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Alexander Birbrair *Editor*

Stem Cells Heterogeneity - Novel Concepts

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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."

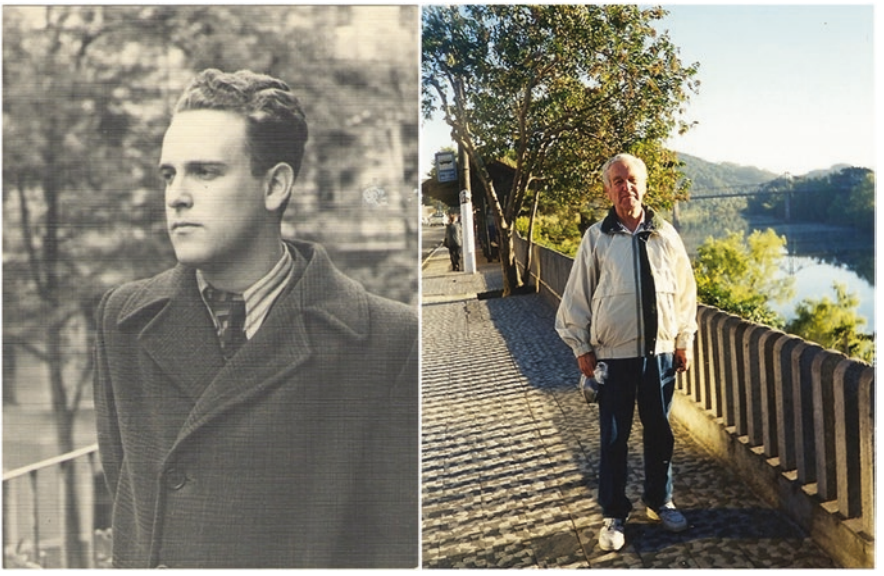
This book, *Stem Cells Heterogeneity - Novel Concepts*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of stem cells to the biology of different organs 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 stem cells' role within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of stem cell 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. Eleven chapters written by experts in the field summarize the present knowledge about stem cell heterogeneity in distinct circumstances.

Alice Jouneau from INRA discusses the heterogeneity in epiblast stem cells. Ricardo Pardal and colleagues from Sevilla University describe stem heterogeneity in the adult carotid body. Salvetti Alessandra and Leonardo Rossi from the University of Pisa compile our understanding of stem cell heterogeneity in planaria. Wa Xian and colleagues from the University of Texas Health Science Center update us with what we know about Barrett's esophagus stem cells. Kiyoshi Ohnuma and colleagues from Nagaoka University of Technology summarize current knowledge on

pluripotent stem cell heterogeneity. Jiri Hatina and colleagues from Charles University address the importance of sarcoma stem cell heterogeneity. Elio A. Prieto González from the Interamerican Open University focuses on heterogeneity of adipose-derived stem cells. Ganokon Urkasemsin and Joao N. Ferreira from Mahidol University introduce our current knowledge about salivary gland stem cells. Weiqiang Wang and Zhong Chao Han from Tianjin Institute of Health and Stem Cells talk about the heterogeneity of human mesenchymal stem cells. Sujit K. Bhutia and colleagues from the National Institute of Technology Rourkela focus on mitochondrial heterogeneity in stem cells. Finally, Dario Pisignano and colleagues from the University of Bari give an overview of the heterogeneity of renal stem cells and their interaction with bio- and nano-materials.

It is hoped that the articles 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 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
Stem Cells Heterogeneity

Alexander Birbrair

Abstract Adult endogenous stem cells are crucial to maintain organ homeostasis due to their particular capacity to originate more specialized cell populations in a coordinated manner based on the body necessity. Extensive studies in a variety of tissues have highlighted the importance of stem cells for the functioning of our organism, including the skin, intestine, stomach, skeletal muscle, bone marrow, and others. Although significant progress has been made in our understanding of stem cell biology, our knowledge about these cells still remains limited due to their complexity and their dynamics. The advancement of our knowledge on these essential cells will have substantial implications in our understanding of tissue homeostasis and disease. Importantly, not all stem cells are alike even within the same tissue. They differ in their cell cycle status, surface marker expression, response to various extrinsic molecules, and distinct lineage outputs after transplant. The expanding literature which backs heterogeneity within stem cells is presently of great interest and brings questions as how stem cell subpopulations are generated, why they exist, and whether stem cells heterogeneity influences disease progression or therapy options. In more recent years, the combination of fluorescent and confocal microscopy with genetic state-of-art techniques, such as fate lineage tracking and single-cell RNA sequencing, enabled remarkable advance in the discovery of multiple novel essential functions for stem cell subpopulations in health and disease, before unexpected. This book provides an overview on our knowledge of stem cell subtypes in different organs under physiological and pathological conditions and discusses the possible origins and consequences of stem cells heterogeneity. 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

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was subdivided into three volumes entitled: Stem Cells Heterogeneity—Novel Concepts, Stem Cells Heterogeneity in Different Organs, and Stem Cells Heterogeneity in Cancer. Here, we offer a selected compilation of comprehensive chapters on what we know so far about heterogeneity within stem cells. More than 30 chapters written by scientists in the field outline our present knowledge on stem cells heterogeneity.

Keywords Stem cells · Heterogeneity · Plasticity · Regeneration · Microenvironment · Niche

Adult endogenous stem cells are crucial to maintain organ homeostasis due to their particular capacity to originate more specialized cell populations in a coordinated manner based on the body necessity [1]. Extensive studies in a variety of tissues have highlighted the importance of stem cells for the functioning of our organism, including the skin [2], intestine [3], stomach [4], skeletal muscle [5], bone marrow [6], and others [7]. Although significant progress has been made in our understanding of stem cell biology, our knowledge about these cells still remains limited due to their complexity and their dynamics. The advancement of our knowledge on these essential cells will have substantial implications in our understanding of tissue homeostasis and disease. Importantly, not all stem cells are alike even within the same tissue. They differ in their cell cycle status, surface marker expression, response to various extrinsic molecules, and distinct lineage outputs after transplant. The expanding literature which backs heterogeneity within stem cells is presently of great interest and brings questions as how stem cell subpopulations are generated, why they exist, and whether stem cells heterogeneity influences disease progression or therapy options. In more recent years, the combination of fluorescent and confocal microscopy with genetic state-of-art techniques, such as fate lineage tracking and single-cell RNA sequencing, enabled remarkable advance in the discovery of multiple novel essential functions for stem cell subpopulations in health and disease, before unexpected. This book provides an overview on our knowledge of stem cell subtypes in different organs under physiological and pathological conditions and discusses the possible origins and consequences of stem cells heterogeneity.

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Chapter 2
Heterogeneity in Epiblast Stem Cells

Alice Jouneau

Abstract Epiblast stem cells (EpiSCs) are pluripotent cells that are derived from mouse embryos at gastrulation stages. They represent the primed state of pluripotency, in which cells are on the verge of differentiation and already express markers of the three primary lineages (mesoderm, endoderm, neurectoderm). EpiSCs display some heterogeneity intra- and inter-cell lines in the expression of some of these lineage markers. We relate this heterogeneity to signalling pathways that are active in EpiSCs, either due to addition of growth factors (FGF2 and activin) in the culture medium, or endogenously active (FGF, Nodal, and Wnt). By modulating Wnt or activin/nodal pathways, cell lines close to EpiSCs but with different properties can be obtained. These signalling pathways are all at work in vivo to pattern the pluripotent epiblast and specify cellular fates.

Keywords Epiblast · Primed · Pluripotency · Heterogeneity · Wnt · Activin/Nodal · EpiSC · Differentiation · Fate · Signalling pathways · Patterning

Introduction

Pluripotency is in vivo a continuous process starting within the inner cell mass at the blastocyst stage and ending at the end of gastrulation. It can be captured in the forms of pluripotent stem cells that can self-renew in vitro indefinitely under defined conditions while maintaining their capacity to differentiate into all cells of an organism. In the mouse, two types of pluripotent cells have been captured, embryonic stem cells (ESCs) and epiblast stem cells (EpiSCs), that represent the naïve and primed state, respectively. ESCs correspond to the initial stage of pluripotency in vivo, while EpiSCs are closer to its end. They considerably differ at many levels, the transcriptome, epigenome, chromatin organization, and even functionally. In spite of that, their pluripotency has been demonstrated in vitro and in vivo, by the

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generation of differentiated progeny belonging to the three primary lineages, mesoderm, endoderm, and ectoderm.

Although conditions to maintain the self-renewal of stem cells in vitro are well established, pluripotency remains a precarious balance, pointing out its inherent ephemeral nature and the natural inclination toward differentiation [1]. This is especially revealed by heterogeneity within cultures of both ESCs and EpiSCs. Here we will review available data on mouse EpiSC heterogeneity and will attempt to relate it with the embryonic origin of the cells and the signalling pathways at work in the EpiSCs and the embryo. We will also show that these signalling pathways have been used to manipulate culture conditions and create divergent cell lines with biased differentiation trends.

Origin, Characterization of EpiSC

Epiblast stem cells (EpiSCs) have been derived from the epiblast of postimplantation mouse embryos, from E5.5 to E7.5 (Fig. 2.1) [3, 4]. All stages of gastrulation are compatible with EpiSC derivation, up to somitogenesis, when the master

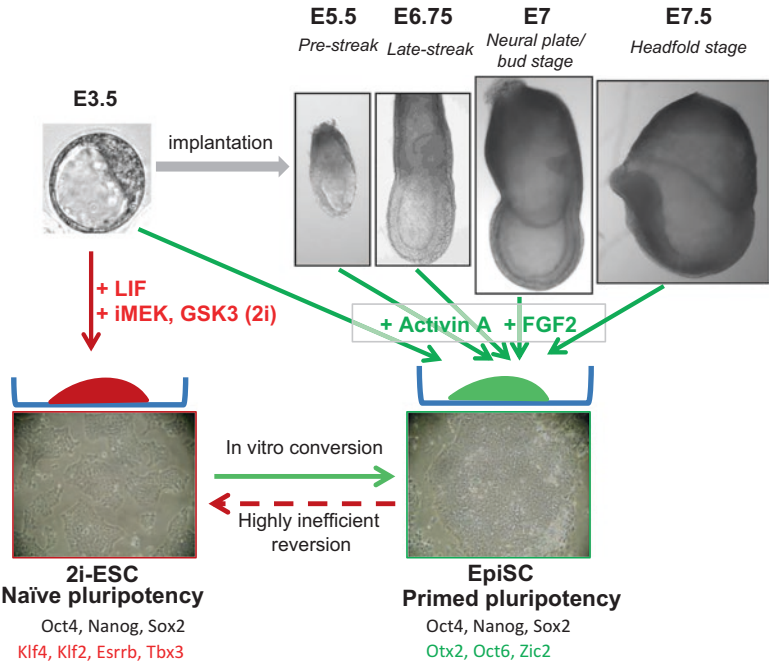

Fig. 2.1 Derivation of mouse pluripotent cells. Stages of embryos are from [2]. Growth factors and small molecules used for derivation are shown in red and green. Specific markers of naive and primed pluripotency are also indicated in red and green, respectively, at the bottom of the figure

pluripotency factor Pou5f1/Oct4 is no longer expressed [5]. Despite this large panel of embryonic stages at the time of derivation, the different EpiSC lines share a very similar transcriptome [6]. Culture conditions are instructive for the derivation of the EpiSCs and require the presence of FGF2 and activin. Activin is either added or produced by feeder cells when stimulated by FGF2 [7]. Using these conditions, EpiSCs have also been derived from preimplantation blastocysts [8] and can also be obtained in vitro directly from ESCs [9–11]. Importantly, the reverse is not true, and no ESCs can be derived from postimplantation epiblast [3]. EpiSCs correspond to a more advanced developmental stage, with a transcriptome resembling that of the late epiblast stage [6] and are hardly able to revert in vitro back to a naïve (ESC) state [12].

EpiSCs grow as flat colonies, resembling human ESCs, with which they also share the signalling pathways that support their self-renewal and some other molecular features, characterizing the primed state of pluripotency [4]. EpiSCs are distinct from ESCs at both molecular and functional level ([13]; and for a review: [14]). They do not express a panel of transcription factors that characterize in the mouse the naïve state of ESCs and early preimplantation epiblast, such as Klf2, Klf4, Klf5, Esrrb, and Tbx3, while still expressing the core pluripotency factors, Oct4, Nanog, and Sox2, albeit at a reduced level except for Oct4. Other transcription factors such as Otx2, Zic2 and Oct6 come into play to regulate gene expression in EpiSCs [15]. EpiSCs also express epiblast specific gene like Fgf5 and many lineage specific genes, priming them for differentiation. Although not able to form chimeras upon injection into blastocysts, as ESCs do, the pluripotency of EpiSCs is demonstrated by teratoma formation and importantly, by their ability to incorporate and differentiate into the three germ layers upon grafting in early gastrulating embryos [6, 16]. However, the ability of EpiSCs to generate the germ cell lineage has not yet been demonstrated in vivo and remains very inefficient in vitro [17].

Heterogeneity of EpiSC

Heterogenous Lineage Markers Expression

In ESCs, many transcription factors have been shown to fluctuate reversibly during cultures, among which, core pluripotency factors such as Nanog. In EpiSCs, fluctuations of core pluripotency factors have not been examined, but immunostaining or in situ hybridization has revealed some heterogeneity in the expression of lineage markers: markers of the primitive streak (Brachyury/T; [18, 19], of mesendoderm (Sox17, Foxa2; [20], of endoderm (Gata4; [18], and of neurectoderm (Sox1; [21].

Brachyury/T is a transcription factor expressed in cells ingressing the primitive streak. Using fluorescent reporters for T, it was shown that about 25–30% of EpiSCs are positive and most of these cells are also positive for OCT4 or NANOG [21, 22].

The equilibrium is restored following isolation of the positive and negative fraction, demonstrating that these cells are not fully committed. However, in the highest expressing fraction of T positive cells, pluripotency declines, as exemplified by the reduced ability to form colonies from single cells and to differentiate into embryoid bodies [23]. Therefore a minor (about 10%) part of the EpiSC population is already differentiating and has lost Oct4 [21]. They may also express more advanced mesendoderm markers as FOXA2 and SOX17 positive cells within EpiSC colonies are negative for OCT4, hence differentiating [20].

This heterogeneity for T expression does not only exist within EpiSCs colonies but also between cell lines. Bernemann and collaborators reported that different EpiSC lines derived independently but under the same culture conditions could display variable levels of T, with some having almost no expression [24]. Interestingly, the lines with no T expression display a higher ability to revert to an ESC-like state when submitted to naïve culture conditions, suggesting that they are in an earlier state of pluripotency (see below).

Sox1 is one of the earliest known marker of the neurectoderm [25]. The use of a reporter for SOX1 has shown that about 25% of EpiSC are positive but only half of this fraction is still expressing Oct4 and thus can convert to SOX1-negative cells. Hence, this population is probably already committed.

Fgf5 is expressed in the postimplantation epiblast and commonly used as a marker of the primed state. In fact, an FGF5 reporter revealed that a minor fraction (5%) of EpiSCs are negative but interconvertible with the positive fraction [26].

Significance of Heterogeneity: Biased Differentiation Ability?

The use of reporter for T or Sox1 allows sorting positive and negative populations, to investigate the status of the subpopulations at the molecular and functional level. Although being pluripotent, T positive EpiSCs have characteristics of primitive streak progenitors, as they express Mixl1, Sox17, and Foxa2 [21, 22]. At the same time, Sox1-positive EpiSCs down-regulate these primitive streak markers. These two contrasting populations show a bias trend during differentiation. After embryoid body differentiation, more cardiac progenitors (mesoderm lineage) are produced from T positive than from Sox1 positive cells. However, both populations are pluripotent as they are able to form neural as well as endoderm progenitors [21]. In another study, T sorted populations were submitted to unbiased differentiation in the presence of only serum, or with serum and BMP4, which promotes both mesoderm and surface ectoderm differentiation (Fig. 2.2). After only 2 days of differentiation, T positive cells only express mesoderm markers, while the negative cells display neurectoderm and epidermis markers [22]. In the presence of BMP4, cells will differentiate according to their initial level of T, toward mesoderm for T-high cells and epidermis for T-low/negative cells.

Early response of EpiSCs to differentiation cues has been examined and has revealed that subpopulations and/or lines upregulate Mixl1 earlier than others,

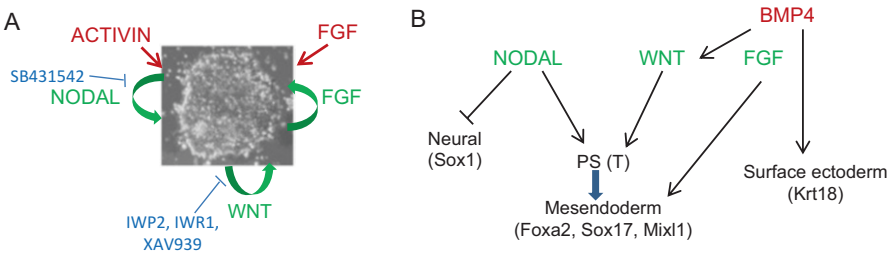

Fig. 2.2 Signalling pathways at work in: (**a**) EpiSCs; (**b**) the gastrulating embryo and their role in lineage specification. Growth factors provided externally (in the culture medium or by the extra-embryonic region (derived from trophectoderm)) are in red, and those produced by epiblast cells are in green

correlated with a higher propensity toward endoderm differentiation, while others upregulate Sox1 earlier and display neural-biased differentiation [6, 27].

Altogether these studies reveal a certain degree of heterogeneity within and between EpiSCs lines, with different subpopulations expressing concurrent lineage markers that are maintained in a dynamic equilibrium. At present, it seems that T expressing EpiSCs remains truly pluripotent, whereas cells displaying neural bias exemplified by Sox1 expression have lost the ability to revert. Single cell transcriptome analysis would help to understand the nature of this heterogeneity of EpiSCs.

Signalling Pathways in EpiSCs as the Main Source of Heterogeneity?

Different signalling pathways are active in EpiSCs, due to either autocrine/paracrine production or to addition of exogenous factors (Fig. 2.2a). They all play roles in maintaining self-renewal but also promote differentiation toward different lineages. Manipulating these signalling pathways permitted the characterization of EpiSC lines with specific properties.

Activin/Nodal (TGFβ) Pathway

Activin and nodal belong to the TGFβ superfamily of growth factors. Activin is added exogenously in the culture medium or, when feeders are used, secreted by them when stimulated by FGF2 [7]. In addition, nodal is also secreted directly by EpiSCs themselves [28]. Activin/Nodal pathway has been shown to promote Nanog expression [29], which accounts for its ability to sustain pluripotency in EpiSCs. Indeed, it allows the maintenance of pluripotency and self-renewal in EpiSCs, as shown by the adverse effect of SB431542, an inhibitor of TGFβ receptors [3]. When

SB is added to EpiSCs in culture, they rapidly differentiate. In the embryo, nodal promotes primitive streak and mesendoderm formation and restrains neural induction (Fig. 2.2b) [25, 30].

FGF Signalling Pathway

Through the activation of the downstream effector ERK, FGF activity is a major driver of the transition from naïve to primed pluripotency [31]. However, the role of FGF in the maintenance of primed epiblast cells is not clear [32]. It is not essential for self-renewal but increases survival at passaging and, together with Nodal, inhibits neural differentiation [28]. FGF signalling is also autocrine as EpiSCs produce different FGF, such as FGF5, FGF4, and FGF8. Among these, FGF8 has an important role in the embryo for axis specification and mesoderm development [33].

WNT Signalling

WNT pathway is endogenously activated in EpiSCs. In the embryo, together with Nodal, it promotes the formation of primitive streak/mesoderm (Fig. 2.2b) [34, 35]. Primitive streak progenitors are under the dependence of WNT signalling and upon treatment of EpiSCs with the WNT signalling inhibitors IWP2, IWR1, or XAV939, the expression of primitive streak markers such as T, Foxa2, as well as definitive endoderm markers are strongly downregulated [19, 21, 23, 28, 36].

Spatial Heterogeneity and Patterning of the Epiblast In Vivo

Hence, the different signalling pathways that are active in EpiSCs, either exogenously added or endogenously produced, have differentiation promoting or inhibiting actions while sustaining self-renewal and proliferation. This somehow reflects the in embryo situation, where these different signalling molecules, together with BMP4, participate to the patterning of the embryo and the generation of lineages during gastrulation (Figs. 2.2b and 2.3a) and see Arnold and Robertson [38] for a review. Differentiation toward neural lineage is considered as a default pathway both in the epiblast and in human ESCs, when Nodal is absent or inhibited [25, 39]. On the other hand, mesoderm and endoderm lineages are induced by WNT and Nodal pathways. BMP4 stimulates two concurrent lineages, epidermis or mesoderm, depending on the status of the cells and the influence of other signalling pathways.

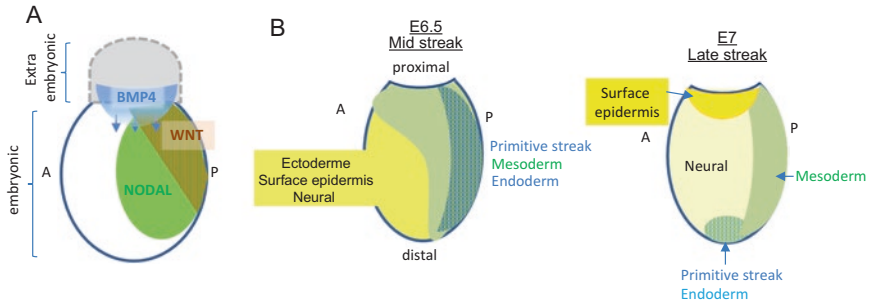

Fig. 2.3 (**a**) Signalling molecules that pattern the epiblast at gastrulation. (**b**) Fate map of the epiblast at two stages of gastrulation; adapted from [37]. A = anterior, *P* = posterior. Proximal and distal are relative to the extra-embryonic region

In the embryo, regionalization of transcription factor expression and gradients of signalling molecules that take place at the onset of gastrulation are crucial for the patterning of the epiblast. Moreover, spatial transcriptome of the mouse epiblast at mid-gastrulation (E7) has allowed defining groups of genes specific to four regions (anterior, posterior, distal, proximal) [40]. Previous studies established the fate maps of these regions by performing clonal analysis and heterotopic grafting (Fig. 2.3b) [37, 41]. These experiments, as well as ectopic transfer under testis capsule, also revealed that epiblast cells from all regions are mostly pluripotent [42]. This has also been confirmed more recently by the equivalent ability of the anterior and posterior epiblast to give rise to EpiSCs after explantation in vitro [5]. As gastrulation proceeds, loss of potency occurs when cells enter the primitive streak ([37] for a review). However, clonal analysis showed that the anterior third of the primitive streak contained a population of cells with stem cell properties, as their descendants both remain in the streak and give rise to the new germ layers [41]. Interestingly, close inspection of the transcriptome of EpiSCs reveals that EpiSCs display similarities with the anterior primitive streak of the late epiblast (neural plate/bud stage [2]), whatever the initial stage of the embryo, E5.5 to E7.5 [6].

Manipulating Signalling Pathways to Generate EpiSCs with Different Properties

Although EpiSCs have demonstrated their pluripotency, the activity of WNT and nodal/activin signalling pathways promotes constant drifting of the cultures toward mesendodermal lineages and progression toward late epiblast fates. Hence, different groups have tried to manipulate signalling pathways to reduce spontaneous differentiation or to reorientate this commitment toward other lineages.

Wnt Signalling

New EpiSC lines have then been derived in the continuous presence of WNT signalling inhibitors such as IWP2 [18, 23] or IWR1 [36]. In this condition, the efficiency of EpiSC derivation is increased, and the cell lines are less prone to differentiation. Gene expression profiling shows the expected downregulation of primitive streak markers in these treated EpiSCs. In one study comparison with the transcriptome of epiblast at different stages suggests that the treatment drives EpiSCs toward an earlier, pre-gastrulating epiblast [23]. Moreover, the ability to form colonies from single cells is increased, as well as the efficiency of reversion back to a naïve state [23, 36]. These lines are pluripotent, according to teratoma formation. Interestingly, human ESCs cultured in the presence of IWR1 inhibitor also display enhanced cloning efficiency and less spontaneous differentiation [36]. This is in agreement with the finding that WNT signalling is indeed active in human ESCs and drives mesendoderm differentiation [43].

Strikingly one study reports the colonization of blastocysts and the formation of chimeras using EpiSCs derived in the continuous presence of IWP2. Such chimera formation was not tested for the other cell lines derived with the same inhibitor [18] or IWR1 [36].

Nodal Signalling

Neuroectoderm fate emerges from the anterior epiblast of the embryo, where no Nodal signalling is present (Fig. 2.3a) [44]. Liu and colleagues tested whether EpiSCs could be maintained in the presence of the Nodal signalling inhibitor SB431542 [45]. They could stably maintain cultures only if FGF2 was added (=EpiSCs$^{S/F}$). Although the transcriptome remains globally similar to that of regular EpiSC, and distinct from neural progenitor cells, EpiSCs$^{S/F}$ are enriched for transcription factors involved in neural and surface ectoderm such as Sox1 and Krt18, respectively, and both Nanog and Oct4 expression are reduced. Accordingly, their global profile is closer to the anterior proximal epiblast, fated to become ectoderm (Fig. 2.3b), than to the anterior distal site (or anterior primitive streak), as regular EpiSCs do. During differentiation of EpiSCs$^{S/F}$, BMP4 strongly induces ectodermal markers, at the expense of mesoderm ones (as for T negative population of regular EpiSC [22]. Hence these new cell lines now display a differentiation propensity toward ectoderm, and their pluripotency may be compromised.

Conclusion

EpiSCs are maintained in vitro in a precarious balance with continuous trend toward mesoderm/endoderm differentiation. When derived and cultured with WNT inhibitor, cultures are described as more stable, hence easier to maintain along passages. However, although these treated cells are slightly less advanced than regular EpiSCs, they are still comparable with the epiblast at the late stages of gastrulation. Such a late epiblast identity can be considered as their main drawback, as it can explain their apparent inability to generate germ cell linage. Indeed, primordial germ cells are specified in the posterior proximal epiblast at early stage of gastrulation, before migrating in the extraembryonic region [46, 47].

Therefore, it could be desirable to obtain in culture stem cells that would correspond to an earlier epiblast state, before any lineage priming, and responsive to any lineage cues, including germ cell lineage. Such state has been recently coined as the formative state by Austin Smith [48]. It would be equivalent to the epiblast at E5, when patterning has not yet started and which transcriptome shows less variability than at E6.5 [49, 50]. In vitro, formative state should be close to the epiblast-like cells (EpiLCs) that are obtained after transfer of ESCs in a medium containing activin and FGF2 for 2 days [47]. However this EpiLC state is in vitro just a transient one and cannot be stabilized. When kept in culture, cells continue their evolution toward EpiSC state. Recently EpiLCs and EpiSCs were subjected to micropatterned differentiation, which somehow mimics the epiblast patterning during gastrulation [51]. Interestingly, EpiLCs display an organized pattern and form both anterior and posterior derivatives of the primitive streak upon addition of activin, FGF, WNT, and BMP4. By contrast EpiSCs only generate definitive endoderm without any organized patterning [52]. This illustrates that the late identity of EpiSCS interferes with their ability to respond to environmental cues in an unbiased manner and that they have lost the plasticity of earlier epiblast cells. Using the same controlled system, it would be interesting to challenge the response of the alternative EpiSCs treated with either Nodal inhibitor or WNT inhibitor. Moreover, propagating the formative state in culture may be challenging, at least in terms of epigenetic stabilization. Indeed the epiblast at E5 is in the process of major epigenetic reorganization, including DNA de novo methylation and genome-wide rearrangement of H3K27me3 and H3K9me3 [53–55]. Hence, such cells may be in a paradoxical state, having a quite homogenous transcriptome but a very heterogenous epigenome. Indeed, a recent study on single-cell methylome revealed the existence of oscillations of DNA methylation that are maximal within E5.5 epiblast cells [56]. Hence, the use of epigenetic drugs, together with manipulation of signalling pathways, may help to stabilize primed pluripotent cells in an earlier epiblast state.

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Chapter 3
Progenitor Cell Heterogeneity in the Adult Carotid Body Germinal Niche

Verónica Sobrino, Valentina Annese, and Ricardo Pardal

Abstract Somatic stem cells confer plasticity to adult tissues, permitting their maintenance, repair and adaptation to a changing environment. Adult germinal niches supporting somatic stem cells have been thoroughly characterized throughout the organism, including in central and peripheral nervous systems. Stem cells do not reside alone within their niches, but they are rather accompanied by multiple progenitor cells that not only contribute to the progression of stem cell lineage but also regulate their behavior. Understanding the mechanisms underlying these interactions within the niche is crucial to comprehend associated pathologies and to use stem cells in cell therapy. We have described a stunning germinal niche in the adult peripheral nervous system: the carotid body. This is a chemoreceptor organ with a crucial function during physiological adaptation to hypoxia. We have shown the presence of multipotent stem cells within this niche, escorted by multiple restricted progenitor cell types that contribute to niche physiology and hence organismal adaptation to the lack of oxygen. Herein, we discuss new and existing data about the nature of all these stem and progenitor cell types present in the carotid body germinal niche, discussing their role in physiology and their clinical relevance for the treatment of diverse pathologies.

Keywords Tissue-specific or somatic stem cells · Germinal niche · Neurogenesis · Angiogenesis · Dopaminergic neuronal cells · Vascular cells · Multipotency · Self-renewal · Proliferation · Differentiation · Carotid body physiology · Peripheral sympathetic nervous system · Neuroblasts · Mesectoderm-restricted progenitors · Neural crest

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Introduction

Somatic stem cells enable functional and structural plasticity of adult tissues. These cells are responsible for tissue repair and maintenance, and in some cases, like in the central nervous system (CNS), they contribute to functional plasticity and adaptability to a changing environment. Neural stem cells within the brain give rise to new neurons and glia throughout life [1], to contribute to seminal functions such as learning and memory [2]. Since their discovery [3], research on neural stem cells has created a dynamic and expanding field that has profoundly inspired neuroscience and regenerative medicine against neurological disorders. Adult brain stem cells have been shown to self-renew and to exploit multipotency by differentiating into glial and neuronal cells [3]. Understanding proliferation and differentiation mechanisms employed by neural stem cells to achieve their functions is critical for our capacity to use these cells in our own benefit.

Adult stem cells reside in specific niches where they are subjected to fine regulation of their behavior [4]. These germinal niches have been shown to include both cellular and noncellular elements, and their integrity and complexity ensure a correct functioning of stem cells and avoid the appearance of potential hyperproliferation and conversion into cancer stem cells [5]. A deep comprehension of germinal niches is also fundamental to understand stem cell biology and behavior and to be able to use these cells in our fight against disease.

Stem cells within germinal niches are not alone, but rather they are accompanied by multiple cell types that participate in the regulation of niche functioning. Most of these escorting cell types are actually derived from the stem cells themselves. They are proliferative progenitors, some of them multipotent and some others more restricted to specific cell lineages, and they contribute to niche functioning both by being part of cell lineage progression and by regulating stem cell behavior. This heterogeneity of progenitor cell types is observed in every germinal niche that is thoroughly characterized in the adult nervous system. For instance, in the subventricular zone (SVZ) of the adult forebrain, multipotent astrocyte-like quiescent stem cells or type B cells give rise to proliferative intermediate progenitors or type C cells, which eventually progress through specification to generate neuroblasts or type A cells [6]. At the end, neuroblasts migrate through the dorsal migratory stream toward the olfactory bulb to generate new interneurons that participate in olfaction plasticity [7]. All these intermediate cells have been shown to modulate stem cell activity and neurogenesis through soluble molecules, constituting a critical component of the niche functioning [8]. Therefore, adult germinal niche functioning relies on the participation of a heterogeneous group of stem and progenitor cells, which manage to survey the environment to be able to respond to maintenance, injury, or physiological adaptation requirements.

Adult germinal niches have also been described in the peripheral nervous system (PNS), where multipotent neural crest-derived stem cells (NCSCs) are able to persist into adulthood to contribute to tissue maintenance, repair, and adaptation. Not as exhaustively characterized as their counterparts in the CNS, adult multipotent

NCSCs have been described to reside in the enteric ganglia [9] and in some other target tissues of NCSC migration, such as the bone marrow [10], cornea [11], heart [12], or skin [13]. Little is known in general about the functioning and regulation of these stem cell niches in the adult PNS, including the characterization of diverse stem and progenitor cell types potentially present.

We have recently described the existence of an enticing germinal niche within a specific area of the PNS, the adult carotid bodies (CB). These are chemoreceptor organs that play a crucial role in the detection of changes in chemical variables in the blood, informing the CNS to enable the triggering of counter-regulatory responses [14]. The most important parameter change perceived by the carotid body is a decrease in blood PO2. In the case of acute hypoxemia, the carotid body immediately increases the firing rate toward the CNS, so that respiratory centers in the brain stem can trigger hyperventilation through a sympathetic discharge [15]. If the stimulus is prolonged, like in high-altitude dwellers, the carotid bodies are crucial facilitating organismal acclimation to the decrease in environmental oxygen [14, 16]. This well-described physiological adaptation response relies on an increase of carotid body size, including a rise in the number of chemoreceptor neuronal cells [17, 18]. The new neurons will permit a continuous stimulation of the respiratory centers in the CNS and hence the maintenance of the respiratory drive during hypoxia. This cellular plasticity, remarkable for an adult neural tissue, was poorly understood until our characterization of the cellular events taking place in the hypoxic organ [19]. We have shown that the carotid body constitutes a captivating germinal niche in the adult PNS, containing a heterogeneous population of stem and progenitor cells that support physiological adaptation of the organ. Herein, we present and review recent data deciphering carotid body cellular heterogeneity, providing markers, and presenting molecular mechanisms involved in carotid body niche functioning. We will also comment on the potential tools our work might offer to control carotid body functioning and the clinical relevance of these tools for the treatment of a growing number of disease conditions.

CB Multipotent Stem Cells and Physiological Adaptation to Hypoxia

The CB is a paired organ situated at the bifurcation of the carotid artery and constitutes a specialized ganglion of the sympathetic nervous system. This organ is formed after migration of neural crest-derived progenitors from the adjacent superior cervical ganglion and is innervated by afferent sensory nerve fibers joining the glossopharyngeal nerve [20]. The CB is also composed by a profuse network of blood vessels necessary for its chemoreceptor activity [21, 22]. The neural parenchyma in this organ is organized in glomeruli of chemoreceptor type I cells (also termed as glomus cells). These are specialized neuronal cells able to detect different chemical stimuli arriving in the blood, such as hypoxia, hypercapnia, acidosis, or

hypoglycemia, and to communicate the situation to sensory nerves through chemical synapsis [23, 24]. The molecular mechanism by which these cells detect the main stimulus, hypoxia, is only recently being thoroughly elucidated [25, 26]. Neuronal glomeruli are surrounded by sustentacular type II cells. These cells express the glial fibrillary acidic protein (GFAP) and were thought to serve as glial cells supporting function and maintenance of neuronal cells [27]. However, we showed a decade ago that these cells function as multipotent neural crest-derived stem cells, being able to activate proliferation in hypoxia and to differentiate into new neuronal cells that will contribute to physiological adaptation of the tissue to the hypoxemic situation [28]. We also showed that these cells do not respond to hypoxia themselves but need communication of the hypoxic stimulus from neuronal cells [29], similar to other nervous system germinal niches where stem cells are also regulated by neuronal activity [30]. Moreover, we exhibited the capacity of CB type II cells to function as multipotent neural stem cells in vitro, being able to grow as spherical colonies, called neurospheres, that contained both differentiated and undifferentiated cells [28].

Two crucial cellular processes are executed in the organ under chronic hypoxia as part of the adaptation program. One is the already mentioned neurogenesis, or production of new glomus cells, to permit physiological adaptation by increasing firing rate to CNS and maintaining respiratory drive. The other process is a profound angiogenesis, or production of new blood vessels in hypoxia, to optimize vascularization of the growing neural parenchyma [31]. We have shown by cell fate mapping that GFAP+ multipotent stem cells participate in both neurogenesis [28] and angiogenesis [32, 33]. Furthermore, by using neurosphere-forming assays in vitro, we have shown that CB stem cells (CBSCs) have the multipotent capacity to differentiate into neuronal cells [28] and vascular cells, including smooth muscle, pericytes, and even endothelial cells [32]. To the best of our knowledge, our data exposes the only example described of an adult population of PNS stem cells able to convert into both neuronal and mesectodermal derivatives in response to physiological stimuli.

In a normoxic-resting situation, GFAP+ CBSCs present cellular protrusions surrounding neuronal cells, a quiescent conformation that optimizes the detection of stimuli coming from glomus cells [29]. However, once activated by hypoxia, these stem cells change their phenotype, switch their filament protein expression from GFAP to nestin, become rounded, and start proliferating [28] (Fig. 3.1a). These

Fig. 3.1 (continued) micrograph showing an example of a GFAP/nestin double-positive cell. EC: endothelial cell. Scale bars: 2 μm and 200 nm in inset (3). (**d**) Transwell migration assay with CB progenitors. Neurosphere-dissociated cells are plated on one side of the porous filter and photographed on the other side after 72 h and crystal violet staining. EPO: erythropoietin at 7 IU/mL. PD: 50 μM of PD98059 (Sigma), inhibitor of EPO signaling. Ab: EPO-neutralizing antibody (1:20; Santa Cruz). (**e**) Migratory index measured in the different conditions shown in (**d**). (**f**) Time-lapse microscopy measurements of nestin+ progenitor cell movement in flat substrate, without (left) or with (right) EPO at the indicated concentration in the culture medium. Trajectories are delineated with ImageJ software. (**g**) Quantification of cumulated distance of the cellular movement shown in (f), but in the indicated conditions. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student's *t*-test

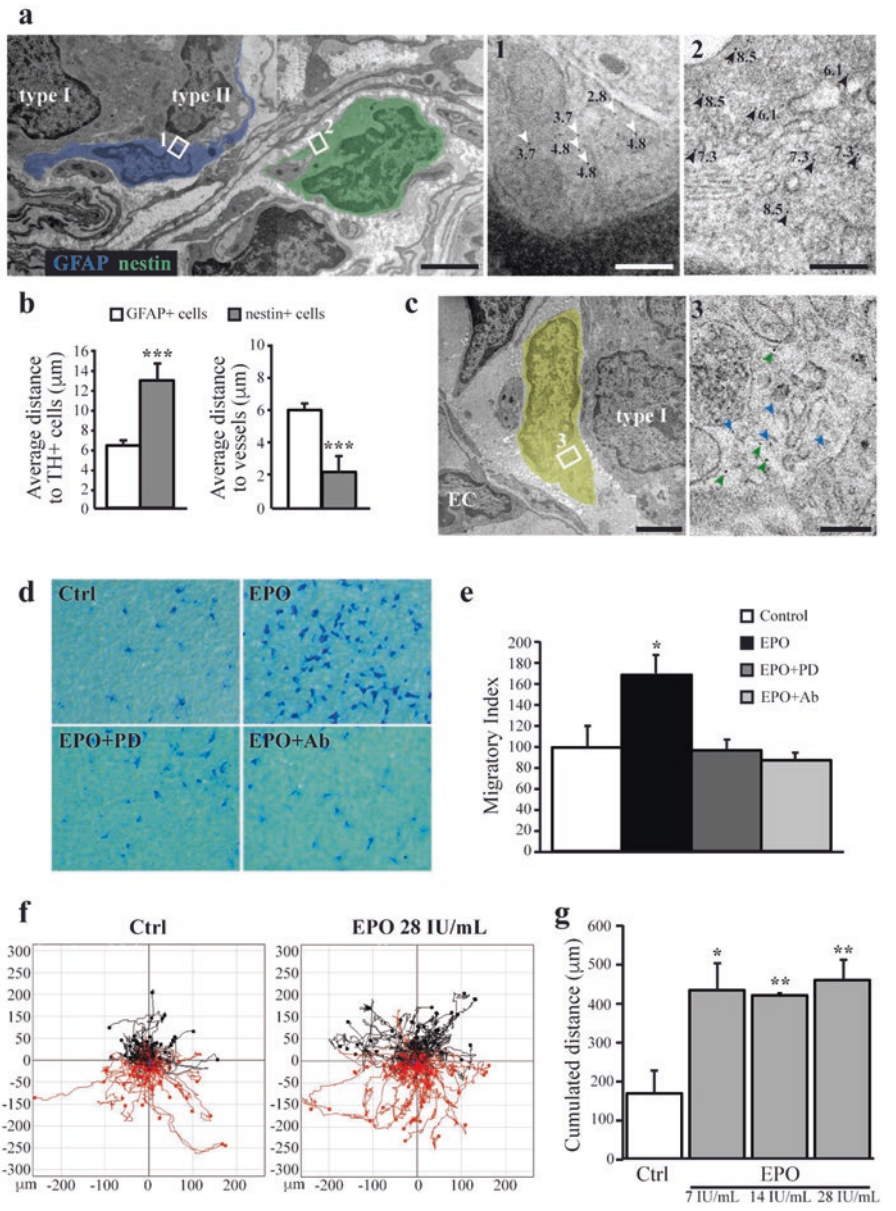

Fig. 3.1 Migratory movements of nestin+ progenitors within the CB parenchyma. (**a**) Composite of two electron micrographs displaying the detection of GFAP+ (blue pseudo-colored) and nestin+ (green pseudo-colored) cells, using gold particle-associated antibodies, within the parenchyma of a normoxic rat CB. Different developmental times gave rise to different gold particle sizes for both stainings (panels **1** and **2**). Scale bars: 2 µm in (**a**) and 200 nm in (a1 and a2). (**b**) Quantification of the distance between GFAP+ or nestin+ cells and the closest TH+ cell (left graph) or the closest endothelial cell (right graph), using confocal microscopy (pictures not shown). (**c**) Electron

nestin+ proliferative stem cells give rise to specified progenitor cells that differentiate into one of the two cellular lineages, neuronal or mesectodermal, to participate either in neurogenesis or in angiogenesis, respectively. Our data suggest that a fair amount of these nestin+ cells remain multipotent and are able to go back to the quiescent GFAP+ phenotype upon stimulus cessation [28], which probably minimizes stem cell depletion.

