expressed, and the whole-mount immunocytochemistry indicated that *Tert*$^{high}$ ZBTB16$^+$ GFRA1$^-$ cell population included the long and short chains of $A_{al}$ undifferentiated spermatogonia. To address the SSC activity in the GFRA1$^+$ and GFRA1$^-$ cells, the *Tert*$^{high}$ KIT$^-$ cells were subdivided into the GFRA1$^+$ and GFRA1$^-$ cells, and the transplantation assay was carried out [49]. The *Tert*$^{high}$ KIT$^-$ GFRA1$^+$ population produced threefold more spermatogenic colonies than the *Tert*$^{high}$ KIT$^-$ GFRA1$^-$ cells, but the important point is that the *Tert*$^{high}$ KIT$^-$ ZBTB16$^+$ GFRA1$^-$ cells contained transplantable SSCs. Furthermore, the number of *Tert*$^{high}$ KIT$^-$ GFRA1$^-$ cells was thrice the number of the *Tert*$^{high}$ KIT$^-$ GFRA1$^+$ cells; therefore, the total numbers of transplantable SSCs of the GFRA1$^+$ and GFRA1$^-$ populations are comparable. Intriguingly, the GFRA1$^-$ cells converted to GFRA1$^+$ following transplantation. These results indicate that transplantable SSCs are not limited in the $A_s$ population, and they suggest that SSC niche factors dictate the conversion from $A_{al}$ to $A_s$ after transplantation.

Although the number of $A_s$ spermatogonia in adult mice is estimated to be about 35,000 [50], the number of transplantable SSCs was estimated to be about 3000 [51]. The discrepancy of the numbers of $A_s$ and transplantable SSCs suggest that not all $A_s$ spermatogonia are transplantable SSCs; in other words, a small subset of $A_s$ represent transplantable SSCs. Furthermore, considering the fact that some $A_{pr}$ and $A_{al}$ possessed SSC activity, by which they could regenerate donor-derived spermatogenesis in the transplantation assay, the proportion of $A_s$ among the 3000 transplantable SSCs would further decline.

## Spermatogonial Stem Cell Definition Based on Genetic Lineage Tracing

Spermatogonial transplantation is a powerful approach to unequivocally identify SSCs existing in any donor cell population by assessing their self-renewal and differentiation activities. In the transplantation assay, however, SSCs are colonized in

the infertile recipient testes, in which the germ cells were depleted by Busulfan, an alkylating agent, or absent due to a congenital genetic defect, such as *Kit* mutation. Because donor-derived spermatogenesis is regenerated in the germ cell-depleted microenvironments, the SSCs identified by the transplantation assay are likely to represent those under a regeneration condition. It is important to understand the SSC identity and behavior under homeostatic conditions such as those in normal adult testes. To address this, a cell lineage-labeling system using transgenic mice has been developed (Fig. 12.4). For labeling candidate SSCs, a transgenic mouse line expressing a tamoxifen-inducible Cre recombinase ($CreER^{T2}$) under the control of the regulatory sequence of a gene of interest is crossed with another transgenic mouse line with a floxed-stop *GFP* or *lacZ* reporter gene under the control of a

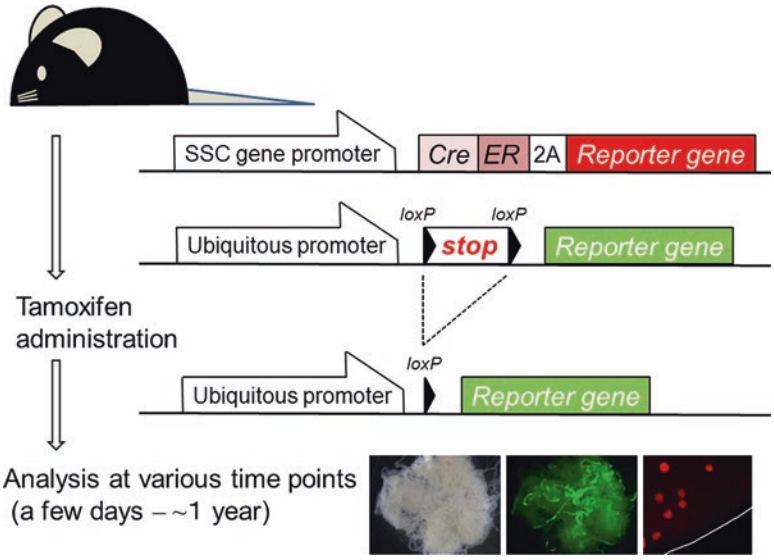


**Fig. 12.4** Schematic representation of a cell lineage-tracing experiment to investigate the SSC identity and behavior. Genetic lineage tracing requires a transgenic mouse line with two constructs, Cre recombinase under the control of the regulatory sequence of a gene of interest (SSC gene promoter) and an inducible reporter. In the Cre recombinase construct, Cre is fused to a tamoxifen-inducible mutated estrogen receptor ($CreER^{T2}$), and a fluorescent reporter (e.g., RFP) is inserted downstream of the SSC gene promoter. Additionally, the two genes are connected by a small 2A peptide sequence that mediates a co-translational cleavage, producing $CreER^{T2}$ and RFP. Alternatively, an internal ribosome entry site (IRES) sequence can be introduced between the $CreER^{T2}$ and RFP sequences to allow the co-expression of the two genes. The RFP enables the identification of SSC candidates, as well as the visualization of the Cre-expressing cells. For a reporter construct, a reporter gene (e.g., GFP) under the control of a ubiquitous promoter is flanked by a *loxP-stop-loxP* (floxed-stop) sequence. When tamoxifen is transiently administered to the mice, $CreER^{T2}$ is translocated to the nucleus and the floxed-stop sequence is removed. The Cre-expressing cells are irreversibly labeled with the reporter gene under the control of the ubiquitous promoter. At various time points after tamoxifen administration, the testes are dissected and analyzed to identify fluorescent spermatogenic colonies. This genetic cell-labeling system can be used in normal and regenerative circumstances