An interesting aspect of the behavior of CB nestin+ proliferative progenitors is their migration capability. By combining electron microscopy with immunodetection of GFAP and nestin, using gold particle-associated antibodies, we can study the shape and position of these cells within the CB parenchyma (Fig. 3.1). We have used different developmental times during silver enhancement procedure, in order to obtain a different gold particle diameter for the detection of GFAP or nestin with ultrasmall gold particle-conjugated antibodies (4.67 ± 0.45 nm for GFAP, $n = 18$ particles; 8.3 ± 1.6 nm for nestin, $n = 37$ particles), allowing us to perform both labeling procedures at the same time (Fig. 3.1a). The position of nestin+ progenitors, compared to GFAP+ cells, is clearly more detached from neuronal glomeruli and closer to blood vessels (see quantification shown in Fig. 3.1b), which suggests a movement process. We even find some cells in transition, positive for both GFAP and nestin, getting detached from glomus cells (Fig. 3.1c). Migration might be part of the specification and differentiation process from CBSCs. In fact, cytokines inducing differentiation into mesectodermal lineage, such as erythropoietin (EPO) (see below), are also potent activators of migration, as evidenced by migration assays performed with nestin+ progenitor cells (Fig. 3.1d–g). We also have preliminary evidence (data not shown) that new neuronal glomeruli are preferentially formed at the periphery of the organ. All data together suggest that both neurogenesis and angiogenesis processes involve progenitor cell migration.

In summary, GFAP+ and nestin+ stem cells are the quiescent and proliferative versions, respectively, of CB multipotent progenitors (CBSCs). They are interchangeable depending on the presence of the hypoxic stimulus and eventually give rise in hypoxia to more specified progenitor cells that complete differentiation into either neuronal or mesectodermal cell lineages. This behavior is typically observed in CB neurospheres, which are constituted by a central core full of nestin+ progenitors and mesectodermal cells, and budding blebs in the surface, full of TH+ glomus cells [28, 33], resembling the activated situation of the CB niche. Interestingly, in addition to multipotent cells, we have discovered specified progenitors from both lineages, with quiescent phenotype, within the normoxic CB parenchyma in vivo. The existence of these restricted progenitors might confer a clear evolutionary advantage to this niche since these cells are able to convert into differentiated cells under hypoxia much faster than multipotent stem cells. We have found specific markers for these restricted progenitors and have studied their biology and their overlapping marker expression with multipotent cells. Herein, we now expose the main characteristics of restricted progenitors from both cell lineages and discuss the physiological and clinical relevance of their existence within the CB.

Neuronal-Committed Progenitors Within the CB Parenchyma

During the past century, different morphometric studies distinguished two classes of glomus cells in the CB, classically termed type A and type B glomus cells. This classification was made attending to ultrastructural parameters, such as the diameter of exocytotic vesicles, the number of mitochondria, or the size of the nucleus, among others [34]. Type A glomus cells displayed more abundant dense-core vesicles and with higher diameter. These cells were frequently in contact with nerve terminals and sinusoidal capillaries, exhibiting the expected aspect of chemoreceptor glomus cells. The proportion between both types of cells seemed to be even, but type B cells were usually in the periphery of type A cell glomeruli and with cytoplasmic extensions surrounding type A cells [35]. We have recently shown that type B glomus cells seem to be immature neuroblast-like cells, ready to convert into fully mature glomus cells (type A) in response to the hypoxic stimulus [36].

CB neuroblasts (CBNBs), or type B glomus cells, share multiple features with mature glomus cells, or type A glomus cells, such as the expression of dopaminergic markers like TH or dopamine decarboxylase (DDC). However, they also display proper characteristics of immature cells [36], such as expression of immature cell markers typical of sympathoadrenal progenitor cells, like HNK-1 [37] or the transcription factor Ascl1 [21], and expression of neuroblast markers like Tuj1 or Ncam2 [38, 39]. Moreover, we have shown that CBNBs do not yet contain a mature hypoxia-responsive machinery, since they are not able to respond to acute exposures to hypoxia in the way mature glomus cells do, despite their membrane expression of ion channels and their responsiveness to other chemical stimuli [36]. We have also shown that CBNBs are smaller in size than mature glomus cells, they have less mitochondria and vesicles and smaller vesicle size, and their position in the glomeruli is peripheral [36]. All these ultrastructural characteristics are in consonance with the classical morphometric studies performed in type B glomus cells [34, 35].

The expression of HNK-1 by CBNBs is particularly interesting, since this cell surface marker allows the prospective isolation of these cells by flow cytometry [28, 36]. HNK-1, also known as CD57, constitutes a surface glycoepitope that is usually attached to a glycoprotein related to cell adhesion, such as NCAM, tenascin, or laminin [37]. HNK-1 is typically expressed by migrating neural crest cells during development, where it has been shown to have a role in migration and cell adhesion to the substrate [40, 41]. Moreover, HNK-1 has also been involved in synaptic plasticity in the CNS [42]. Although the expression of this antigen can vary among species, it has been demonstrated that HNK-1 is expressed in the neural crest of birds, rats, dogs, pigs, and humans, among others, but curiously not in mice [41, 43–45].

An interesting question that arose when characterizing CBNBs was their cellular origin. Numerous studies of carotid body development performed in avian and rat models [46, 47] demonstrated that glomus cell precursors arriving to the CB from the superior cervical ganglion express Tuj1, PGP9.5, NPY, TH, and HNK-1, together with required expression of transcription factors like Ascl1 [20, 21, 48]. We have shown that most of these markers, enzymes, and transcription factors are highly

expressed in the adult CB neuroblast population [36]. These similarities between fetal CB neuronal precursors and adult neuroblasts led us to hypothesize that these adult neuroblastic cells might be descendants of those fetal neuronal precursors. However, our in vitro studies have demonstrated the existence of neuroblasts within CB stem cell-derived neurospheres (see response to referees in [36]), meaning that CB multipotent stem cells might have the capacity to replenish neuroblasts within the adult tissue. The lack of expression of HNK-1 in the mouse CB has so far impeded a formal cell fate mapping study in these neuroblastic cells to solve the question of their cellular origin. Nevertheless, we cannot formally discard the possibility of a mixed origin for adult CB neuroblasts, both from multipotent stem cells and directly from fetal glomus cell precursors.

Another interesting aspect of CBNBs, in fact related to their cellular origin, is the overlapping expression of markers with other undifferentiated cells. We have performed immunohistochemical studies to show the lack of co-localization of HNK-1 with GFAP (Fig. 3.2a), suggesting that CB type II cells do not express the membrane glycoepitope. However, we found partial co-localization between nestin and HNK-1 (Fig. 3.2b), indicating an overlapping between the expressions of the intermediate progenitor marker and the neuroblast marker. We have preliminarily analyzed this overlap by flow cytometry in the normoxic adult CB (Fig. 3.2c) and have exposed that about half of HNK-1+/TH+ neuroblasts seem to express nestin. This result might denote the existence of two subtypes of neuroblasts, with different grades of maturation. In any case, these flow cytometry data suggest that neuroblasts are likely derived from nestin+ intermediate progenitors (nestin+/HNK-1-/TH- cells in the plot of Fig. 3.2c), which are themselves the proliferative version of multipotent stem cells (see above), hence confirming neuroblasts as part of the CBSC lineage.

Finally, we have also studied the neurosphere-forming capacity of CB neuroblasts, compared to other populations of undifferentiated cells in the organ (Fig. 3.2d–h). CB bulk cells were sorted by flow cytometry into three different groups (Fig. 3.2d) and plated to form neurospheres: HNK-1 highly positive cells (HNK H group; mainly composed of neuroblasts), cells expressing low levels of HNK-1 (HNK L group; with a mix of neuroblasts and nestin+/HNK-1+ progenitor cells (Fig. 3.2c)), and HNK-1-negative cells (negative group). Neurospheres obtained from the negative population were bigger in size (Fig. 3.2e, f) and contained almost no differentiation into TH+ glomus cells (Fig. 3.2g, h). These neurospheres resemble those obtained from mesectodermal-restricted progenitors (see below) [33]. On the other hand, neuroblasts (HNK H group) gave rise to smaller neurospheres (Fig. 3.2e, f) composed by almost only TH+ cells (Fig. 3.2g, h), con-

Fig. 3.2 (continued) populations (high, low, and negative), regarding expression of HNK-1, were sorted to form neurospheres. (**e**) Bright-field pictures of neurospheres obtained from the cell populations shown in (**d**). Scale bar: 200 μm. (**f**) Quantification of diameters of the neurospheres shown in (**e**). (**g**) Examples of neurospheres stained with nestin (green) and TH (red), from the three groups shown in (**d**) and (**e**). Scale bar: 100 μm. (**h**) Quantification of dopaminergic differentiation present in neurosphere sections from the study shown in (**d**–**g**). Cell nuclei were counterstained with DAPI. *$p < 0.05$, ***$p < 0.001$, Student's *t*-test

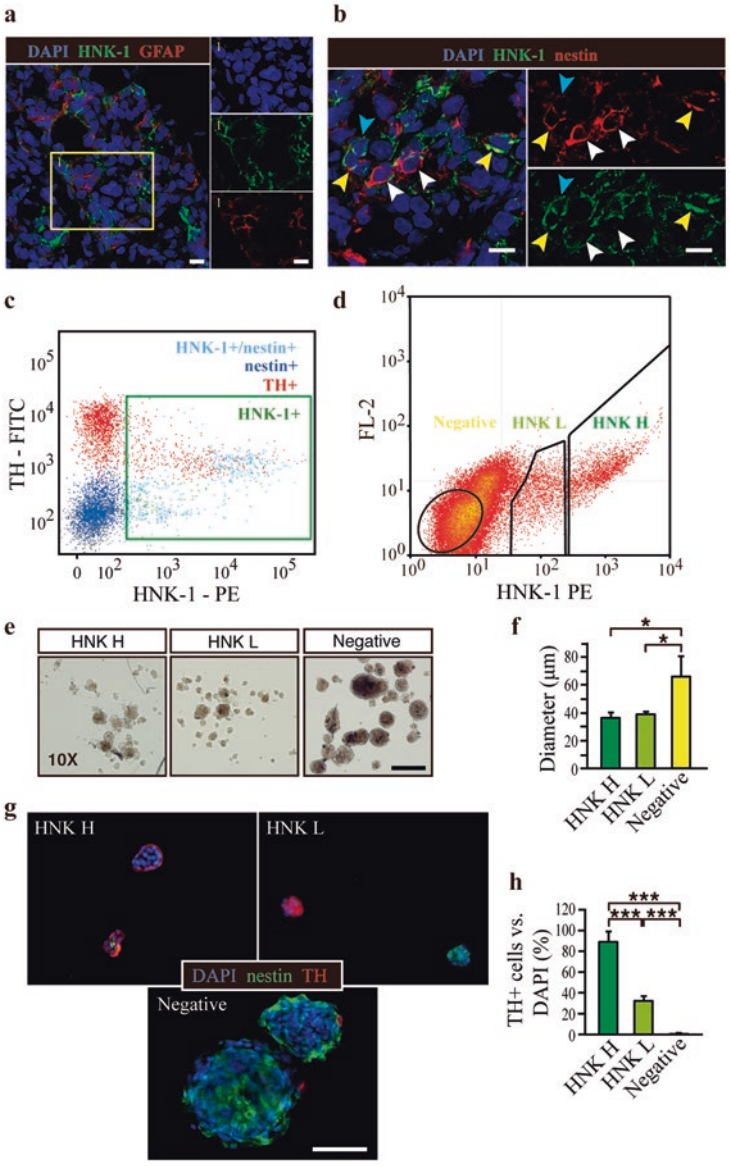

Fig. 3.2 Marker expression in CB neuroblasts. (**a**) Immunohistological detection of HNK-1 (green) and GFAP (red) in a section of a normoxic rat CB. The inset (1) further shows the lack of co-localization. Scale bars: 10 µm. (**b**) Immunostaining for HNK-1 (green) and nestin (red) in a normoxic rat CB slice showing HNK-1+ cells (blue arrowheads), nestin+ cells (white arrowheads), and HNK-1/nestin double-positive cells (yellow arrowheads). Scale bar: 10 µm. (**c**) Flow cytometry plot showing CB bulk cells stained with antibodies against HNK-1, nestin, and TH. Nestin+ cells have been previously gated in blue in order to be visible in the TH vs. HNK-1 plot. (**d**) Cytometric plot showing the staining of CB bulk cells with anti-HNK-1 antibodies. Three different

firming their neurosphere-forming capacity and their high neuronal specification. Neurospheres from HNK L group displayed a small average size and a mix of different capacities for dopaminergic differentiation (Fig. 3.2e–h), corroborating the mix of undifferentiated cells present in this population.

CB Neuroblast Proliferation in Response to Hypoxia

In normoxic-resting conditions, neuroblasts remain quiescent within the CB neural parenchyma. However, the hypoxic stimulus provokes these cells to enter the cell cycle, executing one or two cell divisions and rapidly maturing into glomus cells [36]. This recent description of CBNB proliferation in response to hypoxia contributes to the understanding of CB growth-mediated adaptation to chronic hypoxemia [17, 18]. But it also helps to solve a classical debate about the observation of cell cycle protein expression in CB TH+ dopaminergic cells [31, 49]. We have shown by time-lapse microscopy that mature glomus cells are postmitotic and that neuroblasts are able to divide once or twice rapidly under the hypoxic stimulus [36]. Moreover, we have observed in vivo that neuroblast proliferation takes much less time (3–4 days) than the stem cell production of new glomus cells, which takes 7–10 days to be completed [28, 36]. Hence, the presence of quiescent immature neuronal cells within the CB neural parenchyma may have evolved to permit a faster neurogenesis and hence speedier adaptation to the hypoxic environment. Interestingly, the fact that we find some neuroblasts still expressing nestin, and that we find neuroblasts that divide only once while others divide twice, might indicate the existence of different grades of specification among neuroblasts, which could somehow increase duration and efficiency of this fast neurogenesis.

An interesting question that arose during our studies of CBNB proliferation was if these neuroblastic cells were directly sensitive to hypoxia or they rather need niche signaling to respond to the hypoxic stimulus, similar to the case of multipotent stem cells in the organ [29]. Our in vitro time-lapse microscopy experiments have demonstrated that the hypoxic stimulus per se is sufficient to trigger neuroblast proliferation [36]. Cellular responses to chronic hypoxia depend on the regulation of hypoxia-inducible factors (HIFs) by prolyl-hydroxylases (PHDs). PHDs are oxygen-sensitive enzymes responsible for tagging HIFs for degradation in normoxia [50]. During the lack of O_2, the consequent inhibition of PHDs stabilizes HIFs, which in turn activate the expression of HIF-dependent adaptation genes. We have observed a clear expression of HIF2α in CB neuroblasts, especially increased during hypoxia [36]. In fact, recent works have clearly established a prominent role for HIF2α in CB glomus cell growth and survival during development [51, 52]. Moreover, a new study has shown that inactivation of HIF2α leads to a decrease in hypoxia ventilatory response in adult mice and to the absence of TH+ cell proliferation in the CB, while inactivation of PHD2 has the opposite effect, leading to CB hyperplasia even in normoxia [53]. Although further experiments are necessary, all these data suggest that CB neuroblasts are able to respond directly to hypoxia, by

increasing proliferation in a HIF2α-dependent manner. Nevertheless, we cannot discard the existence of supplementary mechanisms, involving niche signaling, that might modulate the division of CB neuroblasts. Interestingly, all these works also confirm the presence of neuroblasts in the mouse CB, despite the lack of HNK-1 expression.

CB Neuroblast Maturation in Response to Hypoxia

As mentioned above, CBNBs are immature cells unable to respond to an acute exposure to hypoxia, despite the presence of exocytotic vesicles and the expression of membrane ion channels. These cells are not ready to function as chemoreceptor cells in the organ to translate the hypoxic stimulus [36]. Mature glomus cells increase intracellular calcium and mitochondrial production of NADPH in response to acute hypoxia [25, 26], and those responses are basically absent in HNK-1+ neuroblasts [36]. CBNBs do respond, by rising intracellular calcium, to other chemical stimuli, such as high potassium, hypoglycemia, or neuromodulators like ATP or acetylcholine (ACh), meaning that these cells face the specific lack of the hypoxia-responsive machinery. Interestingly, after a short in vitro exposure to low oxygen levels (48 h), CBNBs grow in size, lose HNK-1 expression, and acquire the capacity to respond to acute exposures to hypoxia [36], confirming their conversion into fully mature glomus cells. The specific molecular machinery, involving mitochondria, necessary to respond to hypoxia is only recently being elucidated [25, 26]. However, we have not yet studied formally the expression of the identified hypoxia-responsive molecular elements in CB immature neuronal cells.

In addition to the direct effect of hypoxia on neuroblast maturation, we have observed that these cells also mature in response to different niche signaling [36]. Incubation of CBNBs with purinergic molecules (ATP or UTP) or with ACh for 48 h induces a very similar maturation process than exposure to hypoxia. Moreover, we have shown expression of purinergic receptors by neuroblasts [36], and classical morphometric studies indicated the presence of nicotinic receptors in the membrane of type B glomus cells (neuroblasts) [35]. All these data together confirm that CBNBs have the capacity to mature into fully responsive glomus cells in response to purinergic and cholinergic signals being released mainly by neuronal cells. The role of ATP is particularly interesting since it constitutes the main excitatory neurotransmitter released in the CB during hypoxia. ATP has been involved not only in the chemoreceptor synapse of glomus cells with afferent fibers [54] but also in paracrine communications between type I glomus cells and type II sustentacular cells (multipotent stem cells) [55–57]. We have shown that neuroblasts are a relevant component of the glomerulus and are also participating in this ATP-mediated communication within the niche. Figure 3.4a summarizes the progress of neuronal cell differentiation from multipotent GFAP+ CBSCs, indicating the expression of the different markers and the potential intermediate cell types.

Participation of CBSCs in Hypoxia-Induced Physiological Angiogenesis and the Existence of Mesectodermal-Restricted Progenitors

As stated above, CB acclimatization to chronic hypoxia involves a profound angiogenic process, to provide new vessels that will facilitate irrigation of the growing neural parenchyma [31, 58]. The CB is placed in a region where NCSCs display a clear mesectodermal and vascular potency during development. In fact, the whole wall of carotid arteries and of aorta at the level of the aortic arch is derived from the neural crest, as revealed by cell fate mapping studies using Wnt1-cre transgenic mice [48, 59]. Therefore, it was not irrational to hypothesize that CBSCs might be able to participate in both neurogenesis and angiogenesis in response to the hypoxic stimulus. Hence, we decided to take the risk and test for the ability of adult CBSCs to convert into vascular cell types during hypoxia. To that end, we performed the same cell fate mapping approach that was previously used to prove the role of CBSCs in neurogenesis [28]. By using GFAP-cre/R26R transgenic mice, we showed that an important amount of endothelial cells within the hypoxic organ is derived from GFAP+ CB multipotent stem cells [32]. Our quantification of this stem cell-dependent angiogenic process revealed that approximately one every three new endothelial cells being produced in hypoxia is derived from CBSCs [32]. The other two cells might appear from proliferation of preexisting endothelial cells or from recruitment of circulating endothelial progenitors. Our data also indicated that other vascular cell types, such as smooth muscle cells or pericytes, can also derive from CBSCs [32]. Finally, in vitro studies suggested that vascular differentiation from CBSCs might be potentiated by hypoxia itself (via HIF2α) and by the release of pro-angiogenic cytokines during the hypoxic stimulus [32]. Although the classical source for these cytokines is the vessels, in the case of CB, some of these cytokines such as EPO or endothelin-1 (ET-1) have been shown to be released by neuronal cells [29, 60], constituting a nice example of neuronal activity-dependent instruction of multipotent stem cells. The role of ET-1 is particularly interesting since we showed that this cytokine activates stem cell proliferation after being released by neuronal cells [29] and it also instructs proliferative progenitors to differentiate into the vascular lineage [32, 33]. Taken together, these results indicate that glomus cells promote angiogenesis through instruction of multipotent stem cells into the vascular lineage, while neurogenesis is probably more dependent on the activity of restricted neuroblasts. After revealing the stunning plasticity displayed by CBSCs during hypoxia, we tried to clarify the cellular mechanisms involved in their participation in angiogenesis. Specifically, we explored the possibility of finding intermediate-restricted progenitors belonging to the vascular lineage of CBSCs within the CB parenchyma.

Mesectodermal-Restricted Progenitors Within the Adult CB

When analyzing a microarray of gene expression comparing CB neurospheres with different amounts of neuronal differentiation [33], we identified CD10 (also known as neprilysin) as a cell surface marker highly expressed in non-neuronal cells. We first discarded CD10 as a marker for CB multipotent cells. By performing neurosphere assays with CD10+ and CD10- cells, we found that the marker was labeling a subtype of progenitor cells that had lost the ability to convert into TH+ glomus cells [33]. Typical neurospheres obtained from CD10+ progenitors were larger than normal and had no signs of dopaminergic differentiation (absence of TH+ blebs). Nevertheless, we demonstrated, by cell fate mapping with GFAP-cre/R26R transgenic mice, that these CD10+ progenitors belong to the CBSC lineage; hence they are not just some type of vascular progenitors being recruited from circulation [33].

CD10 is a membrane zinc-dependent metallo-endopeptidase [61] that has been shown to cleave signaling peptides in CNS synapses [62] and in the CB [63]. We have shown that CB mesectodermal-restricted progenitors (CD10+) are present in the normoxic parenchyma, probably preserved in a quiescent state, thanks to the cleavage of pro-angiogenic cytokines by CD10. During the hypoxic stimulus, the expression of CD10 in these cells is downregulated [33], increasing their sensitivity to pro-angiogenic cytokines being released under hypoxia, such as EPO or ET-1. The presence of these restricted progenitors within the resting CB parenchyma might have evolved to facilitate fast angiogenesis in response to the hypoxic stimulus.

We also searched for potential markers that might be more commonly expressed in the whole vascular lineage of CBSCs. In this regard we found CD34 as another typical marker for the vascular lineage within the CB, but less exclusive than CD10 (Fig. 3.3). CD34 has been described as a marker for endothelial progenitors and endothelial cells [64] but also as a marker for mesectodermal progenitor cells of neural crest origin in different tissues and organs [65, 66]. CD34+ cells in the CB belong to the neural crest lineage and are derived from CBSCs, as evidenced by cell fate mapping (Fig. 3.3a, b). We found overlapping expression of CD34 with the differentiated endothelial marker CD31 and with CB vascular progenitor marker CD10 (Fig. 3.3c), confirming that CD34 is a marker for the vascular lineage of CBSCs. However, CD34 seems to start being expressed very early in the specification process, as we find co-expression with GFAP or nestin in some cells (Fig. 3.4d). In fact, the data suggest the existence of some CD34+ cells that have not completely lost their capacity for neuronal differentiation (Fig. 3.3e–h), meaning that CD34 expression starts before multipotency has been completely switched off. The diagram showed in Fig. 3.4b summarizes the progress of endothelial cell differentiation from multipotent GFAP+ CBSCs, indicating the overlapping of the different marker expressions and the potential intermediate cell types that might be having a role in this progression.

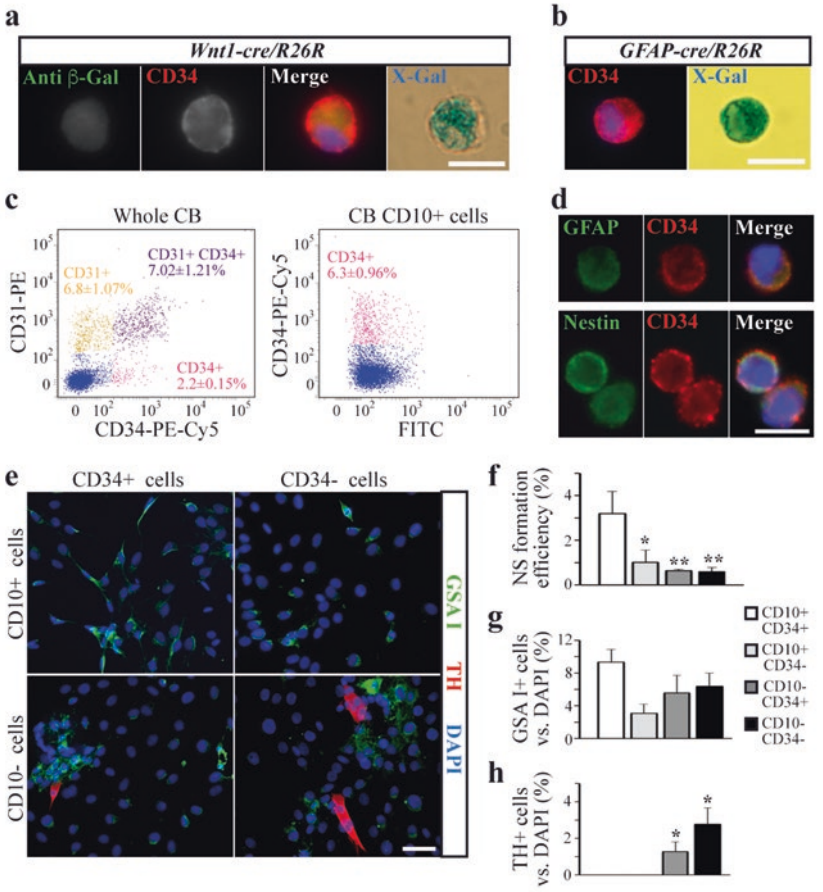

Fig. 3.3 Marker expression in CB mesectodermal-restricted progenitors. (**a**) Example of a CD34+/X-Gal+ cell obtained after cell dispersion of a normoxic CB from a Wnt1-cre/R26R mouse, corroborating the neural crest origin of this type of cells in the CB. Scale bar: 10 μm. (**b**) Example of a CD34+/X-Gal+ cell obtained from the CB of a GFAP-cre/R26R mouse, indicating that this type of cells can derive from GFAP+ CB stem cells. Scale bar: 10 μm. (**c**) Flow cytometry plots showing co-expressions of the vascular markers CD31, CD34, and CD10 in normoxic CB cells. (**d**) Immunocytochemical examples of co-expression of GFAP with CD34, or nestin with CD34, in normoxic CB dispersed cells. Scale bar: 10 μm. (**e**) Neurospheres, obtained from culturing the indicated sorted cells, were plated onto adherent and stained for endothelial cell-specific lectin (GSA I; green), TH (red), and DAPI (blue), to study multipotentiality of the sorted cells. Scale bar: 100 μm. (**f**) Quantification of neurosphere formation from the experiment shown in (**e**). (**g** and **h**) Quantification of the presence of differentiated cells in the cultures shown in (**e**). *$p < 0.05$, **$p < 0.01$, Student's *t*-test

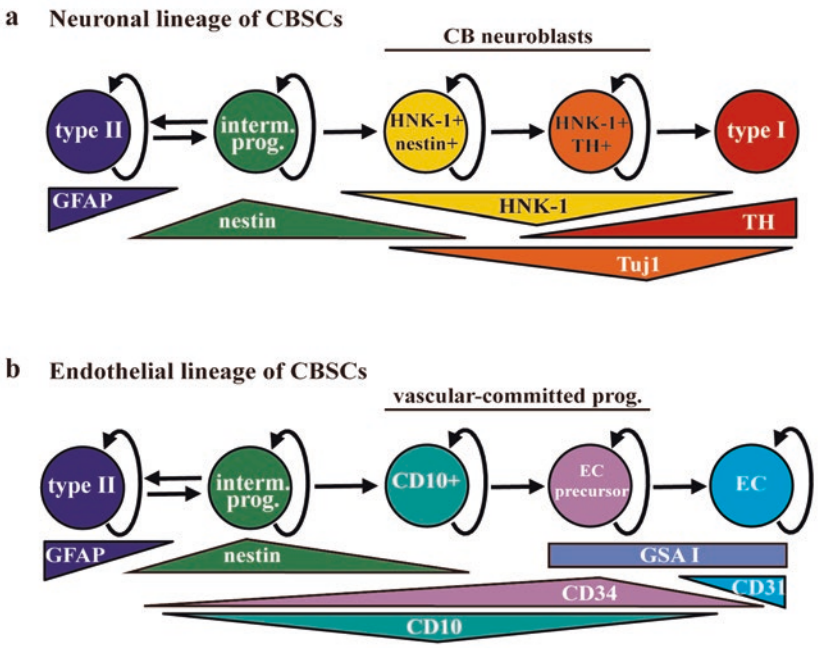

Fig. 3.4 CBSC lineage progression. (**a**) Progression of the neuronal lineage of CBSCs, showing marker expression and potential cell types present in the niche. (**b**) Progression of the endothelial lineage of CBSCs, showing marker expression and potential cell types present in the niche. Curved arrows symbolize proliferation capacity

Clinical Implications and Concluding Remarks

The carotid body has been implicated in the pathophysiology of multiple diseases that course with sympathetic overactivation, generally affecting the cardiorespiratory system. In some of these pathologies, like in hypertension, sleep apnea, chronic heart failure, or some forms of chronic kidney disease, an overactivation of the CB has been proven [67, 68]. In some others, like in obesity, obstructive pulmonary disease, asthma, metabolic syndrome, and diabetes mellitus, the CB is in the spotlight because of having some non-clarified role [67–71]. In the majority of these illnesses, an increase in the size of the CB has been reported [69], very likely associated to its overactivation and to disease progression. Nevertheless, in some cases, overactivation does not necessarily imply parenchyma growth but probably just maturation of neuroblasts without proliferation [36]. Nowadays, the CB constitutes a principal target during the treatment of most of these diseases. In fact, in the case of chronic heart failure and hypertension, resection and denervation of the CB is being tested to try to ameliorate the symptoms [72–74]. However, clinical research in these pathologies is lately focusing on trying to find drugs that would ameliorate overactivation of

the CB, in order to avoid direct surgical resection of the organ [71, 75]. Our work offers multiple options to try to halt this overactivation of the organ. We have characterized the presence of diverse multipotent and restricted progenitor cells within the CB parenchyma and have clarified the mechanisms by which these cells proliferate and differentiate to contribute to tissue growing. By pharmacologically blocking these processes, we should be able to avoid CB growth and hence prevent CB overactivation-dependent symptoms.

A malformation of the CB has also been shown in another series of pathologies related to an impaired development, such as sudden infant death syndrome (SIDS) and congenital central hypoventilation syndrome (CCHS). These diseases have been revealed to course with a reduction in the number of glomus cells and in the number of exocytotic vesicles per glomus cell [76, 77], which may cause malfunction of the organ and the appearance of serious apneas leading to death. Our results on the study of the biology of CB neuroblasts might shed light on the pathophysiology of these diseases.

Regarding a very different type of pathology, an interesting question is whether the proliferative potential of the CB stem cell niche is related to the appearance of paragangliomas in the organ. These tumors are usually benign and resemble the CB of individuals exposed to chronic hypoxemia [17, 78]. Moreover, the incidence of CB paragangliomas increases in high-altitude dwellers [79–81]. However, it has not been established whether there is a relationship between CB germinal niche and tumorigenesis within the organ. Mitochondrial mutations described as the most frequent cause of congenital paraganglioma [82, 83] do not give rise to any type of growth when studied in animal models [84, 85]. On the other hand, a recent work has demonstrated that inactivation of PHD2 in TH+ dopaminergic cells induces paraganglioma-like growth in the mouse CB [53]. Stabilization of HIF2α in dopaminergic cells seems to promote massive proliferation of TH+ cells, accompanied by strong vascularization. These data suggest that there could be a relationship between the cellular mechanisms for CB hypertrophy and the appearance of paragangliomas. In any case, our understanding of CB niche functioning will very likely improve our capacity to treat paraganglioma tumors.

Finally, another clinical aspect that might benefit from our studies on the CB niche is the use of CBSCs in cell therapy against Parkinson disease. CB cell aggregates have been successfully transplanted into the brain for the amelioration of Parkinson symptoms in animal models, due to the release of dopaminotrophic factors by neuronal type I cells [86]. CBSC cultures have been proposed as a strategy to optimize glomus cell transplantation [87]. Our results might help to obtain better yields of glomus cell production in vitro and hence to increase the efficiency of this type of transplants against Parkinson disease.

In summary, the CB has evolved as a remarkable oxygen detector in mammals, containing a stunning germinal niche within the adult PNS, necessary for a correct physiological adaptation to a changing environment. Our recent data on the characterization of diverse stem and progenitor cells present within the CB parenchyma is increasing our understanding of the organ physiology and pathology, will very

likely improve the treatment of a variety of different diseases related to CB malfunction, and will probably influence the use of the CB niche for cell therapy against neurodegenerative disorders.

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Chapter 4
Planarian Stem Cell Heterogeneity

Salvetti Alessandra and Leonardo Rossi

Abstract Planarian (Platyhelminthes, Triclads) are free-living flatworms endowed with extraordinary regenerative capabilities, i.e., the ability to rebuild any missing body parts also from small fragments. Planarian regenerative capabilities fascinated scientific community since early 1800, including high-standing scientists such as J.T. Morgan and C. M. Child. Today, it is known that planarian regeneration is due to the presence of a wide population of stem cells, the so-called neoblasts. However, the understanding of the nature of cells orchestrating planarian regeneration was a long journey, and several questions still remain unanswered. In this chapter, beginning from the definition of the classical concept of neoblast, we review progressive discoveries that have brought to the modern view of these cells as a highly heterogeneous population of stem cells including pluripotent stem cells and undifferentiated populations of committed progenies.

Keywords Planarian · Stem cells · Neoblast · Regeneration · Committed progeny · Platyhelminthes · Piwi · Flatworms · Clonogenic neoblast · σ-Neoblasts · ζ-Neoblasts · Tetraspanin

Planarian Stem Cells: The Neoblasts

The term neoblast, introduced by Harriet Randolph in 1987 [1], refers to cells characterized by certain morphological features and located in a mesenchymal-like tissue filling the space between the various organs, commonly referred as the parenchyma [2]. Neoblasts are small cells (10–12 μm in diameter) with an elevated nucleus/cytoplasmic ratio and a scanty undifferentiated basophilic cytoplasm, rich in ribosomes, in which only a few small mitochondria can be found [3–5] (Fig. 4.1a, b). A unique morphological feature of neoblasts is the presence of amorphous, non-membrane-bound, electron-dense aggregates, called chromatoid bodies whose

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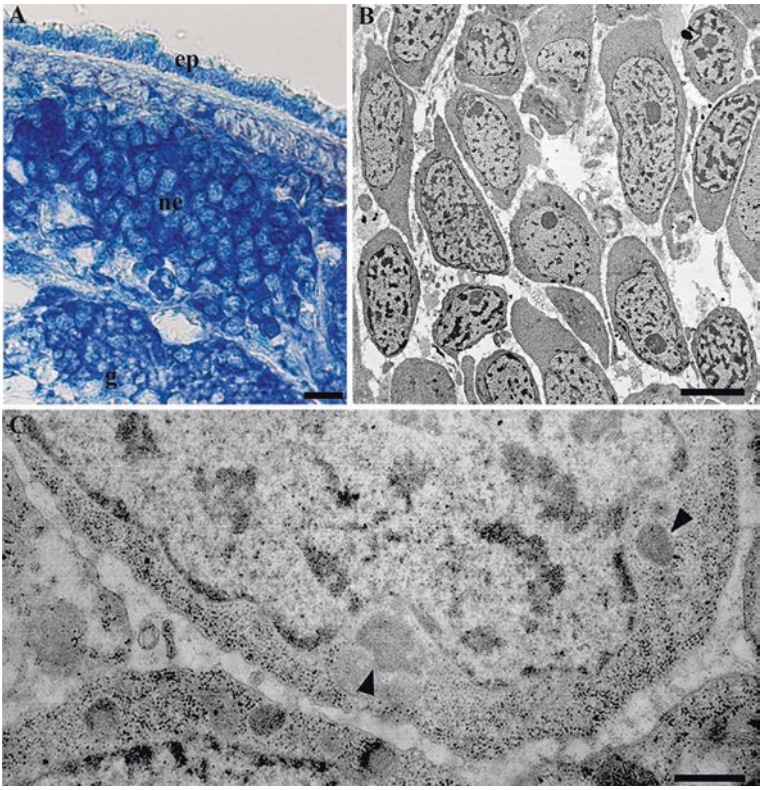

Fig. 4.1 Neoblast morphology. (**a**) Light microscopy of a methylene blue- and toluidine blue-stained cross section of an intact planarian. Neoblasts (ne) are visualized as small cells (about 10 μm) intensely stained in the cytoplasm and located in the parenchyma between the epidermis (ep) and the gut cells (g). Scale bar corresponds to 25 μm. Dorsal is at the top. (**b**) Electron microscopy image showing several neoblasts accumulated beneath the blastema. Scale bar corresponds to 10 μm. (**c**) Electron micrograph showing part of a neoblast showing two chromatoid bodies (arrow heads) in a scanty cytoplasm rich in ribosome. Scale bar corresponds to 1 μm

number and size decrease with cell differentiation (Fig. 4.1c) [6–9]. Chromatoid bodies contain RNA and proteins and are reminiscent of germ granules observed in germline cells of many animals [10]. Indeed, some of the germ granule-specific proteins are also found in chromatoid bodies, and it has been postulated that germ granules and chromatoid bodies share some functions, being both involved in post-transcriptional regulation [11–13] and silencing of the activity of transposable elements [14].

These data are in agreement with the finding that a number of RNA-binding proteins often considered to be restricted to germline functions operate in multipotent progenitors and stem cells of many metazoans [15], including planarians, thus suggesting the existence of a “germline multipotency program” present in multipotent stem cells that generate somatic cell type [16].

Historically, neoblasts were also defined by a behavioral parameter: the ability to divide. In fact, with the exception of spermatogonia and oogonia, all the other cells that exhibit mitotic division in the planarian body were defined as neoblasts. Therefore, neoblasts were considered as the only dividing cells in asexual specimens [17]. This historical point of view is, under some aspects, still actually true, i.e., only undifferentiated cells in the planarian body retain proliferative capability. However, according to recent molecular studies, only a subgroup of the heterogeneous neoblast population is truly involved in mitotic activity (please see below). Being proliferative cells, neoblasts are sensitive to high dose of X-ray (30 Gy for *D. japonica* and 60 Gy for *S. mediterranea*) at which differentiated cells are unaffected. This creates the opportunity to produce "neoblast-free" planarians, a model system widely used by planarian scientist community. Two irradiation-sensitive populations can be visualized by fluorescence-activated cell sorting (FACS) after Hoechst/calcein AM staining: a X1 population that, according to DNA content, is thought to include G2/S/M neoblasts; and a X2 population that is thought to include, together with X-ray-insensitive cells, the G1 neoblasts [18].

Thanks to the unique ability of neoblasts to divide, scientists were able to identify their distribution in the planarian body by analyzing mitosis, incorporation of bromodeoxyuridine, and labeling with the S-phase-specific markers [19–22]. The analysis of the expression pattern of S-phase-associated markers reveals that neoblasts are located in body parenchyma and distributed throughout the entire planarian body with the exception of the anterior part of the head, especially behind the eyes, and the pharynx, the planarian mouth (Fig. 4.2a, b). In the parenchyma, neoblasts preferentially accumulate deep in the body, close to the gut branches (Fig. 4.2i). Depending on the species, neoblasts also accumulate in dorsal clusters along the anterior midline and lateral dorsal lines; these dorsal clusters are only observable in *D. japonica* [21, 22] and not in *S. mediterranea* [23] (Fig. 4.2a, b), the two main planarian species used for molecular studies. The nature of these clustered cells is still puzzling [17], and despite they express genes specific for proliferating cells, they do not actively incorporate bromodeoxyuridine also after 6 days of continuous exposure [21]. These clustered cells behave differently from disperse neoblasts also in other cases. For example, the depletion of the neoblast-specific gene *DjPhb2* strongly reduces the number of spread neoblasts without affecting those located in the dorsal body midline (Fig. 4.2c) [24]. Different behaviors between disperse and clustered neoblasts, found in *D. japonica*, primed a series of researches focused in establishing heterogeneity in neoblast population. Neoblast distribution and species-specific differences have been confirmed by the analysis of the expression of cell cycle not-related genes specific for X-ray-sensitive cells [13, 25–28]. Among the different neoblast-specific genes identified so far, the *S. mediterranea* smdewi1 and its *D. japonica* homologue *DjPiwi-4* are the most common markers used to label a cell as a neoblast at present. Table 4.1 summarizes the requirements nowadays used to define a cell as a neoblast and Fig. 4.3 a review of the main steps of regeneration process.

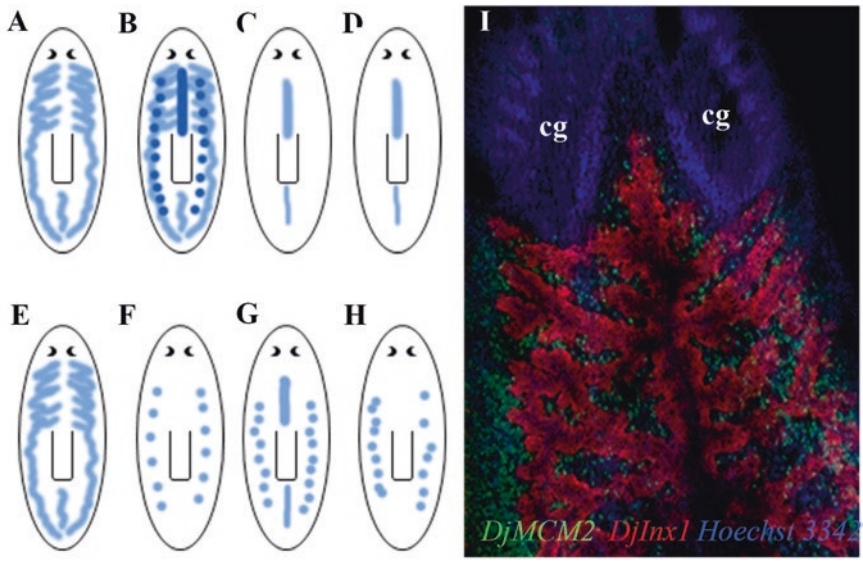

Fig. 4.2 Neoblast distribution in *S. mediterranea* and *D. japonica* visualized by using molecular markers. (**a**–**h**) Schematic drawing of the expression patterns of different markers in different experimental conditions. (**a**) Expression pattern of S-phase-associated genes in *S. mediterranea*. (**b**) Expression pattern of S-phase-associated genes in *D. japonica.* (**c**) Expression pattern of general S-phase neoblast markers in *D. japonica* specimens silenced for the expression of *DjPhb2*. (**d**) Expression of *DjPiwi-1* in *D. japonica*. (**e**) Expression of some *D. japonica* neoblast-specific genes that do not accumulate in the dorsal anterior midline. (**f**) Expression of nanos gene in dorsal presumptive testis-forming region in asexual *S. mediterranea* and *D. japonica*. (**g**) Residual neoblasts visualized by S-phase markers early after low-dose X-ray treatment in *D. japonica*. (**h**) Residual neoblasts visualized by S-phase markers 4 days after low-dose X-ray treatment in *D. japonica*. (**i**) Double fluorescent in situ hybridization of *DjMCM2* (green) and *DjInx1* (gut marker; red) in *D. japonica*. Nuclei are stained in blue with Hoechst 3342. Cg: cephalic ganglia of the central nervous system. Anterior is at the top

Neoblasts Are Heterogeneous, It's a Matter of Fact!

Despite neoblasts share the same morphological features, in early 2000 it appeared clear that neoblasts are a heterogeneous population of cells, "a cell type for all seasons," says Jaume Baguñà in its personal account narrating planarian regeneration between the 1960s and 1990s [33].