ubiquitous promoter [52]. Tamoxifen administration transiently induces the translocation of CreER$^{T2}$ to the nucleus, followed by the removal of the floxed-stop sequence [53]. Consequently, the Cre recombinase-expressing cells, presumably the cell population expressing the gene of interest, are irreversibly labeled with the introduced reporter gene. The progenies of the cell population are permanently labeled with the reporter gene under the control of the ubiquitous promoter. At various time points after tamoxifen administration, the testes from mice administered with tamoxifen are analyzed to identify the reporter$^+$ spermatogenic colonies. If the labeled cells are SSCs, they replenish the existing differentiated germ cells by forming newly generated spermatogonia; thus, patches of spermatogenic colonies expressing the reporter gene are formed. If the labeled cells are non-SSCs, such as transit-amplifying cells, the reporter gene-expressing spermatogenic colonies are transient and will eventually disappear. Additionally, when a second reporter gene is connected to CreER$^{T2}$ by a small 2A peptide sequence or an internal ribosome entry site (IRES) sequence to allow the co-expression of the two genes, identification of Cre recombinase-expressing cells is possible by detecting the second reporter gene$^+$ cells (RFP in Fig. 12.4), and their topographical distribution in seminiferous tubules can be investigated.

This CreER$^{T2}$-mediated cell lineage-tracing system demonstrated that *Gfra1*-, *Id4*-, *Sox2*-, *Zbtb16*-, *Bmi1*-, *Pax7*-, *Nanos2*-, and *Axin2*-expressing cells could give rise to reporter gene-labeled long-term spermatogenesis under homeostatic conditions in adult testes, indicating that the cells expressing these molecules are SSCs in undisturbed testes [14, 15, 32, 38, 54–58]. In addition, *Id4*-, *Bmi1*-, and *Pax7*-expressing cells have been shown to be able to regenerate long-term spermatogenesis under regenerative conditions after irradiation or chemotherapy [55–57]. Transgenic mouse studies or whole-mount immunocytochemistry confirmed that these genes were expressed in $A_s$ spermatogonia. As mentioned earlier, while it was shown that most of the GFP$^+$ cells in the testes of *Id4*-BAC clone transgenic mice were $A_s$ [34], the GFP$^+$ spermatogonia in the *Id4-GFP* knock-in mice were not limited to $A_s$; instead, GFP was broadly expressed in undifferentiated spermatogonia, including $A_{pr}$ and $A_{al}$ [38]. In the third type of *Id4* transgenic mice, that is, *Id4-2A-CreER$^{T2}$-2A-RFP* knock-in mice, the major population of RFP$^+$ cells comprised $A_s$ spermatogonia, but the age of the mice analyzed was not described, and again, flow cytometric analysis was carried out only in juvenile testes (8 dpp) [55]. Nonetheless, the study clearly demonstrated that *Id4*$^+$ undifferentiated spermatogonia are SSCs in normal and regenerative conditions.

The drawback of the Cre-mediated cell-labeling system is that the quantification of the colony-forming cells is difficult. The efficiency of the tamoxifen-induced removal of the floxed-stop sequence is not 100%. The threshold of intracellular tamoxifen concentration to complete the genetic recombination might be affected by the cell types or cell conditions. In addition, the efficiency of successful recombination will be affected by several factors including the promoter activity of the gene of interest, turnover and stability of Cre recombinase, and gene repair machinery available in the target cells. If the target gene is weakly expressed due to the high stability of the gene product, the target cells might not be efficiently labeled with the Cre recombinase.

## Heterogeneity of Spermatogonial Stem Cells

Currently, SSCs are identified by the transplantation assay or cell lineage-tracing experiments. These two methods have revealed that SSCs exhibit heterogeneous phenotypes and that no universal feature covering all SSCs was observed. At the least, almost all SSC activity was detected in undifferentiated spermatogonia, comprising phenotypically and biologically heterogeneous cells. As originally described, undifferentiated spermatogonia display single (isolated), paired, and aligned morphological features. Furthermore, while the expression of ZBTB16 and CDH1 is relatively constant in the $A_s$, $A_{pr}$, and $A_{al}$ spermatogonia, and has been used to identify all undifferentiated spermatogonia, the $A_s$, $A_{pr}$, and $A_{al}$ spermatogonia display a trend of differential gene expression patterns [14]. *GFRA1*, *Id4*, *Bmi1*, *Pax7*, *Nanos2*, *Lhx1*, *Bcl6b*, *Etv5*, *T*, *Sall4*, *Sox2*, *Eomes*, and *Pdx1* are shown to be preferentially expressed in $A_s$, whereas *Pou5f1*, *Ngn3*, *Lin28A*, *Sohlh1*, *Sox3*, and *Rarg* are preferentially expressed in long $A_{al}$ in adult testes [14, 15, 32, 38, 54–57]. It should be noted that the use of different transgenic mouse lines or detection methods including flow cytometry, whole-mount immunocytochemistry, and immunohistochemistry, occasionally resulted in a different conclusion (for the *Id4-GFP* mouse, *Id4-RFP* mouse, *Sox2-GFP* mouse, etc.). In general, undifferentiated spermatogonia expressing $A_s$-oriented genes present high SSC activity in either the transplantation assay or cell lineage-tracing experiments; however, the SSC activity is not limited to the cells expressing $A_s$-oriented genes. The $A_s$-oriented gene$^-$ or $A_{al}$-oriented gene$^+$ undifferentiated spermatogonia from adult testes presented a low level of SSC activity, indicating that SSCs with different gene expression profiles exist. Undoubtedly, the undifferentiated spermatogonia with high SSC activity in developing testes, normal adult testes, and regenerative adult testes appeared to be different. Undifferentiated spermatogonia expressing *Pou5f1* or *Ngn3*, both of which are $A_{al}$-oriented genes in adult testes, exhibit high SSC activity in the transplantation assay using juvenile testis cells or cell lineage-tracing experiments under a regenerative condition, respectively [14, 15, 31, 38, 47]. The possible key factor for generating heterogeneous SSCs is the surrounding microenvironment or the SSC niche. In developing testes, the SSC niche produces abundant growth factors for self-renewing proliferation and less inhibitory factors, which are supposed to be produced from differentiated germ cells, for self-renewal [59]. On the other hand, the SSC niche in homeostatic adult testes produces a moderate amount of mitogenic factors to maintain a stable SSC number. Accordingly, the majority of the SSCs in developing testes are in a mitotic state, while the SSCs in homeostatic conditions are likely to be quiescent or in a slow cycling state. In regenerating testes, the SSC niche again stimulates growth factor production for SSC expansion.