Some evidences support neoblasts heterogeneity:

1. Ultrastructural investigation on X1 and X2 isolated cells revealed that stem cells can be classified in two types according to their morphology [34]. Authors divided neoblasts in "type A" and "type B" according to chromatin ultrastructure, size, and number of chromatoid bodies. In particular, type A neoblasts show a greater amount of euchromatin, a higher number of chromatoid bodies, and a larger size than type B neoblasts. Type A neoblasts are enriched in X1 population, while type B neoblasts are concentrated in X2 population.

Table 4.1 Requirements used nowadays to define a cell as a neoblast

Size	8–12 μm
Ultrastructural features	– High nucleus/cytoplasmic ratio – Presence of chromatoid bodies – Mostly undifferentiated cytoplasm
Cytochemical properties	– Basophilic cytoplasm
Proliferating activity	– Able to incorporate BrdU – Stainable with anti-phospho-histone 3 antibody – Sensitive to high dose of X-ray (neoblasts are totally abolished 24 h after treatment) – Positive for S-phase-specific markers (i.e., MCM and PCNA)
Expression cell cycle not-related genes specific for X-ray-sensitive cells	i.e., piwi, pumilio, bruli, vasa
Expression of chromatoid body-specific genes	Specific DEAD box RNA helicases, tudor homologues, piwi proteins, LSm (like-Sm) RNA-binding proteins

2. The first tangible molecular evidence for the presence of neoblast subpopulations came up with the identification of a *D. japonica* homologue (*DjPiwi-1*) of the *Drosophila* Piwi gene. *DjPiwi-1* is expressed in small cells preferentially clustered along the dorsal midline (Fig. 4.2d). *DjPiwi-1*-positive cells match the neoblast definition, in terms of morphology and X-ray sensitivity [35]. However, they do not participate to regeneration, and, being part of neoblasts accumulated in the anterior midline, they are likely arrested in the cell cycle. On the contrary, several other neoblast-specific genes identified so far are not expressed in the dorsal midline such as *DjH2Az* (histone family, member Z), *DjSam68-like*, and *DjCIP-29* [36] (Fig. 4.2e).
3. Sato and co-workers succeeded in identifying *D. japonica* germline stem cells that specifically express a nano-related gene (*Djnos*) [37]. *Djnos*-positive cells are expressed in the presumptive ovary or testis-forming region of both *D. japonica* and *S. mediterranea* asexual strains (Fig. 4.2f) [37, 38], express the S-phase marker DjPCNA, but do not incorporate BrdU. The germline stem cells are morphologically indistinguishable from the neoblasts but do not contribute to the regeneration process at all [37], and those located in the presumptive testis-forming region might be part of the clustered neoblasts of the dorsal lateral lines observed in *D. japonica*.
4. Neoblasts show different levels of radiosensitivity after treatment with low X-ray doses. After treatment of *D. japonica* specimens with 5 Gy of X-ray, it is possible to observe a gradual loss of the S-phase marker *DjMCM2*-positive cells that reaches a maximum 3–4 days after treatment. From day 1 to 3, not all the neoblasts disappear with the same rate. Indeed, neoblasts spread all over the parenchyma are less radioresistant than clustered neoblasts, and, among them, some neoblasts clustered in the dorsolateral parenchyma have a higher radiotolerance than those clustered anterior to the pharynx along the midline of the planarian body (Fig. 4.2g, h) [39].

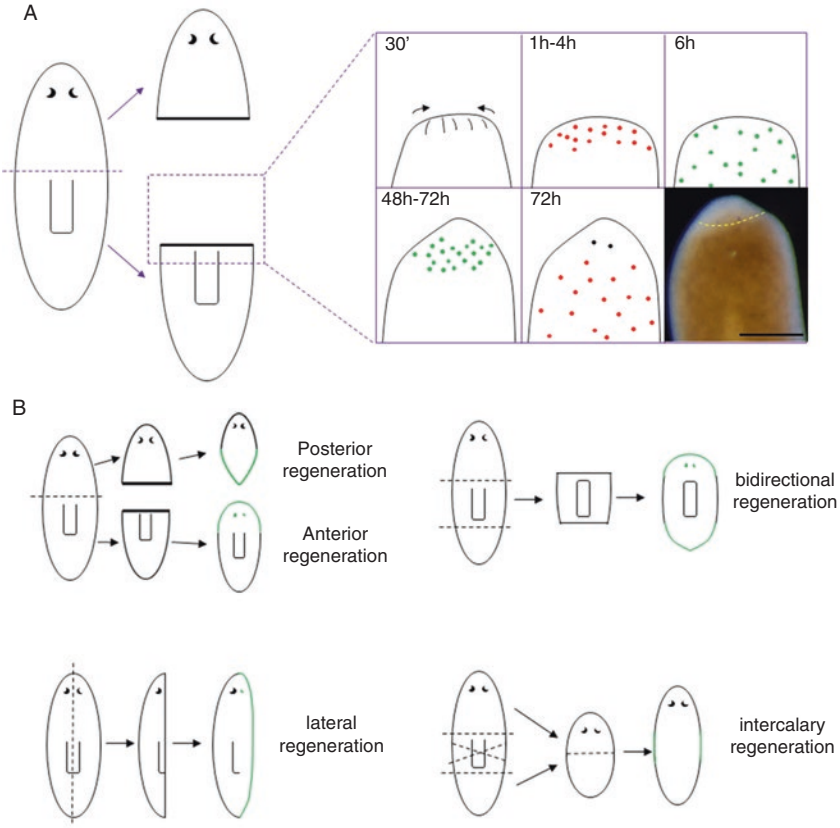

Fig. 4.3 Planarian regeneration. (**a**) After wounding planarian fragments undergo to a stereotyped series of events that lead to the formation of a regenerative blastema, an unpigmented region in which any missing body structure is rebuilt in less than 15 days. Immediately after cut, a strong muscular contraction at the site of wounding occurs to minimize the surface area of the wound [29]. A thin layer of epithelium covers the wound within 30 min. This early epithelization involves both active and passive cell migration phenomena in which the old cells from the dorsal and ventral epidermis facing the wound simultaneously spread to close the wound surface, joining first near the wound center [30]. From approximately 1 h through 4 h postamputation, a high number of apoptotic cells accumulates within an area extending approximately 100 μm from the wound site [31]. Then, neoblasts respond to wounding in a widespread first mitotic peak around 6 h after cut and a second localized mitotic peak in the post-blastemal area from 48 to 72 h after cut [32]. Three days after the cut, a second, systemic increase in apoptotic cell number can be observed [31]. The proposed role for this systemic increase in cell death is that apoptosis promotes a complementary remodeling response that occurs in uninjured tissues to restore anatomical scale and proportion [31]. Accumulation of postmitotic cells leads to the formation of the regenerative blastema, in which de novo tissue morphogenesis occurs. Red dots, apoptotic cells; green dots, mitotic cells; black dots, eyes; yellow dotted line demarcate the blastemal region. (**b**) Schematic drawings of planarian regeneration. In black pre-existing tissues and in green newly produced tissues during regeneration. Scale bar corresponds to 500 μm

5. Neoblasts include a subpopulation of pluripotent stem cells that can form large descendant-cell colonies in vivo. These neoblasts are called clonogenic neoblasts (c-neoblasts) and are able to rescue lethally irradiated hosts producing all the differentiated cell types [40]. Accordingly to an approximate estimation, c-neoblasts represent about 5–20% of isolated neoblast-enriched fractions. Thus neoblast population results divided at least in clonogenic (minority) and non-clonogenic (majority) cells. However, the inability of most neoblasts to perform the rescue process might also be due to the high risk of cell damage in sorting and injection procedures, and so the possibility that all neoblasts are pluripotent cannot be completely ruled out. Interestingly, among the different neoblast-like cells, distinguishable in neoblast-enriched fractions, those with a diameter included between 10 and 12 μm with low cytoplasmic granularity and showing blebs and/or cytoplasmic processes possess the higher engraftment rate success [40].
6. Single-cell transcriptomic analysis reveals the presence of different neoblasts subpopulations and represents the first direct evidence of neoblast heterogeneity (please see below).

The Naïve Versus the Specialized Neoblast Model

The demonstration of the existence of a subpopulation of c-neoblasts opens to several possible interpretation of the concept of neoblast itself. First of all, which is the nature of the non-clonogenic neoblasts? It is known that these cells fulfill some of the requirements for being considered neoblasts: they are *Smedwi-1* positive and X-ray sensitive; however, it is unknown if these cells are irreversibly determined or can change their fate, if they can divide and renew or represent a transient state between pluripotent c-neoblast and differentiated cells. Most of these questions are being to be answered (please see next paragraph); however, different models have been proposed (Fig. 4.4) to combine the activity of clonogenic and non-clonogenic neoblasts during regeneration and normal tissue homeostasis [41]: (a) the naïve neoblast model in which neoblasts are considered a homogeneous population of cells with respect to differentiation potency and renewal capabilities. In this model the specification process would occur in postmitotic progeny cells, (b) the specialized neoblast model in which specialized self-renewing neoblasts exist in the population and produce different lineage-committed nondividing cells, and (c) the mixed neoblast model in which c-neoblasts produce specialized neoblasts that are able to divide and renew itself or differentiate in a group of specialized cells (Fig. 4.4). The existence of c-neoblasts itself demonstrates that at least a small subpopulation of naïve neoblasts exists. However, several lines of evidence also support the specialized model. First, the existence of germline stem cells demonstrates the presence in X-ray-sensitive cells of at least two populations, ones determined toward a specific germ fate. Moreover, a great work has been done in identifying tissue-specific transcription factors that are also expressed in very small populations of tissue precursors positive for *smedwi-1* [42]. This is the case of *ovo* transcription factor required

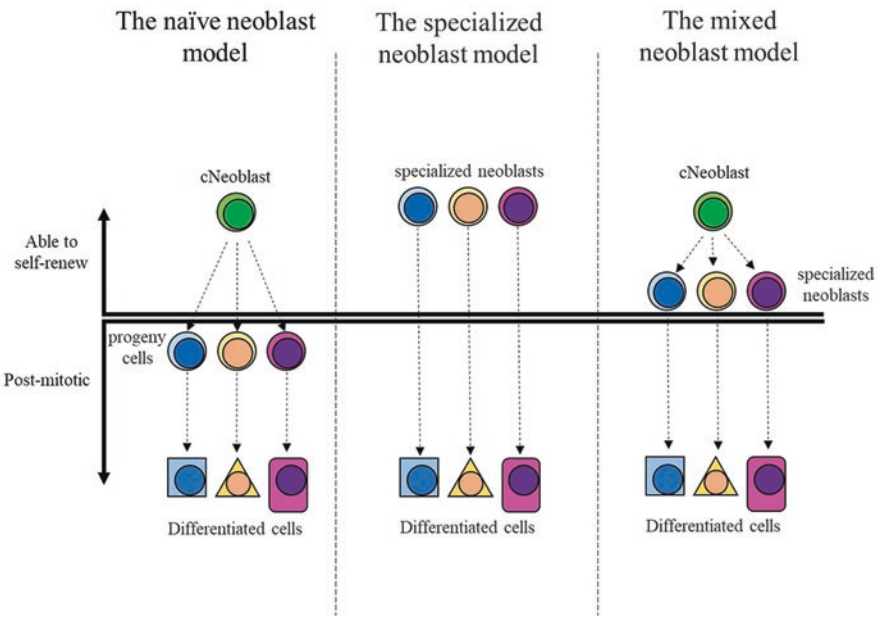

Fig. 4.4 Models proponing different relationships between c-neoblasts, specialized neoblasts, and postmitotic progenies

for planarian eye cell differentiation [43], *six1/2-2* and *POU2/3* required for protonephridia regeneration [44], *lhx1/5* and *pitx 1* required for the maintenance of serotonergic neural identity [45], some bHLH genes required for neurogenesis [46], *Smed-FoxA* required for regeneration of the pharynx [47], *Smed-FoxD* and *Smed-zic-1* required for anterior pole regeneration [48, 49], and *Smed-gata4/5/6* necessary for gut cell differentiation [50](Fig. 4.5). Under some point of view, all these data led to the recognition that while planarians do possess pluripotent c-neoblasts, specification toward numerous tissue types begins at the neoblast level [51]. However, the existence of *smedwi-1*-positive neoblasts that also express tissue-specific transcription factors is a necessary but not a sufficient condition to affirm the existence of subpopulations of self-renewing specialized neoblasts, i.e., the "mixed model." Indeed, the ability of these populations to actively proliferate and renew itself is still unknown, and they might just represent postmitotic undifferentiated progeny cells that retain a certain level of *smedwi-1* expression.

Single-Cell RNA Sequencing Scratched the Surface of Neoblast Heterogeneity

Revolution in molecular biology techniques that typified the last years opened unexpected possibility to determine cell-type-specific transcriptomes. Taking advantage of this possibility, the research group of Peter Reddien at Massachusetts Institute of

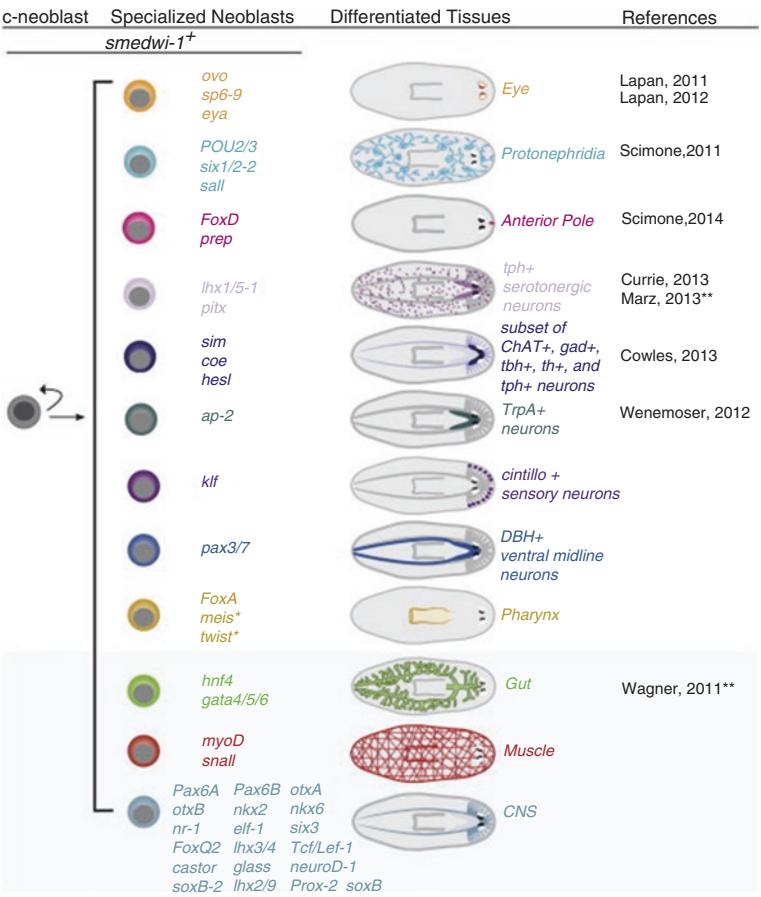

Fig. 4.5 A summary of all known transcription factors expressed in neoblasts and functionally associated with distinct lineages by RNAi analysis is shown in the upper part of the model (white background). The transcription factors expressed in neoblasts and in specific tissues, but that have not been shown to be involved in their specification, are shown in the lower part of the model (blue background). From [42]

Technology embarked in a punctual analysis of single-cell transcriptional profiling to determine the transcriptomes of individual cells from adult planarians. Initially, to molecularly profile individual neoblasts of *S. mediterranea*, they performed parallel single-cell qPCR analyzing 96 genes from each cell [52]. This allowed to identify, among individual neoblasts isolated by (FACS) (X1-fraction; 4C DNA) [18] from the prepharyngeal region of intact worms, two major, roughly equally sized populations: the zeta-class (ζ-class), expressing high levels of a discrete set of genes (e.g., *Smed-zfp-1*, *Smed-g6pd*, *Smed-fgfr-1*, *Smed-p53*, *Smed-soxP-3*, and *Smed-egr-1*) and the sigma-class (σ-class) expressing low levels of those genes but elevated levels of *Smed-soxP-1*, *Smed-soxP-2*, *Smed-soxB-1*, *Smed-smad-6/7*,

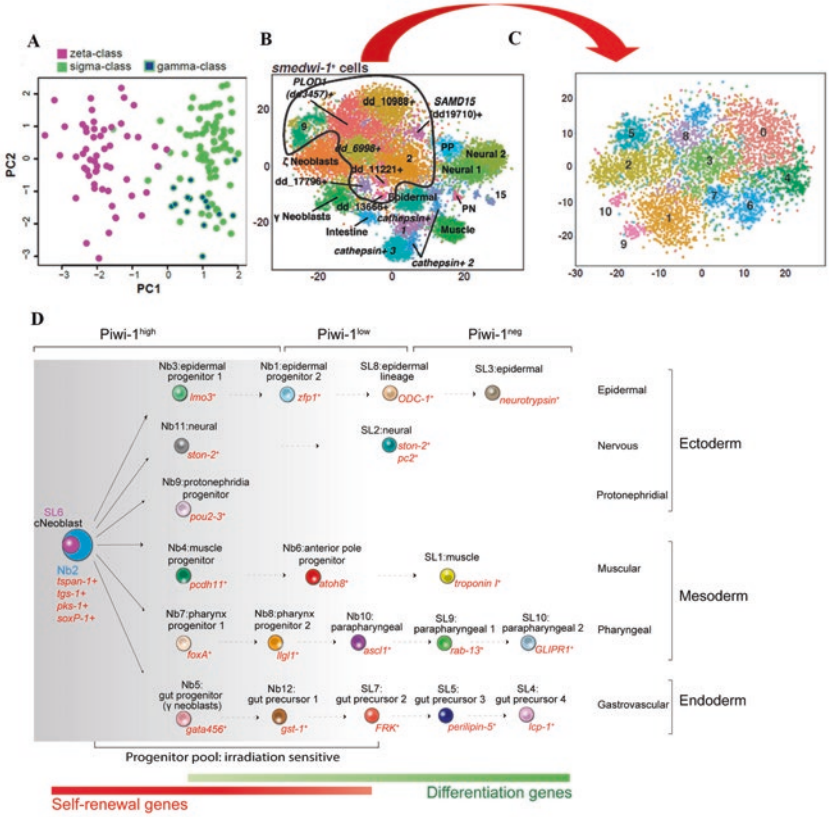

Fig. 4.6 Single-cell transcriptional profiling reveals the existence of several neoblasts subpopulations. (**a**) Principal component analysis (PCA) of the qPCR results on 176 cells from the X1(4C) gate. Each dot represents a cell, colored according to its class. From [52]. (**b**) t-SNE representation of 22 clusters generated from subclustering cells with *smedwi-1* expression ≥ 2.5 [ln(UMI-per-10,000 + 1)]. A boundary indicates *smedwi-1* high clusters further subclustered in (**c**). Identity of numbered clusters is unknown. PP, parenchymal; PN, protonephridia. Intestine cluster is indicated by lower expression of *smedwi-1* and enriched *gata4/5/6* and *hnf-4* expression. (Ct-SNE representation of 11 clusters generated from further subclustering of (**b**). (**b**, **c**) From [53]. (**d**) Proposed lineage composition model of planarian smediwi-1-positive cells. Twelve major classes representing six cell lineages of all three germ layers were found. Nb2 can self-renew and give rise to a wide range of tissue types in single-cell transplantation. This image was taken from [54]

Smed-inx-13, *Smed-pbx-1*, *Smed-fgfr-4*, and *Smed-nlk-1*. A third group of neoblasts, actually a subclass within the σ-class, expressing high levels of *Smed-gata4/5/6*, *Smed-nkx2.2-like*, *Smed-hnf4*, and *Smed-prox-1* was also identified and designated as gamma-neoblasts (γ-class) (Fig. 4.6a). With the exception of *Smed-prox-1*, the other three transcripts have been previously linked to the planarian intestine [40, 55], suggesting that γ-neoblasts includes gut progenitors. σ-class and ζ-class remain present throughout the cell cycle confirming that these two classes reflect two separate populations not defined by cell cycle state. Spatial distribution of the two classes

is not restricted; σ and ζ neoblasts are indeed intermingled and roughly of equal abundance. Although both neoblast classes are stably present throughout regeneration, the increase in neoblast proliferation early after wounding can be largely attributed to mitotic activity of σ-neoblasts indicating that wound-site accumulation of neoblasts mainly involves this class. Animals devoid of ζ-neoblasts, obtained by silencing *smed-zfp-1*, were still able to generate a blastema following amputation, thus demonstrating that σ-neoblasts undergo mitosis in the absence of the ζ-neoblasts and remain capable of mounting a normal regenerative response. Interestingly, in *smed-zfp-1* RNAi, epidermal lineage markers [56] and several transcripts associated with epidermis, cilia, and secretory cells were severely reduced, while transcripts characteristic of the eye, brain, intestine, protonephridia, and muscle were unaltered. This allowed to conclude that ζ-neoblasts give rise to a lineage that ultimately is involved in the maintenance and/or differentiation of an epidermal cell type. Transplantation experiments of neoblasts obtained from *smed-zfp-1* RNAi into lethally irradiated hosts revealed that the σ-class is collectively pluripotent and regenerates the ζ-class. ζ-class cells thus represent a population of cells that continually arise from σ-neoblasts, and the σ- to ζ-class shift in transcriptional profile begins directly upon entry into S-phase. Recently, Lai and co-workers [57] provided independent evidence that σ-neoblasts are the only population of *smedwi-1*-positive cells capable of multiple rounds of cell division and hence self-renewal. In particular, they abrogated condensin complex by RNAi and observed some σ-neoblasts that do not die after mitosis fails continue to attempt to replicate, self-renew, and accumulate replicated DNA. On the contrary, they never observed enlarged ζ- or γ-neoblasts, suggesting they do not go through multiple rounds of division and are postmitotic after a single round of division. Although further direct evidences are necessary to understand renewal capabilities of the different neoblast subclasses, these data suggest that a subset of σ-neoblasts during S-phase gains either zeta- or gamma-expression signatures, proceed through M-phase, and then give rise to zeta- and gamma-class cells which are postmitotic cells, thus no more considerable proper neoblasts despite they still express neoblast markers.

Recently, the Peter Reddien group highly improved single-cell transcriptome analysis by applying single-cell RNA sequencing method Drop-seq to determine the transcriptomes for 66,783 individual cells from adult planarians [53]. Cells sharing gene expression were then grouped into nine broad tissue classes whose identity was established by using known tissue markers. Finally, subclustering of each broad tissue type, in isolation, enabled separation of cells into the cell populations constituting each tissue. This allowed to identify more than 150 subclusters including differentiated cells, precursors, and neoblasts. To identify neoblast subclasses, they subclustered 12,212 cells with *smedwi-1* expression of ≥2.5 [ln(UMI-per-10,000 + 1)]. Clustering resulted in the identification of several subclasses (Fig. 4.6b), only some of them showing the expression of S- or G2-phase markers (among them the previously described γ- and ζ-neoblast classes). Clusters marked by a G1/G0 cell cycle status (i.e., not expressing S or G2 markers) displayed expression of various differentiated tissue markers suggesting that these cells could be

transition states for those lineages. To focus on pluripotent c-neoblasts, the authors selected cells expressing high levels of *smedwi-1* excluding γ- and ζ-neoblasts and subclustered, in isolation, this set of cells. Some of the newly obtained subclusters were remnant of those obtained by general clustering of all the *smedw-1*-positive cells and co-expressed tissue-specific markers (or belonged to γ- or ζ-classes). On the contrary, other clusters (namely, clusters 0, 3, 7, and 8) were largely devoid of tissue-specific markers and thus possibly include true c-neoblasts (Fig. 4.6b, c). The idea that *smedwi-1* differential expression levels might represent a discriminatory parameter for subclassifying neoblasts has been the driving force of a revolutionary recent paper from the Alejandro Sánchez Alvarado group [54]. Using super-resolution microscopy, authors identified a subpopulation of high-expressing *smedwi-1*-positive cells (Piwi-1^{high}) encompassing 41% of the total number of *smedwi-1*-positive cells. Piwi-1^{high} cells are mainly cycling cells included in the X1 fraction and are completely eliminated 1-day post-high-dose X-ray treatment. Piwi-1^{high} cell markers are promptly upregulated during early regeneration steps. Thus allowing to conclude that high levels of both *smedwi-1* gene expression and PIWI-1 protein positively correlate with a neoblast subpopulation encompassing a defined spectrum of functional states, including c-neoblasts. By single-cell RNA profiling of Piwi-1^{high}-enriched fractions, the authors subclassified these cells into 12 subpopulations (Nb1 to Nb12) including γ- and ζ-neoblast classes as well as subgroup of cells expressing differentiated tissue markers and representing lineage progenitors (Fig. 4.6d). Interestingly, the expression of the σ-class marker *soxP-1* did not segregate in a specific cluster, and, albeit at different levels, its expression was detected in six neoblast cell clusters, suggesting that *soxP-1* expression might be akin to *smedwi-1* in showing quantitative differences across cells. To identify neoblast subcluster(s) which might include c-neoblasts, the authors analyzed all the clusters (Nb1 to Nb12) using different criteria to identify which one(s) may contain pluripotent stem cell activities. Only the Nb2 cluster satisfied all selection criteria. Using a polyclonal antibody against the extracellular domain of a Nb2-specific tetraspanin (TSPAN-1) and FACS technology, they isolated Nb2 cells and, by single-cell transplantation into lethally irradiated planarians, demonstrated a rescue rate of 23.2%, in marked contrast with a 2% rescue rate for general X1(FS) cells and no rescue for TSPAN-1-negative cells. This data revolutionizes our understanding of neoblast biology and complexity and strongly indicates that Nb2 cells encompass pluripotent, self-renewing stem cells that can be prospectively isolated using the membrane-associated protein TSPAN-1.

Neoblast *2.0*

Giant steps have been done in the last 10 years in planarian research due to the development of transcriptomic and genomic resources, highly efficient hybridization protocols, cell sorting strategies, and gene silencing technologies. From being a classic model for regenerative studies, planarians actually represent an ultimate

system for studying several stem cell-related issues from classical cell determination and differentiation processes to aging [58], stem cell niche [59], epigenetic regulation of pluripotency [60], and mechanisms for standing starvation [61]. All these issues rotate around a unique property: the existence of a population of adult pluripotent stem cells. The long journey that has been done to discover and characterize planarian stem cells makes necessary to reconsider the significance of the term neoblast also to integrate molecular, cellular, and ultrastructural data with the discovery of high heterogeneity in this population of cells. First, if we agree to consider as neoblasts all the cells that express *smedwi-1* (or *DjPiwi-4*), it is a matter of fact that not all of them are pluripotent stem cells, and some evidences (to be supported by further studies) suggest that not all should be considered stem cells being indirectly demonstrated that ζ- and γ-neoblasts are postmitotic cells [57]. This might be also the case for *smedwi-1*-positive cells included in clusters not expressing S- or G2-phase markers or expressing low level of smedwi-1 and committed toward a specific lineage [53, 54]. So, it seems quite clear that neoblasts include pluripotent stem cells (c-neoblasts; inside the Nb2 cluster) on one side and lineage-committed postmitotic cells on the other side. Unclear is what happens in the middle! Indeed, the existence of more than one cluster of cells showing high *smedwi-1* levels and expression of S- and G2-phase markers has been proved [53], but still no direct evidence exists of their ability to self-renew. Thus it is still not possible to know if some of these clusters might represent transition states, with restricted differentiation potency, with an intermediate position in the path between c-neoblasts and committed progeny.

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Chapter 5
The Cellular Origin of Barrett's Esophagus and Its Stem Cells

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Abstract The incidence of esophageal adenocarcinoma is rapidly increasing in Western countries. This is despite the introduction of sophisticated endoscopic techniques and our ability to readily monitor the presumed precursor lesion known as Barrett's esophagus. Preemptive approaches, including radiofrequency ablation (RFA), and photodynamic therapy (PDT) for Barrett's esophagus and dysplasia are achieving dramatic initial results. Although the long-term efficacy of these nonspecific ablative therapies is awaiting longitudinal studies, reports of recurrences are increasing. More targeted therapies, particularly directed at the stem cells of Barrett's esophagus, demand knowing the origin of this intestinal metaplasia (IM). The prevailing concept holds that Barrett's esophagus arises from the "transcommitment" of esophageal stem cells to produce an intestine-like epithelium. An alternative explanation derives from the discovery of a discrete population of residual embryonic cells (RECs) existing at the gastroesophageal junction in normal individuals that expands and colonizes regions of the esophagus denuded by chronic reflux. These RECs form IM within days of esophageal injury, suggesting a novel mechanism of tumorigenesis.

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A corollary of this work is that the Barrett's stem cell is distinct from that of the squamous epithelium and, once identified, will form the basis of new preemptive strategies for addressing Barrett's and its related neoplasia.

Keywords Esophageal adenocarcinoma · Barrett's esophagus · Intestinal metaplasia · Residual embryonic cells · Adult stem cells · Stem cell cloning · Preemptive therapeutics · Cancer precursor · High-throughput screening · Cancer prevention

Intestinal Metaplasia and Carcinogenesis

Intestinal metaplasia is the ectopic growth of intestine-like tissue in the esophagus or stomach and has been implicated in the development of untreatable esophageal adenocarcinoma in the West and gastric adenocarcinoma in the East. Its rise is tightly linked with chronic inflammation due to acid reflux disease in the case of esophageal adenocarcinoma and chronic *H. pylori* infections for gastric adenocarcinoma. Unlike many cancers, the rates of esophageal adenocarcinoma have increased dramatically in the United States and are now five times what they were in 1950. At the same time, treatments for this cancer are still largely palliative, and thus tremendous efforts have focused on potential strategies of treating or preventing intestinal metaplasia. Such strategies are entirely dependent on understanding the origins of intestinal metaplasia.

Intestinal metaplasia at the distal esophagus was first observed and defined by Dr. Barrett and therefore was named as Barrett's esophagus [1]. Barrett's esophagus is a remarkably common condition and can progress to esophageal adenocarcinoma. The clinical challenges presented by Barrett's and adenocarcinoma are enormous but distinct. Managing the large Barrett's population is complicated by relatively low rates of progression, complex and low-yield endoscopic monitoring protocols, and nonselective ablation options. Conversely, most esophageal adenocarcinoma patients are diagnosed with disseminated disease at their index visit, and the cancer is poorly responsive to therapy. This is a common paradox seen with many carcinogenic sequences, with a disconnect between a common and gradually developing precursor model and a malignant endpoint that emerges suddenly without warning. This highlights gaps in our understanding of the natural history of the decades-long evolution of cancer from precursor lesions and underscores the need for cogent and predictive cellular models of these processes to further discovery of effective therapeutics.

Gastric intestinal metaplasia implicated in gastric adenocarcinoma [2] and perhaps other metaplastic precursor lesions that precede certain pancreatic and bladder cancers are not dissimilar to Barrett's esophagus. As a group these metaplastic precursors give rise to some of the most aggressive and poorly responsive human cancers. If the observations presented here for Barrett's esophagus prove to be generalized across these lesions, new concerted strategies for targeting the stem cells of metaplastic precursor lesions could prevent large numbers of lethal cancers.

Current Theories of Cellular Origin of Barrett's Esophagus

The Rise of Barrett's Metaplasia and Esophageal Adenocarcinoma

Barrett's esophagus was described nearly 60 years ago as an unusual growth of tissue that resembled small intestine epithelia in place of the normal squamous tissue of the esophagus. It is now clear that this "intestinal metaplasia" is triggered by gastroesophageal reflux disease (GERD), a seemly benign condition experienced by nearly 20% of individuals in Western populations. About 10% of those with GERD develop Barrett's metaplasia, and once formed this intestine-like growth does not regress even with pharmacological suppression of acid reflux. Significantly, Barrett's metaplasia confers an estimated 50–150-fold increase risk of developing esophageal adenocarcinoma, and therefore Barrett's is considered an essential precursor of this cancer. A remarkable if disturbing statistic of esophageal adenocarcinoma is its dramatic fivefold increased incidence in the past 60 years in developed countries. The other dismal statistic is the average 1-year life expectancy following diagnosis. Given that treatments for late stages of this disease are challenging and largely palliative, much effort has been focused on understanding the earlier, premalignant stages of these diseases for therapeutic opportunities.

Origins of Barrett's Metaplasia: The Debate

If the premalignant stages of esophageal adenocarcinoma represent the only tractable solution to this disease, it is essential to understand the origin of Barrett's metaplasia to develop new therapeutic strategies. However, the ontogeny of Barrett's metaplasia remains an intriguing mystery with various hypotheses involving *trans*-commitment of resident stem cells, migration from lower gastrointestinal sites, the reparative emergence of submucosal glands, dissemination from bone marrow, and opportunistic growth of residual embryonic cells pre-existing at gastroesophageal junction. However, the field has not reached a consensus on the very important question of the origin of Barrett's metaplasia, in large part due to holes in the various arguments that have prevented the rise of one concept over the other. Without such unity, it is difficult to decide on key elements to target in a therapeutic program designed to prevent the development of irreversible metaplasia.

Migration of Gastric Cardiac Epithelium

The most obvious potential source for Barrett's was the migration of gastric cardiac epithelium in a process of repairing gastroesophageal reflux-mediated damage to the adjacent esophageal epithelium. Some of the studies using canine model suggests the possibility of upward growth of gastric cardiac epithelium in the pathogenesis of Barrett's esophagus. In this study, the distal esophageal mucosa was denuded and allowed to regenerate in the presence of gastroesophageal reflux [3]. Later on, the observation of humans after esophagogastrostomy [4] also indicates that the distal esophageal mucosa sometimes regenerates with a columnar mucosa consistent with an upward growth of the junctional epithelium. However, Barrett's epithelium includes a variety of epithelial types and histological appearances that present as islands of columnar epithelium, which is distinct from the gastric cardia. These findings cannot be explained on the basis of proximal migration of cardiac epithelium alone. Gillen and colleagues [5] challenged the migration theory of Barrett's pathogenesis using a canine model. In their experiments, they denuded the distal esophagus mucosa with a ring of squamous epithelium left intact between the denuded area and stomach in order to prevent the migration of gastric epithelium of the cardia region. Following acid reflux, they observed the development of a columnar mucosa in distal esophagus, and thus this study supports that the columnar epithelium did not originate from the gastric cardiac epithelium but is metaplasia. Taken together, this otherwise convenient migration model of Barrett's esophagus has been challenged by experimental evidence and questioned by the absence of intestinal features in gastric cardiac epithelium. More complex models have been proposed since its demise.

Reparative Emergence of Submucosal Glands

Esophageal submucosal glands (ESMGs) and ducts have been proposed to serve as a potential source of progenitor cells that respond to esophageal injury [6, 7]. While Barrett's esophagus always originates at the GE junction, in the human esophagus, the ESMG distribution is heterogeneous, but, in general, similar concentrations of ESMGs are present in proximal and distal regions [8, 9]. Importantly, the stem cells of ESMG and ducts leading from ESMGs to surface epithelium express p63. This gene [10] encodes a p53-like transcription factor of which expression was specific to stem cells of stratified epithelia including the epidermis, esophagus, as well as mammary and prostate glands. In contrast, p63 expression is absent in the stem cells of Barrett's esophagus [11]. Together with the distinct cell types and histology between intestinal-like structures of Barrett's and submucosal gland, no convincing evidence supports the basal stem cells in ESMG are the cellular origin of Barrett's esophagus.

Colonization of Bone Marrow Stem Cells

Probably the most radical theory was the colonization of the acid-damaged esophagus by circulating, multipotent bone marrow stem cells. The evidence in favor of this model was based on the incorporation of cells into IM from genetically tagged bone marrow transplants. However, although bone marrow stem cells have been linked to mesoderm-derived tissues, their potential to form epithelial populations, typically of ectoderm or endoderm origins, has not been established. Moreover, the pattern of incorporation of bone marrow cells in Barrett's glands was not consistent with any role as progenitors to this metaplasia. It doesn't explain the site of origin of Barrett's esophagus exclusively at the gastrointestinal junction.

Transcommitment of Esophagus Squamous Cells

By far, overall, the dominant concept for the origin of Barrett's esophagus centers on the notion that acid reflux induces the esophageal squamous stem cells to switch their fate to generating columnar epithelia with intestinal characteristics [12]. The initial empirical basis for the transcommitment hypothesis was not from Barrett's esophagus at all but rather from a transgenic mouse model that yielded gastric IM. In brief, Sagano et al. generated a transgenic mouse in which caudal homeobox 2 (Cdx2), a gene implicated in intestinal cell differentiation, was expressed from a promoter active in gastric parietal cells [13]. These mice showed evidence of gastric IM. As Cdx2 expression was reported in precursors of both gastric and esophageal adenocarcinoma, these findings were extrapolated to explain the origins of Barrett's esophagus from esophageal squamous stem cells. Accordingly, multiple efforts expressed Cdx2 in murine esophagus and in human esophageal epithelia in vitro, although little evidence has emerged to support the concept that Cdx2 could "transcommit" esophageal cells to IM. The concept of stem cell transcommitment suggests a stem cell that normally gives rise to one set of differentiated cell types is altered to yield progeny with alternative cell fates. Attempts at transcommitting human esophageal epithelial cells to Barrett's esophagus have employed bile salts and low pH of gastric fluids, although again the effects were limited. Given the near-epidemic rates of Barrett's esophagus in Western populations, it might have been imagined that one of these treatments could coax esophageal stem cells to Barrett's esophagus. By way of counterexample, it was recently demonstrated that human airway stem cells readily transcommit to squamous metaplasia. Squamous metaplasia in the trachea and bronchi is tightly linked with a history of smoking and is thought to be a precursor to squamous carcinoma in the lung. However, it was unclear whether squamous metaplasia arose from rare, squamous stem cells among airway epithelial stem cells or from airway stem cells altered by carcinogen exposure. Using clonal lineages of tracheobronchial stem cells, Kumar et al. [14] showed that simple changes in growth conditions could direct 100% of these stem cells to

assume two different fates: airway epithelium, including ciliated and goblet cells, or stratified squamous differentiation. Interestingly, stem cells from squamous tissue such as skin, esophagus, and cervical epithelium have been analyzed for two decades, and yet no reports of such facile cell fate alterations have arisen. Thus, it seems that esophageal stem cells, as well as those of other squamous tissues, do not have an intrinsic capacity for transcommitting to columnar metaplasia that would explain the abundance of Barrett's cases. If acid-induced epithelial injury leads to squamous cell transcommitment and etiology of Barrett's metaplasia, it follows that acid suppression may prevent such metaplasia. However, no data exist to demonstrate prevention of BE using PPI therapy [15] nor antireflux procedure decreased the incidence of esophageal adenocarcinoma [16].

Enemies from Within: Residual Embryonic Cells at the Squamocolumnar *Junction*

The weaknesses in the above four models for the cell of origin of Barrett's esophagus and their limited predictive value signaled the need for additional models. The research using p63ko mouse model as an extreme damage model of the squamous epithelium in mouse proximal stomach showed that the metaplasia occurs in the proximal stomach of p63ko mouse broadly and it matches human Barrett's at the whole-genome gene expression level [17, 18]. The bioinformatics analysis from all other tissues in the gastrointestinal (GI) tract revealed that Barrett's esophagus was distinct from small intestine and, therefore, not a simple transcommitment to intestinal fate. Importantly, the cellular origin of Barrett's-like structures was examined by tracking back through embryogenesis in this mouse; it was apparent that even E14 p63ko embryos have a highly proliferative metaplasia, whereas the wild-type counterparts showed the expected, early squamous epithelium in the esophagus and proximal stomach. One day earlier, however, at E13, the culprit cells were identified as a simple columnar epithelium lining the proximal stomach and expressing metaplasia markers such as Car4 and Krt7. This line of cells along the basement membrane had to represent the "ground state" of the metaplasia in both the p63ko and the wild-type embryos, which of course begged the question as to why the wild-type embryos did not also develop metaplasia. The answer came from the analysis of the p63+ stem cells in the esophagus of the wild-type E13 embryos. These cells first appear as a small population of cells sequestered in the esophagus away from the Car4 cells lining the proximal stomach. One day later at E14, this p63+ population of squamous stem cells had greatly expanded in numbers and begun a posterior migration to and underneath the line of Car4 cells. In the process of undermining these Car4 cells, the p63+cells displaced the Car4 cells from basement membrane. These displaced Car4 cells showed considerably less proliferation than those more proximally distributed, which were still attached to the basement membrane, consistent with a wealth of studies on epithelial cell biology. This displacement phenomenon does not happen in the p63ko embryos because p63 is required for the

regenerative expansion of these squamous stem cells populations in the first place. Aside from the undermining process in wild-type embryos, which revealed why they do not develop metaplasia, it was the tracking of displaced Car4 cells through development that revealed the origins of Barrett's in the adult. In particular, following the Car4 cells in the wild-type embryos through E15-E18, Car4+ (Krt7+) cells ride above the stratifying squamous cells until they were sloughed off at E17. However, a group of these Car4/Krt7+ cells, designated here as residual embryonic cells (RECs), remained precisely at the squamocolumnar junction of E18 embryos. In contrast, p63-null embryos showed extensive development of a Krt7+ metaplasia without evidence for a squamous population of cells (Fig. 5.1). Even in adult mice, RECs were a consistent feature of this junction. If RECs were retained at the junction, it was possible that they represent a source of cells for the initiation of Barrett's esophagus. This hypothesis was tested by monitoring the activity of these Krt7+ cells in a mouse model in which the esophageal epithelium was damaged by the conditional expression of diphtheria toxin A subunit. Significantly, damage to the adjacent squamous tissue of the esophagus triggered a rapid expansion and anterior march of these Krt7 cells from the SCJ to the esophagus. Although collateral damage of other squamous tissues in the Krt14− driven Cre recombinase in this experiment precluded analysis beyond 10 days, this model provided support for the notion that RECs are the source of Barrett's esophagus (Fig. 5.2). Yamamoto et al. addressed the key question which is how predictive the mouse models were for human disease. Using a stem cell cloning technology, the authors cloned the multipotent stem cells of human Barrett's esophagus and showed that SOX9, a member of the SOX [*S*ry-related high-mobility group (HMG) b*ox*] family of HMG DNA-binding domain transcription factors, expresses in these stem cells. Furthermore, the Barrett's esophagus stem cells lack p63 expression, which distinguishes them from the stem cells of the esophageal squamous epithelium and submucosal glands [11]. Taken together, these data usher in the concept that a unique, prexisting population of junctional cells are the immediate source of Barrett's esophagus and thus represent a novel target of prospective therapies to eliminate the Barrett's stem cell and the risk they pose for progression to dysplasia and lethal esophageal adenocarcinoma. (Fig. 5.3)

The discovery of RECs as the cellular origin of Barrett's esophagus has been extended to the analysis of human papilloma virus (HPV)-induced cervical cancer [17]. It is known, for instance, that HPV infects the cells of the entire cervix, though it has been unclear why cervical cancers always arise in the so-called transition zone between the cervix and the endocervical epithelium. A detailed analysis of this junction as performed and identified a discrete population of cells specifically expressing many of the genes that were found in Barrett's intestinal metaplasia. Furthermore, cryo- or electro-ablation of this population of cells eliminates them permanently, and the procedure is also thought to remove the risk for HPV-induced cervical carcinoma. Thus overall this work solved the mystery as to why cancers in the cervix are confined to the transition zone and provides support for ablative technologies for preventing both cervical carcinoma and upper GI tract cancers though eliminating metaplastic lesions.

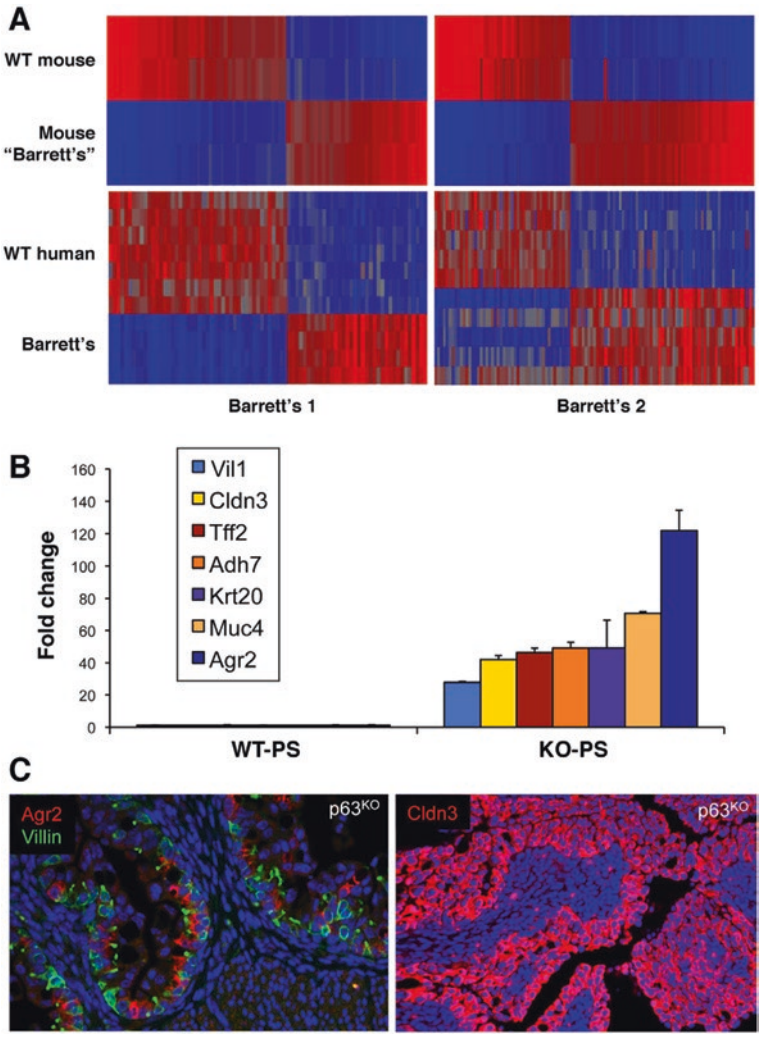

Fig. 5.1 Bioinformatics linking p63ko metaplasia with human Barrett's. (**a**) Heatmaps of whole-genome expression microarray data comparing differentially expressed genes in wild-type and p63ko proximal stomach with normal patient esophagus and those with Barrett's esophagus. (**b**) Histogram of fold changes of key Barrett's markers in proximal stomach of wild-type versus p63ko E18 embryos. (**c**) Immunofluorescence micrograph showing expression of Agr2, Cldn3, and villin, key markers of human Barrett's esophagus, in metaplasia of p63ko mouse. Figure 5.1 is reprinted with permission from Xian et al. [19]

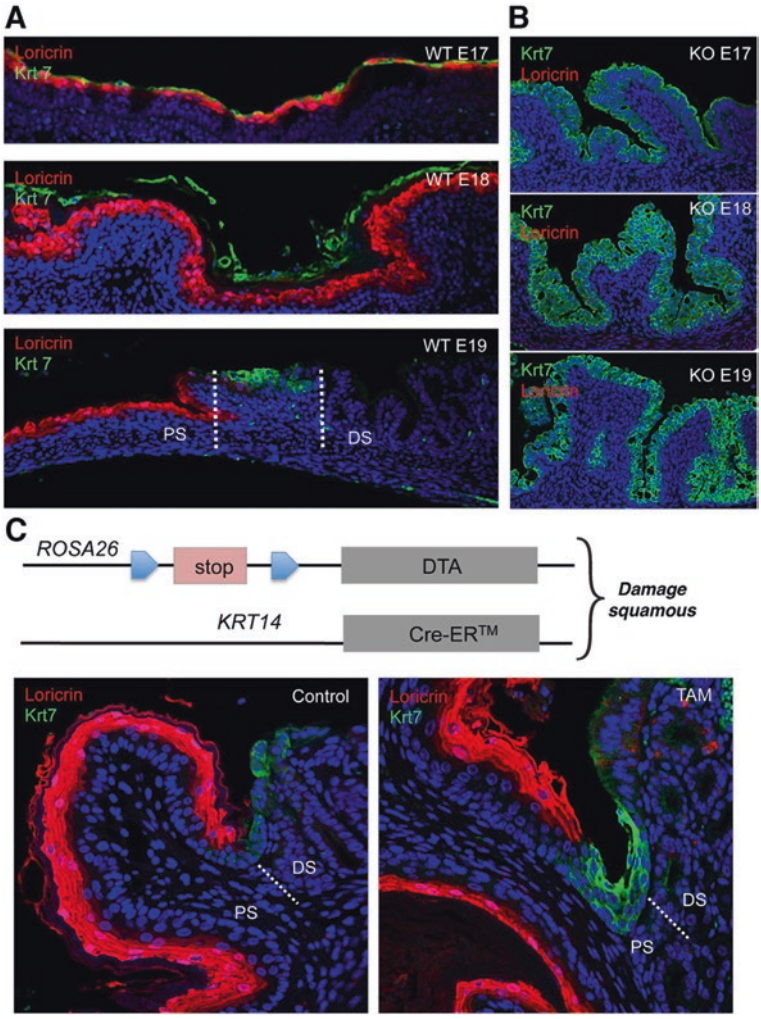

Fig. 5.2 Dynamics of RECs in wild-type and p63ko mice. (**a**) Co-labeling of squamous (antiloricrin, red) and RECs (anti-Krt7, green) in wild-type embryos showing suprasquamous disposition of RECs at E17, sloughing of RECs at E18, and retention of RECs at the squamocolumnar junction at E19. (**b**) Corresponding labeling of loricrin and Krt7 in p63ko embryos showing the absence of squamous cells and the development of a Barrett's-like metaplasia in late embryogenesis. (**c**) Schematic of mouse strain in which diphtheria toxin A subunit is conditionally expressed in Krt14-expressing squamous stem cells. Left, section through the squamocolumnar junction staining with anti-loricrin showing squamous tissue and anti-Krt7 showing junctional RECs. Right, section through junction of mouse after 7-day expression of DTA in squamous tissue showing redistribution of Krt7 cells toward squamous epithelium. Figure 5.2 is reprinted with permission from Xian et al. [19]

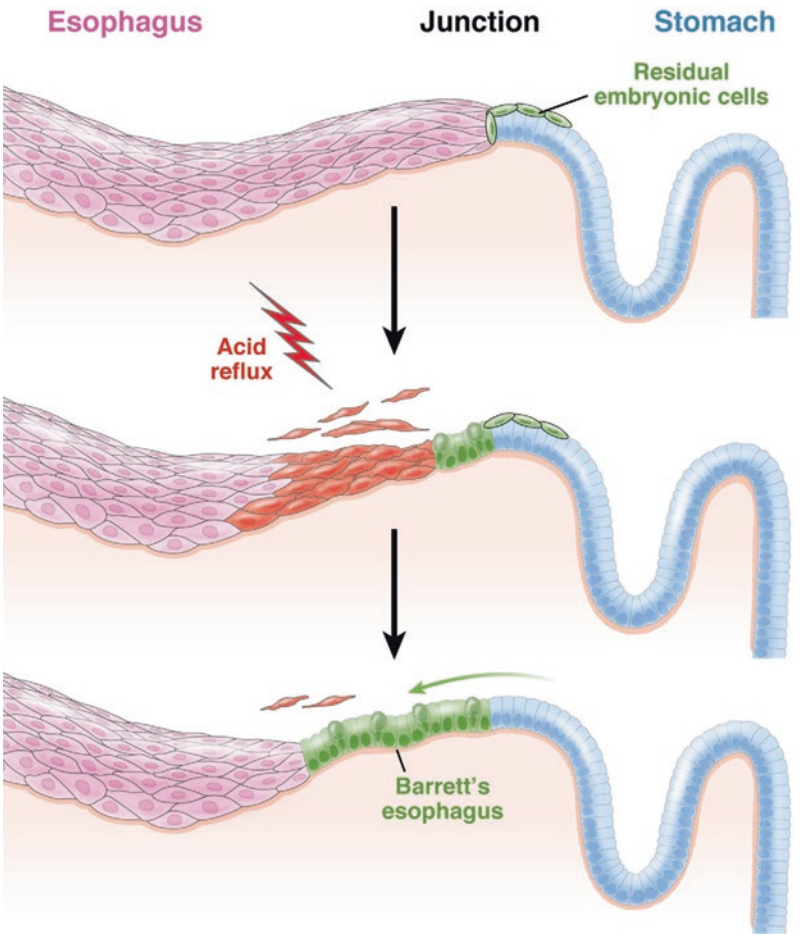

Fig. 5.3 Opportunistic expansion of RECs in response to gastroesophageal reflux disease. RECs are shown at the junction of the esophagus and stomach in normal adults and expand along the basement membrane vacated by the reflux-induced death of squamous esophageal cells to form Barrett's esophagus. Figure 5.3 is reprinted with permission from Xian et al. [19]

Targeting Stem Cells in Barrett's Esophagus

Barrett's mucosa is metaplastic columnar epithelium that has replaced the native squamous epithelium, thereby providing greater resistance to the effects of gastroesophageal reflux. In the meanwhile, it is also believed to serve as precursor lesion of esophagus adenocarcinoma based on clinical studies and basic research [20]. Barrett's is thought to predate the appearance of adenocarcinoma by one or more decades and overall progresses to esophageal adenocarcinoma (EAC) at a rate of 0.2–1% per year [21]. EAC is a highly lethal cancer whose incidence has quadrupled in the past four decades [22–24]. Efforts at chemotherapy and surgical

resection have not appreciably altered survival rates for this cancer, and therefore much hope is placed on early detection and therapeutic eradication of advanced stages of Barrett's esophagus before it can progress to EAC [25–27]. Since then efforts to preempt the progression of dysplastic Barrett's to adenocarcinoma employ non-specific technologies such as radiofrequency ablation to remove surface epithelia harboring this intestinal metaplasia [28, 29]. While remarkably effective especially in focused centers, recurrences of Barrett's and dysplasia, as well as the emergence of EAC, remain problematic [30–32]. These recurrences may be due to the survival of hypothetical Barrett's stem cells in post-ablation mucosa, suggesting potential advantages of specifically targeting this stem cell population as part of a broader therapeutic approach to reducing rates of EAC. The existence of stem cells underlying the regenerative growth of Barrett's esophagus has recently been established [11]. The existence of stem cells from normal columnar epithelia such as intestine has been firmly demonstrated by multiple in vivo and in vitro studies [33]. Especially recently there has been a new technology developed to capture and maintain ground-state stem cells in culture in their most immature form, which allow us to study their physiological and pathological roles in great detail [34] (Fig. 5.4). Using this technology, the ground-state stem cells from patient-matched, endoscopic biopsies of esophagus, Barrett's, and stomach were isolated and representative, single-cell-derived clonal lines or "pedigrees" from each were established.

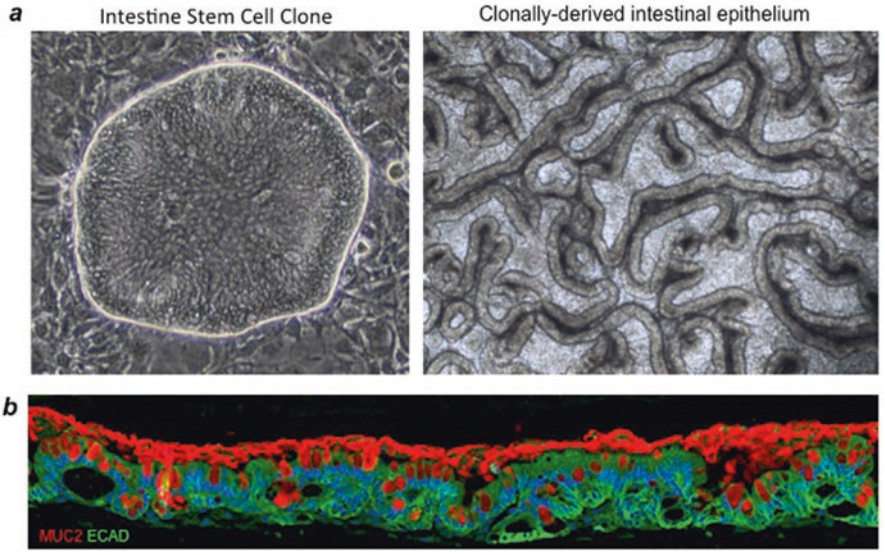

Fig. 5.4 Cloning adult stem cells from human Intestine. (**a**) *Left panel*, representative image of single-cell-derived intestinal stem cell colony on irradiated 3T3 feeder cells. *Right panel*, in vitro intestine-like structures in air-liquid-interface differentiation model. (**b**) Cross section of ALI structures showing stem cells differentiated into goblet cells that express mucin 2 detected by immunofluorescence (red). E-cadherin stained all epithelial cells (green) and Dapi-stained nuclei (blue)

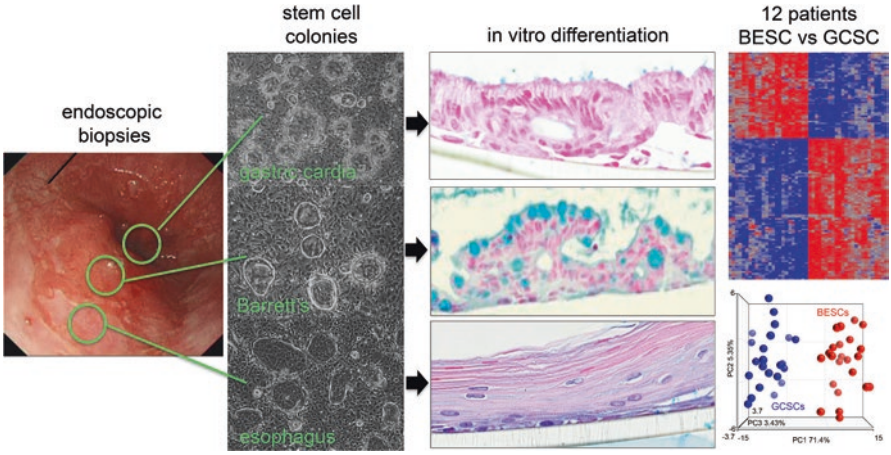

Fig. 5.5 Cloning Barrett's stem cells from endoscopic biopsies. One millimeter endoscopic biopsies were selected from the esophagus, Barrett's, and gastric epithelium and processed for generating stem cell colonies. Single-cell-derived "pedigrees" were expanded and differentiated in vitro to yield the indicated three-dimensional epithelia. Patient-matched stem cells from Barrett's (BESC) and gastric cardia (GCSC) yield a consistent differential gene expression pattern that can also be seen in principal component analysis of whole-genome expression. Figure 5.5 is reprinted with permission from Xian and Mckeon [35]

Importantly these pedigrees from the esophagus, stomach, and Barrett's possess all of the canonical features of stem cells including (1) long-term self-renewal, (2) multipotent differentiation, and (3) absolute commitment to the respective lineages from which they were derived. Extensive analyses of the esophageal, stomach, and Barrett's stem cells from a cohort of Barrett's cases, as well as the cognate epithelia derived from them, demonstrated that Barrett's stem cells are distinct from those of the esophagus or the stomach suggesting Barrett's as a unique developmental entity with its own stem cells (Fig. 5.5).

These patient-matched series of stem cells from Barrett's cases also allowed a clonal analysis of the genomic changes each had undergone in these patients. Structural variations in the form of copy number variation (CNV) and exome sequencing for single nucleotide variation (SNV) revealed a spectrum of changes in the Barrett's stem cells across this patient cohort. Most patients showed stereotyped sets of mono- or biallelic deletions at fragile sites impacting genes such as the INC4A locus including p16, and FHIT and WWOX, and in general have nearly the full complement of deletions as reported for the typical esophageal adenocarcinoma (EAC) [36]. Several of the Barrett's cases also showed more ominous amplifications of proto-oncogenes (c-Myc, Myb) and receptor tyrosine kinases (FGFR), as well as mutations in p53 and other genes mutated in EAC. Finally, a third of the Barrett's stem cells of these cases showed, like their counterparts in the esophagus stomach, little in the way of CNV or SNV, suggesting that clinically defined Barrett's can arise without driver mutations or alterations in any form. The ability to Barrett's

epithelium to establish without a protracted phase of mutational maturation is consistent with the rapid appearance of a Barrett's-like epithelium in our p63-null mice and in the overall notion that Barrett's arises from a preexisting population of cells at the gastroesophageal junction.

If the long-term regenerative growth of Barrett's is indeed dependent on a stem cell distinct from those that support the local esophageal and gastric epithelium, these differences should render Barrett's stem cells selectively targetable. Present standard-of-care for dysplastic Barrett's relies on mucosal resections and physical ablation via radiofrequency ablation and cryogenics, which are expensive, time-consuming, and not without morbidities including strictures and recurrent disease [37]. The adaptation of cohorts of patient-matched stem cells of Barrett's and local epithelia may enable moderate and even high-throughput testing of small molecule, biologics, and even immunological approaches to the selective eradication of the Barrett's stem cells in a manner that would spare those of normal epithelia to take up the slack. It can be imagined that these patient-matched stem cells can be used in a high-throughput screening configuration and are well along the road to identifying compounds that selectively compromise Barrett's stem cells of any mutational profile as well as others that selectively kill Barrett's stem cells with advanced profiles [35]; (Fig. 5.6). We anticipate that such studies, if validated both in vitro and in vivo, could yield small molecules and biologics that could be used in combination with endoscopic mucosal resections and physical ablation modalities to improve patient care and outcome. The patient-specific esophageal stem cells that are cultured in vitro are valuable resources for autologous transplantation to repair distal esophagus following the eradication of Barrett's epithelium and its stem cells. Thus, the combination of chemoprevention therapy and regenerative medicine may lead to the

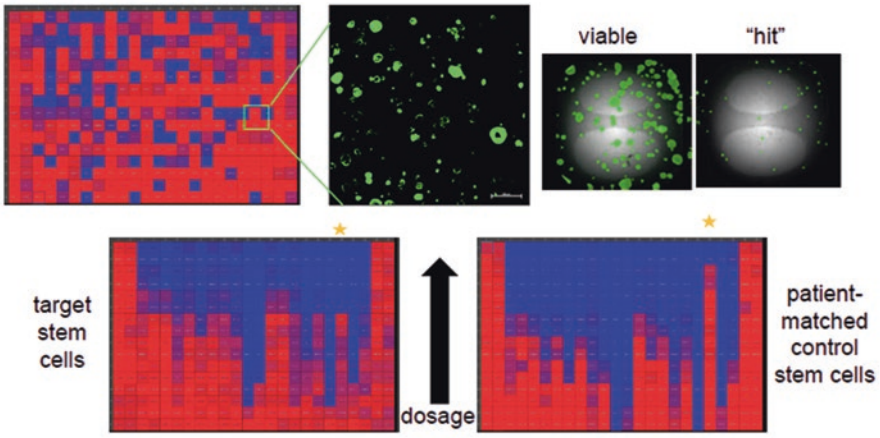

Fig. 5.6 Adaptation of patient-matched Barrett's and gastric stem cells to screening formats. Green fluorescent protein-labeled Barrett's esophagus and gastric cardia stem cells in 384-well format screened with known and experimental drugs. Bottom, dose-response analysis to identify potential therapeutic windows. Figure 5.6 is reprinted with permission from Xian and Mckeon [35]

extinction of this precancerous lesion and subsequently dramatic reduction of the incidence of esophageal adenocarcinoma. This requires the adult stem cells cloning technology being widely adapted to clone and establish Barrett's and esophageal stem cell lines and performance of high-throughput screening on a wide range of Barrett's stem cells with a spectrum of mutational profile, using small animal model such as canine model to further test the hypothesis, cooperation, and coordination of various clinical centers for clinical trials. Nevertheless, demonstrations of Barrett's esophagus and possibly all intestinal metaplasia as developmentally unique entity with its own stem cells and cancer can initiate through opportunistic growth of these metaplastic stem cells without driver mutation are important advances in cancer biology. It begins a new era of the research in the management of precancerous lesion and overall strategies of cancer prevention.

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Chapter 6
Pluripotent Stem Cell Heterogeneity

Yohei Hayashi, Kiyoshi Ohnuma, and Miho K. Furue

Abstract Pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, show heterogeneity with respect to their pluripotency, self-renewal ability, and other traits. PSC heterogeneity may exist among cell lines, among cells within a line, and among temporal states of individual cells. Both genetic and epigenetic factors can cause heterogeneity among cell lines. Heterogeneity among cells within a cell line may arise during long-term culturing even when a PSC cell line is derived from a single cell. Moreover, the expression levels of genes and proteins in PSCs fluctuate continuously at a frequency ranging from a few hours to a few days. Such heterogeneity decreases the reproducibility of research. Thus, methods related to the detection, reduction, and control of heterogeneity in experiments involving human PSCs need to be developed. Further, the presupposition that PSCs are highly heterogeneous should be taken into account by all researchers not only when they plan their own studies but also when they review the studies of other researchers in this field.

Author contributions: Sections "Genetic Variability Among PSC Types," "Epigenetic Differences Among PSC Lines" and "Heterogeneity Among Each Cell in a Cell Line," (YH); Sections "Introduction," "Temporal Fluctuation of PSCs," and "Perspective and Conclusion" (KO), Sections "Imaging Methods for Detecting PSC Heterogeneity" and "Quality control of heterogeneous hPSCs" (MKF).

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Introduction

In this chapter, we present six points of view on pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Fig. 6.1). Since a PSC may potentially develop into any type of cell (pluripotency) and proliferate limitlessly (self-renewal), PSCs are a promising source for gaining an understanding of early embryonic development and applying such understanding to regenerative medicine and drug discovery. However, in this regard, heterogeneity of PSCs is an important issue that remains unresolved and therefore in need of further research.

Many studies have indicated that PSCs may be heterogeneous. Although PSCs share a similar undifferentiated stem cell phenotype, each line has slightly different properties, such as the propensity for differentiation. Such line-to-line heterogeneity stems from not only the genetic background of the cells (Section "Genetic Variability Among PSC Types") but also from epigenetic modifications (Section "Epigenetic Differences Among PSC Lines"). Moreover, heterogeneity also exists among cells within a PSC line (Section "Heterogeneity Among Each Cell in a Cell Line"). Cell-to-cell heterogeneity is also caused by dynamic (temporal) changes in gene expression levels in a cell (Section "Temporal Fluctuation of PSCs"). Thus, PSC heterogeneity exists among lines, among cells within a line, and among temporal states in individual cells.

A difficulty arises in that PSC heterogeneity may lead to poor reproducibility in cell quality or cell processing, especially in the case of human PSCs (hPSCs) includ-

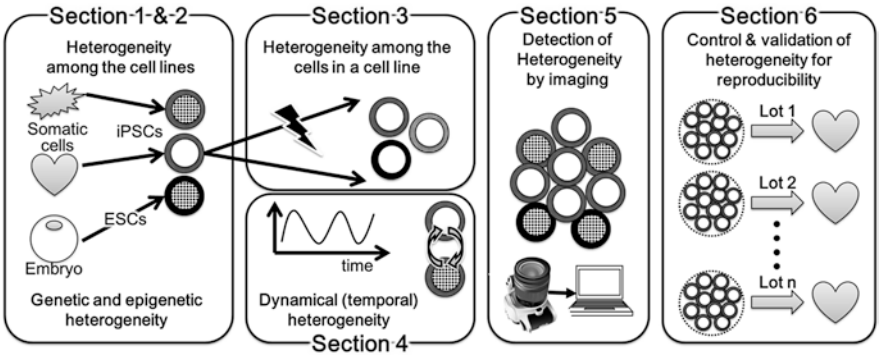

Fig. 6.1 Schematic representation of PSC heterogeneity

ing human ESCs (hESCs) and human iPSCs (hiPSCs). In order to utilize hPSCs as a tool in regenerative medicine or pharmaceutical research, stable and robust culturing is required. Many techniques have been proposed to overcome the issue of reproducibility. Microscopic imaging techniques are the most powerful methods that are available to detect hPSC heterogeneity (Section "Imaging Methods for Detecting PSC Heterogeneity"). Moreover, validation, standardization, and regulation of hPSC heterogeneity should be achieved prior to their implementation as tools in regenerative medicine and drug screening (Section "Quality Control of Heterogeneous hPSCs").

Genetic Variability Among PSC Types

The causes of variability among PSC types may be categorized as (i) genetic and (ii) epigenetic factors [1]. Reasons for heterogeneity among PSC types are summarized (Fig. 6.2). Each reason is further explained below.

Donor Variation (Differences in Genetic Backgrounds)

Normally, hESCs and other embryo-derived PSCs may be obtained from any type of tissue of any type of individual, which allows for the generation of large quantities of genetically diverse cells. Since genetic diversity forms the basis of drug efficacy, toxicity, and adverse reactions, care must be taken in collecting PSCs from variable genetic backgrounds for use in drug development [2]. Genetic variations can affect stem cell behavior in vitro via variabilities in gene expression and in signaling pathways [1]. One study demonstrated that the donor genetic background accounted for more functional differences between hiPSCs, than either the donor cell type or the derivation method [3]. The authors compared hepatic differentiation

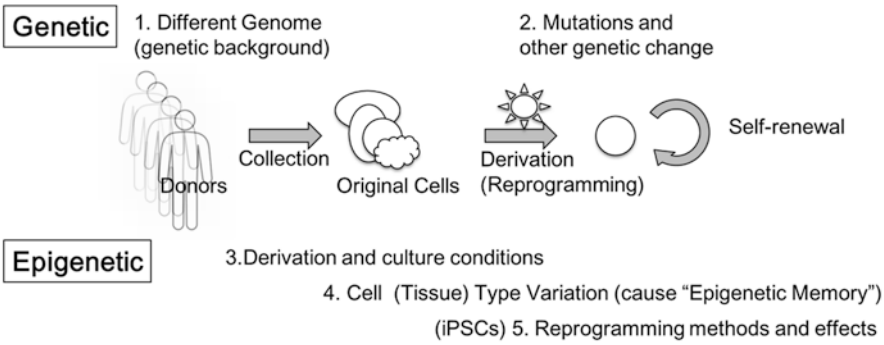

Fig. 6.2 The causes of heterogeneity among PSC types

efficiency among hiPSCs from peripheral blood and dermal fibroblasts from the same donor or different donors and reported that variations in hepatic differentiation were largely attributable to donor differences, rather than to the original cell type. These findings suggested that genetic variation among the normal population may contribute substantially to the variability observed in PSC behavior, compared to genetic mutations acquired during the reprogramming or to long-term culturing of PSCs.

Mutations and Other Genetic Changes During Derivation of PSCs

Two types of mutations and other genetic changes may occur during derivation of PSCs. One is clonal selection of existing somatic mutations, and the other is the occurrence of de novo mutations during PSC derivation.

Clonal Selection of Existing Somatic Mutations

Since each PSC line is derived from a single cell or a few founder cells, clonal selection of existing somatic mutations causes cell line variability, even among cells from the same donor. Several studies reported that much of the genetic variation found among PSCs was due to pre-existing variation in the cloned, original somatic cells [4–9]. PSC clones carried existing somatic mutations or largely random genetic changes. However, some pre-existing genetic variation can either facilitate or inhibit reprogramming of iPSCs, which are preferentially propagated by selective advantages or disadvantages [10]. Although, to our knowledge, no proof of preferential propagation has been obtained from a single donor carrying mosaic genetic variations, some mutations have been reported to affect iPSC derivation [11]. Mutations, which cause altered DNA repair (e.g., ataxia-telangiectasia, Fanconi anemia, and DNA ligase IV (LIG4) syndrome) [12–14], premature aging (e.g., Hutchinson-Gilford progeria syndrome and Néstor-Guillermo progeria syndrome) [15, 16], altered telomere homeostasis (e.g., dyskeratosis congenita) [17–19], mitochondrial respiratory dysfunction [20–22], chromosomal abnormalities [23, 24], and fibrodysplasia ossificans progressiva [25, 26], have all been shown to affect efficiency of reprogramming somatic cells into iPSCs.

By using this process, some researchers have successfully derived both normal and mutant PSCs from the same donor carrying mosaic mutations. NLRP3-mutant and nonmutant iPSC lines were generated from two chronic infantile neurologic cutaneous and articular (CINCA) syndrome patients with somatic mosaicism [27]. Nonmutant iPSCs may function as natural isogenic control cells in disease modeling and drug development. They found that mutant cells were predominantly responsible for pathogenesis because only mutant iPSC-derived macrophage cells

showed the disease-relevant phenotype. These results indicated that mosaicism in a donor may be useful for analyzing somatic mosaicism using hiPSC technology.

De Novo Mutations During Derivation of PSCs

Although the studies mentioned above indicated that a majority of PSC mutations pre-existed in founder somatic cells, some studies reported that the rate of de novo mutations was also increased during iPSC derivation [28, 29]. One study compared point mutation profiles between ESCs and iPSCs under closely identical conditions from the same mouse strain, and demonstrated that the rate of point mutations in iPSCs was much higher than that in ESCs [29]. A recent study reported that these mutations preferentially occurred in structurally condensed lamina-associated heterochromatic domains, but were underrepresented in protein-coding genes and in open chromatin regions, including transcription factor binding sites [30]. Other reports describing the effect of genetic changes in iPSC derivation use thorough genomic analysis [31, 32], and the genetic variants were generally benign in contrast to disease-causing single-nucleotide polymorphisms (SNPs). However, a harmful de novo mutation may have a critical effect on the application of PSCs in regenerative medicine. A fine case in point is the first clinical trial using human iPSC-derived cells, where one patient could not undergo transplantation due to concerns regarding genetic changes that had occurred in the iPSCs [33].

Although the molecular mechanism by which reprogramming-induced mutations are introduced remains to be elucidated, mutation signature analysis indicated that oxidative stress associated with reprogramming may be a likely cause of point mutations [30, 31]. There is an urgent need for methods which may be used to decrease the rate of de novo mutations during the derivation and prolonged culturing of PSCs for use in clinical applications.

Epigenetic Differences Among PSC Lines

Types of PSCs

PSCs may be obtained from different sources. Two types of hPSCs are widely used: hESCs derived from the inner cell mass (ICM) of the blastocyst [34] and hiPSCs generated by introducing key transcription factors into somatic cells [35, 36]. There is much disagreement regarding the differences between hESCs and hiPSCs. However, both hESCs and successfully reprogrammed hiPSCs generally have similar gene expression patterns, differentiation potentials, and epigenetic signatures [37–39].

The Effect of Culture Conditions (Intracellular Signal State)

Although conventional hPSCs in bFGF-dependent culture conditions have been widely used, an alternative pluripotent state with different signal dependence has attracted much attention. The derivation of mouse epiblast stem cells (mEpiSCs) indicated that pluripotent cells may exhibit developmental stages [40, 41]. mESCs derived from the preimplantation inner cell mass represent the "naïve" stage, and mEpiSCs derived from the post-implantation epiblast represent the "primed" stage. mESC self-renewal has been achieved through exposure to the leukemia inhibitory factor (LIF) [42–44]. Addition of extracellular signal-regulated kinase (MEK) and glycogen synthase kinase 3 (GSK3) inhibitors (2i) in defined medium allowed the cells to attain a homogeneous ground state [45]. On the other hand, mEpiSCs are cultured in a medium containing bFGF and activin/nodal/TGF, which is similar to conventional human PSC culture medium. Although some early studies used LIF in feeder-free media for undifferentiated hESCs [46, 47], LIF and its downstream STAT3 signaling pathway were shown to be dispensable for maintaining primed human and primate PSC self-renewal in several independent studies [48–50]. Thus, currently, defined hPSC culture media usually do not contain LIF; however, it was reported that media for naïve hPSCs contained LIF and not bFGF [51–54]. STAT3 activation was reported to be crucial in reprogramming human PSCs to the naïve state [55]. A recent study also showed that LIF promoted X chromosome reactivation, which was one of the characteristics of naïve pluripotency in female hPSCs [56]. These studies indicated that naïve hPSCs required different culture conditions and signaling activation status compared to primed hPSCs. Based on their similarities in cytokine requirement and signal dependence, conventional hPSCs represent a primed state, similar to mEpiSCs [57]. Differences in the cytokine requirements and signal dependency under PSC conditions lead to variation in PSC behavior. It is commonly observed that differentiation efficiency obtained by an established method is dependent on the specific maintenance of the culture conditions of PSCs.

Recent studies highlighting different culture conditions demonstrated the different developmental potentials of PSCs. A study demonstrated the generation of mouse expanded potential stem cells, which can contribute both to the embryo proper and to the trophectoderm lineages in a chimeric mouse production assay [58].

Cell (Tissue)-Type Variation and "Epigenetic Memory"

Somatic cell nuclear transfer (NT) and transcription factor-based reprogramming can generate PSCs, which are designated as NT-ESCs and iPSCs. Through different mechanisms and kinetics, these two reprogramming methods mostly reset genomic methylation, an epigenetic modification of DNA that influences gene expression. The resulting PSCs may have different properties in their epigenetic status. Many studies have reported that iPSCs derived from adult tissues harbor residual

epigenetic characteristics of their somatic tissue of origin, which causes their preferential differentiation propensity toward lineages related to the donor cell while restricting alternative cell fates [59, 60]. Such "epigenetic memory" is harmful in terms of biased differentiation properties; however, a skewed differentiation potential may be advantageous in certain types of research and clinical applications. One study demonstrated that pancreatic beta-cell-derived iPSCs maintained an open chromatin structure at key beta-cell genes, together with a unique DNA methylation signature that distinguishes them from other PSCs. These iPSCs also demonstrated an increased ability to differentiate into insulin-producing cells both in vitro and in vivo, compared with other PSCs [61]. "Epigenetic memory" of the donor tissue could be reset by differentiation and serial reprogramming via treatment with chromatin-modifying drugs [59] or telomerase overexpression [62].

Reprogramming Methods

It is believed that different reprogramming methods may give rise to variable PSCs in terms of their epigenetic status, pluripotency, and other functionalities. Using mouse PSCs, one study demonstrated that differentiation and methylation of NT-ESCs were more similar to conventional ESCs than were iPSCs. Therefore, the authors proposed that NT was more effective in establishing the ground state of pluripotency than iPSC reprogramming, which could leave an epigenetic memory of the tissue of origin that may influence the results of directed differentiation [59]. By contrast, one study demonstrated that differentiated cells derived from isogenic human iPSCs and NT-ESCs showed comparable lineage gene expression, cellular heterogeneity, physiological properties, and metabolic functions [63]. Genome-wide transcriptome and DNA methylome analysis indicated that iPSC-derived cardiomyocytes and endothelial cells and iPSC-ECs were similar to isogenic NT-ESC counterparts. Although iPSCs and NT-ESCs shared the same nuclear DNA and carried different sources of mitochondrial DNA, they claimed that molecular and functional characteristics of cells differentiated from PSCs are primarily attributed to genetic composition rather than the reprogramming method.

In order to avoid unnecessary misunderstandings stemming from cell line heterogeneity, standardized PSC lines are used as a partial solution for general research purposes. Also, using as many cell lines as possible is crucial to achieve plausible results. However, for rare diseases, it is sometimes hard to generate sufficient PSC lines. In these cases, using isogenic control cells to neutralize the genetic background effect may be effective in ensuring that the cellular phenotypes stem from the mutation. Our study also demonstrated that variations in hepatocyte differentiation efficiency could be predicted by studying the gene expression profile of undifferentiated hPSCs [64]. Development of such prediction methods may be crucial for avoiding misinterpretations caused by cell line heterogeneity.

Heterogeneity Among Each Cell in a Cell Line

Heterogeneity among cells in a cell line may be acquired during long-term culturing even when a PSC cell line is derived from a single cell (Fig. 6.3). In detecting cell-to-cell variation and heterogeneity, single-cell genome, epigenome, and transcriptome sequencing technologies serve as powerful tools to dissect comprehensive heterogeneity and to identify distinct characteristics, even within a PSC line in the same dish [65].

Genetic Heterogeneity Among Each Cell in a PSC Line

Chromosomal and Subchromosomal Changes

The International Stem Cell Initiative (ISCI) analyzed 125 hESC lines and 11 hiPSC lines, from 38 laboratories worldwide, for genetic changes occurring during culture [66]. In this study, most lines remained karyotypically normal between early and late passages, but some lines tended to acquire karyotypic changes after prolonged culture, commonly affecting chromosomes 1, 12, 17, and 20. Subchromosomal structural variants also appeared sporadically. No common variants related to culture were observed on chromosomes 1, 12, and 17, but a minimal amplicon in chromosome 20q11.21 occurred in >20% of the lines. In the following study, PSC lines containing this amplicon were shown to have higher population doubling rates, attributable to enhanced cell survival through resistance to apoptosis [67]. Overexpression of BCL2L1 (BCL-XL isoform), with a locus situated within the minimal amplicon, provides control cells with growth characteristics similar to those of CNV-containing cells, whereas inhibition of BCL-XL suppresses the growth advantage of CNV cells, establishing BCL2L1 as a driver mutation.

Other studies also reported that the karyotypic heterogeneity generated by mosaic aneuploidy may contribute to the reported functional and phenotypic heterogeneity of hPSCs lines, as well as their therapeutic efficacy and safety following transplantation [68, 69].

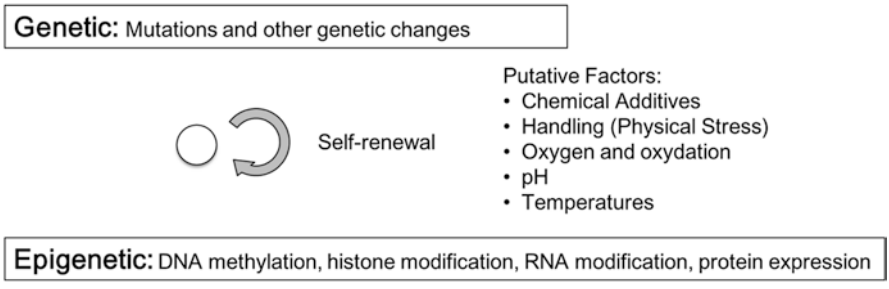

Fig. 6.3 Factors influencing heterogeneity of PSCs among each cell in a cell

Copy Number Variations (CNVs)

During derivation and maintenance culture of PSCs, subtle changes in genomic DNA may occur. One study demonstrated that 17 different hESC lines maintained in different laboratories identified 843 CNVs of 50 kb–3 Mb in size [70]. Twenty-four percent of the loss of heterozygosity (LOH) sites and 66% of the CNVs changed in culture between early and late passages of the same line. Thirty percent of the genes detected within CNV sites had altered expression compared to samples with normal copy number states, of which >44% were functionally linked to cancer. These results suggested the importance of monitoring the genomic DNA status during PSC culturing for research and clinical purposes.

Epigenetic Heterogeneity Among Each Cell in a Cell Line

Heterogeneity of DNA Methylation Among Each Cell

A hiPSC line comprises a heterogeneous population characterized by variable levels of aberrant DNA methylation [71]. These aberrations are induced during somatic cell reprogramming, and their levels are associated with the type of hiPSC source cell. Heterogeneity of DNA methylation status in the hiPSC population was reduced during prolonged culture to a level similar to that of hESCs. The ISCI reported that, in a large scale analysis using mainly hESCs, DNA methylation patterns changed haphazardly with no link to the time in culture [66].

The Effect of Cell Cycle Stages on PSC Behaviors

Undifferentiated PSCs display an unusual mode of cell cycle regulation, with shorter G1 and G2 phases. When individual PSCs are exposed to differentiation stimuli, they remodel the cell cycle so that the length of G1 and overall cell division times increase. Heterogeneous responses of individual stem cells to pro-differentiation signals result in asynchronous differentiation. The reason for different cells in the same culture responding in a nonuniform manner to identical exogenous signals remains unclear. Recent studies demonstrated that the cell cycle position may directly influence lineage specification and suggested that cells in G1 were uniquely "poised" to undergo cell specification [72, 73]. G1 cells served as a "Differentiation Induction Point," which may explain the heterogeneity of stem cell cultures.

Temporal Fluctuation of PSCs

PSCs are not temporally stable and fluctuate between multiple states. Although the cell lines are stable, the expression levels of PSC-related genes and proteins fluctuate dynamically at a frequency ranging from a few hours to a few days (Fig. 6.1).

Since the fluctuation does not specifically occur in vitro but may also occur in vivo, its dynamics are considered to be closely related to maintaining the "stemness" of the cells during embryonic development. In this section, we introduce PSC heterogeneity based on temporal expression dynamics.

Temporal Fluctuation of PSC-Related Gene Expression

Temporal fluctuation of Nanog is a well-known phenomenon. Nanog is a homeobox protein, which is thought to be one of the most important transcription factors for maintaining pluripotency in PSCs including ES and iPS cells of human and other animals [74–76]. Hence, it was believed that all PSCs may homogeneously express a high level of Nanog as well as other PSC-related transcription factors. However, it was shown that Nanog of mouse and human PSCs displayed mosaic expression, although both high- and low-Nanog cells expressed high levels of other PSC-related transcription factors [77, 78]. Later, it was shown that low-Nanog cells are not a stable subpopulation. Sorting and culturing of low-Nanog cells resulted in the production of high-Nanog cells eventually [79]. Gene expression tracking techniques using single molecule in situ fluorescence hybridization also showed that the gene expression of estrogen-related receptor beta (*Esrrb*), which is co-expressed with Nanog, also changes with time [80, 81]. Moreover, time-lapse imaging of the Nanog fluorescent protein fusion ESC line showed that Nanog expression may fluctuate over several generations [82].