The dynamic exchange of undifferentiated spermatogonia can occur in regenerative conditions. In the normal adult testes, under homeostatic conditions, undifferentiated spermatogonia expressing $A_s$-oriented genes (e.g., *Ngn3*$^-$ *Gfra1*$^+$ cells) self-renew and maintain spermatogenesis; however, under regenerative conditions,

spermatogonia expressing $A_{al}$-oriented genes (e.g., *Ngn3*$^+$ *Gfra1*$^-$ cells) re-express the $A_s$-oriented genes and acquire SSC activity [14, 38]. In addition, the transplantation assay, which mimics a regenerative condition, detected SSC activity in *Ngn3*$^+$ *Gfra1*$^-$ cells. *Ngn3*$^+$ *Miwi2* (*Piwil4*)$^+$ *Gfra1*$^-$ *Kit*$^-$ spermatogonia, which are mostly $A_{pr}$ and $A_{al}$ and possess characteristics of transit-amplifying cells, are not responsible for homeostatic spermatogenesis. However, this cell population is crucial for regeneration after injury in the adult testes. The transplantation assay demonstrated that the *Ngn3*$^+$ *Miwi2*$^+$ *Gfra1*$^-$ cells indeed have a robust reconstitution activity [60].

Collectively, several lines of evidence indicate that SSC activity is not limited to $A_s$; the $A_{pr}$ and $A_{al}$ retain or regain SSC activity. Furthermore, live cell imaging experiments have indicated that the differentiation process from $A_s$ to $A_{al}$ spermatogonia is not unidirectional, because $A_s$ or $A_{pr}$ spermatogonia could be occasionally generated from $A_{al}$ by fragmentation [15]. Another intriguing research has reported that KIT$^+$ A1 spermatogonia became transplantable SSCs after culturing in vitro, indicating a possible dedifferentiation from the differentiating spermatogonia to SSCs [61]. These studies should be confirmed using different experimental settings, and the molecular mechanisms underlying these findings should be elucidated.

## Concluding Remarks and Future Directions

In the past two decades, several SSC marker genes or $A_s$-oriented genes expressed in SSCs have been identified by the transplantation assay and cell lineage-tracing experiments (Table 12.1). Unfortunately, the functional roles of the majority of these genes, including *Id4*, *Bmi1*, *Pax7*, *Sox2*, *Eomes*, and *Pdx1*, in SSCs, have not been determined. However, *Gfra1*, which is the receptor of GDNF, is an exception. GDNF is an essential growth factor for SSC self-renewal, and directly drives the self-renewal of SSCs, followed by binding to GFRA1 [62, 63]. *Bcl6b*, *Lhx1*, *Etv5*, *T*, and *Nanos2* have been suggested to be involved in GDNF-signaling in cultured SSCs [64–66]. The list of genes expressed in undifferentiated spermatogonia is increasing, but the functional interaction between these genes and their identified gene products is poorly understood. Recent studies using single-cell transcriptomic analysis of fresh testicular cells and undifferentiated spermatogonia enriched for SSCs have revealed that the expression patterns of all $A_s$-oriented genes are not correlated with each other [38, 67–70]. Presumably, the gene expression patterns in SSCs are more dynamic and unstable than previously thought. Future studies should address and elucidate the biological significance of the complex omics data, including not only the transcriptome but also methylome, proteome, and metabolome data obtained from undifferentiated spermatogonia. Furthermore, the deterministic roles of different SSC niches in developing, homeostatic, and regenerating testes in SSC heterogeneity should be clarified. Elucidating the functional roles and interactions of characteristic gene products and finding their causal regulators from the SSC

**Table 12.1** Cell surface and intracellular molecules directly demonstrated to be expressed in spermatogonial stem cells via two experimental approaches

| | Transplantation assay | | Cell lineage-tracing experiment | |
|---|---|---|---|---|
| | Juvenile | Adult | Homeostasis | Regeneration |
| GFRA1/*Gfra1* | [28] | [49] | [15] | [15] |
| THY1 | [30] | [25, 30] | | |
| CDH1 | | [23] | | |
| MCAM | | [27] | | |
| ITGA6 | | [22, 30] | | |
| CD9 | | [26] | | |
| *Pou5f1*[a] | [31, 47] | [38][b] | | |
| *Miwi2*[a] | | [60] | | |
| *Id4* | [40] | | [55] | [55] |
| *Sox2* | | [32] | [32] | |
| *Zbtb16* | | | [38] | |
| *Bmi1* | | | [56] | [56] |
| *Pax7* | | | [57] | [57] |
| *Nanos2* | | | [54] | |
| *Axin2* | | | [58] | |
| *Ngn3*[a] | | | | [14] |

[a]Expressed in $A_{al}$ spermatogonia, transit-amplifying cells, in adult testes
[b]Higher SSC activity in *Pou5f1*$^-$ cells than *Pou5f1*$^+$ cells

niches will provide new insights into SSC biology and clarify the universal identity of SSCs, that is, characters that could be conserved among various mammalian species including humans, amidst their heterogeneity.

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# Chapter 13
# Sources, Identification, and Clinical Implications of Heterogeneity in Human Umbilical Cord Stem Cells


**Frank G. Lyons and Tobias A. Mattei**

**Abstract** Heterogeneity among different subpopulations of human umbilical cord mesenchymal stem cell (hUCMSCs) lines is an ubiquitous phenomenon, with such variability being related to several factors including the identity of the individual donor, tissue source (Wharton's jelly vs. umbilical cord blood), culture conditions, as well as random variations in the cloning expansion process. In this chapter, we provide a general overview on the sources as well as available experimental techniques for proper identification of heterogeneity in hUCMSCs. Finally, we provide a brief discussion on the current scientific evidence regarding the potential superiority of subpopulations of hUCMSCs for specific clinical applications. Taking into account the exponential growth on the available experimental data on hUCMSCs in the past few years, this chapter is not intended to be comprehensive in nature, but rather is intended to provide a general overview about the central role which the topic of heterogeneity has in both basic science and clinical research in umbilical cord stem cells.