Other PSC-related genes such as *Rex1*, *Hes1*, *Stella*, and *Zscan4* are also known to fluctuate temporally [83–85]. Similar to *Nanog*, all these genes fluctuate temporally, except *Zscan4*. Zinc finger and SCAN domain containing 4 (ZSCAN4), which has functions related to telomere elongation and genomic stability, is specifically expressed in the two-cell stage mouse embryo and in ESCs [86]. The expression of *Zscan4* is intermittent; it is expressed in only 5% of the population at a time but in all cells eventually [87–89]. All these reports indicated that expression of many PSC-related genes is not stable but fluctuates temporally during the maintenance of their population.

Temporal Expression May Be Essential for Maintaining Pluripotency

The question may arise as to whether temporal fluctuation is an in vitro-specific artifact. The inner cell mass of the blastocyst is the origin of mouse and human ESCs. Although mouse PSCs share the same properties with the inner cell mass, human and monkey PSCs share the same properties with the epiblast, which arises from the inner cell mass [41]. It is reported that Nanog and other PSC-related gene

expression in the mouse inner cell mass and monkey epiblast may not be homogeneous but mosaic [77, 90, 91]. Since it is well-known that the fate of cells in the inner cell mass remains undetermined and that the cells have powers of regulation, mosaic expression may not be stable but changeable. Thus, although there is no direct evidence that PSC-related gene expression temporally fluctuates in vivo, fluctuation may not be an in vitro-specific artifact.

Issues of whether all main genes corresponding to PSCs temporally fluctuate, as well as whether all fluctuating genes correspond to pluripotency, may need to be clarified. *Oct3/4* (*Pou5f1*), which is a master gene of pluripotency similar to Nanog, is known to have a rather stable expression level in mouse and human PSCs [82, 92]. *Zscan4*, not showing any correlation with *Rex1*, is thought to be correlated with telomere shortening, irrespective of the pluripotency [89]. Thus, temporal fluctuation of these genes may closely be correlated with PSC population maintenance. However, it is not always correlated to the maintenance of pluripotency.

Two hypotheses have been suggested to explain why temporal fluctuation is displayed by so many PSC-related genes. The first is that fluctuation is required for preparing subpopulations that are origins of multiple lineages. Reportedly, a gene being expressed at a high level at a specific time indicates its readiness for differentiation into a specific lineage [84, 93–95]. For example, mouse ESCs expressing low and high levels of *Hes1* tended to differentiate into neural and mesodermal cells, respectively [84]. The second hypothesis is that higher "flexibility" is required to keep the cells in a state of larger "stemness" (pluripotency). Since there are so many genes that temporally fluctuate, the networks of these dynamics should be considered to understand the mechanisms involved. Many reports indicated that fluctuation is essential for maintaining pluripotency and that gene network dynamics should be considered to understand the complex dynamics [96–98]. These cells are able to develop into all types of cells, suggesting that PSCs are most "flexible" cells.

Whether fluctuations in gene expression levels of the PSC gene are important in maintaining PSC pluripotency is debatable [82, 99–101]. To clarify mechanisms of temporal fluctuation in gene dynamics, gene expression feedback control techniques, which enable regulation of the gene expression level to fit its preset function, such as the patch clamp technique of electrophysiology, are required.

Similarity Between Sister Cells at the Onset of Differentiation

We studied PSC heterogeneity related to temporal expression dynamics [102]. The main purpose of our experiment was to determine whether the origin of heterogeneity was caused by the production of many subpopulations (Fig. 6.4a) or by an increase in each cell's heterogeneity (Fig. 6.4b). The former and the latter are related to the first and the second hypothesis we described in the last subsection, respectively. We focused on the heterogeneity, or to be exact, similarity, between sister cells derived from a mother ESC at the onset of differentiation. Cells at the onset of differentiation are expected to resemble those in vivo, since the cells emerge from

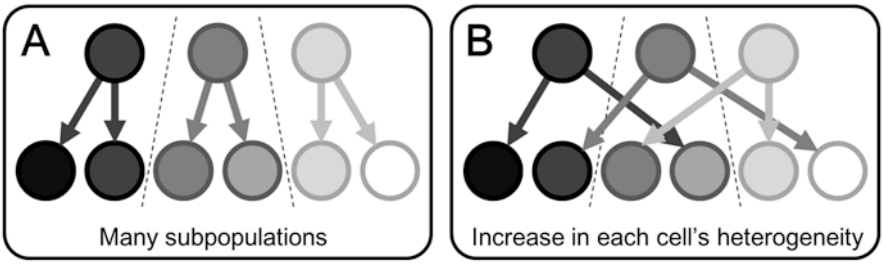

Fig. 6.4 Schematic of two possible mechanisms causing heterogeneity

the artificial self-renewal condition in vitro, to start differentiation. The cells are required not only to proliferate but also to differentiate into many lineages at the onset of differentiation.

Green fluorescence protein expression driven by the *Nanog* promoter (Nanog-GFP) was used to monitor the state of cells. Nanog-GFP is active only in undifferentiated cells [103]. Population analysis using flow-cytometry showed that the peak width of the cell population broadened at the onset of differentiation, suggesting that the population of ESCs had reached heterogeneity. Single-cell analysis via time-lapse imaging was used to compare the difference in Nanog-GFP intensity of the sister cells to those of randomly chosen non-sister cell pairs, which were used as an unsynchronized control. The Nanog-GFP-level difference between sister cells was smaller than that between non-sister cells in the maintenance medium, but not at the onset of differentiation. Similar results were obtained in the Nanog-GFP mouse iPS cell line, suggesting that the sister cells were close to each other in the maintenance medium but were not close at the onset of differentiation.

The results suggested that the cells produced different cells. For example, the high-Nanog-GFP cells are produced from both high- and low-Nanog-GFP cells. Thus, there is a possibility that the PSCs do not produce many subpopulations with similar properties but increase temporal fluctuation of each cell to produce heterogeneity at the onset of differentiation (Fig. 6.4b). After increasing heterogeneity, some external factors such as cell-cell interaction may be necessary to produce differences between each lineage.

In this section, we introduced PSC heterogeneity based on temporal and intermittent expression dynamics of PSCs. The cell state is dynamic, and such dynamics may be important for maintaining pluripotency.

Imaging Methods for Detecting PSC Heterogeneity

Whereas previous studies have revealed genetic instability and heterogeneity in hPSCs, the true extent of genetic variation in hPSCs is only likely to become apparent when whole-genome sequence analysis is performed [104]. A number of

candidate genes for aberrant phenotype were reported [66, 67, 105]; however, morphology is a practically important criterion that is used to continuously evaluate the undifferentiated state and heterogeneity of hPSCs. Healthy undifferentiated hPSCs form a typical colony that appears as tightly packed, round cells with large nuclei and notable nucleoli without spaces between cells [106, 107]. Altered propensity of aberrant hPSCs, such as rapid cell growth, may affect their colony appearance, compared with that of healthy parent hPSCs. Several studies demonstrated that morphology is correlated to hPSC quality [106, 108–112]. Therefore, morphological analysis may be valuable for evaluating the heterogeneity of hPSCs.

Recent developments in image analysis have facilitated the evaluation of cellular growth and status using non-labelled as well as labelled images [107, 111–116]. Time-lapse live-cell phase-contrast images acquired using a culture observation system revealed that hPSCs exhibit variation in colony and cell areas (Fig. 6.5) [116]. Using this system, we developed a non-labelled imaging method for calculating hPSC growth using formulae to calculate the cell number in hPSC colonies driven from hPSC colony areas and number of nuclei [116]. Although the relationships between the colony areas and nuclei numbers are linear, equation coefficients are dependent on the cell line used, colony size, colony morphology, and culture conditions. When culture conditions are improper, the change in cell condition can be detected by these morphological analysis using phase-contrast images (Fig. 6.6) [116]. This indicates that image analysis may be used to quantify heterogeneity.

Machine learning including pattern recognition, automated recognition, and autonomous prediction is gaining recognition as a potential analysis tool. Tokunaga et al. [112] reported that supervised machine learning pattern recognition can distin-

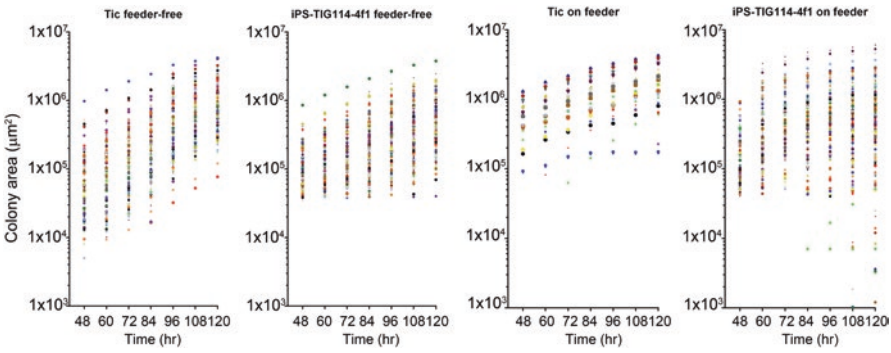

Fig. 6.5 Colony areas during culture. The hiPSC lines, Tic, or iPS-TIG114-4f1 cells were seeded using feeder-free culture conditions or KSR-based conventional culture conditions with feeder cells in 6-well-plates and cultured for 5 days. After the cell clumps had settled on the plate surfaces (48 h), phase-contrast images of these cells were acquired every 12 h and analyzed using the culture observation system and software. Cell clump areas of >0.25 μm^2 were recognized as undifferentiated hiPSC colonies for analysis. (**a**) Colony areas for Tic feeder-free cell culture. (**b**) Colony areas for iPS-TIG114-4f1 feeder-free cell culture. (**c**) Colony areas for Tic on feeder. (**d**) Colony areas for iPS-TIG114-4f1 on feeder. Modified from Fig. 2 and Supplementary Fig. 1 in STEM CELLS TRANSLATIONAL MEDICINE 2015;4:1–11

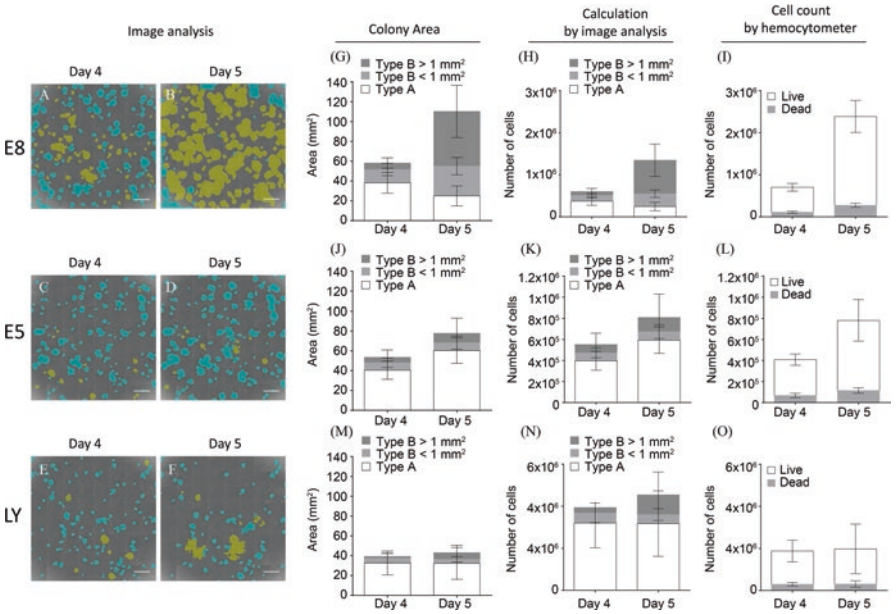

Fig. 6.6 Detection of change in cell growth by the imaging system. Tic cells were seeded on Matrigel in TeSR-E8 medium. After 3 days, cells were cultured for more than 2 days in TeSR-E8 medium (**a**, **b**, **g**–**i**), E5 medium that did not contain insulin, fibroblast growth factor-2, or transforming growth factor-b, or TeSR-E8 medium with an inhibitor of phosphatidylinositol 3 kinase of LY294002 at a concentration of 20 mM (**e**, **f**, **m**–**o**). (**a**–**f**) Phase contrast images were analyzed using the imaging analysis system. Type A colonies were recognized as blue and type B colonies as yellow. (**g**, **j**, **m**) The colony areas were extracted from the images and analyzed for type A or type B colonies <1 mm^2 or >1 mm^2. (**h**, **k**, **n**) Cell numbers calculated using the equation with the ratio of type A or type B colonies. (**i**, **l**, **o**) The cells dissociated by trypsin/EDTA were counted using a hemocytometer. Scale bars = 2 mm. Modified from Fig. 5 in STEM CELLS TRANSLATIONAL MEDICINE 2015;4:1–11

guish completely reprogrammed iPSCs from improperly reprogrammed cells. Generally, iPSC colonies are manually selected based on their morphology via expert observation. Utilizing representative iPSC colony images of incompletely reprogrammed iPSCs selected by experts, it is possible to train computers to classify colonies according to morphological patterns. This finding confirms that cell morphology is a valuable marker, which noninvasive computation image analysis can evaluate as hPSC quality. We reported that the non-labelled live-cell image analysis can classify morphological heterogeneity of hPSC colonies based on the statistical analysis of images with unbiased morphological parameters [107]. Colony morphology of an aberrant hPSC subclone (#12 trisomy) and parent cell line was compared. Whereas most of the population of the aberrant hPSC subclone appeared to have partially differentiated colony morphology, most of the population of a parent cell line seemed to show typical embryonic stem cell-like morphology. Classification utilizing statistical analysis of colony images revealed morphological

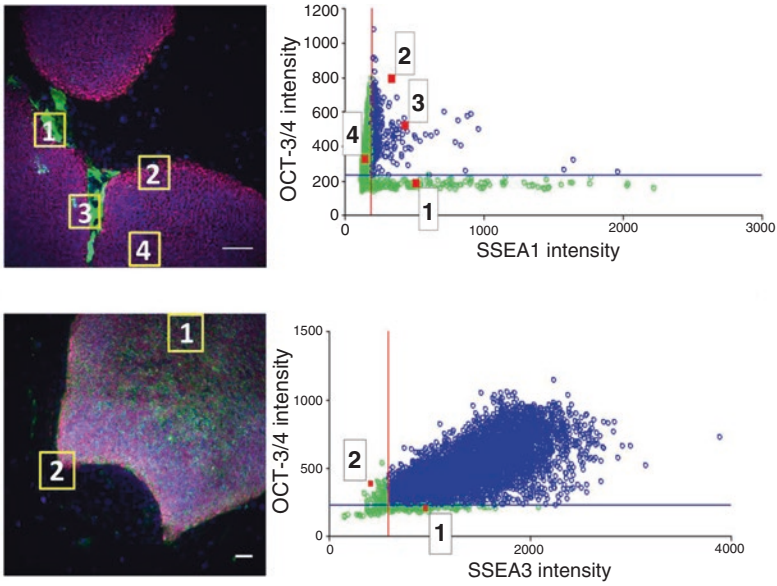

Fig. 6.7 Localization of stem cell markers in hiPSC 201B7 colonies analyzed for expression profiles. Representative cells stained with Oct3/4 and SSEA1 (**a**, **b**) and with OCT3/4 and SSEA3 (**c**, **d**) were tracked back to the images from plots. (**a**, **c**) Merged image of immunostaining. A representative field of the images taken by an imaging cytometer is shown. Regions containing each target cell are marked with a yellow rectangle. Scale bar, 100 μm. (**b**, **d**) Analyzed fluorescent intensity profile of each cell in the field. Red plots represent target cells. Fluorescent images stained with SSEA1 or SSEA3 with OCT-3/4 antibody and Hoechst33342. The nucleic region of each target cell is marked as cyan Modified from Fig. 4 in In Vitro Cell. Dev. Biol.—Animal (2017) 53:83–91 DOI 10.1007/s11626-016-0084-3

heterogeneity in both cell lines that was similar to heterogeneity in gene expression profiles. The analysis indicated that the aberrant subclone had unhealthy colonies at a ratio of 27.3% that were characterized by the loss of clear colony edges, a comparatively flatter cytoplasm, and a low nucleus-to-cytoplasm ratio. The parent line had unhealthy colonies at a ratio of 13.3%, suggesting that non-label image analysis of morphology may predict unhealthy state by monitoring variations in hPSC heterogeneity.

Flow cytometry is the central methodology for canonical markers of stem cells [117], but recent developments in imaging analysis encourage the use of immunocytochemistry. This is because two-dimensional imaging cytometry enables evaluation of the heterogeneity of undifferentiated state hPSCs to analyze localization and morphological information regarding immunopositive cells in the culture [118]. Whole images of cells in a culture vessel acquired by the image analyzer can demonstrate the population ratio and staining intensity of the cells with positional information. Two-dimensional imaging cytometry reveals spatially heterogenic expression of the hPSC markers in undifferentiated hPSCs (Fig. 6.7) [118].

Quality Control of Heterogeneous hPSCs

As a result of the heterogeneities described above, the quality of hPSCs used in research may cause concern. Because hPSCs are generally adapted for in vitro culture, they tend to have increased growth and less apoptotic cell death [70, 119–123]. When adapted aberrant hPSCs appear in the cell population, they rapidly replace the parent cell population, resulting in altered propensity for survival and retention of an undifferentiated phenotype [121]. Therefore, it is recommended that the quality of hPSCs be routinely checked during culturing for a long period. The International Stem Cell Banking Initiative (ISCBI) established in 2007 with funding from the International Stem Cell Forum (http://www.stemcell-forum.net/) provided guidance for banking and supplying of human embryonic stem cell lines for research purposes in 2009 [117] and points to be considered prior to clinical applications in 2015 [124]. These papers which describe best practices for the banking of hPSCs may also be valid for basic research using hPSCs in laboratories. An international study conducted on characteristic phenotypes of hPSCs by the ISCI summarized typical surface marker profiles and stem cell-related marker genes [76]. Based on these studies, the following five measurements are recommended to evaluate the quality of hPSCs in laboratories.

Cell Morphology

Cell morphology is an important criterion, as described above. When culturing cells obtained from a supplier such as a cell bank, it is necessary to acquire phase contrast images of cultured cells at the earliest possible time and compare them with the images provided by the supplier. Ideally, it is recommended to acquire cell images at every passage. At minimum, it is necessary to acquire both images of undifferentiated and differentiated cell areas every five passages at weak and strong enlargements to confirm whether the cells are able to maintain their original morphology.

Growth Rate

Growth rate is an important characteristic because an increase in growth rate may indicate transformation [124]. Changing culture conditions affects the growth rate. Just after the cells are thawed, they grow slowly. Three or four passages following thawing may be a suitable time to calculate the growth rate. It is necessary to calculate growth rate, at least every five passages after that. Although it is ideally recommended to count cell numbers of the culture vessel at every passage, dissociation into a single cell in the presence of an ROCH inhibitor is not always used under all culture conditions. Either counting cell numbers separately or monitoring live cell images is recommended, as described above.

Characterization of Stem Cell Marker Genes and Antigen Expression

Characterization of stem cell marker genes and antigen expression provides useful fundamental information on cell state and the variability and consistency of cultures [124]. Although an assay using the whole-genome expression arrays may be valuable in a cell bank where analyzed data from various cell lines are accumulated, analysis of extensive information derived from the array would be difficult in a small laboratory and also less cost-effective. A PCR array for human stem cell markers or selective typical genes, such as typical genes, Nanog, Oct 4, DNMT 3B, TDGF, GABRB3, GDF3 [117], and also BCL2L1 as an anti-apoptotic gene [66], may be useful in a laboratory. Quantitative analysis by flow cytometry is generally used for a typical surface marker set: SSEA-1 (negative or very low), SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. As recent developments in digital imaging technology have enabled the acquisition of an entire surface image of a culture vessel, immunocytochemical analysis with imaging has also become a useful tool to provide quantitative characterization, reflecting even a minute aberrance without losing spatial and morphological information of the cells [118, 125, 126]. Alkaline phosphatase-positive colony-forming assays are useful for quantitation of stem cell lines [127]. However, it should be taken into consideration that hPSC cell cultures vary in gene and antigen expression from one passage to another [76].

Genotype

Several genotyping techniques may be available in the laboratory as well as in the cell bank [124]. Karyotyping by Giemsa banding is the most common technique used to identify changes in chromosomal numbers as well as translocations and rearrangements. Spectral karyotyping or fluorescent in situ hybridization can also be performed in the laboratory. While a specific expensive instrument is not necessary for these analyses, proficient techniques for karyological study are required. Ideally, it is recommended to analyze the genotype of hPSCs every five passages. At a minimum, the genotype of hPSCs should be determined after every ten passages.

Comparative genome hybridization (CGH) microarray or multiple single-nucleotide polymorphism (SNP) analysis may be suitable for the laboratory, and the number of cells analyzed may be critical for sensitivity to abnormal clones [76]. Deep sequencing or whole-genome sequencing can be performed at large institutes or universities with well-equipped facilities [128–130]. However, the sensitivity and accuracy of these methods should be considered, and the results should also be interpreted with caution.

Differentiation Potential

Assessing the ability of stem cells to form teratomas in SCID mice has long been considered the "gold standard" for confirming pluripotency of cells [131]. However, this may not be realistic in the research laboratory, as the teratoma assay is time-consuming and costly. An alternative method is the formation and characterization of embryoid bodies [132–134]. Recently, various differentiation protocols for specific lineages using defined culture conditions have been developed. Determination of specific differentiation induction methods to measure differentiation potential is easy to perform in the laboratory.

Considering progressive phenotypes of aberrant clones, these measurements are recommended at least every ten passages. However, performing all of these measurements is time-consuming and costly. It is recommended that cryopreserved stocks of cells intended for use be prepared at the earliest possible time and analyzed once in the laboratory to assure that reliable stocks can be obtained [135, 136] and that cells newly cultured from the cryopreserved stock are used within 3 months to minimize the effects of heterogeneity from prolonged culture.

Perspective and Conclusion

In this chapter, we focused on the heterogeneity of PSCs from six points of view (Fig. 6.1). PSCs are heterogeneous. Such heterogeneity unfortunately decreases reproducibility of research. Thus, research related to verification, reduction, and regulation of heterogeneity is important, especially for hPSCs. However, as heterogeneity is closely related to pluripotency, a fundamental property of PSCs, there is a possibility that complete elimination of heterogeneity may also cause the elimination of pluripotency. Under the present circumstances, the presupposition that PSCs are highly heterogeneous should be taken into account by all researchers not only when they plan their own studies but also when they review the studies of other researchers in this field.

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Chapter 7
Sarcoma Stem Cell Heterogeneity

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Abstract Sarcomas represent an extensive group of divergent malignant diseases, with the only common characteristic of being derived from mesenchymal cells. As such, sarcomas are by definition very heterogeneous, and this heterogeneity does not manifest only upon intertumoral comparison on a bulk tumor level but can be extended to intratumoral level. Whereas part of this intratumoral heterogeneity could be understood in terms of clonal genetic evolution, an essential part includes a hierarchical relationship between sarcoma cells, governed by both genetic and epigenetic influences, signals that sarcoma cells are exposed to, and intrinsic developmental programs derived from sarcoma cells of origin. The notion of this functional hierarchy operating within each tumor implies the existence of sarcoma stem cells, which may originate from mesenchymal stem cells, and indeed, mesenchymal stem cells have been used to establish several crucial experimental sarcoma models and to trace down their respective stem cell populations. Mesenchymal stem cells themselves are heterogeneous, and, moreover, there are alternative possibilities for sarcoma cells of origin, like neural crest-derived stem cells, or mesenchymal committed precursor cells, or – in rhabdomyosarcoma – muscle satellite cells. These various origins result in substantial heterogeneity in possible sarcoma initiation. Genetic and epigenetic changes associated with sarcomagenesis profoundly impact

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the biology of sarcoma stem cells. For pediatric sarcomas featuring discrete reciprocal translocations and largely stable karyotypes, the translocation-activated oncogenes could be crucial factors that confer stemness, principally by modifying transcriptome and interfering with normal epigenetic regulation; the most extensively studied examples of this process are myxoid/round cell liposarcoma, Ewing sarcoma, and synovial sarcoma. For adult sarcomas, which have typically complex and unstable karyotypes, stemness might be defined more operationally, as a reflection of actual assembly of genetically and epigenetically conditioned stemness factors, with dedifferentiated liposarcoma providing a most thoroughly studied example. Alternatively, stemness can be imposed by tumor microenvironment, as extensively documented in osteosarcoma. In spite of this heterogeneity in both sarcoma initiation and underlying stemness biology, some of the molecular mechanisms of stemness might be remarkably similar in diverse sarcoma types, like abrogation of classical tumor suppressors pRb and p53, activation of Sox-2, or inhibition of canonical Wnt/β-catenin signaling. Moreover, even some stem cell markers initially characterized for their stem cell enrichment capacity in various carcinomas or leukemias seem to function quite similarly in various sarcomas. Understanding the biology of sarcoma stem cells could significantly improve sarcoma patient clinical care, leading to both better patient stratification and, hopefully, development of more effective therapeutic options.

Keywords Sarcoma · Liposarcoma · Ewing sarcoma · Chondrosarcoma · Synovial sarcoma · Osteosarcoma · Mesenchymal stem cells · Sarcoma stem cells · Sarcoma cells of origin · Genetic and epigenetic plasticity · In vitro sarcoma progression models · Sox-2 · p53 · pRb · Wnt/β-catenin pathway · Dickkopf

Sarcomas represent an unusually wide, extensive, and heterogeneous group of tumors, whose sole common denominator is that they originate from mesenchymal cells. They could be divided according to various criteria. The most traditional histopathologic classification divides sarcomas into two large groups according to the type of tissue of primary manifestation, namely bone sarcomas, including osteosarcoma and chondrosarcoma, and soft tissue sarcomas, including liposarcoma, fibrosarcoma, undifferentiated pleomorphic sarcoma, leiomyosarcoma, rhabdomyosarcoma, and a large group of other pediatric sarcomas [1]. Besides, we can classify sarcomas according to genetic criteria, and, again, we can distinguish two large groups: sarcomas with largely normal karyotypes and discrete structural chromosomal changes and sarcomas with complex karyotypes and pronounced karyotypic instability. The first group includes especially pediatric sarcomas, which rely for the most part on reciprocal translocations to activate specific oncogenes (Table 7.1). The adult sarcomas – including osteosarcoma, chondrosarcoma, most liposarcomas, fibrosarcoma, angiosarcoma, leiomyosarcoma, and undifferentiated pleomorphic sarcoma – carry usually very complex karyotypes with numerous structural and numerical alterations [2, 4]. Researchers have only begun to understand the complex mechanisms behind this karyotypic instability. Especially dedifferentiated

Table 7.1 Most frequent structural chromosomal aberrations found in sarcomas[a]

Tumors	Cytogenetic events	Genes involved/fusion
Fibrosarcoma, infantile	t(12;15)(p13;q26)	*ETV6-NTRK3*
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SS18-SSX1, SSX2*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWSR1-ATF1*
	t(2;22)(q32.3;q12)	*EWSR1-CREB1*
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FOXO1*
	t(1;13)(q36;q14)	*PAX7-FOXO1*
Mesenchymal chondrosarcoma	del(8)(q13;3q21.1)	*HEY1-NCOA2*
Alveolar soft-part sarcoma	t(X;17)(p11;q25)	*ASPSCR1-TFE3*
Lipoma	12q15 rearrangement	*HMGA2* rearrangement
Ewing sarcoma	t(11;22)(q24;q12)	*EWSR1-FLI1*
	t(21;22)(q22,q12)	*EWSR1-ERG*
	t(7;22)(p22;q12)	*EWSR1-ETV1*
	t(17;22)(q12;q12)	*EWSR1-E1AF*
	t(2;22)(q33;q12)	*EWSR1-FEV*
	t(16;21)(p11;q22)	*TLS(FUS)-ERG*
	Inversion of 22q	*EWSR1-ZSG*
Myxoid/round cell liposarcoma	t(12;16)(q13;p11)	*FUS-DDIT3*
	t(12;22)(q13;q12)	*EWSR1-DDIT3*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWSR1-NR4A3*
	t(9;17)(q22;q12)	*TAF15-NR4A3*
Dermatofibrosarcoma protuberans/ giant cell fibrosarcoma	t(17;22)(q22;q13), supernumerary ring chromosomes encompassing chr 17 and 22	*COL1A1-PFGFB*
Inflammatory myofibroblastic tumor	t(2;19)(p23;p13)	*TPM4-ALK*
	t(2;17)(p23;q23)	*CLTC-ALK*
	inv(2)(p23;q13)	*RANBP2-ALK*
Angiomatoid fibrous histiocytoma	t(2;22)(q32.3;q12)	*EWSR1-CREB1*
	t(12;22)(q13;q12)	*EWSR1-ATF1*
	t(12;16)(q13;q11)	*TLS(FUS)-ATF1*
Low-grade fibromyxoid sarcoma	t(7;213)(q34;p11)	*FUS-CREB3L2*
Sclerosing epithelioid fibrosarcoma	t(11;16)(p11;p11)	*FUS-CREB3L1*
Hemosiderotic fibrolipomatous tumor	t(1;10)(p11;q24)	*MGEA5-TGFBR3*
Epithelioid hemangioendothelioma	t(1;3)(p36;q25)	*WWTR1-CAMTA1*
Soft tissue myoepithelioma	t(1;22)(q23;q12)	*EWSR1-PBX1*
GCT of tendon sheath	t(1;2)(p13;q37)	*COL6A3-CSF1*
Solitary fibrous tumor	inv(12)(q13;q13)	*NAB2-STAT6*
Nodular fasciitis	t(17;22)(p13;q13)	*MYH9-USP6*
Pseudomyogenic hemangioendothelioma	t(7;19)(q22;q13)	*SERPINE1-FOSB*
Soft tissue angiofibroma	t(5;8)(q15;q13)	*AHRR-NCOA2*
CIC-DUX4 sarcoma	t(4;19)(q35;q13)	*CIC-DUX4*

(continued)

Table 7.1 (continued)

Tumors	Cytogenetic events	Genes involved/fusion
	t(10;19)(q26;q13)	*CIC-DUX4L10*
BCOR-CCNB3 sarcoma	inv(X)(p11.4;p11.22)	*BCOR-CCNB3*
Phosphaturic mesenchymal tumor	t(2;8)(q35;q11)	*FN1-FGFR1*
Leiomyoma (uterine)	t(12;14)(q15;q24) or deletion of 7q	*HMGA1 (HMGIC)* rearrangement
Synovial sarcoma	t(X;18)(p11;p11)	*SS18-SSX1 or SS18-SSX2, SS18-SSX4*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1-WT1*
Endometrial stromal tumor	t(7;17)(p15;q21)	*JAZF1-SUZ12*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWSR1-NR4A3*
	t(9;17)(q22;q11)	*TAF15-NR4A3*
	t(9;15)(q22;q21)	*TCF12-CHN*

[a]Source: References [1–3]

liposarcoma (one of four major liposarcoma groups [5]) features a constantly changing karyotype involving the so-called neochromosomes – giant, sometimes ring-shaped, chromosomal structures that accumulate most of the amplified oncogene loci originating from different chromosomes and result from multiple complex mechanisms, such as chromothripsis and breakage-fusion-bridge cycles [6].

Mesenchymal Stem Cells

Many of the mesenchymal tissues that can be affected by sarcomas undergo continuous remodeling and renewal much like epithelia, and it comes as little surprise that there is a similar hierarchical cellular organization. Supposedly sitting on the top of this cell hierarchy are mesenchymal stem cells (MSCs), which behave much like other adult stem cells, i.e., they can self-renew and differentiate into the respective downstream cell types. For MSCs, trilineage differentiation potential in vitro is considered a sort of a definition criterion. Upon appropriate stimulation, MSCs can enter osteogenic, adipogenic, and chondrogenic differentiation [7, 8]. The true differentiation potential of MSCs is broader, however. At least in vitro, they are able to enter the neurogenic differentiation pathway as well. Of course, they also differentiate into fibroblasts, the major constituent of *lamina propria* of most, if not all, epithelial tissues. This fibroblastic differentiation pathway can take a special form – the carcinoma-associated fibroblasts (CAFs) – providing a supportive stroma found in practically all carcinomas. Another differentiated cell type originating from MSCs is endothelium, a differentiation pathway exploited by tumors of various origins as well, yielding tumor vasculature.

MSCs can be isolated and propagated from a lot of tissues in the body, two prototypical sources being bone marrow and white adipose tissue. These cells are,

nonetheless, not identical and differ in terms of both their relative differentiation abilities and epigenetic genome regulation [9, 10]. These biological differences extend into different nomenclatures: The bone marrow-derived MSCs (BM-MSCs) have been recently proposed to be called skeletal stem cells [11], while the white adipose tissue MSCs are traditionally called adipose tissue-derived stromal cells (ASCs). A notable exception among mesenchymal tissues as to for their exclusive derivation from MSCs is the skeletal muscle, which carries its own stem cell population, the satellite cells. Remarkably, the satellite stem cell niche also adopts a MSCs-like population, again carrying a special name, the fibroblast-adipocyte precursor (FAP) [12]. To make the things even more complicated, there is a separate adult stem cell population, the neural crest-derived stem cells. These cells have descendant cell types, such as specialized neurons, glial cells, and melanocytes, but they can also differentiate into the full spectrum of mesenchymal cell types [13]. In conclusion, there is a pronounced and rather extensive heterogeneity among stem cell populations of normal mesenchymal tissues.

Molecular Biology of MSC Stemness and Differentiation

As introduced above, MSCs have one of the broadest differentiation capacities among adult stem cells, each of the various differentiation programs dominated by specific signals resulting in the activation of specific transcription factors. Transcription factors crucial for osteogenic differentiation are Runx2 and directly downstream positioned Osterix. Among the signals, bone morphogenetic proteins are prominent, resulting in specific Smad activation; notably, a direct Smad-Runx2 protein-protein interaction has been described. Adipogenic differentiation results from the transcription factor succession C/EBPα–C/EBPβ–PPARγ. As to the chondrogenic differentiation, Sox-9 is regarded as a master transcription factor [14].

Crucial from the point of view of sarcoma initiation and development, MSC stemness and differentiation seem to be regulated by an intricate network, whose essential players are classical tumor suppressor proteins p53 and pRb on one hand and key stemness regulators SIRT-1 and Sox-2 on the other (Fig. 7.1). First of all, p53 is a general stemness inhibitor, a function not limited to MSCs [15]. Mechanistically, a part of this stemness inhibition relies on direct as well as indirect transcriptional repression of both *SIRT-1* (two p53 binding sites in the promoter plus a binding site for the p53 downstream transcription factor HIC-1, as well as p53-inducible miRNA34-mediated silencing) [16, 17] and *Sox-2* (mediated mainly by p53-activated miRNA145) [18–20]. *SIRT-1*, a longevity gene, codes for NAD^+-dependent protein deacetylase, which, by virtue of this specific posttranslational modification, regulates the activity of numerous cellular proteins. Among them is p53, with deacetylation resulting in blocking of its nuclear translocation and the significant diminution of transcription activation potency, at least in embryonic stem cells [21]. Sox-2 is another direct SIRT-1 target, but in this case, the effect is exactly opposite: Deacetylation promotes the nuclear localization and transcrip-

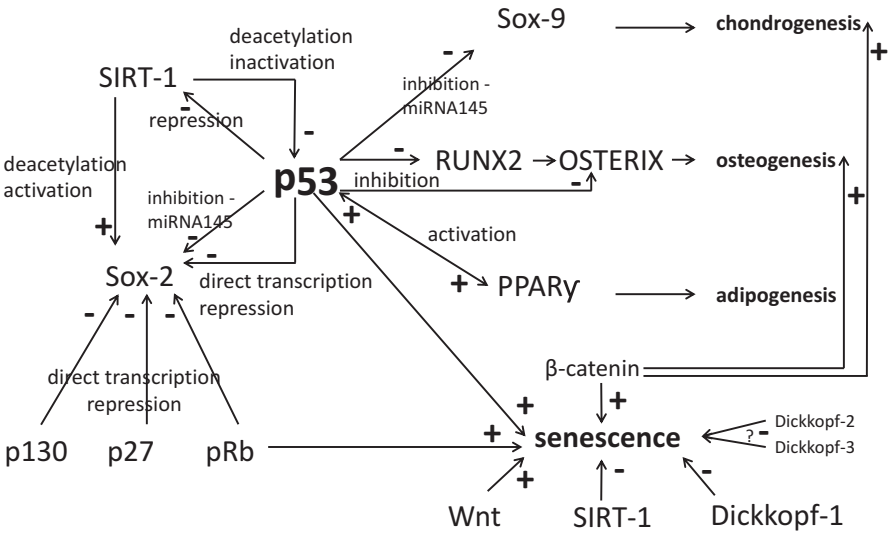

Fig. 7.1 Molecular circuitry operating in stemness regulation in MSCs and sarcoma stem cells. See text for detailed explanation

tional activation of downstream genes [22]. Besides SIRT-1 and p53-mediated regulation, *Sox-2* is transcriptionally repressed in primary fibroblasts by an unusual complex involving cell cycle inhibitor p27 and retinoblastoma family proteins pRb and p130; notably, p53 potentiates this effect [23]. In addition to stemness regulation, p53 is also crucially involved in MSC differentiation. Functional p53 seems to be a necessary prerequisite for successful adipogenic differentiation, in part via mitochondria-generated reactive oxygen species, in part via a direct mutual positive regulation with PPARγ [24]. On the other hand, p53 inhibits both osteogenic (in part by directly repressing the *Runx2* gene and inhibiting Osterix activity through a direct protein-protein interaction) [25] and chondrogenic (via the miRNA145-mediated silencing of Sox-9) differentiations [18, 19, 26].

Some of the tumor suppressor activities of p53 and pRb consist in their actions on cellular life span, restricting it and promoting a special type of cell cycle arrest – senescence. Indeed, MSCs – especially of human origin – are not immortal, at least in vitro, and succumb to senescence arrest sometimes after just a few passages; inasmuch these properties lie in the nature of MSCs or result from culture stress (the bone marrow is a frank hypoxic area, whereas MSCs are usually cultured in normal oxygen conditions) is still not entirely clear. Anyway, senescence could be regarded as an antitumor barrier, overcoming of which could be an important step in tumorigenesis. Interestingly, apart from p53 and pRb, there seems to be one more player impacting both MSC differentiation and senescence, namely, the Wnt/β-catenin pathway. The activation of β-catenin has been reported to be part of both osteogenic and chondrogenic differentiations; on the other hand, this pathway also

accelerates the senescence of MSCs; MSC immortalization can be promoted by Wnt inhibitor Dickkopf-1 (and by SIRT-1) [27, 28].

MSC stemness regulation and sarcomagenesis are associated in various ways. Both SIRT-1 and Sox-2 are poor prognosis factors across various sarcoma types [29, 30]. Diverse sarcomas can be found in increased frequency in affected families suffering from both *p53* (Li-Fraumeni syndrome) and *pRb* (hereditary retinoblastoma) germ line mutation-driven hereditary cancer syndromes [31]. One of the genes carried within the amplified region underlying neochromosome generation in dedifferentiated liposarcoma is *MDM2* coding for a direct p53 inhibitor [32]. Our own transcriptomic analysis of two separate progression models of murine soft tissue sarcoma, the JUN fibrosarcoma progression series [33] and the 3T3-L1 – LM3D liposarcoma progression series [34], revealed that Dickopf-2 and Dickkopf-3, as well as additional published inhibitors of canonical Wnt/β-catenin signaling including Adenomatosis polyposis coli down-regulated 1, Fibulin-5, Maternally expressed 3, and Integrator complex subunit 6, are all upregulated during sarcoma progression. Strikingly, this seems to be accompanied by upregulation of the Receptor tyrosine kinase-like orphan receptor 2 (Ror2), suggesting a switch from the canonical Wnt signaling to the noncanonical Wnt5a-Ror2 pathway [34a].

Sarcoma-Initiating Cells and Sarcoma Stem Cells

Keeping in mind the above-outlined development of mesenchymal tissues reflecting an intrinsic cellular hierarchy that starts from mesenchymal stem cells and follows a given differentiation path through progenitors to terminally differentiating cells, it is quite natural to expect that part of this hierarchy would be preserved in sarcomas. Accordingly, we can assume the existence of sarcoma stem cells that both self-renew and differentiate, much like in carcinomas and leukemias. Of course, only some sarcoma types – for example, osteosarcoma, chondrosarcoma, liposarcoma, leiomyosarcoma, or rhabdomyosarcoma – allow to follow a certain differentiation pathway reminiscent of physiological differentiation. Other sarcoma types – diagnosed as undifferentiated pleomorphic sarcoma or simply just spindle cell sarcoma – do not show any easily recognizable differentiation pattern. In fact, the residual differentiation capacity might not be easily discernible even in high-grade tumors of the former sarcoma types. In such cases, the tumorous (pseudo) differentiation can take the form of diversity in functional aspects, like clonogenicity, tumorigenicity, therapeutic resistance, motility, and invasiveness. Various questions about sarcoma stem cells – like those about their abundance, properties, self-renewal, and differentiation ability – should be clearly separated from questions about sarcoma-initiating cells, which are cells that incur the first mutagenic insult ultimately yielding a sarcoma.

In this respect, the various types of mesenchymal stem cells would be apparent candidates [35–37]. Several reasons support this conclusion. First, again drawing lessons from various carcinoma and leukemia stem cells, the path from a respective

tissue stem cell (mesenchymal for sarcomas) to its derivative cancer stem cell is simpler and more direct than the path assuming cancer initiation in a more differentiated cell, because the various stemness mechanisms are already operating. In addition, the reliance of sarcomas and sarcoma stem cells on Sox-2 and SIRT-1 is a good argument for their derivation from mesenchymal stem cells. We should not forget, however, that MSCs are not a uniform cell type and BM-MSCs clearly differ from ASCs and probably from MSCs isolated from other sources, NCSCs notwithstanding. Another argument is that MSC-specific expression signature has been identified in some sarcomas and it could be especially clearly revealed (together with the restauration of the full multilineage differentiation potential in vitro) by performing an experimental reversion of sarcoma cells, for example, by an shRNA-mediated knockdown of driver fusion oncogenes in translocation-derived sarcomas. Finally, MSCs are amenable to both spontaneous transformation and purposeful genetic manipulation resulting in sarcomas (see below). This last argument should not be overemphasized, however, because a similar outcome could also be arrived at by the in vitro transformation of normal fibroblasts, that is, differentiated mesenchymal cells [38].

In addition, even though it could be feasible to infer the origin of simpler translocation-dependent sarcomas, this task can be much more complicated in karyotypically complex sarcomas. Indeed, available models suggest alternative possibilities. For example, at least in mouse models, a probable osteosarcoma-initiating cell is an osteogenic progenitor, not MSC [39, 40]. What is more, it could be shown that a human fibrosarcoma cell line could be converted to a liposarcoma by a forced expression of a key liposarcoma oncogene *CHOP* (*DDIT3*) [41]. And, of course, rhabdomyosarcomas derive almost certainly from satellite cells [42, 43]. We can thus conclude that there are many potential candidates for the sarcoma cell of origin, including various MSCs and NCSCs and various other mesenchymal cell types.

Modeling Sarcomagenesis in MSCs

Assuming the MSC origin of at least a great part of sarcomas, we can directly use our knowledge of MSC biology – including our ability to differentiate MSCs along a desired path, together with our increasing understanding of underlying biology of diverse sarcomas – to build MSC-based models of sarcoma development. This endeavor can have several forms.

First, we can perform in vitro differentiation of MSCs, identify differentially expressed genes, proteins, or pathways, and relate them to the corresponding sarcoma type. Two studies illustrate well this point. In the first of them, primary human BM-MSCs were subjected to chondrogenic differentiation, and a specific chondrogenesis expression signature was identified. When the gene expression profiles of chondrosarcoma samples of different grades were confronted with the chondrogenesis expression signature, it turned out that all the grade III and grade II

metastatic cases clustered together close to the undifferentiated MSCs, whereas grade I and nonmetastatic grade II chondrosarcomas were more similar to late differentiation stages of MSCs approaching mature chondrocytes [44]. This result rather convincingly shows that stemness preservation (or regain) can represent an important contribution to metastatic competence. In addition, the chondrogenesis gene expression signature identified could be of a direct clinical utility, especially for the prognosis of grade II chondrosarcoma patients.

A conceptually similar approach has been applied to liposarcoma. The liposarcoma itself represents a complex diagnosis consisting of at least four distinct tumor types: dedifferentiated, pleomorphic, myxoid/round cell, and well-differentiated [5]. It could be shown that each of them corresponds, at least in terms of their specific gene expression profile, to a different stage of adipogenic differentiated MSC. Again, this result might have an immediate diagnostic value. Dedifferentiated and pleomorphic liposarcomas thus feature the expression of typical MSC markers – like CD44, CD54, and hepatocyte growth factor – while myxoid/round cell and well-differentiated liposarcomas adopt the expression of typical fat markers, namely, adiponectin, leptin, and lipoprotein lipase [45]. This approach made also possible the identification of genes and pathways typical of either path of liposarcomagenesis. Although their clinical utility has yet to be demonstrated, an intriguing candidate pathway compromised in both dedifferentiated and pleomorphic liposarcoma cases is insulin signaling, a very well-defined proadipogenic signaling pathway, which could be amenable to various pharmacological modulations [46]. It should be stressed, nevertheless, that especially dedifferentiated liposarcoma is a very complicated tumor type, for which the cell of origin is largely unclear (it could be a MSC at an early point of adipogenic differentiation or progressing well-differentiated liposarcoma) and whose genome, as mentioned above, is extremely unstable with unprecedented consequences for gene expression, stemness, and clinical behavior (see below).

If we embrace the idea that most sarcomas may originate from MSCs, a logical next step is to undertake an attempt at deriving sarcoma models by their targeted manipulation. In the last decade, several valuable sarcoma models have been established in this way, revealing several general rules of sarcoma development. First, it turned out that rodent (especially murine) MSCs are distinctly more susceptible to initiate sarcomagenesis than their human counterparts; indeed, murine and rat MSCs are even prone to spontaneous sarcomagenesis upon prolonged in vitro culture, which is practically never observed in human MSCs. We can only speculate about biological reasons for this difference. It is known for quite a long time that mouse adult tissues constitutively express telomerase and the murine cells are thus immortal upon appropriate cell culture conditions, eliminating the senescence barrier (see above). In addition, most – if not all – experiments have been performed on MSCs isolated from various inbred mouse strains, and we can assume a random fixation of various mutant alleles during the inbreeding process. The existing senescence barrier – probably among other mechanisms – makes human MSCs intrinsically resistant to sarcomagenesis, and usually this is the first obstacle to be overcome in order to convert human primary MSCs into desired sarcoma cells. A standard

approach is to introduce viral oncogenes that eliminate the p53- and pRb-mediated senescence arrest (HPV *E6* and *E7* oncogenes or SV40 large T antigen, respectively), complemented by the stable overexpression of the gene coding for catalytic subunit of telomerase (hTERT) [47, 48]. Even these MSC derivative cell lines (called 3 hit MSCs – E6, E7, and hTERT) were not susceptible to spontaneous sarcomagenesis, and two additional genetic steps turned out to be necessary, namely, c-Myc stabilization by virtue of SV40 small t antigen expression and a permanent mitogenic stimulation by the forced expression of a constitutively active *Ha-Ras* oncogene [47, 48]; *Myc-Ras* is a traditional cooperating oncogene pair, defined by its joint ability to transform rat embryonic fibroblasts [49]. The resulting 5 hit MSCs finally yielded undifferentiated pleomorphic sarcomas when injected into a severely immunocompromised mouse [50].

A separate question is which factors promote particular types of sarcomagenesis. This seems to be a very complex issue encompassing several points, such as the source of MSCs (BM-MSCs vs. ASCs), their species origin (mouse or human), and of course the genetic changes either spontaneously accumulated or purposefully introduced. Especially, p53 deficiency (alone or combined with pRb deficiency) can initiate various sarcoma types depending on other factors. In mouse BM-MSCs, spontaneous p53 mutations have been associated with fibrosarcoma development [51], whereas mouse *p53−/−* ASCs were transformed toward leiomyosarcoma [50], and the combined deficiency of both $p16^{INK4a}$ and $p19^{ARF}$ (two tumor suppressor proteins encoded by a single locus and acting via pRb and p53 pathways, respectively [49]) coupled to c-Myc overexpression in mouse BM-MSCs triggered osteosarcoma development [52]. The knowledge and availability of translocation-activated fusion oncogenes provided additional possibilities of specifically directing sarcomagenesis along a desired pathway. The FUS-CHOP oncoprotein, specific for myxoid/round cell liposarcoma, provided a particularly revealing example. A purposeful expression of FUS-CHOP in mouse ASC of *p53−/−* background [50], in 4 hit (HPV E6, E7, hTERT, and SV40 small-t) or 5 hit (+ $Ha\text{-}Ras^{V12}$) human BM-MSC backgrounds [50], or in HT1080 fibrosarcoma cells [41], respectively, was able to divert the pathway of sarcomagenesis from leiomyosarcoma, undifferentiated pleomorphic sarcoma, or fibrosarcoma to liposarcoma. It seems, therefore, that this type of liposarcomagenesis involves at least two principal causal factors: a general tumorigenic transformation of cells (provided by the recipient cells that are already competent to various types of sarcomagenesis) and a limited, corrupt, and incomplete lipomatous differentiation provided by the FUS-CHOP translocation oncoprotein.

Ewing sarcoma provides another example of a sarcoma that relies on a translocation oncogene imposing its effect on target cells. From a certain point of view, its biology seems to be opposite to myxoid/round cell liposarcoma, with sarcomagenesis resulting from a specific dedifferentiation or reprogramming toward a primitive stem cell phenotype. Indeed, in transgenic mice, a conditional *p53* deletion in embryonic limb bud cells led predominantly to osteosarcoma (i.e., a tumor featuring an intrinsic partial differentiation ability) [53], while if combined with *EWS-FLI-1*

translocation oncogene overexpression, this partial differentiation was lost, leading to Ewing sarcoma-like tumors [54]. Like FUS-CHOP, EWS-FLI-1 alone does not transform human adult BM-MSCs (unlike mouse MSCs), but it is able to impose a gene expression profile reminiscent of Ewing sarcoma [55]. Intriguingly, the degree of matching between these gene expression profiles was even greater if pediatric instead of adult BM-MSCs were used. A further increase was achieved when *EWS-FLI-1*-transduced pediatric human BM-MSCs were cultured in medium used to raise induced pluripotent stem cells [20], quite clearly classifying Ewing sarcoma as a stem cell- or reprogramming-type malignancy.

On the other hand, it is not yet clear whether MSCs are the cells of origin for Ewing sarcoma. Independent experiments with *EWS-FLI-1*-transduced NCSCs also showed a strong concordance with the Ewing sarcoma gene expression profile [56], leaving the question of Ewing sarcoma's cell of origin open. A similar question with a very similar dilemma is also pending for synovial sarcoma [57, 58].

Sarcoma Stem Cell Heterogeneity

From all the discussion above, we can take the existence of sarcoma stem cells as, if not certain, then certainly highly probable. Various approaches have been adopted to identify and isolate sarcoma stem cells (Table 7.2), which are, by and large, identical to those applied in various carcinomas, lymphomas, and leukemias, including a group of "obligate" stem cell markers, like CD44, CD90, and CD133 [80]. Such approaches suffer from an inherent weakness that they are based on an *a priori* assumption that positive cells equal stem cells, which is not always true. Specific for certain sarcomas could be cell surface markers that define normal mesenchymal stem cells.

Another possibility would be a marker-free approach essentially aimed at identifying chemoresistant cancer and normal stem cells, like side population (SP) sorting directed toward cells with a high expression of ABC efflux membrane transporters, especially ABCB1 and ABCG2, or Aldefluor assay targeting cells specifically overexpressing detoxification enzymes of aldehyde dehydrogenase family, especially ALDH1A1 and ALDH1A3 [81]. Several experimental models in which sarcomagenesis could be followed in a stepwise manner showed that sarcoma development and progression were associated with an increase in the stem cell fraction, expressed as both SP- [79] and Aldefluor-positive cells [65]. This association could be interpreted as sarcomas representing indeed stem cell tumors. Interestingly in this context, the Wnt inhibitor Dickkopf-1 has been reported not only to be crucial to overcoming senescence but also to increase ALDH1A1 expression and thus to promote sarcoma stemness [82]. In addition, specific stem cell targeting could be one mechanism of action of a relatively new antisarcoma chemotherapeutic drug trabectedin [83].

Table 7.2 A survey of stem cell markers exploited to identify and enrich for sarcoma stem cells

Marker	Biological function	Sarcoma type	References
CD133 (prominin-1)	Surface glycoprotein with five transmembrane domains localizing to membrane protrusions	Synovial sarcoma, osteosarcoma, rhabdomyosarcoma, Ewing's sarcoma, liposarcoma, chondrosarcoma	[59–63]
ALDH(1) (aldehyde dehydrogenase)	Group of enzyme catalyzing the oxidation of intracellular aldehyde to carboxylic acid	Osteosarcoma, Ewing's sarcoma, liposarcoma, fibrosarcoma, synovial sarcoma, chondrosarcoma, rhabdomyosarcoma, myxoid/round cell liposarcoma	[59, 61, 63–66]
Nestin (neuronal stem cell)	Type VI intermediate filaments protein	Rhabdomyosarcoma, osteosarcoma, fibrosarcoma (only in sphere-forming cell subpopulations)	[60, 61, 63]
CD184 (also as C-X-C chemokine receptor type 4 – CXCR4 – or fusin)	Alpha-chemokine receptor specific for stromal-derived factor-1 (SDF-1)	Osteosarcoma, synovial sarcoma	[60, 67]
CD117 (mast/stem cell growth factor receptor—SCFR; c-Kit proto-oncogene)	Receptor tyrosine kinase	Osteosarcoma	[59, 60, 68]
CD29 (integrin beta-1)	Adhesion molecule and extracellular matrix receptor	Osteosarcoma	[69]
CD49f (integrin alpha-6)	Adhesion molecule and extracellular matrix receptor	Osteosarcoma	[60]
STRO-1 (stromal cell precursor surface antigen)	Cell surface marker protein expressed on mesenchymal stem cells	Osteosarcoma	[59, 60, 68]
SSEA-4 (stage-specific embryonic antigen-4)	Glycosphingolipid expressed on embryonic stem cells	Osteosarcoma	[60]
CD57 (HNK1 - human natural killer-1 or LEU7)	Cell surface protein expressed on NK cells and neuroendocrine tumors	Ewing's sarcoma	[60]
LGR5 (leucine-rich repeat-containing G-protein-coupled receptor 5) also as G-protein-coupled receptor 49 (GPR49) or G-protein-coupled receptor 67 (GPR67)	Member of the Wnt signaling pathway; R-spondin receptor	Ewing's sarcoma	[60]

(continued)

Table 7.2 (continued)

Marker	Biological function	Sarcoma type	References
FGFR3 (fibroblast growth factor receptor 3) also as CD333	Receptor tyrosine kinase	Rhabdomyosarcoma	[60]
NANOG	Transcription factor involved in self-renewal of undifferentiated embryonic stem cells	Ewing's sarcoma, osteosarcoma, rhabdomyosarcoma	[60, 70, 71]
Sox2 also as SRY (sex-determining region Y)-box 2	Transcription factor involved in self-renewal of undifferentiated embryonic stem cells	Osteosarcoma, Ewing's sarcoma, rhabdomyosarcoma, synovial sarcoma	[60, 71]
Oct-4 (octamer-binding transcription factor 4) also as POU5F1	Transcription factor involved in self-renewal of undifferentiated embryonic stem cells	Osteosarcoma, Ewing's sarcoma, rhabdomyosarcoma	[60, 66, 70, 72]
CBX3 (chromobox protein homolog 3)	Component of heterochromatin, binds DNA and other proteins and receptors	Osteosarcoma	[73]
c-Myc	Transcription factor activating proliferation and apoptosis	Rhabdomyosarcoma	[71, 74]
Pax3 (paired box gene 3)	Transcription factor involved in muscle development	Rhabdomyosarcoma	[71, 75]
CD105 also as endoglin (ENG)	Involved in TGF-β signaling, cytoskeletal organization, and migration	Osteosarcoma	[76]
CD44	Cell surface glycoprotein expressed on mesenchymal stem cells important in cell-cell interactions and cell adhesion and migration; can interact with many ligands (HA, osteopontin, collagens, etc.)	Osteosarcoma	[69, 76]
CD146 also as melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18	A surface glycoprotein involved in cell adhesion, a receptor for laminin alpha 4	Fibrosarcoma, undifferentiated pleomorphic sarcoma, osteosarcoma	[77]
ABCG2 (ATP-binding cassette subfamily G member 2)	Protein transporting various molecules across extra- and intracellular membranes	Osteosarcoma, undifferentiated pleomorphic sarcoma	[61]

(continued)

Table 7.2 (continued)

Marker	Biological function	Sarcoma type	References
ABCA5 (ATP-binding cassette, subfamily A member 5)	Protein transporting various molecules across extra- and intracellular membranes	Osteosarcoma	[73]
Side population (dye exclusion)	Multiple ABC efflux pumps, including ABCB1 and ABCG2	Osteosarcoma, Ewing's sarcoma, synovial sarcoma, undifferentiated pleomorphic sarcoma, fibrosarcoma	[59, 78, 79]

Genetic and Epigenetic Plasticity of Sarcoma Stem Cells

An obvious question is inasmuch stemness of potential sarcoma stem cells is a "heritage" from an initiated normal stem cell (in this case most probably a mesenchymal stem cell) or whether it results directly from the action of sarcoma oncogenes. Two karyotypically simple translocation-dependent sarcomas could be rather instrumental to illustrate the latter possibility.

Synovial sarcoma is initiated by t(X;18)(p11;q11) translocation, resulting in a fusion protein between SS18 (whose gene is located on chromosome 18) and one of the translocation partner proteins SS1, SS2, or, rarely, SS4 (collectively called SSX, encoded by multiple homologous genes located on chromosome X) [84]. It has been reported that synovial sarcoma cells, without any sorting or selection, exhibited a high degree of stemness: Clonogenicity (sarcosphere formation) and tumorigenicity were comparable to those achieved by stem cell marker sorted populations in other tumors [85]. The SS18-SSX chimeric proteins encompass several transcription regulatory and protein-protein interaction domains, but, notably, no DNA-binding domain. The actual notion is that SS18-SSX engages a plethora of protein interaction partners, leading to complex changes in gene expression and finally resulting in accentuated stemness. Well-documented protein-protein interaction takes place between SS18-SSX and epithelial-mesenchymal transition (EMT)-triggering transcription repressors Snail and Slug, which prevents their binding to the E-cadherin gene promoter and results in pseudoepithelial transdifferentiation observed in some synovial sarcomas [85a]. Many SS18-SSX interaction partners involve chromatin modifier proteins, with a complex epigenetic reprogramming as a direct consequence. For example, it has been reported that SS18-SS2 directly interacts with the Polycomb-group complex 1 components Bmi1 and Ring1B, resulting in Bmi1 destabilization and the consequent derepression of a large group of Polycomb-silenced developmental genes. SS18 itself is a component of the mSWI/SNF-BAF chromatin remodeling complex, and its replacement by SS18-SSX fusion oncoproteins leads to complex changes in gene expression, among others to the erasure of the repressive histone mark H3K27me3 at the *Sox-2* locus [86, 87].

Strikingly, both of these effects are achieved also by the EWS-FLI-1 fusion oncoprotein underlying Ewing sarcoma, but via different mechanisms. Unlike

SS18-SSX, EWS-FLI-1 acts on its own as a direct transcription factor, via the ETS-DNA-binding domain supplied by the FLI-1 translocation partner [88]. According to recent results, EWS-FLI-1 can directly compete with the Polycomb repressor complex 1 (or, more probably, transcription factors mediating its recruitment, like YY-1) for binding to particular loci – notably certain *HOX* genes, especially posterior *HOXD* genes – resulting in their derepression [89]. In addition, it likely acts as both direct and indirect activator of Sox-2. This latter regulatory function is based on its direct transcriptional repression of miRNA145, encoded by a p53-responsive gene and directly targeting Sox-2 (as well as Oct-4). Intriguingly, EWS-FLI-1 is itself a target of miRNA145-mediated silencing, its repression thus stabilizing EWS-FLI-1 itself as well, a regulatory circuit already described for the relationship between Oct-4 and miRNA145 in embryonic stem cells [20].

Not surprisingly, all the epigenetic regulations crucially depend on the entire regulatory context resulting from signals acting on the cell. In the above-discussed experimental analyses of both synovial sarcoma and Ewing sarcoma, variations in cell culture media played important roles. In vivo, such a regulatory context would probably differ from cell to cell, generating a heterogeneous cell population with variable expressions of stemness traits in each individual sarcoma cell.

What is the situation in karyotypically complex sarcomas? As already mentioned above, dedifferentiated liposarcoma is an example of tumor type with an unusually high degree of genetic and epigenetic instability. This instability manifests at all thinkable levels. Karyotypic instability is mainly represented by neochromosomes, giant or ring chromosomes accumulating amplified segments of various chromosomes. Their origin is not clear, but the consensus is that they are triggered by the originally extrachromosomal amplification of a specific amplicon at 12q. Among the genes amplified are *MDM2* (coding for a direct p53 inhibitor, as mentioned above), *CDK4*, and *YEATS2* (coding for an acetylated and crotonylated histone reader). Via repeated breakage-fusion-bridge cycles, the 12q amplicon triggers a progressive genome destabilization, including structural chromosomal aberrations [90]. Notable among them is the translocation between *HMGA2* and *CPM* genes. *HMGA2* codes for a nonhistone chromatin protein involved in global gene expression regulation. The translocation removes the 3′-part of the *HMGA2* gene, resulting in two principal effects. First, it leads to the production of a shortened protein, and, second, because the removed 3′-part of the gene contains at least three target sequences for the let-7 miRNA, this shortened HMGA2 protein is grossly overexpressed [91]. Intact HMGA2 expressed at a normal level promotes adipogenic differentiation; an overexpressed full-length or shortened protein abolishes it instead, however. In addition, overexpressed HMGA2 has particular gene expression consequences. Among the genes it specifically induces, prominent is the *SS1*, one of the synovial sarcoma translocation partners (see above), eventually promoting stemness [92]. Copy number alterations in dedifferentiated liposarcoma underlie the overexpression of some additional genes with presumed roles in stemness (*c-JUN* oncogene, mesenchymal stem cell factor gene *TUFT1*) or chromatin organization (heterochromatin factor gene *CBX1*) [90]. There can also be more traditional epigenetic aberrations, like the promoter hypermethylation of the gene encoding the

key adipogenic transcription factor C/EBPα or of the *miRNA193b* gene [93]. One of the miRNA193b targets is the fatty acid synthase – an important cancer metabolic enzyme that (as described for leiomyosarcoma) at the same time interacts with various histone modification enzymes and modulates their activity [94], producing a sort of feed-forward loop in epigenome destabilization. Last but not the least, some point mutations found in liposarcoma can produce similarly widespread epigenetic consequences to those caused by more extensive changes described above. About 8% of dedifferentiated liposarcoma cases harbor point mutations (mostly missense) in the gene encoding histone deacetylase 1 [93]; striking in this respect is the finding that HDAC inhibitors might specifically target sarcoma stem cells (as described for the osteosarcoma model) [95].

These various mechanisms of dedifferentiated liposarcoma genome instability create an unprecedented level of genetic and epigenetic plasticity and impacts numerous genes implicated in differentiation and stemness regulation. This situation sets a completely new stage for research on cancer stem cell heterogeneity. Traditionally, it has automatically been assumed that a cancer stem cell achieves its stemness either from an initially mutated normal stem cell or as the direct molecular consequence of an initiating mutation. Once established, cancer stemness can be passed to some daughter cells, resulting in a more or less stable cancer stem cell pool, and it can be lost only by differentiation. Isolated populations of cancer stem cells, like side population cells or stem cell marker sorted cells, thus provide quasi-pure stem cells, but for a certain time only – until they are diluted by differentiation. In the context of the huge genetic and epigenetic plasticity, like that found in dedifferentiated liposarcoma, stemness might be understood in a completely different way. Stem cells might correspond simply to cells that at a given moment accumulate a sufficient number of stemness-promoting and differentiation-inhibiting mutations and epigenetic changes; in other words, stemness can be understood as a defined actual functional state rather than a quasi-fixed cell type. Some of such stem cells may differentiate, others can simply lose their stemness-promoting and differentiation-inhibiting genetic and epigenetic changes as a direct consequence of genetic and epigenetic plasticity, and still others, originally non-stem cells, can regain these changes by the same token. Stem and non-stem cells are thus continuously and bidirectionally changing, mixing, and merging.

Microenvironmental Influence on Sarcoma Stem Cells

All the discussion on sarcoma stem cells pursued by now concentrated largely on cell-autonomous mechanisms. We know, however, that tissue homeostasis is regulated by the cross talk between tissue-specific stem cells and their microenvironment, and, in a similar way, signaling from tumor niches may play relevant roles in the regulation of sarcoma stem cells [96–98]. Among the different subtypes of sarcoma, the relevance of the interaction between microenvironmental components and cancer (stem) cells has been especially well described in osteosarcoma [99].

Osteosarcoma cells closely interact with local microenvironmental cell types, such as stromal cells (MSCs and cancer-associated fibroblasts), osteoblasts, osteocytes, osteoclasts, or chondrocytes, as well as with immune infiltrates mainly composed of T lymphocytes and macrophages [97, 100]. The interaction between these multiple players results in the production of signaling factors that contribute to either favoring or decrease of stemness properties in osteosarcoma [97]. Thus, signaling mediated by fibroblastic growth factor (FGF)-Sox2 axis [101], transforming growth factor β (TGF-β) [102], the Hippo signaling regulator YAP1 [103], or NOTCH1 [40], among others, was reported to promote stemness in osteosarcoma. On the other hand, signals with proven pro-osteogenic activities, like those dependent on bone morphogenetic proteins (BMP) [104] or WNT factors [101], seem to decrease sarcoma stem cell frequency and to promote osteogenic differentiation. In addition, extracellular matrix components of the tumor microenvironment have also been reported to interact with sarcoma stem cells. This is the case of hyaluronan, which may promote stemness properties in tumor cells through the binding with its receptor, the cancer stem cell marker CD44 ([105] – see Table 7.2). We can assume that details of composition of this complex tumor microenvironment differ in each individual tumor, resulting in a wide spectrum of osteosarcoma stemness modulation.

Notice that within the context of osteosarcoma (and possibly other sarcoma types as well), MSCs can thus be viewed not only as possible cells of origin but also as a stromal supporting type. Indeed, MSCs are the cell type with rather precisely described interactions with osteosarcoma stem cell subpopulations. MSCs may be activated by the acidic conditions generated by osteosarcoma cells and these tumor-conditioned MSCs favor osteosarcoma stemness and chemoresistance via IL6-NF-κB signaling [106]. Moreover, MSCs may increase chemoresistance of osteosarcoma cells through the activation of IL6/STAT3 pathway [107].

Several locations within the bone microenvironment where pro-stemness signaling is particularly active have been proposed as suitable niches for osteosarcoma stem cells [96] (Fig. 7.2): (i) the perivascular niche, which was described as the most likely location for the most immature MSCs and therefore may also constitute a niche for sarcoma stem cells originated by transformed MSCs [108]; (ii) the hypoxic niche, which is an important stemness-promoting environmental condition in bones [109]; and (iii) the endosteal niche, which is a signal-rich environment where tumor cells interfere with the bone remodeling process, establishing a “vicious cycle” that favors osteoclast-mediated osteolysis and the subsequent release of calcium and growth factors (FGF, TGF-β, IGF1, BMP, etc.), which support stem and tumorigenic properties [97]. In any case, these three prototypical osteosarcoma niche types differ in their detailed molecular mechanisms of stem cell support, plausibly resulting in a niche-dependent osteosarcoma stem cell heterogeneity.

The detailed knowledge of the microenvironment in maintaining tumor homeostasis has encouraged the development and testing of therapies aimed to counteract pro-tumoral signals, including pro-stemness signals, from the microenvironment [96]. Consequently, several therapeutic strategies have been recently developed to target the role of the tumor-promoting osteoclast activity [110, 111], to reduce the vascularization of tumors [112], and to enhance the immune response against tumors [113, 114].

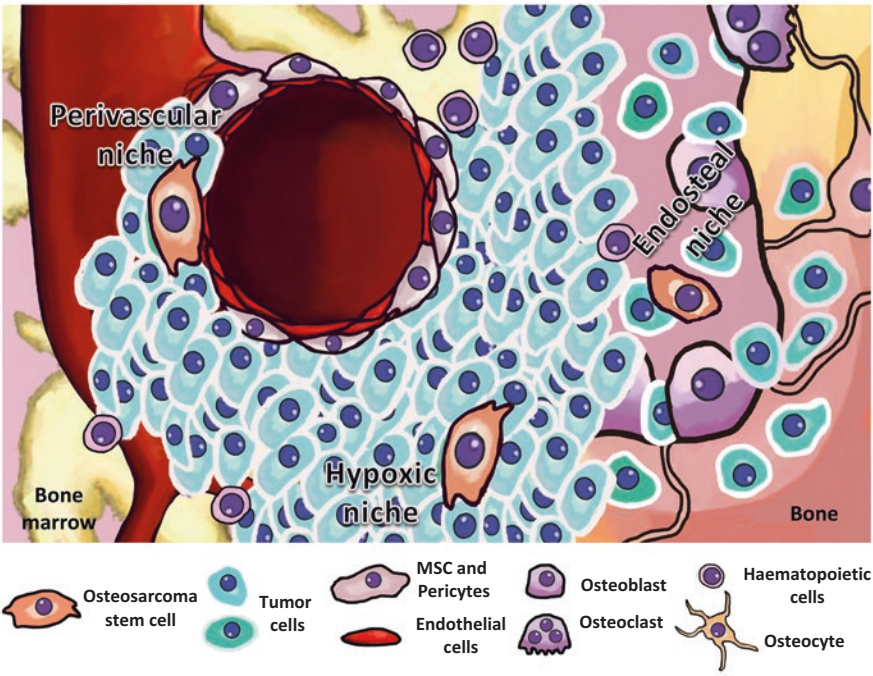

Fig. 7.2 Osteosarcoma stem cell niches. Figure shows the most relevant cell types of the bone microenvironment that may interact with osteosarcoma cells. Suggested locations for osteosarcoma stem cells include the perivascular niche, the endosteal niche, and the areas of poor vascularization (hypoxic niche) (adapted from [96]

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Chapter 8
Heterogeneity in Adipose Stem Cells

Elio A. Prieto González

Abstract Adipose stem cells (ASCs) are the basis of procedures intended for tissue regeneration. These cells are heterogeneous, owing to various factors, including the donor age, sex, body mass index, and clinical condition; the isolation procedure (liposuction or fat excision); the place from where the cells were sampled (body site and depth of each adipose depot); culture surface; type of medium (whether supplemented with fetal bovine serum or xeno-free), that affect the principal phenotypic features of ASCs. The features related to ASCs heterogeneity are relevant for the success of therapeutic procedures; these features include proliferation capacity, differentiation potential, immunophenotype, and the secretome. These are important characteristics for the success of regenerative tissue engineering, not only because of their effects upon the reconstruction and healing exerted by ASCs themselves, but also because of the paracrine signaling of ASCs and its impact on recipient tissues. Knowledge of sources of heterogeneity will be helpful in the standardization of ASCs-based procedures. New avenues of research could include evaluation of the effects of the use of more homo1geneous ASCs for specific purposes, the study of ASCs-recipient interactions in heterologous cell transplantation, and the characterization of epigenetic changes in ASCs, as well as investigations of the effect of the metabolome upon ASCs behavior in culture.

Keywords Heterogeneity · Mesenchymal · Adipose stem cell · Adipose tissue depot · Stromal vascular fraction · Stemness · Xeno-free · Immunophenotype · Gene expression analysis

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Introduction

The discovery that adipose tissue represents an interesting source of multipotent stem cells (adipose stem cells [ASCs]) has led to many studies exploring the clinical potential of these cells in cell-based therapies. Recent advances have allowed a better understanding of the secretory capacity of adipose tissue and the role of adipokines in the development of obesity and associated disorders, giving a new dimension to the study of adipose tissue biology in normal and diseased states. Adipose tissue is a mesenchymal tissue that comprises several cell types, such as preadipocytes, adipocytes, fibroblasts, endothelial cells, monocytes, macrophages, and lymphocytes [1].

Stem cells are present in numerous tissues; these cells are capable of self-renewal, are viable for long periods, and can differentiate into various lineages according to the microenvironment in vivo or the culture conditions in vitro (see Fig. 8.1). When these cells are exposed to factors that stimulate their growth and differentiation, they can become adipocytes, osteocytes, or chondrocytes. This capacity is the basis of stem cells' success in regenerative procedures for healing improvement or tissue regeneration. Stem cells are classified according to their tissues of origin: (1) embryonic stem cells (ESCs), (2) fetal stem cells (FSCs), and (3) adult stem cells [1].

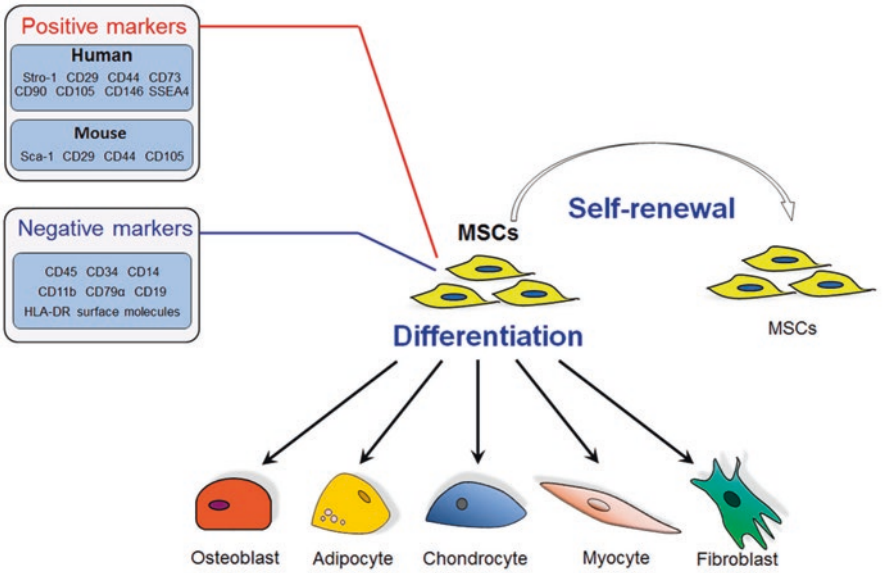

Fig. 8.1 Pluripotency of mesenchymal stem cells (MSCs). There are both positive and negative markers for identifying MSCs. MSCs possess the characteristics of being self-renewing and differentiating into multiple cell types, including osteoblasts, adipocytes, chondrocytes, myocytes, and fibroblasts. Reproduced from Hu L, Chong Y, Fan Z, Arshad A, Jianhua M, Airong Q (2018) Mesenchymal Stem Cells: Cell Fate Decision to Osteoblast or Adipocyte and Application in Osteoporosis Treatment, Int. J. Mol. Sci. Section Biochemistry, Molecular and Cellular Biology 19(2), 360; https://doi.org/10.3390/ijms19020360

ASCs separated, by their adherence to plastic surfaces, from adipose tissue (obtained by different methods) that can reasonably be considered as belonging to the same population, have been named differently and the nomenclature includes such terms as adipose derived adult stem cells (ADASs), adipose derived adult stromal cells (ADSCs), adipose stromal cells adipose mesenchymal stem cells (AdMSCs), preadipocytes, adipose derived stromal stem cells, and processed lipoaspirate cells. The consensus term proposed for the plastic-adherent, cultured and pluripotent cells is adipose derived stromal/stem cells, but in recent articles the ASCs denomination is still being used for adipose stem cells [2, 3].

In order to avoid confusion about terminology, we will refer to multipotent precursor cells from adipose tissue stroma as adipose stem cells (ASCs) or adipose-derived stem cells (ADSCs).

Adipose stem cells of embryonic origin show great multilineage potential, but their isolation poses concerns about the ethics of the procedures used to obtain these cells; this has fueled research that is oriented to obtain cells that, naturally or through reprogramming, could substitute for ESCs in tissue regeneration.

Adult or postnatal stem cells were first isolated from bone marrow (BMSCs)) and showed good results in tissue repair. Bone marrow cells consist of two subpopulations, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

Adipose Derived Stem Cells. Adipose Tissue and Stromal Vascular Fraction

Adipose derived stem cells (ADSCs) have been recognized as an alternative to bone marrow and FSCs for their use in regenerative medicine, owing to their multipotentiality and relative abundance, features that are essential for good results in cell-based therapy. Approximately 75 million cells can be isolated from 200 mL of processed lipoaspirate in a culture period of less than a week. This means that in a single liposuction procedure it is possible to obtain nearly 375 million ASCs per donor. It has been estimated that 70 million cells are contained in a cubic centimeter of bone marrow aspirate [4].

The higher amount of adipose tissue that can be obtained from a single donor, as compared with what is extracted in a bone marrow aspiration, and the fact that the proportion of stem cells in adipose tissue is also greater, points to adipose tissue as an eligible source of stem cells for therapeutic practice [5].

ADSCs exhibit greater proliferative capacity than bone marrow-derived MSCs, but are similar in colony-forming efficiency. However, bone marrow-derived MSCs are more easily differentiated toward osteogenic and chondrogenic lineages, but are equal to ADSCs in differentiation potential into adipocytes. Moreover, adipose tissue-derived stem cells are more powerful immunomodulators than stem cells derived from bone marrow [6]. These features, which have been repeatedly con-

firmed, are the basis of the increased utilization of ADSCs in stem cell-based regenerative transplant protocols [7, 8]. ADSCs were mentioned for the first time in the work of Zuk et al. [9, 10] and MSC immunological markers ($CD29^+$, $CD44^+$, $CD71^+$, CD90, $CD105^+$, CD49D/CD31, $CD3^-$, $CD4^-$, and CD106) were detected.

ASC therapy does not pose complex ethical problems, as the cells are easily isolated with procedures that are less invasive than those for isolating MSCs from bone marrow [11]. The immunomodulatory properties of ASCs are a feature that contributes to the attractiveness of these cells for transplant-based treatments.

Heterogeneous ADSCs (also referred to as ASC) are isolated from the aqueous fraction obtained after the enzymatic digestion of lipoaspirate; this aqueous fraction is known as the stromal vascular fraction (SVF) [12]. Despite differences between cells in the intact adipose tissue and those in the SVF, the SVF is defined by the National Cancer Institute as *"A population of stromal vascular fraction (SVF) cells derived from autologous adipose tissue, with potential tissue regenerative activity. SVF cells are obtained through liposuction and contain multiple cell types, including adipose-derived stem cells (ADSCs), mesenchymal and endothelial progenitor cells, leukocyte subtypes, lymphatic cells, pericytes, and vascular smooth muscle cells. The SVF cells are processed in such a way as to contain a reproducible and consistent composition of heterogeneous cells. Upon processing and administration, the adipose-derived SVF cells can differentiate into different tissue types, support neovascularization, replace cells and repair injured issue"*. (https://ncit.nci.nih.gov/ncitbrowser/pages/home.jsf?version=18.08d) The SVF also contains a collagen matrix, with nerves, blood, and lymphatic vessels [12–14]. After isolation from the SVF and subsequent culture and expanding, there remains a more homogeneous cell population that expresses MSC markers (CD90, CD73, CD105 and, CD44), but no hematopoietic ones (CD 45, CD34). This population successfully differentiates into adipogenic or osteogenic lineages that can be used for reconstruction or tissue repair. Following plastic-adherent selection, the SVF cells yield between 0.25 and 0.375 million ASCs per milliliter of human lipoaspirate [15, 16].

There are many other tissues and organs from which MSCs have been identified and isolated, such as brain, umbilical cord blood, peripheral and menstrual blood, periosteum, muscle, skin, gut, liver, spleen, and lung. There are no widely normalized procedures for the processes of MSC isolation to expansion and differentiation, and there is no fully shared definition of the features of a mature differentiated cell population [2, 3, 17, 18].

When cultured, after a few hours ASCs can be identified as spindle-shaped cells with a fibroblast-like appearance. As culture time increases, they differentiate according to the conditioning medium; when cells differentiate toward adipocytes positive staining for Oil Red O is observed, whereas when cells differentiate toward osteocytes or chondrocytes, the staining is positive for Alcian blue or Alizarin red [19].

Adipose tissue (AT) is a regulator of energetic homeostasis, owing to its participation not only as a triglyceride reservoir and a key factor in thermogenesis; but also owing to its involvement in glucose and lipid metabolism. AT influences insulin sensitivity, hormone responsiveness, and the secretion of adipocytokines, which act

in appetite control and the inflammatory response, among many other functions. Increased adipose tissue, which can lead to obesity, is the initial event that, through the generation of insulin resistance, can induce alterations in glucose metabolism, leading to hyperglycemia, hyperinsulinemia, impairment of pancreatic function, diabetes, dyslipidemias, and atherosclerosis [20].

However, not all adipose tissues, found in the same body places, consist of the same kinds of adipocytes and behave in the same way. There are two main types of adipose tissue, categorized as: white adipose tissue (WAT) and brown adipose tissue (BAT). This categorization is based on the color and the distribution of fat inside the cells composing the tissues. WAT cells show a single intracellular drop of fat, referred to as unilocular distribution, whereas in BAT cells the fat distribution is multilocular [2, 21].

WAT beneath the skin is called subcutaneous white adipose tissue (SWAT); this tissue is distributed in the subcutaneous regions of the body, such as in the abdomen, buttocks, and thighs. WAT is also located in Bichat's fat pad, the retro orbital space, hands, and feet, as well as in other structures that need cushioning or need to be held in place. SWAT is subdivided into superficial adipose tissue and deep adipose tissue, and MSCs from these sources may be different [22]. ASCs isolated from subcutaneous regions show more osteogenic potential than that obtained from the deep layer adipose tissue. In line with this, more osteogenic marker genes are expressed in these ASCs than in ASCs derived from other compartments of adipose tissue, such as visceral white adipose tissue (VWAT). However, adherent cells isolated from SWAT and VWAT showed comparable proliferation capacity and adipogenic potential [23].

SWAT is the main regulator of energy metabolism, accumulating triglycerides that deliver non-esterified fatty acids (NEFAs) to energy production; SWAT avoids the development of ectopic fat depots, protects against dermal infections, and provides thermal isolation and defense against mechanical traumas [2, 24]. Visceral WAT can be found deeper in the body cavity in different locations. There are six visceral depots where VWAT can be found: omental, mesenteric, perirenal, gonadal, epicardial, and retroperitoneal. There is some controversy about the common origin of SWAT and VWAT, because there is evidence indicating that the main source of VWAT is the lateral plate of the mesoderm, but there is also VWAT of mesothelial origin. This different origin of at least a proportion of VWAT cells is in line with the strikingly divergent consequences of subcutaneous obesity and visceral or abdominal visceral obesity [25]. Whereas increased SWAT is less harmful and is even considered to be protective, VWAT is predominantly associated with the development of metabolic syndrome [20, 26].

BAT is found in the human body at the beginning of life in cervical, perirenal, periadrenal, axillary, paravertebral, and supraclavicular regions, but is transformed into WAT in the adult. Despite this distribution, BAT has been considered to have little metabolic significance in the adult, although there is evidence that BAT is still functional in supraclavicular, cervical, axillary, paravertebral, and suprarenal regions [27].

In addition to these adipose tissues, a third type has been identified in rodents and has been called beige adipose tissue. Beige or brite (from brown and white) adipocytes are located within WAT but are more akin to those in BAT. This new type of adipose cells express thermoregulatory protein uncoupling protein 1 (UCP-1), and morphologically the cells are like brown adipocytes. Another kind of adipocytes are those that grow in the same niche as bone marrow, the bone cavity, and this tissue is referred to as marrow adipose tissue (MAT); the adipocytes in MAT are descendants of stem cells from a different origin than that of WAT or BAT [28].

Heterogeneity of adipose tissue is a complex issue that goes beyond this first recognized division in WAT and BAT, when classification of adipose tissue is based in different criteria, like localization, immunological markers, the gene expression profile, and the secretome [24, 29].

Human and animal WAT exhibit differences in biochemical and endocrine properties, such as in insulin sensitivity and signaling, adipocytokine secretion, lipolytic activity, and sensitivity to hormones [2]. The two types of adipose tissues also exhibit differences in gene expression [1, 29].

ASCs represent a dynamic population of cells that are influenced, as is every cell, by their genetic composition and the environment, and they exhibit differences according to their precise locations in the body; these variations are related to proliferation capacity and stemness (see Fig. 8.2). When WAT derived ASCs, obtained from SWAT were compared with ASCs obtained from VWAT, it was found that the SWAT-derived ASCs showed greater proliferation than the ASCs obtained from VWAT and were more easily differentiated to adipose or osteogenic lineages [2].

Other authors attribute more adipogenic characteristics and fewer requirements for growth factors for SWAT than for VWAT; VWAT cells have been called "antiadipogenic" and show greater requirements for growth factors to differentiate; this behavior has to be added to the list of differences between ASCs according to their specific fat depot. Differences between adipocytes from these fat depots are shown in gene expression signatures, differentiation capacity, sensitivity to growth factors, and biochemical regulation in health and disease [2, 22, 30]. V-ASCs, i.e., ASCs from VWAT have been found to proliferate more than S-ASCsi.e., ASCs from SWAT, but S-ASCs express greater adipogenic capacity than V-ASCs [31].