**Keywords** Umbilical cord stem cells · Stem cell heterogeneity · Stem cell therapy · Mesenchymal stem cells · Heterogeneity · Umbilical cord bank · Stem cell harvest · Stem cell differentiation · Cloning expansion · Immunomodulation

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## Introduction

Human umbilical cord mesenchymal stem cells (hUCMSCs) are an attractive source for future therapeutic applications in multiple clinical fields. Advantages over other traditional tissue sources include the non-invasive harvest from tissue which would otherwise be disposed in the postpartum period, relative high cell yields, and phenotype-mirroring of mesenchymal stem cells from other established sources [1–3].

Umbilical cord stem cells can be obtained from either the Wharton's jelly and/or the umbilical cord blood. In humans, the umbilical cord is typically 40–60 cm in length and is enclosed by an outer amniotic epithelium within which a single vein and two arteries run. Between the outer epithelium and the vessels lies a mucoid connective tissue, the so-called Wharton's jelly. This tissue yields vascular support (in an adventitial-type role) and also protects such vessels from kinking and twisting along the length of the cord from placenta to the growing fetus [3]. This jelly-like connective tissue, which was first described by Thomas Wharton in 1656, has no neural tissue and is also avascular, displaying some contractile capability provided by the presence of mesenchymal stem cell–derived myofibroblasts [1]. Importantly from a preclinical research perspective, such features are unique to humans, as in other species the mucoid connective tissue contains other blood vessels. Moreover, the three main vessels in the human umbilical cord are devoid of adventitia, having only tunica intima and tunica media layers, as the role of the tunica adventitia role is fulfilled by the Wharton's jelly. Ultimately, from a functional standpoint, the Wharton's jelly seems to play an essential role in maintaining the structural stability of the three main vessels responsible for exchange of nutrients, oxygen, and $CO_2$ between the placenta and the fetus [4].

Mesenchymal stem cells (MSCs) were first identified and isolated from bone marrow [5, 6]. However, such harvesting process is somewhat complex from a technical standpoint, with the added potential for considerable morbidity related to the donor site [2]. Since these initial studies, multiple other tissue sites have been studied, each demonstrating variable degree of efficacy as sources of MSCs; each of them, however, involving complex harvest, isolation, and expansion processes, as well as at least some degree of procedural morbidity [6–13]. Embryo-derived stem cells provide an obvious solution to these limitations by yielding copious amounts of phenotypically pluripotent stem cells without the morbidity of bone marrow or other tissue harvest [14]. However, this source of stem cells is associated with quite profound ethical concerns which have ultimately prevented the expansion of research with such cell types [2]. An exciting scientific breakthrough that was believed to have the potential of overcoming such limitations associated with embryonic stem cells was the technique of reprogramming adult cells to a primitive pluripotent phenotype, the so-called induced pluripotent stem cells (iPSCs) [15]. However, initial promising results have been countered by evidence of mutation and genetic variability in genes with known causative effects in cancer [16, 17], raising serious questions about the safety profile of such new cell engineering methods.

As previously described, umbilical cord–derived stem cells have established advantages over other stem cell sources. First, cells are isolated from the umbilical cord, which would be otherwise discarded in the postpartum period as a matter of routine; no additional invasive procedure is required to harvest stem cells from the umbilical cord after birth. Next, the process of cell isolation from the cord tissue is relatively simple due to the straightforward anatomic and structural nature of the human umbilical cord. Finally, the resultant cells' phenotype parallels that of other MSC cell sources [1, 2, 4, 18].

When harvesting tissue from the umbilical cord there is the option of using either the Wharton's jelly or umbilical cord blood as tissue sources. Traditionally, MSCs have been derived from connective tissues such as bone or fat. Interestingly, some initial studies reported that human umbilical cord blood was not a viable source for MSC isolation [19, 20]. Subsequent studies, however, demonstrated that not only can MSCs be derived from full-term cord blood but also that it may produce a higher cell yield compared to bone marrow and adipose tissue sources [10, 21, 22].

The exponential growth of stem cell banks in the past years may also raise the possibility of employing an individual's own source of stem cells. However, despite the widespread belief in a dichotomous paradigm regarding stem cells (in which a cell "is" or "is not" essentially a stem cell), it seems clear that such a "stemness" property of cells would be better understood as a spectrum of pluripotency, allowing for several degrees of differentiation capacity. In such a scenario, stem cell heterogeneity among different stem cell lines can be viewed as the norm and not the exception, with such variability being related to several factors, including the identity of the individual donor, tissue source, culture conditions, as well as random variations in the cloning expansion process [23] (Fig. 13.1). Taking into account such multitude of variables, even if focusing specifically in the subgroup of mesenchymal stem cells isolated from umbilical cord tissue (which would encompass MSC lines obtained from either the Wharton's jelly or the umbilical cord blood) [24], a wide variation of cell behavior can still be expected. Therefore, the development and validation of reproducible means for isolating homogenous lines of MSCs has been considered as an indisputable prerequisite in order to translate current research into predictable and successful therapeutic strategies. Therefore, it is no surprise that the progressive realization of the heterogeneous nature of the samples yielded by most MSCs' isolation protocols has somewhat tampered initial experimental enthusiasm. There have been some complaints regarding the slow pace of translation of MSC scientific advances toward effective therapeutic modalities. MSC heterogeneity has been proposed as an explanation for translational failure, and such a phenomenon, which up to now has been more extensively investigated in bone marrow sources of MSCs, is nowadays a well-established and recognized theme in stem cell research [25, 26]. As stem cell heterogeneity has become something of a barrier to advance translation into reproducible therapeutic protocols, considerable efforts within the stem cell scientific community have been observed in the past few years in order to address such issue [27, 28].