There have been analyses of differences in cell fate between ASCs from subcutaneous fat and those from visceral fat. It has been shown that ASCs isolated from SWAT SVF differentiate more readily than those obtained from VWAT. Macotela et al. found that the differentiation requirements of these types of cells were far from being similar [29]. In their study, isolated cells from mouse vascular fraction were sorted by fluorescence-activated cell sorting (FACS), and adipocyte precursor cells were negative for CD45, CD31, and Ter 119 and positive for CD34 and SCA1. After 7 days of treatment in the presence of the typical induction protocol that included rosiglitazone, the confluent culture showed that more than 90% of ASCs from SWAT were positive for lipid accumulation and the expression of markers specific for adipocytes. It is noteworthy that about 10% of cells from the subcutaneous depot differentiated into adipocytes, even in the absence of hormone inductor.

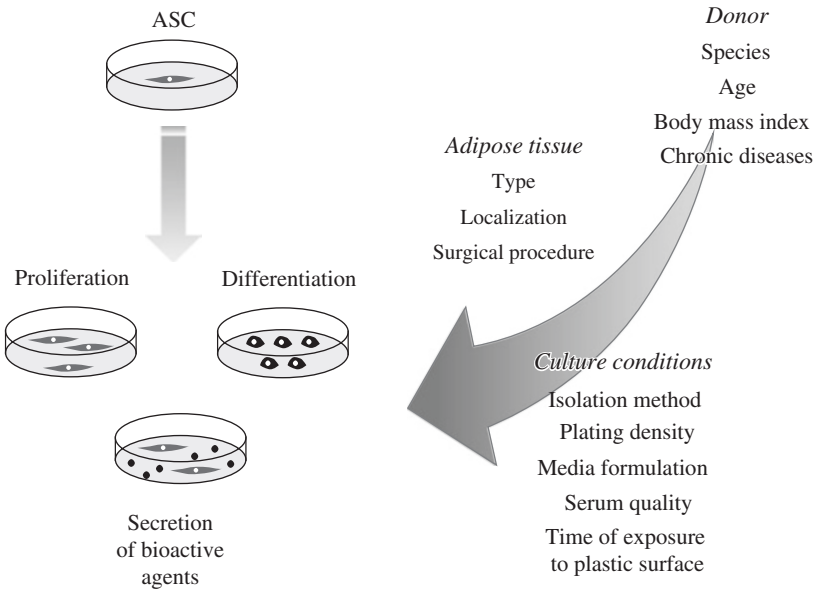

Fig. 8.2 Sources of variations in adipose stem cells (ASCs) are related to biological and procedural characteristics. Several factors influence the relevant features of ASCs' proliferation capacity, differentiation potential, and secretion of immunoactive and trophic molecules. Reproduced from Kocan B, Maziarz A, Tabarkiewicz J, Ochiya T, Banas-Zabczyk A (2017). Trophic Activity and Phenotype of Adipose tissue Derived Mesenchymal Stem Cells as a Background of their Regenerative Potential. Stem Cells Int, 2017; 1653254, doi: 10.1155/2017/1653254

Conversely, less than 20% of ASCs from VWAT exhibited characteristic adipocyte features with the same treatment protocol.

Bone morphogenetic factor (BMP)-4 is a known inducer of MSCs into adipocyte, muscle, and bone lineages, and the requirement for this factor was almost null for ASCs from SWAT. In this study, (28) gene expression in ASCs from SWAT was several times greater than that in ASCs from VWAT; this expression was relevant for markers of adipogenic differentiation such as *Fabp4* (fatty acid binding protein), 730-fold greater in ASCs from SWAT; *Slc2a4* (Glut 4), 300-fold greater; *Adipoq* (adiponectin), 730-fold greater; and *Lep* (leptin), 20-fold greater compared with the gene expression in ASCs from VWAT. When ASCs from VWAT were treated with BMP-4, there was a lower rise in the expression of *Fabp4* (480-fold), *Slc2a4* (160-fold), *Adipoq* (660-fold), and *Lep* (21-fold). When BMP-4 was added to cultures 2 days before adding induction cocktail (IC), a small increase in the differentiated cell number was obtained in ASCs from subcutaneous depots, while in VWAT cells, differentiation rose to 90% [29].

There are reports of heterogeneity in gene expression between subcutaneous and gluteal depots. A group of 131 genes are expressed differently in subcutaneous abdominal adipocytes than in gluteal adipocytes, including two that are molecular signatures of visceral adipocytes, i.e., homeobox genes HOXA5 and NR2F1, which

are upregulated in subcutaneous abdominal fat, showing a 2.5-fold change compared with that in gluteal adipocytes; this seems to show that subcutaneous abdominal adipocytes have a closer relationship with visceral fat than with gluteal adipose cells [32].

Heterogeneity Related to Differences in ASCs Isolated from the Same Depot

Determining the location of stem cells in adipose tissue is a difficult task because of the absence of precise markers of undifferentiated ASCs, as occurs with MSCs [17]. However, immunohistochemical and immunofluorescence techniques have helped to identify the microenvironment for ASCs as the perivascular zone, which is also abundant in pericytes and of course in endothelial cells [33]. In a refining of the search for the precise place of pericytes within adipose tissue, it was concluded that there are two distinct perivascular precursors of ASCs: the pericytes around capillaries and microvessels and the adventitial cells around arteries and veins [34, 35].

There are several reports about ASCs in the perivascular space in capillaries and in the adventitial layer in greater vessels. These cells have been identified as bearing $CD34^+$, $CD31^-$, $CD140\beta^-$, and α smooth muscle actin (smA), while others have found ASCs in the external adventitious ring, which express $CD90^+$, $CD34^+$, $CD31^-$, $CD146^-$, and smA^-. Traktuev et al. found ASCs expressing $CD34^+$, $CD31^-$ markers in microvascular walls, attributing to this ASC population functions related to the maintenance of blood supply, specifically in the angiogenesis and stabilization of a vascular network by human ASCs and endothelial cells in adipose tissue implants [36]. Another study reports that stem cells isolated from the perivascular region of human white adipose tissue express $CD34^+$, $CD31^-$, $CD146^-$, $CD45^-$ markers that are utilized to identify MSCs. There are other markers expressed by stem cells in the perivascular region besides those accepted for MSCs. These markers are CD146 neuroglial proteoglycan 2 and CD140β. There are reports of 146^+ cells isolated in the perivascular region that have shown multilineage differentiation in culture. These cells could be representative of another population, being dissimilar in one marker, but coinciding in some markers [35, 36].

Based on surface markers, pericytes have been classified into two categories: $CD31^-/CD45^-/CD34^+/CD146^-$ cells (adventitial stromal/stem cells) and $CD31^-/CD45^-/CD34^-/CD146^+$ cells (pericytes [PCs]). In addition to classification according to these markers, adventitial stromal/stem cells and PCs are classified by maturity, according to aldehyde dehydrogenase (ALDH) activity, a functional marker of primitivity, which is utilized as an indicator of the development of a multiple connectivity network. Intensity of staining with Aldefluor was utilized to classify the viable ASC (CD34+/CD45−/CD31−/CD146) and pericyte populations PC (CD146+/CD34−/CD45−/CD31−) into Aldefluor-dim (ALDHdim) and Aldefluor-bright (ALDH-br). The cell subclasses express four levels of maturity,

which is another expression of their heterogeneity: (a) ALDHbr ASC (most primitive); (b) ALDHdim ASC; (c) ALDH-br PC; and (d) ALDHdim PC (least primitive). This finding is suggestive of a developmental hierarchy in the population of MSCs in the perivascular niche [37]. The presence or absence of a single marker in cells obtained in the same histological place points to the heterogeneous nature of that population and to the fact that if its fate is to be differentiated in several cell subpopulations that can be found at different developmental stages in that region.

Kilinc et al. studied the SVF from 58 patients and identified four major subpopulations: adipose derived stem cells (ADSCs), hematopoietic stem cells (HSC progenitors), and adipose tissue endothelial cells and pericytes (ATEC/PCs) [38]. Biomarkers expressed in the SVF corresponded to the cell subpopulations as follows: adipose tissue ($CD34^{high}$, $CD45^{-}$, $CD31^{-}$, $CD146^{-}$; ADSC ($CD34^{low}$, $CD45^{+}$, $CD206^{+}$, $CD31^{-}$, $CD146^{-}$) and HSC progenitors ($CD34^{high}$, $CD45^{-}$, $CD31^{+}$, $CD146^{+}$); ATEC/Pc ($CD45^{-}$, $CD34^{-}$, $CD31^{-}$, $CD146^{+}$). These findings constitute further evidence of heterogeneity in cells from the niche surrounding the vessels within adipose tissue. It is necessary to remark that these authors concluded that the relative proportion of ADSCs to HSC in the SVF was the main feature that determined the amount of secreted paracrine healing factors in the SVF, thus modulating the therapeutic capacity of transplanted cells.

Heterogeneity in the SVF is one of its main distinguishing characteristics. Hematopoietic progenitor cells and pericytes expressed several features that were close to ADSCs, as they could grow in adherent culture and show mesenchymal multipotency. However, it should be stressed that plastic-adherent cells from the SVF, which originated the ADSCs and expanded mesenchymal stem cells, were grown from a conventional ADSC subset. The importance of the interactions of cells in the SVF cannot be underestimated and should be analyzed in the light of the influence of angiogenic and immunomodulatory factors in cell grafting and development [39, 40].

Yoshimura et al. have reported a profile that intersects with that mentioned above [41]; namely, (ASC/ADSC) $CD31^{-}$, $CD34^{+}CD45^{-}$, $CD90^{+}$, $CD105^{-}$, $CD146^{-}$); endothelial progenitor cells ($CD31^{+}$, $CD34^{+}$, $CD45^{-}$, $CD90^{+}$, $CD105^{low}$, $CD146^{+}$); pericytes that are profiled separately from endothelial cells, ($CD31^{-}$, $CD34^{-}$, $CD45^{-}$, $CD90^{+}$, $CD105^{-}$, $CD146^{+}$); and blood-derived cells ($CD45^{+}$). These authors report a general picture of the proportion profiles of these cells that can be found in the SVF: the percentages are: $CD2^{+}$ 11%, $CD11a^{+}$ 18%, $CD14^{+}$ 29%, $CD31^{+}$ 49%, $CD45^{+}$ 57%, and $CD90^{+}$ 60%). A different composition of the SVF was found by Astori et al. [42]; namely, $CD14^{+}$ 11%, $CD31^{+}$ 2%, $CD34^{+}$ 7%, $CD45^{+}$ 9%, $CD90^{+}$ 29%, and $CD146^{+}$ 47%.

It is evident that sometimes there were wide divergences between the proportions of $CD31^{+}$ $CD45^{+}$ or $CD90^{+}$ cells identified by these two groups of researchers. It is worth noting that, in addition to the heterogeneous nature of SVF cell composition, among many known and unknown intervening variables are donor heterogeneity, the isolation and identification procedures, and even the precise body location—and within it, the depth of tissue—from which the sample was taken [43, 44].

Heterogeneity Related to Procedures

ASCs have been isolated through lipoaspiration and block resection of tissue. The procedures used affect the growth, survival, and cell yield. There is a consensus that a higher yield of viable ASCs is obtained by liposuction than through resection [2, 45].

Microaspiration of fat (micro fat harvesting) has also been reported to be more efficient than the usual procedures, as expressed in higher yields, greater viability, greater adhesion rates, and greater secretion of growth factors, such as insulin-like growth factor (ILF) and platelet-derived growth factor (PDGF) [46]. Comparison of procedures indicates that a higher surface-to-volume ratio is obtained with micro-cannulas than that obtained with standard cannulas.

Baer and Geiger [17] reviewed the literature on the heterogeneity of sources for ADSCs; their review showed that more than 85% of the cells in the SVF that were adherent to plastic expressed $CD31^-$, $CD34^+$, $CD45^-$, and $CD146^-$. Stem cell properties have been linked by other authors to a phenotype comprising $CD31^-$, $CD34^+$, $CD45^-$, and $CD105^+$ cells. An important profile correspondence with potential differentiation toward mesenchymal lineages is that of $CD31^-$ vs $CD31^+$, when in 2005 it was reported by Boquest et al. that only $CD31^-$ cells had multilineage differentiation in culture [47].

It has been stated that inconsistency in determining the precise characteristics of ADSCs in the SVF results in a lack of strict definition of the characteristics of an ADSC. The variety of different stem cells in the SVF may be one of the reasons for this lack of a precise defining pattern, and this variety could also be a consequence of shifts in the phenotype of ADSCs in the first hours after adherence in culture, as a sort of Heisenberg uncertainty principle.

The International Society for Cellular Therapy has presented criteria for the identification of MSCs after isolation from the source; namely, adherence to the plastic surface of culture plates, an immunological profile that should be positive for CD90, CD73, and CD105 in more than 95% of the MSCs and negative for CD34, CD45, CD11b or CD14, CD19 or CD79α; human leukocyte antigen (HLA) DR expression; and the ability to differentiate into adipocytes, osteoblasts, and chondrocytes [48].

ASC yield, differentiation capacity, and doubling time was compared in cells obtained from resection, tumescent liposuction, and ultrasound-assisted liposuction, and it was shown that the number of viable cells in the SVF was not influenced by the particular procedure. ASCs obtained from the above procedures that expressed the consensus markers yielded the same numbers of viable cells that successfully differentiated into adipogenic, chondrogenic, and osteogenic lineages. However, ultrasound-assisted liposuction resulted in lower ASC proliferation, as well as prolonged doubling time [49].

Heterogeneity Related to Donors

There is no unique method for ASC isolation, or for assessing differences arising from distinct procedures. But there are also variations attributable to cell donors. Cell characteristics are affected by the donor's body mass index (BMI), age, sex, health condition, ethnic origin, unhealthy habits, and drug consumption [50]. The procedure used for lipoaspiration or liposuction, the time elapsed between tissue excision and the start of the specific isolation protocol, and the precise area from which tissue is obtained also influence ASC characteristics [17, 51–54].

The proliferation and differentiation of ASCs isolated from one donor might differ from these features of ASCs obtained from another donor. The ASC population obtained from each adipose tissue sample is heterogeneous with respect to the differentiation stage; the proportion of these cells is reported to vary with donor age, but also between subjects of the same age [55].

Inter-individual heterogeneity has been attributed to the sampling method, but it could be the result of different sampling times. The pathological background and the current health status of the donor might influence the relevant characteristics of ASCs. Modifications in the DNA methylation profiles of the genes for transcription factors Runx2 and PPARG influence the osteogenic and adipogenic differentiation of ASCs. As methylation patterns differ between cells harvested from different donors, it is considered that epigenetic modulation is another source of variation between ASCs [56].

BMI of Donors

The effect of BMI on the multilineage differentiation of ASCs is controversial. For some authors, BMI is negatively correlated with the number of stromal cells that can be obtained per gram of tissue, and their differentiation capacity; Van Hamerlen et al. found an inverse relationship between BMI and the number and differentiation capacity of adipocytes in 30 obese women [57]. Aust et al. found the same negative effect of BMI on critical features in cultured ASCs [15]. These results have been confirmed by in vitro studies that also found less proliferation and differentiation as the adipose mass increased [58–62].

Other authors report that BMI was positively correlated with osteogenic differentiation capacity and also with adipogenic differentiation. Also, a BMI above 30 was associated with enhanced adipogenic differentiation compared with that in cells isolated from individuals with a BMI below 25 [63]. In ASCs from obese subjects there are modifications in telomere length and telomerase activity [64]. Altered mitochondrial function results in the increase of oxidative species and cellular deregulation. These alterations must be considered when regenerative treatment is intended to be based upon ASCs obtained from obese donors [65].

In this sense, there is a report revealing that when ASCs obtained from obese individuals were co-cultured with monocytes the inflammasome was activated, as characterized by enhanced interleukin (IL) secretion. These ASCs favored STAT3 over STAT5 transcription factor binding on STAT binding sites from the IL-17A/F gene. Moreover, the conditioned media from ASCs plus monocytes inhibited adipocyte differentiation and impaired the insulin-mediated inhibition of lipolysis. Remarkably, IL-17A secretion also occurred in SVFs obtained from obese but not from lean subjects [66].

BMI is one of the contributors to ASC heterogeneity. Obese adipose tissue that is under cytoplasmic stress—which modifies its secretome—could be negatively associated with those aspects that are relevant for ASC therapeutic effect; however, the reports invoking a favorable relationship with BMI must not be ignored. This controversy could be related to the secretome of obese adipose tissue, which is abundant in cytokines and growth factors that produce a plethora of actions [2], and these actions may possibly provide the explanation for the variations in results (see Fig. 8.3).

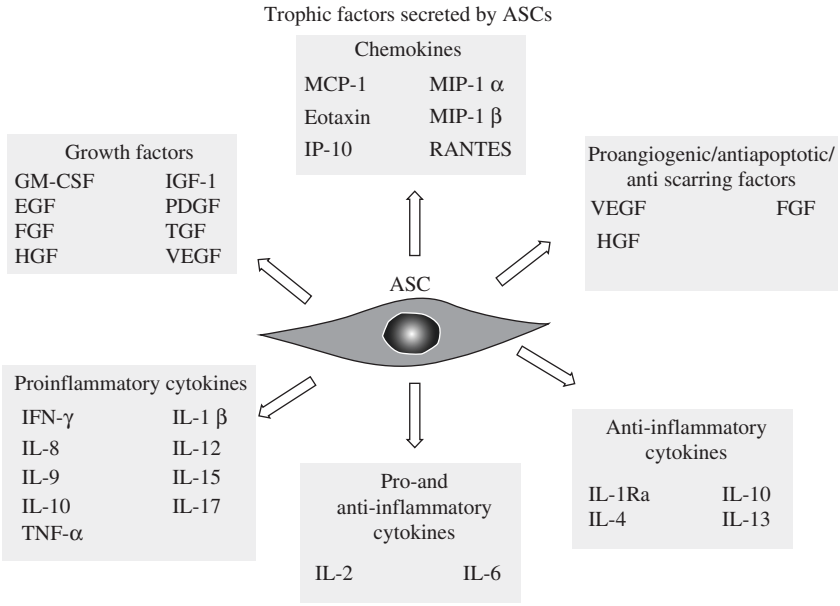

Fig. 8.3 Factors derived from ASCs as an additional source of variations. Several kinds of molecules released by ACSs are relevant to their biological effects and contribute to the intrinsic variations that characterize ASC-based treatment. Reproduced from Kocan B, Maziarz A, Tabarkiewicz J, Ochiya T, Banas-Zabczyk A (2017). Trophic Activity and Phenotype of Adipose tissue Derived Mesenchymal Stem Cells as a Background of their Regenerative Potential. Stem Cells Int, 2017; 1653254, doi: 10.1155/2017/1653254

Adipose tissue stem cells are involved in adipogenic, angiogenic, and secretory functions in obese hypertrophic tissue. Some features of subcutaneous adipose tissue that are shown after massive weight loss mimic the behavior of this tissue when it was hypertrophic, and this behavior has been called “adipose memory”. This behavior is related to the imbalance caused by cytoplasmic stress, and is expressed as an inflammatory secretome with a profound impact on glycolipid metabolism. The use of ASCs from formerly obese patients poses the question of whether the reversibility of these cellular patterns in these potential donors affects the therapeutic use of their ASCs [67].

Adipose tissue from women who have had bariatric surgery expressed differences in SVFs, with distinct differences in the composition of the SVFs and differences in ASCs from different body fat depots. Subcutaneous depots were richer in adipocytes, visceral depots were the most pro-inflammatory, while pre-peritoneal depots were the least inflammatory, despite their internal origin [68].

Pre-peritoneal adipose tissue from obese patients revealed low pro-inflammatory properties, despite the tissue being an internal adipose depot. Conversely, ASCs from visceral adipose tissue were the most pro-inflammatory, secreting cytokines such as IL-6, monocyte chemoattractant protein (MCP)-1, and granulocyte colony-stimulating factor (G-CSF) [68]. Therefore, ASCs from subcutaneous, visceral, and pre-peritoneal adipose depots could differentially contribute to the chronic inflammatory scenario of obesity, and their properties must be considered as a relevant variable if they are to be used for therapeutic purposes [69].

In healthy overweight individuals, superficial and deep WAT were compared in relation to several characteristics, and showed differences that indicated a certain heterogeneity within the same adipose compartment that could be fueled by the overweight. Leptin transcript levels and also the expression of metabolic genes, such as hormone-sensitive lipase, were higher in superficial WAT than in deep WAT. In the flow cytometry study of Boulet et al., no differences between superficial WAT and deep WAT were detected in the numbers of progenitor cells, endothelial cells, or macrophages, but the number of CD3+ T-lymphocytes was higher in deep WAT than in superficial WAT, whereas the adipogenic potential was lower in the deep depot [70]. The adipogenic potential of the SVF from deep WAT was lower than that of the SVF from superficial WAT, but after the isolation of progenitor cells, the differences in adipogenic potential were abolished. Differences in the numbers of T-lymphocytes were interpreted by the authors as being responsible for the lower adipogenicity in the deep WAT. This proposed link between adipogenic potential and lymphocyte infiltration can be considered as another source of heterogeneity related to the cell microenvironment in overweight subjects.

There is a consistent literature that points to an effect of BMI on the proliferative and differentiation potential of ASCs, besides BMI having an effect on several features involving gene expression, surface markers, and immunogenicity; there is a growing body of evidence that obligates us to consider body weight as a relevant factor when choosing appropriate sources of ASCs [62].

Age of Donors

There are controversial reports on the effect of age on ASC proliferation and differentiation features. Dufrane reported that subcutaneous native adipose tissue was not affected by the donor's age, in terms of cellular senescence and yield of isolated ASCs [71]. In addition, the constant messenger (m) RNA level of osteocalcin and alkaline phosphatase after osteogenic differentiation remained unaffected by donor age. This author further stated that, in experiments aiming to promote angiogenesis by vascular endothelial growth factor (VEGF) release in hypoxic conditions, the secretome of ASCs was also unaffected by age, concluding that the use of adipose cells for bone tissue engineering was not compromised by the donor's age. This result coincides with those of Fickert et al. [72]. Mohamed-Ahmed et al., when evaluating a group of pre-adolescents and adolescents, did not find any age-related differences in the proliferation and differentiation of isolated ASCs [73].

Conversely, in a study by Choudhery et al. [74], in which ASCs from young (<30 years), adult (35–50 years), and aged (>60 years) individuals were compared in relation to the gene expression of the senescence markers SA-βgal p16 (INK4a) and p21, and in relation to superoxide dismutase (SOD) activity, as well as proliferation and differentiation capacity and senescence features, the results showed that ASCs from donors over 50 years old exhibited senescent characteristics and less viability after a hydrogen peroxide challenge, and greater differentiation capacity than that in the ASCs from the group below 40 years old. These authors also reported a lower number of ASCs per gram of tissue and fewer colony-forming units (CFUs) in the older group [74]. Coincidently, it was found that adipogenicity was more effectively induced in cells obtained from donors aged below 30 years compared with findings in cells isolated from donors aged from 30 to 70 years [63]. These results are indicative that donor age is an important factor that contributes to ASC heterogeneity and behavior in culture and probably contributes to the therapeutic success rate of ASC transplantation.

Differences between the results of the studies by Mohamed-Ahmed et al. and Choudhery et al. could be attributable to the different age range considered in the two studies; the study by Mohamed-Ahmed et al. utilized adolescent donors, and involved non-senescent cells (owing to the narrower age range and overall lower age of the group), while in the study of Choudhery et al., where age was negatively associated with ASC proliferation and differentiation, the age range was wider and they included aged subjects, where ASC senescence would be expected [73, 74].

Nugraha Setyawan et al. evaluated differences between ASCs obtained from young and old donors, using a co-culture with porcine oocytes [75]. They found that, regarding confluence, viability, and cell size, ASCs were similar in the older and younger donors, with the sole exception being higher fibroblast growth factor 2 (FGF2) expression in the stem cells from younger subjects.

Although ASCs are found in subjects across all ages, increasing donor age is often reported as exerting a negative effect on SVF cell yield and proliferation capacity. Cellular senescence is expected to increase with age. Senescence is related

to a higher number of SA-β-gal-positive cells, more reactive oxygen species (ROS) produced in mitochondria, and the expression of p21. Other features that are observed in senescent ASCs are low responsiveness to inducing factors and less cell migration, which is considered to be a consequence of impaired chemokine receptor expression, such as that of CXCR4 and CXCR7, in aged cells [76].

When comparing ASCs, muscle-derived stem cells, and bone marrow-derived stem cells from young and older human donors, only the bone marrow MSCs from the latter showed senescence features, low chondrogenic response, and diminished proliferation potential. Moreover, all three MSC types from older subjects resulted in reduced cell yield and adipogenic potential, while osteogenesis and clonogenicity were not affected by age. This result is particularly revealing because it points to a different impact of age on alternative differentiation pathways, not only between MSCs from diverse origins, but also within cells from the same compartment, such as ASCs, where age impairs adipogenic capacity, but does not affect potentiality for differentiation toward bone and cartilage [77].

Coincidently, Kornicka et al., who evaluated oxidative damage and antioxidant response in ASCs from elderly subjects, observed that ROS levels, as well as levels of nitric oxide, were higher in cells from aged donors [78]. While the activity of SOD, an antioxidant enzyme whose substrate is superoxide anion (a deleterious oxygen-derived free radical) was significantly reduced. These results are indicative of oxidative stress. Moreover, cells from aged donors, along with showing less proliferative potential, expressed senescence biomarkers such as p53 protein upregulation, β-gal activity, and enlarged morphology. The relationship between oxidative stress (and cell damage), senescence, and proliferation shed some light on the causes of impaired behavior in cultures of ASCs obtained from older patients.

When analyzing heterogeneity related to donor age, it is evident that, despite phenotypic similarities between the SVF and ASCs, there are different behaviors inherent to their age that show variations which involve aspects that are relevant for stem cell-based therapy, such as yield, viability, proliferation capacity, ability to differentiate, immunomodulation, and migration into the target site [76, 79–81].

These results indicate that donor age is a variant that must be considered when planning cell-based therapies. Increased donor age is consistent with the presence of aging changes in ASCs that affect a repertory of genes whose functions are related to proliferation potential, which is known to decrease with age. The transcriptome of aging human ASCs is more stable than that of age-matched fibroblasts. Age-related changes in ASCs involve cell cycles with a shortened G1 phase, and at the translational level, increased nascent protein synthesis. There is evidence that the chronobiology and aging mechanisms of ASCs are distinct from those of differentiated cells [82]. A non-homogeneous effect over the differentiated cell fate is another remarkable feature that could involve not only alternative genes but also epigenetic modulation [83, 84].

Protocols differ between laboratories in such a way that cell populations can be widely different; this difference in cell composition results in compound samples that make initial cultures, as well as the cell fate upon culture, heterogeneous. These

cells can experience certain difficulties in differentiation. Among the factors that result in further differentiation differences are differences in the plate density after seeding, the material utilized in culture flasks, the rigidity of the substrate used for cell growth, the culture media, the type of serum supplementation (whether human or bovine), the use of defined media with the addition of growth factors, the incubation atmosphere, and the use of antibiotics, among other factors. Media composition exerts an important effect on the expression of genes for transcription factors that are closely related to the stem cell phenotype, such as Oct-4. NANOG, Rex 1, and Sox-2. This points to the relationship between the culture medium and the permanence of stem cells in the undifferentiated stage and the retention of their pluripotency [85, 86].

Heterogeneity Related to Culture Conditions

Some authors consider that many of the above mediating factors are impossible to standardize. It is quite clear that variables regarding human cell donors are inherently non-standardized, but normalized procedures could help in lowering the variable-associated entropy.

Biomaterial Scaffolds

First, let us analyze how the effect of the rigidity or stiffness of the surface on which cells are cultured can change the proliferation and differentiation of ASCs. ASCs that grow on a stiff surface are prone to differentiate into cells that express markers of bone lineage, but when the ASCs are cultured on a softer substrate, the expressed markers are myogenic; however, if the surface is even softer, ASCs differentiate toward a neuronal phenotype. Also, the nature of the coating over the plastic in culture dishes drives cells to change differentiation markers, as has been observed with fibronectin- or collagen-coated surfaces [17], or with those covered by collagen hydroxyapatite, which favored differentiation toward bone cells in a mouse model [87] or titanium-coated surfaces that help in driving cells toward an osteogenic phenotype [88].

Riis et al. [89], reported that ASCs were cultured in five different media: StemPro, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% human platelet lysates (HPL), or α-minimum essential medium (A-MEM) supplemented with 5% HPL, 10% HPL, or 10% fetal calf serum (FCS). Culture media were compared, focusing on their effects upon cell proliferation; CFUs; and cell attachment, morphology and size, granularity, and immunophenotype. ASCs cultured in StemPro did not survive after the first passages, while those that were cultured in A-MEM proliferated faster than those cultured in DMEM. HPL improved cell size, granularity, and proliferation compared with these features in the cells

cultured in FCS. CFUs were appropriate in all media, with the exception of Stem Pro. In FCS-cultured ASCs, higher levels of CD73 and CD105 were found, whereas in HPL-cultured ASCs, there were high levels of CD146. Flow cytometry, which was applied to detect surface biomarkers after seven passages, allowed the identification of four ASC subpopulations, all positive for CD73, CD90, and CD105, but which differed in CD146 and CD271 expression. These results are indicative of how greatly the differences in culture media influence the behavior and surface marker expression of ASCs, and indicate that attention should be paid to culture media as a source of ASC heterogeneity in culture [89].

The use of ASCs in therapy requires a high number of cells with intact proliferation and differentiation capacity. Usually the procedures for expansion were based on two-dimensional cell culture on plastic surfaces, using medium supplemented with serum. These procedures have been useful for years, but now it has become clear that cell proliferation is not as high as needed and the differentiation potential is reduced after several passages. Two-dimensional cultures do not provide a topographical microenvironment that is similar to the three-dimensional (3D) frameworks that are found in vivo and that exert a relevant influence over cellular processes. That is why novel approaches have been developed, relying on a 3D framework of diverse chemical composition, which is intended to resemble the natural microenvironment of the cells. These approaches can also provide a new opportunity to modulate cell growth and differentiation [90].

Advances in biomaterials permit the design and development of strategies for ex vivo cell expansion that are based on biomaterial scaffolds. The combination of those biomaterials with a battery of inductors, can lead cells to differentiate toward a specific lineage. Deeper understanding of stem cell biology and the rationale of biomaterial application in culture are expected to increase the efficacy of stem cell clinical applications in humans. There is a new wave of biomaterials that are engineered to modify the characteristics that can modulate stem cell fate in culture; as well, these biomaterials can be applied to regeneration, repair, and tissue development. Among the characteristics of biomaterials that determine the differentiation route of ASCs in culture are chemical functionality, porosity, wettability, and mechanical properties such as stiffness, topography, and biodegradability [91]. There is a recent report on the high level of pluripotency-associated markers, such as Oct3/4, Sox-2, Nanog, and Myc, in mouse bone marrow MSCs cultured on a microgroove surface; further, these MSCs showed more pronounced proliferation capacity and overall cell yield than those that were grown in a plastic flask [92].

Another research line of biomaterials, organized as tridimensional natural-like scaffolds, is based on the use of extracellular matrix (ECM). Previously decellularized ECMs are placed in culture to provide a framework that improves ASC yield, proliferation, and maintenance of undifferentiated phenotype. It is known that cells are highly sensitive to their microenvironment. This culture system will substitute for tissue culture polystyrene (TCP) surfaces that do not succeed in the creation of a local environment that allows natural cell behavior. There is evidence regarding cell responsiveness to ECM that involves physicochemical features which modulate cell proliferation and differentiation. This evidence has been confirmed in

ECM derived both from bone marrow and adipose tissue, i.e., real ECM is able to mimic the cellular niche for the cells and also favors their natural-like behavior. ECMs promoted the proliferation of MSCs, and the effect was more pronounced when the cell origin was the same as that of the cultured cells. ECM from bone marrow favored osteogenic differentiation, whereas adipose-derived ECM favored adipogenic lineage. Both ECM frameworks influenced the cell morphology toward their respective origins without being restricted by the origin of the cultured MSCs [92–94].

When the protein composition and the structural and mechanical features of bone marrow and adipose ECMs were compared, it was found that the protein composition, i.e., fibronectin and collagen, was very similar, but the proteins differed in their architectural properties, such as fiber orientation and surface roughness in the matrix; as well as differing in their mechanical properties, such as elastic response or storage modulus and surface energy. This result can be interpreted as indicative of the importance of the physical properties of ECM in the cultured cells, and it indicates that decellularized ECM can recreate the microenvironments of natural niches [95].

There have been tests of other matrices, such as that of denatured type I collagen (DC); this DC matrix was reported to be better than TCP in maintenance of the expression of undifferentiated adipogenic markers on ASCs, particularly during the final passages [96].

An interesting approach is based on a fragmin/protamine nanoparticle-coated substratum with human platelet-rich plasma that has shown good results in the retention of ASC multilineage potentiality and proliferation capacity [97].

It has also been reported that mouse MSCs grown on bone marrow-derived ECM, in DMEM plus 10% FBS, proliferated more than cells growing on uncoated surfaces, and maintained their stemness, as manifested by their higher potential for adipogenic, osteogenic, and chondrogenic differentiation. Further evidence of greater stemness in ECM- coated culture was shown by the high expression levels of peroxisome proliferator-activated receptor gamma (*PPARγ*), adipocyte protein 2 (aP2), CCAAT/enhancer-binding protein (C/EBP), Runx2, osteocalcin, Sox9, aggrecan, and collagen II, compared with levels in [98].

There is an increasing body of evidence connecting the role of mechanical force transduction to stem cells, in terms of the regulation of their growth and differentiation. The ECM is essential for transducing forces through cell surface sensors, the cytoskeleton, and the nuclear envelope. Transcriptional activators such as Yes associated protein (YAP) and the protein scaffold PDZ binding motif (TAZ) pathway and variations in nuclear shape and deformability have been invoked as effectors of mechano-sensing influence in MSC differentiation. There is a report on actomyosin that connects the nucleoskeleton with cytosqueleton and regulates nuclear shape, through variations in tensile forces that link cytoskeletal mechanical stress with the responses of MSCs in culture [99, 100].

The relevance of the relationship between external forces, ECM architecture, and chemical composition must not be underestimated, because it is a way to change the differentiation path for ASCs in culture by modifying culture surfaces, as well as

modifying the medium and supplements. The approach to standardize ASC expansion procedures must take into consideration the scaffolds of the cells' growth in all their chemical and mechanical complexity.

In conclusion, the physical properties of a cell culture surface and the utilization of ECMs of different origins, with their intrinsic architectural and chemical variations, can exert not only biochemical, but also mechanical influence, that, through cell mechano-sensors, introduces inter-cell heterogeneity that is independent of other sources of variation. These influences on the culture conditions, if considered and controlled, can modulate the main features that are relevant for ASC therapeutic use.

Culture Conditions, Media, and Supplements

Culture conditions are not an unimportant variable in affecting ASC proliferation, differentiation, and potentiality. Differences in culture medium composition are determinants of ASC proliferation, maintenance of stemness, and differentiation. The addition of antioxidants or antibiotics to the media increases proliferation in ASCs, but there are still doubts about the probable impairment these agents can provoke on the undifferentiated status of mesenchymal stem cells. The type of culture medium is relevant for the kind of cells desired and the desirable stem properties of the cells upon isolation. For instance, DMEM has been utilized for the maintenance of ASCs. But there are differences between DMEM from different sources, and such variations in the precise composition must be taken into consideration. Glucose concentrations in DMEM vary from those close to the physiological value to higher concentrations that fuel proliferation rates. However, elevated glucose concentrations could affect the differentiation capacity of ASCs [101–103].

Changes in the culture conditions, despite the phenotypic closeness of cells after isolation, can shift the cell profile to several subpopulations, consequently leading to great differences in the proliferation and differentiation of cells available for therapeutic procedures [104].

An example of variations related to culture was provided by Baer and Geiger [17]. When they tested five media for ASC culture, they found that the morphology and expansion of these cells were strongly affected by the media used, with some media changing the expression of transcription factors (Nanog, Sox-2, Rex-1, nestin, Oct-4), and affecting the secretion of hepatic growth factor.

Serum supplementation is another source of variation between laboratories. Some protocols are based upon the utilization of serum from different natural sources, such as FCS or bovine serum, while others rely on the addition of several growth factors, e.g., PDGF, epidermal growth factor (EGF), basic fibroblast growth factor (FGFb), the transforming growth factor β superfamily (TGF β1, 2, and 3), insulin growth factor (IGF), VEGF , and the Wint growth factor family [3, 105].

Fetal bovine serum (FBS), which is widely utilized as a medium supplement, is a reagent of animal origin that is not totally characterized, and poses risks to the cell recipients; thus it must be considered a risky option when human clinical therapy is under discussion. Therefore, a preferential objective in the search for a defined medium is one that can sustain the stemness, proliferation, and differentiation of ASCs with efficacy that is the same as or superior to that of bovine serum [106].

A defined medium, along with greater in-depth knowledge of gene expression patterns, genomic stability, and the secretome, is a condition with which to reduce heterogeneity and promote normalized and safer stem cell-based treatment protocols.

The use of serum-free culture medium is safer for human clinical applications than the use of supplemented serum. When chemically pure and defined growth factors are utilized, there are no bio-contaminants that could be transmitted together with the ASCs during therapeutic procedures. Other features that hamper the use of growth factors are batch-to-batch variations and the possibility of immunological reactivity [107].

There is a need to further standardize culture procedures, as well as to standardize the utilization of defined media supplemented with xeno-free growth factors, to further increase the precision, predictability, and safety of the therapeutic use of ASCs.

Other studies have focused on the comparison between fetal and allogeneic human serum. Results points to variations in ASC proliferation, inducible differentiation capacity, and gene expression between cells cultured with serum of bovine or human origin. In the work of Lindroos et al., significant differences were found between ASC cultured in seven serum-free (SF) and xeno-free (XF) media, compared with ASC cultures in both FBS and allogeneic human serum (alloHS) [107]. Evaluation was based on the efficacy of XF and SF in supporting good cytological morphology standards, accepted ASC surface markers, and favorable proliferation rates in ASCs. Cells in XF medium showed significantly higher proliferation rates than ASCs grown in FBS, and the XF-cultured cells conserved their differentiation potential and expressed the accepted surface markers of ASCs. It is thought that Xeno-free medium will become more common for clinical applications of stem cells, because of its lower risks of immune reactions and presence of contaminants, but the effects of XF upon gene expression and cell biological behavior in culture, which could induce ASC heterogeneity, thus influencing the safety of clinical procedures, must be further investigated.

The use of ASCs as a therapeutic option for immunological conditions has led to the evaluation of medium formulations that do not include FBS, owing to the risks associated with FBS involvement in the culture of cells for human applications. The effects of culture media on ASCs were studied, utilizing three different media for comparison: HPL-supplemented media, serum-free media/xeno-free FDA-approved culture medium (SFM/XF), and FBS. The immunomodulatory capacity of ASCs, both resting ASCs and those primed with interferon γ, was compared. Proliferation and differentiation were also evaluated. Interestingly, HPL reduced

immunosuppressive potential, while FBS and SFM/XF did not, either in the naïve or in the primed cells. None of the three supplemented media affected proliferation or differentiation negatively, and proliferation and differentiation were particularly increased with HPL. SFM/XF, but not HPL, is an effective and productive alternative when growing ASCs for therapy use [108]. Considering that HPL is a known and widely utilized inductor of growth and differentiation for ASCs [109], this negative result of HPL in relation to ASC immunomodulatory capacity is an example of the huge variation in responses with different protocols, ASC sources, and culture media.

There is a growing body of results with defined media and SFM/XF that reflects continuous advances in the ability of these formulations to sustain an adequate proliferation rate and differentiation potential in ASCs; maintaining their stemness, with similar cell viability and phenotypic expression shown with the two media. These are reasons to evaluate the application of SFM/XF in tissue engineering, as it shows the same or even greater efficacy than FBS-supplemented media [110–114].

Heterogeneity Related to Differentiation Fate

Genes Associated with Stemness

It is not yet well understood about how determinants of ASC differentiation are controlled inside gene regulatory networks. Gene expression analysis, both genome-wide and targeted at specific gene subsets, has increased our knowledge and comprehension of the molecular pathways that are essential for ASC self-renewal and differentiation. The pattern of gene expression, which changes from the beginning of culture in vitro, differs in cells that maintain stemness and those that are committed to pre-lineage and lineage differentiation. This is a complex picture that adds more heterogeneity to isolated cells, despite their being of the same type of AT or even from the same depot (see Fig. 8.4).

There is a network hierarchy in ASCs derived from both SWAT and VWAT in the control of gene expression for the embryonic stem cell markers NANOG (homeobox protein NANOG), SOX2 (SRY (sex determining region Y)-box 2), and Oct4. In this network NANOG seems to be in a hierarchically higher position than SOX2 (SRY (sex determining region Y)-box 2) and Oct4. Global gene expression analysis has allowed the examination of the effect of (octamer-binding transcription factor) 4Oct4, a key gene that increases the expression of differentially regulated genes of pluripotency markers, such as NANOG, SOX2, and KLF4, and markers of undifferentiated stem cells, such as FOXD1, CDC2, and EPHB1. Downregulated genes in ASCs include FAS, TNFR, COL6A1, JAM2, FOXQ1, FOXO1, NESTIN, SMAD3, SLIT3, DKK1, WNT5A, BMP1, and GLIS3, which are implicated in differentiation processes [115]. Similar results are also reported that implicate micro (mi) RNAs in adipocyte differentiation [116].