In this chapter, we provide a general overview on the possible sources of heterogeneity in hUCMSCs, including inter-individual, tissue-dependent, and cloning/

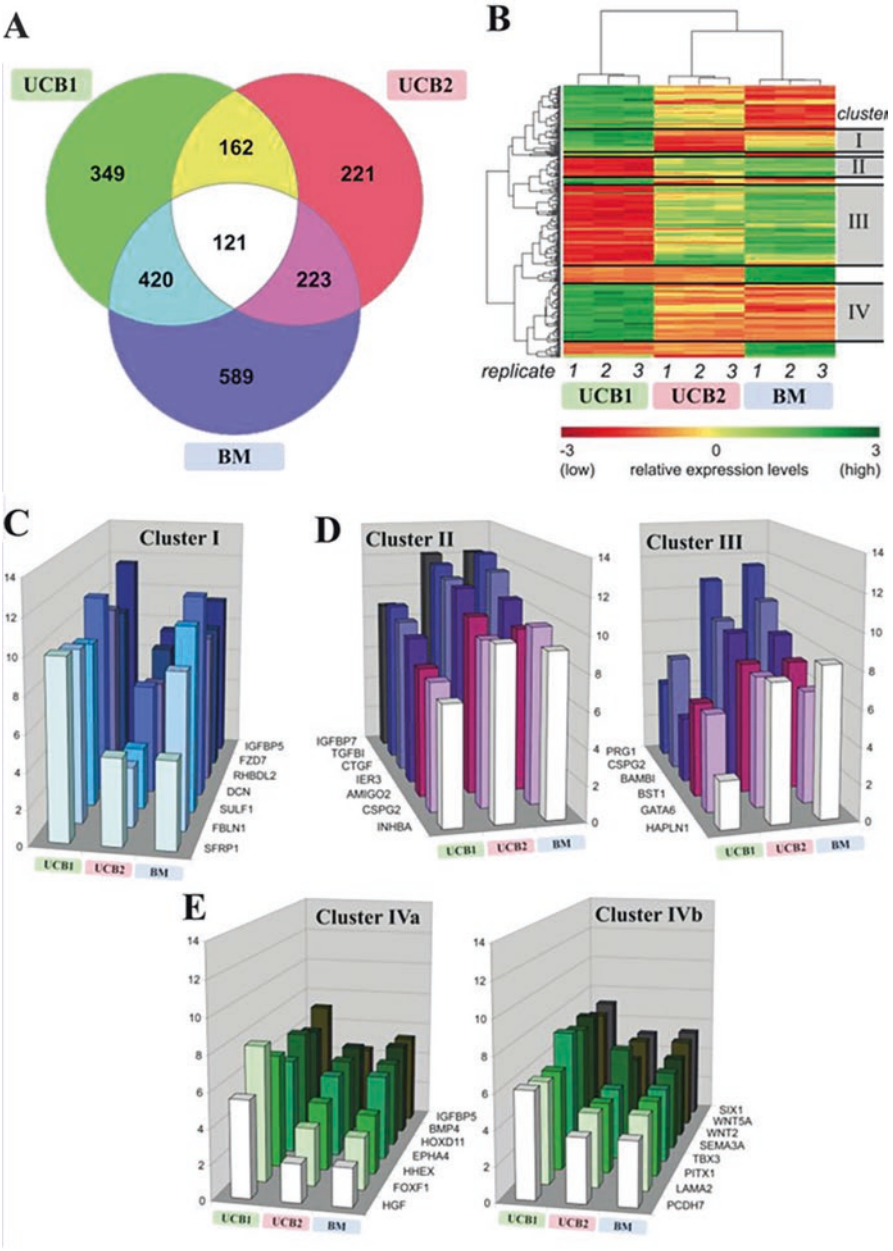


**Fig. 13.1** Microarray analysis of UCB1, UCB2, and BM MSCs identified clusters of genes with differential expression. (**a**) A Venn diagram is shown, representing the distribution 2085 probe sets found to be differentially expressed on the HG-U133A at a FDR of 0.01. (**b**) Heat map display of mean centered and standardized gene expression patterns. The patterns were detected by hierarchical clustering of 290 probe sets differentially expressed between UCB1, UCB2, and BM MSCs at

culture-dependent variability. In sequence, we focus on the available experimental techniques for proper identification of heterogeneity in hUCMSCs. Finally, we provide a brief discussion on the current scientific evidence regarding the possible superiority of subpopulations of hUCMSCs for specific clinical applications. As expected, this chapter is not intended to be comprehensive in nature but to provide a general overview about both the ubiquity of heterogeneity as well as the key role of such topic in both basic and clinical research in umbilical cord stem cells.

## Sources of Heterogeneity

### *Inter-individual Heterogeneity*

Several factors including age, gender, and phenotype have already been established as potential inter-individual causes of MSC heterogeneity [26]. Han et al. compared four tissue sources (bone marrow, adipose tissue, umbilical cord, and placenta) from 22 individuals. Significant variability in proliferative ability, in spite of identical culture methods, was noted across individual samples [27]. Even within the same individual, considerable heterogeneity was observed across the four different tissue sources in spite of consistent methodology and conditions. Another study examined isolation techniques of umbilical cord–derived MSCs using either one of the two standardized enzymatic protocols or an explant protocol, all uniformly designed and performed. These authors found not only differences in cell proliferation profile among the distinct techniques but also unique responses of each hUCMSCs sample to the same technique [29].

These findings are supported by another recent study examining the therapeutic viability of human UC blood-derived MSCs for angiogenesis in the setting of vascular diseases. The authors found that donor-related factors had the single largest influence on therapeutic results, and most importantly, that donor-related factors were non-modifiable by employing established techniques of ischemic preconditioning as an attempt to upregulate vascular endothelial growth factor (VEGF) expression [30]. After co-culturing human umbilical cord endothelial cells (HUVEC) with hUCMSCs in Matrigel, vascular tube formation and HUVEC migration were measured using standard means. Substantial differences in capillary length and cell migration were observed among different donor groups (Fig. 13.2). Based on such results, the authors emphatically state the point that there is a "strikingly variable behavior among MSC from different donors," further emphasizing the increasingly

**Fig. 13.1** (continued) a FDR of 0.001. The relative levels of gene expression are depicted with a color scale, where red represents the lowest and green the highest level of expression. (**c**–**e**) Diagrams illustrating genes selected from clusters with significant increased expression in UCB1 compared to UCB2 MSCs (**c**, Cluster I), and decreased (**d**, Clusters II and III)) or increased (**d**, Cluster IV) expression in UCB1 compared to UCB2 and BM MSCs. Reproduced with permission from Markov et al. [23]

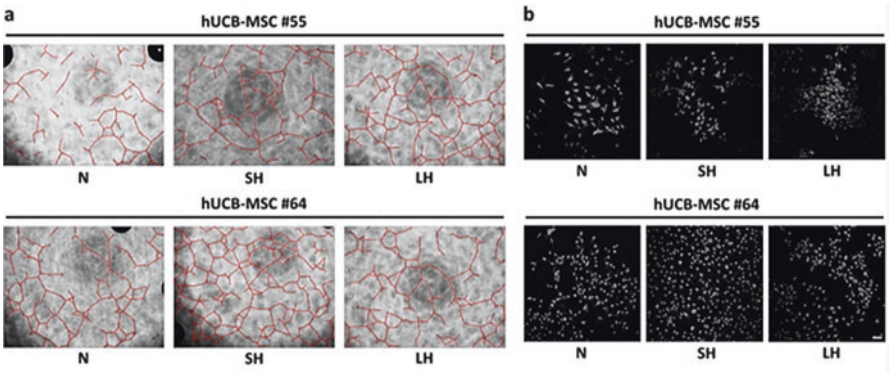


**Fig. 13.2** In vitro angiogenesis capacity is altered by culture conditions and donor. (**a**) Capillary-like tube formation assay of HUVECs cocultured with hUCB-MSC donor #55 or donor #64. Tube formation was quantified by total tube formation length and branch junction. (**b**) Migration assay using HUVECs cocultured with hUCB-MSCs. Reproduced from Kang et al. [30]. License to reproduce image in accordance with publisher guidelines; available at http://creativecommons.org/licenses/by/4.0/. No changes have been made to the original

and pressing need to improve our understanding about the factors affecting stem cell heterogeneity. Figure 13.3 visually demonstrates the considerable gene expression profile variability among the six individual donor tissue sources in such study.

Interestingly, it has been shown that despite such possible inter-individual heterogeneity, most samples of isolated umbilical cord blood MSCs consist of two different populations of progenitors, both positive for CD29 and CD105, denoting their MSC lineage. The first consists of cells with innate neurogenic potential, expressing both pluripotent stem cell markers (such as Oct4, Nanog, Sox2 and ABCG2) as well as the neuroectodermal marker nestin. In propitious conditions, such cells can be easily expanded and differentiated into neurons. The remaining population of cells, however, require an extensive exposure to a combination of growth factors to transdifferentiate into neurons. The authors of such a study proposed that the ratio between these two subpopulations in a batch may be a useful way to determine its innate neurogenic potential [31].

It has also been shown that inter-individual heterogeneity may depend not only on the unique genetic background of the specific donor but also on their overall health status. It has already been demonstrated, for example, that hUCMSCs obtained from donors who suffered from gestational diabetes mellitus may display both premature aging and mitochondrial dysfunction [32]. Similarly, another study demonstrated that several obstetric factors that represent surrogate markers of healthy full-term infants (including birth weight, the number of amenorrhea weeks, placental weight, normal pregnancy, and absence of preeclampsia) had a significant correlation with hUCMSCs proliferation capacity [33].

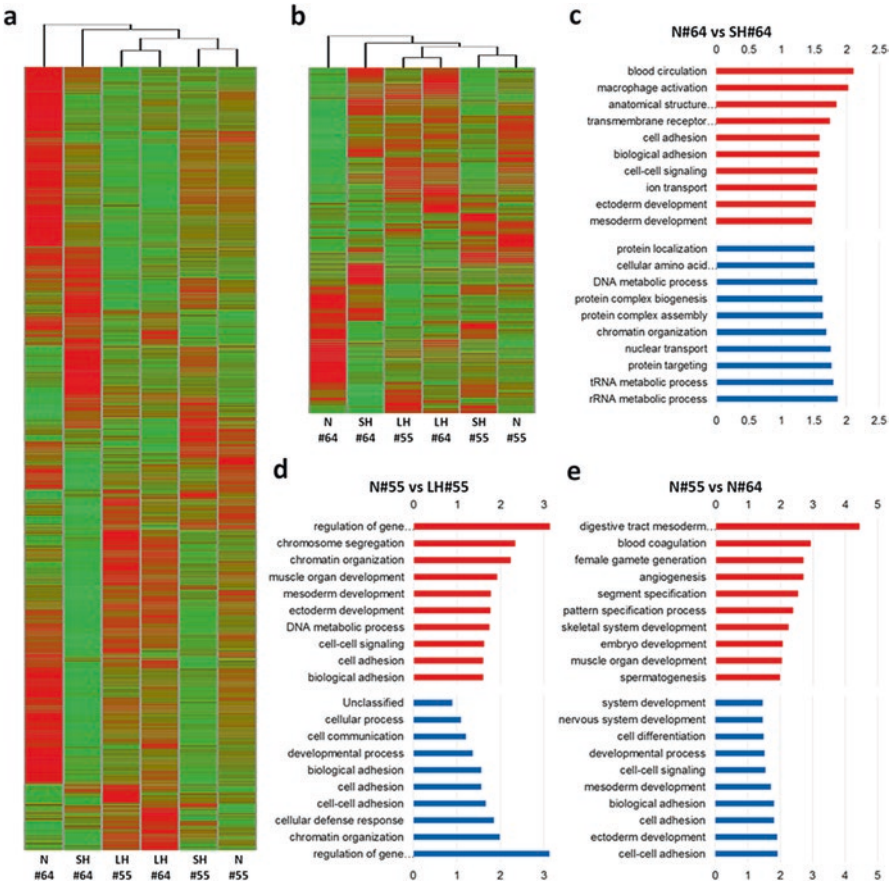


**Fig. 13.3** Global gene expression analysis showing donor-dependent changes in gene expression patterns (**a**–**b**). The global genome heatmap with hierarchical cluster analysis shows similarities of genes at an expression level of more than 100 genes related to the HIF-1 and VEGF signaling pathways (**a**–**b**). (**c**–**e**) GO function-enrichment analysis indicated the top ten categories related to the upregulated genes (red) and the downregulated genes (blue) in N#64 versus SH#64 (**c**), N#55 versus LH#55, (**d**) and N#55 versus N#64 (**e**). The data was analyzed using the PANTHER Classification System. Reproduced from Kang et al. (supplementary Figure S4 of the original article) [30]. License to reproduce image in accordance with publisher guidelines; available at http://creativecommons.org/licenses/by/4.0/. No changes have been made to the original

## *Tissue-Dependent Heterogeneity*

In the first years of hUCMSCs research, there was an intense debate about a supposed "ideal" source of umbilical cord MSC, with several groups passionately defending the Wharton's jelly as the richest source of pluripotent cells, while other groups, based on specific applications, defended the superiority of cells obtained from the umbilical cord blood [24, 34]. Nevertheless, such a debate, albeit

understandable, especially taking into account each groups' unique experience with a specific source of umbilical cord stem cells, seems to be as misguided as the general discussion about the possible superiority of hUCMSCs over other types of stem cells, such as embryonic or bone marrow MSCs. Embryonic stem cells apart, as ethical and legal implications seemed seem to have significantly hampered the initial enthusiasm with such source of stem cells, the heterogenous behavior of subpopulation of cells from other sources may actually prove to be an advantage, as it is quite likely that research on specific therapeutic applications may uniquely benefit from a specific source of cells [35]. In other words, it is quite likely that the ideal source of MSC may strongly depend on the intended therapeutic purposes. For example, despite several theoretical general advantages of hUCMSCs over bone marrow MSC [16, 17], a study exploring the potential of stem cell therapy in ischemic tissues demonstrated that bone marrow and placental chorionic villi MSCs may be preferred in clinical applications for therapeutic angiogenesis as both lines demonstrated upregulation of several angiogenic genes in comparison with adipose tissue and umbilical cord MSC, with bone marrow MSCs displaying high levels of VEGF, while placental chorionic villi MSCs secreted high levels of HGF and PGE2 [36]. Another study, comparing the angio/vasculogenic and immunomodulatory capacity of four MSC sources found variable outcomes among the examined tissue sources. In terms of angiogenic capacity, placental tissue source was more potent than umbilical, bone, and adipose sources [27]. A functional biomarker, CD106, was further identified across all four tissue cell sources, and higher expression of such marker strongly correlated with greater proangiogenic and immunomodulatory potency. The authors propose such type of cell surface proteins as possible means of identifying homogenous cells within a heterogenous stem cell population.

## *Heterogeneity During Cloning Expansion*

It has already been demonstrated that the process of stem cell isolation may lead to a significant heterogeneity in the obtained cell lines. In one study, Paladino et al. [29] studied the response of eight hUCMSCs sources to three established cloning protocols. The first involved infusion of type I collagenase into umbilical cord veins to isolate resident MSCs; the second protocol minced the umbilical cord veins before culture with type I collagenase; the third method involved direct culture of minced umbilical cord veins without enzymatic digestion. After each of these three methods the authors proceeded with an established expansion process as per protocol [37–39]. All samples were compared in terms of population doubling, time to senescence, cell morphology, cell surface markers, and differentiation capacity. Depending on the protocol employed, samples exhibited different doubling rates, time to senescence, and cell morphology but maintained the same cell surface marker profile (a confirmation of preservation of the MSC profile) (see Fig. 13.4). Ultimately, variations in cell proliferation and overall time to senescence were especially sensitive to how cells were isolated and expanded from umbilical cord samples.

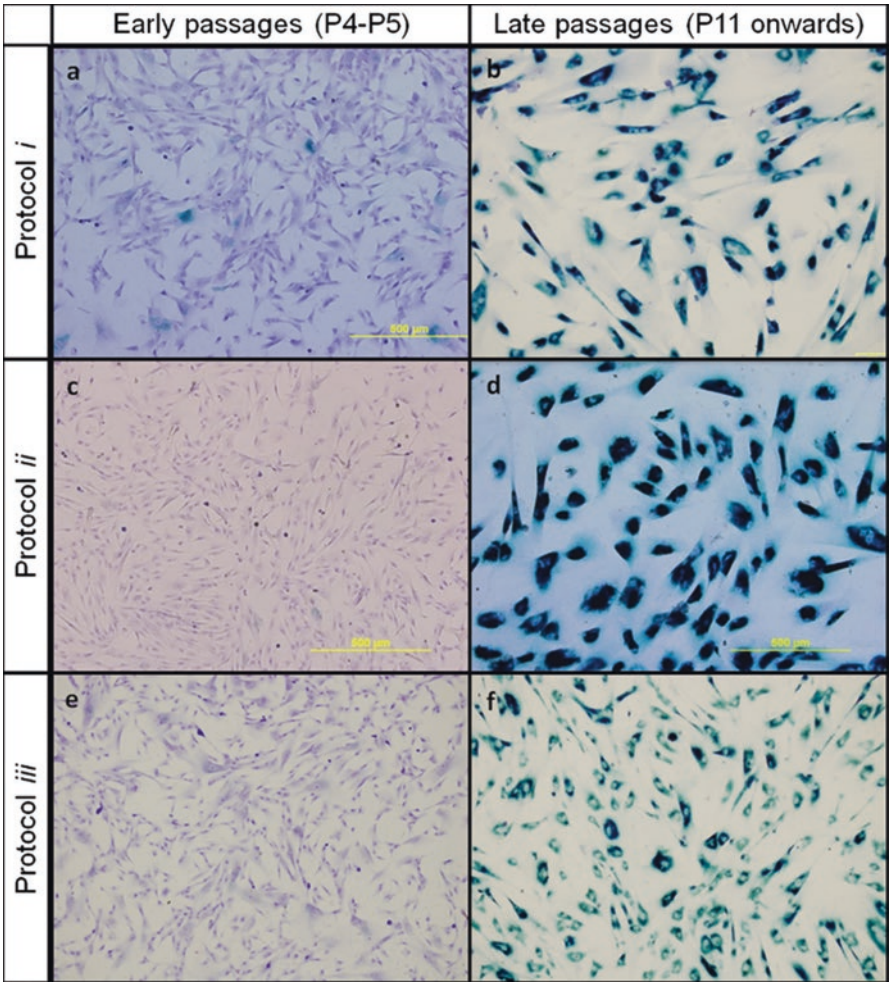


**Fig. 13.4** Beta-galactosidase staining of UC–MSCs to assess replicative senescence. MSCs collected in early and late passages, (**a**, **b**) protocol i; (**c**, **d**) protocol ii; and (**e**, **f**) protocol iii. Young cells stained purple with crystal violet (**a**, **c**, **e**) for better viewing and senescent cells stained green when positive for the b–galactosidase (**b**, **d**, **f**). Reproduced with permission et al. [29]

Despite multiple sources of initial heterogeneity, it has been shown that in vitro expansion of hUCMSCs tends to be associated with a robust process of clonal selection, with an exponential decrease in the diversity of different population and selection of a few single clones over time. Such waves of clonal selection, which has been shown to occur in the very early passages during initial hUCMSCs expansion, may ultimately constitute one of the major determinants upon the overall differentiation capabilities of the final cells [40].

As the process of in vitro clonal expansion is an indispensable step for obtaining hUCMSCs for clinical applications, these results suggest the necessity of not only better understanding of such selection processes but also the preeminent necessity developing methods of fine-tuning it, so that specific clones may be selected depending on the expected clinical benefits which are intended to be achieved with the stem cell therapy. As mentioned earlier, the CD106 cell surface marker (also known as vascular cell adhesion molecule) has been proposed as a possible tool for identification of specific subpopulations of MSCs [27]; however, further studies on the reliability of this, as well as other, markers are still necessary before such type of identification process becomes a standardized protocol across different research laboratories.

## Identification of Heterogeneity

It has already been shown that different clonal subpopulations of MSCs may demonstrate a very homogenous appearance under the microscope [40]. Therefore, it seems clear that more refined methods, such as flow cytometry, fluorescence microscopy, or deep sequencing, are necessary for proper identification of heterogeneity in hUCMSCs. Some recent studies have also proposed new techniques for proper detection of heterogeneity, such as multicolor lentiviral barcode labeling [40]. Although the overall safety implications of such retroviral labeling process is still unknown, especially when considering hUCMSCs which are intended for clinical applications, it seems to ultimately represent an ingenious means of following clonal dynamics during the in vitro MSC expansion. Other reported experimental forms for marking and tracking hUCMSCs which have been described in the literature are CRISPR/Cas9-mediated genome modifications and miRNA screening [40].

## Clinical Implications of Heterogeneity

Although there has been an exponential growth in in vitro experimental studies with stem cell therapies, proper identification and control of heterogeneity, a somewhat neglected topic until very recently, seem to be indispensable steps for successful translation of early promising laboratory results to the clinical arena. Cognizant of such dilemma, several animal studies have been performed not only for determination of the possible benefits of stem cell therapy but, especially, for the identification of specific hUCMSCs lines which may be associated with such therapeutic benefit.

For example, a recent experimental study exploring the immunomodulatory potential of hUCMSCs after traumatic brain injury (TBI) in mice determined that CD45 (as identified in mononuclear cells) constituted a surrogate marker of high expression of typical mesenchymal markers, and, therefore, may be used as a predictor of hUCMSCs units' quality for therapeutic interventions in TBI [41].

Similarly, when exploring the potential of hUCMSCs to retinal degeneration, researchers have demonstrated that a smaller, fibroblast-like, and faster growing subset of hUCMSCs demonstrated superior therapeutic potential which could be attributed to its stronger antiapoptotic effect, sensitivity to paracrine trophic factors, and differentiation potential into retinal pigment epithelium [42].

## Future Trends

Several studies have demonstrated that, apart from individuals with very high likelihood of requiring a treatment involving hUCMSCs, private umbilical cord banking does not seem to be cost-effective from a populational standpoint. Additionally, even in high-income countries, such type of service would likely be restricted to only a small minority of economically privileged individuals.

In fact, it has been estimated that in order to become cost-effective, either the total overall costs of umbilical cord blood banking should be less than US$262 or the likelihood of a child needing a stem cell transplant should be greater than 1 in 110, both of which are quite unlikely scenarios, especially taking into account the high costs of harvesting and stem cell storage as well as the still small number of therapeutic strategies employing hUCMSCs that may be expected to become clinically available in the next few decades [43].

Therefore, if hUCMSCs are expected to become part of the available therapeutic armamentarium for a variety of different diseases, it is likely that, similar to available blood or bone marrow transplant banks, the best alternative would be the development of general public banks. In such a scenario, the development of practical and affordable techniques for proper identification of hUCMSCs heterogeneity as well as for donor–receiver matching (going beyond the simplistic criteria based solely on human leukocyte antigen—HLA) seems of paramount importance in order to provide the most adequate cells for each individual and intended therapeutic application. Overall the therapeutic uncertainty associated with MSC heterogeneity has been already extensively described in the context of research strategies for cancer, vascular disease, autoimmune disorders, musculoskeletal disease, immunotherapy, and neural regeneration [19, 23, 26, 30, 44–46]. However it is important to realize that the issue of hUCMSCs heterogeneity also plays a critical role in the area of tissue banking for future therapeutic uses [29].

## Conclusions

It seems clear that a full appreciation and understanding of the sources and consequences of hUCMSCs heterogeneity is of paramount importance for the development of standardized protocols for isolation and cell culture that may be reliably and reproducibly applied in future translational efforts. Heterogeneity related to

inter-individual and tissue source variability, as well as those inherently associated with currently available techniques for isolation and expansion are, at the present, non-standardizable factors. Finally, although heterogeneity has been usually depicted as a barrier toward translating successful experimental strategies to the clinical practice, it may actually represent a strength of hUCMSCs, especialy if future studies are able to further refine the available methods for identification and selection of desired clonal lines. Taking into account the wide spectrum of behavior of hUCMSCs, it seems likely that the ability to experimentally manipulate and direct clonal expansion toward unique cell lines may further enable the development of more targeted therapeutic interventions across an equally wide spectrum of diseases.

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