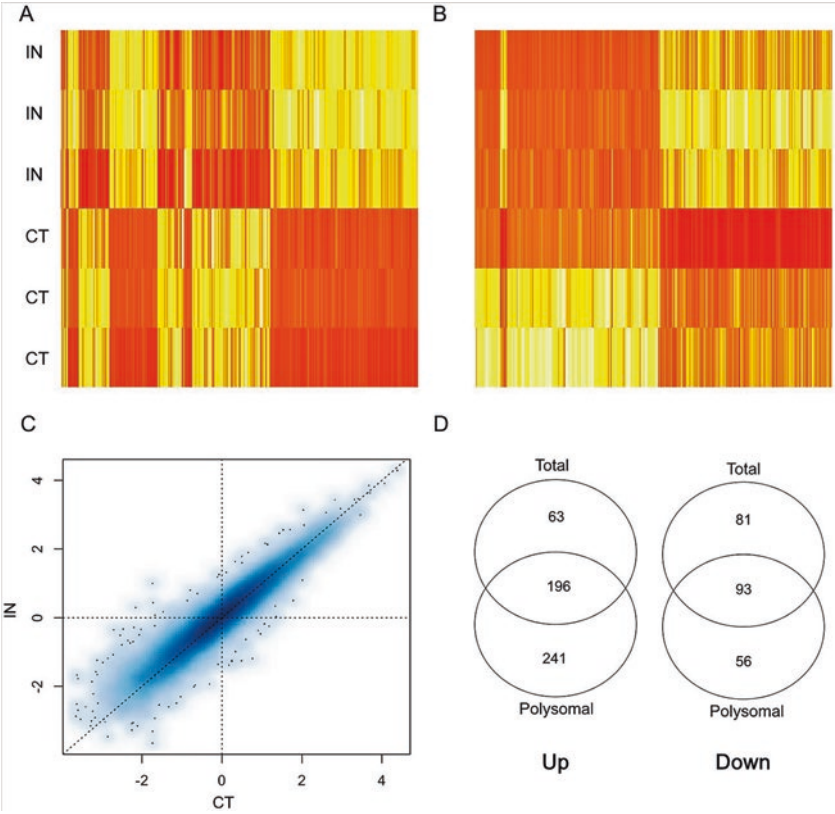

Fig. 8.4 Heterogeneity of gene expression in ASCs with and without osteogenic induction through the evaluation of total RNA versus the polysomal RNA fraction. Diagrams of differentially expressed genes (DEGs) in osteogenic-induced (IN) and non-induced (CT) cells. Heatmaps of the DEGs are shown for the polysomal RNA fraction (**a**) and for the total RNA fraction (**b**). Data represent different samples in the rows (three biological replicates for IN and for CT) and color intensities represent gene upregulation (*red*) and downregulation (*yellow*). (**c**) Scatterplot of log10 RPKM (reads per kilobase per million) values, representing each gene in the polysomal fraction (median number of RPKM, mapped reads from all patients for CT and IN samples). (**d**) Venn diagrams picturing set relationships between DEGs in the polysomal and total RNA fractions for upregulated genes (*top*; *left*) and for downregulated genes (*bottom*; *right*). Reproduced from Robert AW, Angulski ABB, Spangenberg L, Shigunov P, Pereira IT, Bettes PLL, Naya H, Correa A, Dallagiovanna B, Stimamiglio MA. (2018) Gene expression analysis of human adipose tissue-derived stem cells during the initial steps of in vitro osteogenesis. Scientific Reports 8:4739. www.nature.com/scientificreports. doi: 10.1038/s41598-018-22991

Human adipose-derived stem cells in serum-free medium (hADSC) proliferated at a higher rate than osteogenically differentiated cells (hOS-ADSC); the hADSCs produced osteocalcin after 21 days. Relative gene expression showed stable expression of MSX2, RUNX2, and BGLAP over time on different cell culture surfaces.

In ASCs undergoing adipogenic differentiation, the following genes are overexpressed: adipogenic-related genes such as PPARγ, adipocyte protein 2 (AP2), adipose tissue-specific secretory factor (ADSF), sterol regulatory element-binding protein 1C (SREBP1C), lipoprotein lipase (LPL), adiponectin (ADIPOQ), and glucose transporter type 4 (GLUT4).

High-glucose-containing media suppressed osteogenic differentiation and downregulated the expression of the osteogenic genes runt-related transcription factor 2 (RUNX2), collagen type I (COL1A1), osteonectin (ON), and osteocalcin (OCN) in mouse BM-MSCs and MG63 cells [88].

Gene expression on days 1, 7, 14, and 21 post-induction showed genes that could be early-stage differentiation markers. Those genes that were differentially expressed in each day accounted for 128, 218, 253, and 240, respectively. Overexpressed genes were related to diverse functions, such as blood vessel development; leukocyte migration; and tumor growth, invasion, and metastasis. Among the downregulated genes, the more abundant were those implicated in immune response-related processes, e.g., KLF15, LMO3, and FOXO1, while ZBTB16 transcription factors, that is involved in cell cycle progression and interactions with a histone deacetylase, were upregulated during the differentiation process. An array of genes including transcription factors, regulators of adipose carbohydrate metabolism and intranuclear hormone receptors like CEBPA, PPARG, ZNF117, MLXIPL, MMP3, and RORB showed lower expression on days 14 and 21, which is the time when adipocytes mature. ASC differentiation into an adipocyte phenotype results from the execution of a gene expression program involving thousands of genes. Microarray analysis performed on total RNA before and after days 7 and 21, post-adipocyte differentiation, revealed that cells at each stage exhibited very similar expression profiles, while great differences were detected between developmental stages [117].

More than 14,000 transcripts underwent variations during differentiation and approximately 6000 transcripts were changed between 7 and 21 days in culture. With a cutoff of +/− twofold change, 1350 transcripts were upregulated, while 2929 showed lower expression on day 7. When early and late stages in culture were compared, 1107 transcripts were found to be overexpressed, while 606 genes were downregulated [118].

MicroRNAs (miRNAs) represent a new signature with which to define the differentiation of ASCs, and they contribute to differences found between ASCs; miRNAs were found to be differentially expressed, with most of these miRNAs being placed near obesity-related chromosomal regions. There are 42 differently expressed miRNAs (meta-signature miRNAs) in mature adipocytes; miRNAs are specific for adipogenesis and several of them are correlated with the BMP signaling pathway, cell differentiation, WNT signaling, the insulin receptor signaling pathway, MAPK signaling, the cell cycle, and lipid metabolic processes [119]. An miRNA, miR-31, was found to directly bind to the 3′-untranslated region (UTR) of C/EBP-α to inhibit its expression in ASCs, while other entities, such as long non coding RNA (lncRNA) TINCR, miR-31, and C/EBP-α, made up a feedback loop to modulate adipogenic differentiation [120]. There are reports on miRNAs that regulate MAPK signaling

and p16 and MARP3, as well as ASC migration [121]. Other studies have implicated miRNA in osteogenic differentiation [122]. The analysis of both mRNA and miRNAs expression profiling in ASCs also points to an important modulatory role for the miR-30 family at the beginning of the process of differentiation toward mesenchymal lineages [123].

Concluding Remarks

Data reviewed in this chapter can be interpreted as indicating an inherent source of heterogeneity among ASCs before and particularly after the induction process has been initiated. Heterogeneity in ASCs is the result of the interplay of several sources of variations. As long as the specific alterations in these variation sources become known, the efficacy, security, and reproducibility of ASC-based therapeutic protocols will increase, along with the expected impact of these protocols in regenerative medicine.

The heterogeneity of ASCs will be an intense research topic because of the need for more standardized and secure protocols. Increasing our knowledge of the source of the variation in ASCs' behavior in culture and after transplantation is an inescapable objective to pursue. In the future, analysis of the ASC secretome and the interactions of ASCs with metabolomics and nutrition; the evaluation of ASC-associated epigenetic modulation in the patient's cells after heterologous procedures; and the better characterization of donor plus recipient phenotypes could constitute remarkable research avenues. Tissue engineering and repair require better control of ASCs, so that these cells can continue to be used as a principal tool in regenerative therapy.

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Chapter 9
Unveiling Stem Cell Heterogeneity Toward the Development of Salivary Gland Regenerative Strategies

Ganokon Urkasemsin and Joao N. Ferreira

Abstract Epithelial damage in the salivary gland (SG) resulting in irreversible dry mouth can be commonly induced by gamma radiation therapy. This radiation depletes the SG stem/progenitor cell niche slowing healing and natural gland regeneration. Biologists have been focused in understanding the development and differentiation of epithelial stem and progenitor cell niches during SG organogenesis. These organogenesis studies gave insights into novel cell-based therapies to recreate the three-dimensional (3D) salivary gland (SG) organ, recapitulate the SG native physiology, and restore saliva secretion. Such therapeutical strategies apply techniques that assemble, in a 3D organotypic culture, progenitor and stem cell lines to develop SG organ-like organoids or mini-transplants. Future studies will employ a combination of organoids, decellularized matrices, and smart biomaterials to create viable and functional SG transplants to repair the site of SG injury and reestablish saliva production.

Keywords Exocrine glands · Salivary glands · Radiotherapy · Sjögren's syndrome · Hypofunction · Dry mouth · Xerostomia · Regenerative medicine · Tissue engineering · Epithelial cell · Progenitor cell · Stem cell · Three-dimensional cultures · Bio-printing

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Introduction

Salivary gland damage resulting in irreversible dry mouth (or xerostomia) can be commonly induced by radiation therapy for head and neck cancers (HNC). Xerostomia is also usually observed in several systemic diseases, particularly autoimmune, such as graft-versus-host disease, Sjögren's syndrome, granulomatous diseases, and uncontrolled diabetes among others [1].

Xerostomia is a major complication of radiation therapy (RT), which can target more than 500,000 new cases of head and neck cancer that develop every year worldwide. Since saliva is required for food digestion, lubrication, and buffering effects and for protection against environmental hazards, xerostomia can cause various life-disturbing adverse effects, such as progressive caries, unbearable pain, oral fungal infections, speech deficits, taste loss, and swallowing impairment, which greatly impair patients' oral and systemic health [2]. A multicenter randomized controlled trial by Nutting et al. [3] showed that the prevalence of xerostomia (grade 2 and above) can go up to approximately 40% in 12 months even after novel RT modalities are used (such as salivary gland-sparing or intensity-modulated radiation therapy). When the radiation field (during RT) lays on the salivary glands (SG), radiation injury is elicited on secretory epithelial cells, blood vessels, and adjacent nerves [4, 5]. Salivary glands consist of two types of secretory epithelial cells: 80% acinar and 20% ductal. Following RT, patients lose the majority of acinar cells with the surviving secretory cells being primarily ductal; consequently, RT will irreversibly impact salivary secretion and cause inflammatory damage and fibrosis on the long term. This radiation damage further depletes the SG stem/progenitor cell niche deterring healing and natural gland regeneration [4, 6–8]. Yet, no effective therapy has been devised to treat RT-induced xerostomia, and current treatment strategies are confined to the minimization of SG radiation damage or to the administration of artificial salivary substitutes and saliva secretion stimulators [2, 4].

Radiation-induced xerostomia can be an irreversible lifelong condition that will significantly affect the quality of life of cancer patients. Thus, novel and effective therapeutical strategies for SG hypofunction are required. Due to the depletion of the stem cell pool during RT damage, stem/progenitor cell therapies are vital to engender new SG secretory tissues and repair the damaged ones, for the restoration of salivary flow in xerostomia patients [9, 10].

Several biological therapies have been proposed in the last decade for SG regeneration at preclinical stages (the most relevant are summarized in Tables 9.1 and 9.2), which can involve the following biotechnology strategies:

1. Implantation of SG stem/progenitor cells as salispheres into the irradiated gland to replace the functionally damaged cells [9, 24].
2. Transplantation of adult stem cells (e.g., mesenchymal stem cells) with different differentiation cues in mono- or co-culture systems [25, 26].
3. Tissue engineering techniques combining cells with or without environmental cues in 3D biomaterial constructs [21, 27].

Table 9.1 Advantages and limitations of different stem cell culture techniques for salivary gland (SG) repair or regeneration

Culture model	Advantages	Limitations	References
Mouse salisphere cultures with C-KIT-positive cells for transplantation	• Restoration of submandibular gland homeostasis and salivary flow (~55%)	• Lack of clinical prospect due to use of nonhuman cell lines	[9, 11]
Mouse SG salisphere-derived single cells enriched in CD24/CD29	• Fourfold expansion after seven passages	• Long-term cultures (13 passages) can produce karyotypic changes • Lack of clinical prospect	[12]
Co-cultures of mouse fetal epithelium and MSC to generate SG organ germs	• Development of salivary gland-like morphology in 3 days • Uses mesenchymal-epithelial instructive interactions as a template	• Potential differentiation toward divergent lineages after extended in vitro culture due to cellular heterogeneity • Lack of clinical prospect	[13]
Expansion of human SG cells in monolayer culture	• Enhances in vitro expansion of human SG cells • Induces polarization of human SG cells	• Lack of long-term cellular functionality (<9 days) • Lack saliva qualitative studies • Lack of clinical prospect	[14, 15]
SG-derived clonal stem cells expanded by modified sub-fractionation culture	• Genetic and differentiation characteristics similar to bone marrow MSC • Express tight junction markers (i.e., ZO-1)	• Lack of clinical prospect	[16]

MSC mesenchymal stem cells

4. Epithelial cells can potentially be assembled as a 3D organotypic spheroid culture with capabilities to grow and mature into a secretory organ-like appearance (or organoid) [22].

Stem and Progenitor Cells

The first proof of concept study on autologous transplantation of SG cells to functionally rescue salivary hypofunction used in vitro floating spheroid-like cultures of mouse SG progenitor cells, named salispheres [9]. In vitro salisphere cultures have been shown to enrich for SG stem/progenitor cell populations that include KIT (C-KIT, CD117), Sca-1, and Mushashi-1 (Table 9.1) [9]. KIT-expressing (KIT+) progenitors are also found in other epithelial organs beside the SG, such as the prostate gland and lungs, where KIT+ progenitors have remarkable regeneration capabilities [28, 29]. In a salisphere study in mice, 100–300 KIT+ donor-derived cells isolated from the salisphere cultures were sufficient to form both new acini and saliva-transporting ductal structures, restoring the morphology and function of irradiated SG (Table 9.1) [9].

Table 9.2 Advantages and limitations of different biomaterials used in salivary gland (SG) tissue engineering (TE) constructs for SG repair or regeneration

Biomaterial	TE technique	Advantages	Limitations	References
Collagen type I	3D matrix loaded with salispheres of human SG progenitors	• Differentiation of SG progenitors • Long-term self-renewal ability	• Lack of clinical prospect due to presence of xenogeneic substrates	[17]
Matrigel + Collagen	3D matrix with mouse SG salisphere-derived $CD24^{hi}/CD29^{hi}$ single cells	• Differentiated into distinct ductal/lobular organoids with multiple SG cell lineages • Restoration of salivary flow (~46%)	• Require long-term cultures • Lack of clinical prospect	[12]
Matrigel/ Perlecan domain IV peptide	Culture of human SG cells	• Differentiation into self-assembled acini expressing tight junction and water channel proteins	• Lack of proper acinar cell polarity	[18]
PLGA	3D nanofibers construct loaded with SG epithelial cells	• Supports growth, proliferation, and survival of SG cells • Facilitates self-assembly of SG cells to 3D structure	• Lack of 3D branching and proper tight junctions • Lack of saliva flow studies	[19]
PLGA	Lithographically-based patterning with rat SG epithelial cells	• Supports apicobasal polarization • Improves epithelial differentiation	• Long-term in vitro culture • Lack of saliva flow studies	[20]
PLGA coupled with chitosan and laminin-111	Nanofibers for SG epithelial cell proliferation	• Supports apicobasal polarization and maturation of the SG epithelial tight junctions	• Long-term in vitro culture • Lack of saliva flow studies	[21]
HA (2.5D/3D)	3D organotypic culture of human SG cells	• Develop functional 3D spheroids in long-term in vitro cultures with alpha-amylase expression	• Lack of in vivo saliva flow studies	[22]
Laminin-111	3D clusters of mouse SG cells in feeder layers of hair follicle-derived MSC with laminin	• Organization of SG cells in clusters with multilumen formation • Hair follicle-derived MSC feeder layers support SG cell growth	• Lack of saliva flow studies • Lack of clinical prospect	[23]

HA hyaluronic acid, *PLGA* poly(lactic-co-glycolic acid), *MSC* mesenchymal stem cells

Regrettably, human major SG biopsies hold a very limited number of KIT+ progenitor cells [17]. Also, due to the heterogeneity of the KIT+ cell population, further studies have included co-expression of other putative salivary stem cell markers, such as CD24 (HSA) and CD49f (Itga6) [11]. KIT+ cells co-expressing CD24 and CD49f showed an enhanced functional recovery compared to a heterogeneous KIT+ population, which indicates that this subpopulation of KIT+ cells is enriched for SG stem/progenitor cells (Table 9.1) [11]. It is yet to be determined whether human KIT+/CD24+/CD49f+ cells have similar stem−/progenitor-like functions. Nevertheless, recently, SG sphere-derived single cells expressing high CD24 and CD29 markers ($CD24^{hi}/CD29^{hi}$) could be expanded ex vivo by fourfold after seven passages [12], though karyotypic changes (chromosome doubling) were noticed after passage 3. The same research group placed the same spheres in a 3D matrix mixture with Matrigel and collagen, and spheres differentiated in vitro into organoids with ductal/lobular structures. Upon in vivo transplantation of differentiated spheres into an irradiated mouse model, salivary flow was restored to ~46% (of pre-irradiated levels). Interestingly, undifferentiated spheres also partially restored the salivary flow, which denotes these cells may be secreting microenvironment cues that are stimulating the repair of the remaining gland [12]. Despite the abnormal chromosome number, tumor formation was not observed within 4 months (120 days) post-RT. Though, long-term follow-up studies are necessary to confirm tissues are tumor-free. Furthermore, enrichment of C-KIT+ cells within the $CD24^{hi}/CD29^{hi}$ and the CD24+/CD49f+ subpopulations showed similar salivary flow outcomes [12]. The formation of acini and ductal-containing organoids from single cells is an important achievement for the field. However, this salisphere model cannot be translated into humans as it is yet to be demonstrated whether similar salispheres can be obtained from human SG biopsies and in particularly from elderly patients. Salisphere formation is in fact problematic in SG of old age mice [17].

To overcome the above limitations of salisphere-based cultures, our laboratory tested novel 3D spheroid bio-printing cell assembly systems incorporating human dental pulp stem cells expressing KIT+ with high expansion capabilities and binding/tagging them with magnetic nanoparticles [30–36]. Interestingly, KIT is clearly expressed in neural crest progenitors found in the dental pulp of human permanent teeth [37, 38]. These progenitors are termed human dental pulp stem cells, and our research group is enriching them to move SG cell-based therapies from mice to clinically relevant human SG organoids.

Consequently, methodologies for cryopreservation and biobanking of these progenitors have been established. Neumann and others [39] have developed a stem cell biobanking setup where salivary gland integrin α6β1+ cells have been cryopreserved in the long term without affecting their functional and genetic stability, serving as a future therapy in cancer patients. Furthermore, it is crucial to understand how progenitors proliferate and expand particularly during organogenesis. Several researcher groups have demonstrated that KIT and fibroblast growth factor receptor 2b (FGFR2b) signaling are essential for progenitor survival and expansion in the fetal submandibular gland, lung, pancreas, tooth, and skin [40–42].

Moreover, other putative markers that can be used to isolate SG stem/progenitor cells include K5 (Cytokeratin 5), CD29 (Itga1), CD133 (Prom1), Sca1, CD44, CD34, CD90 (Thy1), CD105, CD9, and CD81, but only few populations were proven to actively restore damaged glands [9, 11, 43–45]. Yet, the KIT+ cell population still appears to have the highest stem−/progenitor-like potential in mice. Analysis of regenerated SGs after transplantation of enriched KIT+ progenitor cells shows restoration of tissue homeostasis following irradiation whereby upon an increase in cytokeratin markers of epithelial ductal cells (K7, K8, K14, and K18) and in stem cell markers (KIT, CD133, CD24, and CD49f) induces normalization of vasculature and reduces fibrosis [9, 11]. Other populations of epithelial progenitor/stem cells have been found to be required for glandular branching in the developing mouse model, which are positive for K5+ [43, 46]. These K5+ progenitor cells are from neural crest and may have the potential for SG regeneration by supporting gland innervation [47].

A major obstacle in stem/progenitor cell therapies is the limited lifespan of the cells obtained from in vitro cultivation systems, hence needing to be used within a short time window. Thus, other cell culture systems and cell sources are necessary for the regeneration of salivary glands as well as systems to enrich sufficient numbers of autologous SG progenitor cells. Cell culture systems have been recently established on human minor salivary gland epithelial cells (phmSG) to achieve the maintenance of these cells in an acinar-like phenotype after optimizing growth conditions [48]. These phmSG cells displayed progenitor cell markers (K5 and Nanog) as well as acinar-specific markers such as α-amylase, cystatin C, TMEM16A, and NKCC1. After beta-adrenergic receptor stimulation, phmSG cultures exhibited calcium ion mobilization and formed an epithelial monolayer with transepithelial electrical resistance (TER) and polarization.

This study raises the question on whether the limited available number of human minor SG cells can generate enough saliva to ameliorate the irreversible hyposalivation found in several patients (post-RT, Sjögren's, etc.). Major SG transplants (i.e., from the parotid gland) may be a more feasible option to accomplish higher salivary secretion rates [17], but they are not always available. To generate a reasonable salivary flow, larger in vitro salivary tissues are needed and, consequently, new cell sources capable of generating high cell numbers (in short-term passaging) and a matrix-rich environment [12]. Adult stem cell sources can potentially offer predictable high expansion rates, and due to their heterogeneity, they can be combined into organotypic cultures to generate larger organoids capable of restoring the salivary flow.

Adult Stem Cells

Recently, intraglandular bone marrow-derived (BM) transplants using either mesenchymal stem cells (MSC) or BM bioactive lysates have been shown to induce paracrine pro-survival effects on remaining SG tissues and to potentially induce

site-specific multi-lineage transdifferentiation toward a more functional SG tissue architecture [25, 26]. For example, intraglandular transplantation of BM-MSC improves saliva production, reduces apoptosis, and increases microvessel density in irradiated mice, and transdifferentiation into acinar cells was observed [26]. Highly homogenous bone marrow clonal MSC (BM-cMSC) have recently shown potential to regenerate the SMG, although currently, the regenerative mechanisms are not well understood [25]. Earlier studies in mice have shown that granulocyte colony-stimulating factor-mobilized BM-derived cells can partially regenerate and also functionally restore an irradiated SG [49]. In addition, an in vitro study using BM stem cells (BMSCs) that are co-cultured with neonatal rat parotid acinar cells using a double chamber system showed an increase in the induction of acinar-specific α-amylase expression in BMSCs [50]. This observation indicates that BMSCs can transdifferentiate into acinar-like cells. Yet, transdifferentiation of BMSCs into acinar-like cells was found to occur only in 50% of the cells after co-culturing for 2 weeks. Further studies are still needed to test the secretory function of these acinar-like cells from bone marrow sources. Transdifferentiated BMSCs have not convincingly showed a proper secretory function in vivo.

Interestingly, studies using human adipose-derived mesenchymal stem cells (hAdMSCs) via systemic administration exhibit improved salivary flow rates 4 months after radiation therapy [51]. hAdMSC-transplanted SGs showed lesser tissue fibrosis and epithelial acinar apoptosis and higher secretory mucin and amylase levels. At 4 weeks, a large number of infused hAdMSCs were detected in vivo and were found to have differentiated, whereas in vitro, only low number of co-cultured hAdMSCs (13–18%) were found to transdifferentiate into salivary epithelial-like cells [51]. More recently, soluble signals from feeder layers of hair follicle-derived MSC (that were mitotically inactive) coupled with laminin-111 substrates supported the formation of clusters of mouse submandibular gland cells with multiple lumens [23]. This was a successful attempt to improve the differentiation and organization of SG cells, though its clinical applicability is yet to be demonstrated.

Nonetheless, three-dimensional (3D) tissue/organ constructs are still required to integrate multiple BM-derived tissues and cell lines in biomaterial constructs or extracellular matrices (ECM) under specific growth factor conditions in order to generate whole SG organ-like structures or organoids.

Three-Dimensional Tissue Engineering Strategies

A recent advancement in SG regenerative medicine showed that a bioengineered gland made from embryonic epithelium and mesenchyme can be transplanted into an adult mouse to produce a whole functional SG [13]. This bioengineered SG was composed of a variety of progenitor and stem cells, including cell from epithelial, mesenchymal, endothelial, and neuronal origins. More interestingly, the SG reconnected with the existing ductal system and possessed functional activity. The new

SG was able to secrete saliva, protect the oral cavity from bacteria, and restore swallowing functions.

Thus, future research may translate these bioengineering strategies to animal models with salivary glands that have more structurally and functionally similarities to the human SG. Further studies may also focus on the usage of stem cells or adult salivary progenitors with high expansion capabilities in 3D scaffolds in order to form a bioengineered construct that grows into a functional gland in the adult microenvironment.

Salivary gland tissue engineering requires three essential components: (1) the stem/progenitor cells that retain epithelial progenitor biomarkers typical of the native salivary gland (SG), (2) the extracellular matrix (ECM) proteins that can orchestrate the differentiation of progenitor cells into functional structures, and (3) a biocompatible and biodegradable three-dimensional (3D) scaffold that can hold these components together to recreate the microenvironment found in the native SG [27].

Since dynamic cell-ECM interactions are essential in processes such as epithelial ductal formation/branching, a recent strategy has been to engineer scaffolds that structurally and functionally resemble native ECM architecture. Three-dimensional (3D) collagen matrices have been used for homing salisphere stem/progenitor cells which form epithelial ductal structures with mucin-positive acini, indicating their capability to differentiate in response to the ECM environment [12]. Various biomaterials such as collagen type I, Matrigel, and other animal-derived products have been showing promising results in the differentiation and organization of human SG cells [12, 14, 15]; nevertheless, these biomaterials are not human-compatible. Thus, tissue engineering-based research is gearing toward the creation of xeno-free biomaterials, which can eventually be transplanted into humans.

Recently, researchers have started to utilize the soft hyaluronic acid (HA) hydrogels, which are human-compatible, as biocompatible substrates for SG tissue engineering [52]. When encapsulated in HA hydrogels, human SG cells can grow into organized spheroid structures that merge and proliferate to form larger acini-like structures with a central lumen and are maintained for long term in these gels in vitro [52]. These in vitro 3D acini-like structures also secrete α-amylase, express β-adrenergic and muscarinic receptors that activate protein transport, and induce calcium oscillations upon treatment with cholinergic stimulants. Furthermore, these 3D spheroids continue to secrete α-amylase when hydrogels were implanted in vivo in an athymic rat model [22]. However, these latter 3D structures have reversed polarity suggesting that further environmental cues from the ECM and the myoepithelial cells may be needed to reverse inside-out acini and correct the polarity.

Culture of salivary gland progenitor cells on human perlecan domain IV peptide has been shown to support the formation of 3D acini-like salivary units that express α-amylase [18]. It will be useful to incorporate the perlecan IV domain peptide into biomaterial scaffolds to mediate differentiation and correct polarity and directional secretion of the 3D salivary gland cell cultures in the future.

Other research groups have used poly(lactic)-co-glycolic acid (PLGA), an FDA-approved constituent in implantable dental and orthopedic devices, as a synthetic

material to show that it can support the attachment, proliferation, and survival of salivary gland epithelial cells [19]. The same group further shows that nanofiber PLGA scaffolds can support development and morphogenesis of intact fetal SMG organ cultures and promote natural self-organization of dissociated SMG cells into branched SG-like structures [53]. However, adult SG cells grown on flat polymeric substrates fail to form a complex 3D branching structure and are unable to assemble tight junctions that are needed for unidirectional flow of saliva. To overcome this, recent studies generated lithographically based micropatterning curved "craters" that mimic the physical structure of the basement membrane, which have increased both the surface area and allowed apicobasal polarization and differentiation of salivary gland epithelial cells [20]. An increase in aquaporin-5, a water channel protein marking acinar differentiation, was also detected in SG cells cultured on higher curvature scaffolds. Further studies with PLGA nanofibers coupled with laminin-111 and chitosan showed that laminin-111 promotes the formation of mature epithelial tight junctions and apicobasal polarization, and on the other hand, the chitosan antagonizes this phenomenon [22].

Taken together, current cell-based therapies and tissue engineering studies have provided a promising outlook to regenerate SG and restore the saliva secretory function. However, in order to test these techniques in humans, several hurdles need to be surpassed. To overcome these hurdles, further research steps should include: (1) the elimination of xenogeneic elements from transplants for feasible human use to comply with good manufacturing practices, (2) a thorough assessment of histocompatibility barriers, (3) an evaluation of long-term transplant survival and saliva secretion in larger animal models with a better SG human resemblance (i.e., pigs), and (4) an assessment of tumor sensitivity to bioengineered transplants in SG cancer models. Recent studies indicate that the above research steps are currently being pondered [18, 22, 54, 55].

Novel 3D Bio-printed Magnetic Nanotechnologies for SG Regeneration

Biomedical researchers have been moving toward cell culture technologies in 3D to better recapitulate native cellular environments and ultimately develop organotypic cultures [18, 22]. Novel bio-printing nanotechnologies have been recently developed using magnetic patterning or levitation in which cells bind with a magnetic nanoparticle assembly overnight to render them magnetic (Fig. 9.1) [30–36]. This nanoparticle assembly includes gold, iron oxide, and poly-L-lysine, which can easily tag via electrostatic interaction different cell types at the plasma membrane level. When resuspended in medium, a mild external magnetic field can concentrate and magnetically bio-print cells at the bottom of a cell-repellent plate, where they assemble to form larger 3D spheroids or organoids (Fig. 9.1). The resulting dense cultures can synthesize ECM and can be analyzed similarly to other 2D/3D culture systems, using assays/techniques such as cytotoxicity assays, immunohistochemical

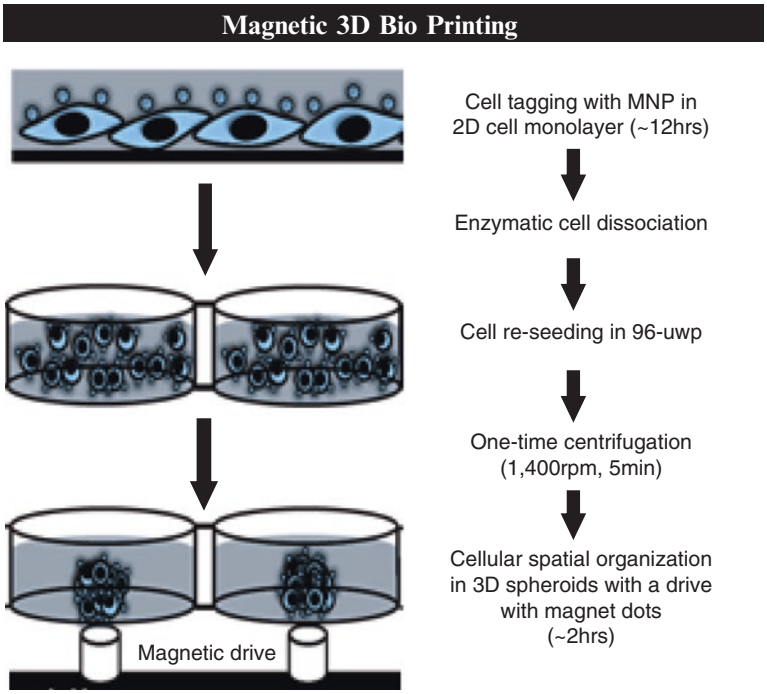

Fig. 9.1 Flowchart with biofabrication steps required for the formation of 3D spheroids by magnetic 3D bioprinting. *MNP* magnetic nanoparticles, *uwp* ultra-low attachment well plate

analysis, Western blotting, and other biochemical assays [56]. These 3D bio-printed systems have been recently found to recapitulate the native ECM from several tissues such as fat, lung, aortic valve, blood vessels, and breast and glioblastoma tumors [30–36]. Further, our research group has showed that these magnetic bioprinting systems can develop SG epithelial organoids with innervation, secretory function upon cholinergic stimulation, and epithelial polarity [57]. The apicobasal polarity in epithelial cells is essential to overcome the challenges related to the directionality of salivary flow. These SG-like organoids will provide a better understanding of human SG physiology in vitro and in vivo, in homeostatic and disease states, although these organoids still lack a robust vascular network [57]. The use of natural ECM can be a plausible alternative that can be accomplished by decellularizing organs with detergents followed by reseeding primary SG cells onto the ECM core [27, 58]. A decellularized SG can be further tested in combination with 3D bio-printed SG epithelial organoids.

The in vitro biofabrication of human SG-like transplants or organoids is crucial to:

1. Generate scaled-up xeno-free biocompatible 3D structures that provide the native architecture with environmental cues to support cell growth [30, 31, 56],

differentiation, and biointegration in the remaining tissues (after damage) to restore homeostasis and functionality [57, 58].
2. Establish methodologies to generate SG-like organoids for scale-up production. These methodologies may need in vitro co-culture systems to integrate in a 3D architecture the cellular complexity of all human SG compartments, such as the acinar and ductal epithelia, myoepithelia, and the networks of parasympathetic nerves and lumenized ducts and vessels [55].
3. Lastly, test new surgical techniques in vivo with ex vivo bio-printed SG transplants/organoids to promptly repair SG damage particularly after RT.

Conclusion

Organotypic 3D bioengineered culture systems are on the rise in regenerative medicine. These novel systems are essential to recapitulate the different cellular components of the SG and create an artificial gland for restoration of secretory function. Researchers have used floating salisphere culture systems combined or not with biomaterial 3D constructs to mimic native environments. Nevertheless, these models provided limited cellular expansion, poor acinar epithelial polarization, and skewed directionality of salivary flow. Bio-printing strategies in 3D using human cells are an avenue that has revealed promising outcomes in several types of tissue including exocrine glands [35, 57].

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Chapter 10
Heterogeneity of Human Mesenchymal Stromal/Stem Cells

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Abstract Increasing evidence has shown that mesenchymal stem cells (MSCs) isolated from body tissues are heterogeneous while being examined in vitro and in vivo. Besides some common characteristics, MSCs derived from different tissues exhibit unique biological properties. In addition, the therapeutic effects of MSCs may vary widely due to their heterogeneity and the technical differences in large-scale ex vivo expansion. In this chapter, the heterogeneity of MSCs will be discussed in three levels: the individual donors, the tissue sources, and the cell surface markers.

Keywords Mesenchymal stem cells · Heterogeneity · Surface markers · Biological property · Cell therapy · Subpopulation · Regenerative property · Immunomodulatory ability · Individual donor · Tissue source

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Introduction

In 1970, Friedenstein et al. found a group of osteoprogenitor cells in bone marrow that were capable of developing fibroblast colonies in vitro and ectopic bone formation in vivo [1]. Further investigation demonstrated that these adult bone marrow stem cells, named as mesenchymal stem cells (MSCs), can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including the bone, cartilage, fat, tendon, muscle, and marrow stroma [2]. Later findings suggest that the ability of MSCs to alter the tissue microenvironment via secretion of soluble factors may contribute more significantly than their capacity for transdifferentiation in tissue repair [3]. Moreover, MSCs mediate immune modulation by interacting with innate and adaptive immunity [4]. The promising features of MSCs, including their regenerative properties and immunomodulatory ability, have generated great interest among researchers whose work has offered intriguing perspectives on cell-based therapies for various diseases. By July 2018, 677 MSC-based clinical trials are registered on clinical.org, either completed or ongoing.

In 2006, heterogeneous procedures for isolating and cultivating MSCs among laboratories have prompted the International Society for Cellular Therapy (ISCT) to issue criteria for identifying unique populations of these cells [5]. However, the isolation of MSCs according to ISCT criteria has produced heterogeneous, non-clonal cultures of stromal cells containing multipotent stem cells, committed progenitors, and differentiated cells. The intrinsic differences and large-scale preclinical amplification have led to distinct biological properties of the MSC population, which may partly explain the differences in the outcomes of the clinical trials with MSCs. More precise molecular and cellular markers to define subsets of MSCs and to standardize the protocols for expansion of MSCs are urgently needed.

The present chapter will discuss the heterogeneity of MSCs with reference to four major aspects: heterogeneity among various individual donors, different tissue origins, differential cell surface markers, and different microenvironment and culture conditions. The schematic diagram of MSCs heterogeneity is demonstrated in Fig. 10.1.

Heterogeneity Among Individual Donors

Plenty of studies have shown that there is heterogeneity in MSCs among different individuals. For instance, Phinney et al. analyzed the heterogeneity of MSCs isolated from posterior iliac crest marrow aspirates of 17 healthy donors and found that MSCs populations showed dramatic differences in growth rates, levels of alkaline phosphatase enzyme activity, and levels of bone-specific gene induction [6]. Significant strain differences were also noted in the properties of mouse MSCs [7]. In addition, Peltzer et al. compared adult bone marrow MSCs with perinatal tissues-derived MSCs (cord blood, umbilical cord, amnion, and chorion) on their in vitro

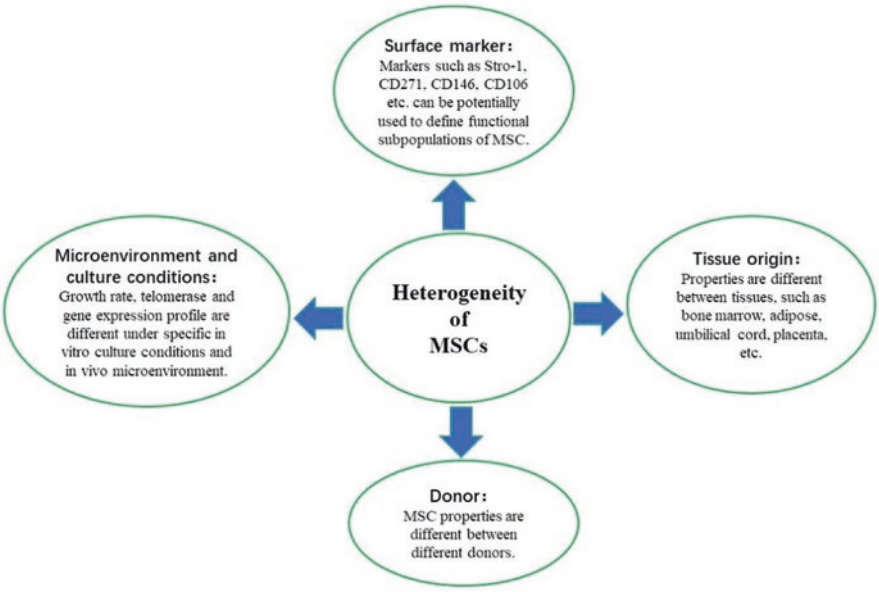

Fig. 10.1 Profile of MSCs heterogeneity

immunomodulatory activities under different priming conditions such as interferon gamma or tumor necrosis factor alpha, and the results showed contrasted effects of cytokine priming embedded in an important between-donor variability [8]. Our unpublished results also demonstrated the heterogeneity in the ability of differentiation and cytokine secretion of MSCs derived from the same kind of tissues but different individuals. Moreover, the age of donor [9] and the method of amplification in vitro [10] also affect the heterogeneity of MSC populations. Kang et al. suggest that sensitivity to hypoxic conditions is different between human umbilical cord blood MSCs originating from different donors and this difference affects the contribution to angiogenesis. The bioinformatics analysis of different donors under hypoxic culture conditions identified intrinsic variability in gene expression patterns and suggests alternative potential genetic factors, ANGPTL4, ADM, SLC2A3, and CDON, as guaranteed general indicators for further stem cell therapy [11].

Heterogeneity Among Different Tissue Origins

MSCs derived from different tissues demonstrated heterogeneity of MSCs properties. In 2006, we established a protocol to isolate abundant MSCs from human umbilical cords (UC-MSCs) with a 100% success rate. The biological characteristics of UC-MSCs were further determined and compared with normal adult bone marrow-derived MSCs (BM-MSCs). We found that UC-MSCs shared most of the

characteristics of BM-MSCs, including fibroblastic-like morphology, immunophenotype, cell cycle status, adipogenic and osteogenic differentiation potentials, and hematopoiesis-supportive function. However, in comparison with BM-MSCs, the UC-MSCs had a higher proliferation capacity and lower levels of expression of CD106 and HLA-ABC. Furthermore, UC-MSCs had a higher percentage of neuron-specific enolase-positive cells than BM-MSCs after neuronal induction [12]. Baksh D compared the proliferation and multilineage differentiation potential of MSCs derived from umbilical cord and bone marrow, which were referred to as human umbilical cord perivascular cells (HUCPVCs) and bone marrow MSCs (BMSCs), respectively. HUCPVCs showed a higher proliferative potential than BMSCs and were capable of osteogenic, chondrogenic, and adipogenic differentiation. Interestingly, osteogenic differentiation of HUCPVCs proceeded more rapidly than BMSCs. Additionally, HUCPVCs expressed higher levels of CD146, a putative MSC marker, relative to BMSCs [13]. Furthermore, the heterogeneity of human MSCs from bone marrow (BM), adipose tissue (AT), and Wharton's jelly (WJ) was evaluated in terms of proliferation, in vitro differentiation (osteogenic, adipogenic, and chondrogenic potential), expression of cell surface markers, and protein secretion using Luminex and ELISA assays. Cell proliferation was higher for WJ-MSCs, followed by AT-MSCs. WJ-MSCs secreted higher concentrations of chemokines, pro-inflammatory proteins, and growth factors. AT-MSCs showed a better pro-angiogenic profile and secreted higher amounts of extracellular matrix components and metalloproteinases [14].

We identified human MSCs from adult bone marrow (ABM), fetal pancreas (FPan), and umbilical cord (UC), and their abilities to support megakaryocyte (MK) differentiation from $CD34^+$ hematopoietic progenitor cells (HPCs) were comparatively studied. FPan-MSCs and UC-MSCs showed the ability to promote megakaryocytopoiesis, while ABM-MSCs expanded more MK progenitor cells from $CD34^+$ HPCs [15]. Hsiao et al. investigated the paracrine factor expression patterns in MSCs isolated from adipose tissue (ASCs), bone marrow (BMSCs), and dermal tissues [dermal sheath cells (DSCs) and dermal papilla cells (DPCs)]. Specifically, mRNA expression analysis identified insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor-D (VEGF-D), and interleukin-8 (IL-8) were expressed at higher levels in ASCs compared with other MSCs populations, whereas VEGF-A, angiogenin, basic fibroblast growth factor (bFGF), and nerve growth factor (NGF) were expressed at comparable levels among the MSCs populations examined. Analysis of conditioned media (CM) protein confirmed the comparable level of angiogenin and VEGF-A secretion in all MSCs populations and showed that DSCs and DPCs produced significantly higher concentrations of leptin. Functional assays examining in vitro angiogenic paracrine activity showed that incubation of endothelial cells in ASCs resulted in increased tubulogenic efficiency compared with that observed in DPCs. Using neutralizing antibodies, they concluded that VEGF-A and VEGF-D were two of the major growth factors secreted by ASCs that supported endothelial tubulogenesis. Therefore, ASCs may

be preferred over other MSCs populations for augmenting therapeutic approaches dependent upon angiogenesis [16].

Though no significant differences in growth rate, colony-forming efficiency, and immunophenotype were observed between MSCs derived from the bone marrow, adipose tissue, the placenta, and umbilical cord blood, MSCs derived from bone marrow and adipose tissue shared not only in vitro tri-lineage differentiation potential but also gene expression profiles. While there was considerable inter-donor variation in DLX5 expression between MSCs derived from different tissues, its expression appears to be associated with the osteogenic potential of MSCs [17]. Similarly, Stubbendorff et al. compared the phenotype, proliferation rate, migration, immunogenicity, and immunomodulatory capabilities of human MSCs derived from umbilical cord lining (CL-MSCs), umbilical cord blood (CB-MSCs), placenta (P-MSCs), and Wharton's jelly (WJ-MSCs). Differences were noted in differentiation, proliferation, and migration, with CL-MSCs showing the highest proliferation and migration rates resulting in prolonged survival in immunodeficient mice. Moreover, CL-MSCs showed a prolongation in survival in xenogeneic BALB/c mice, which was attributed to their ability to dampen TH1 and TH2 responses. Weaker human cellular immune responses were detected against CL-MSCs and P-MSCs, which were correlated with their lower HLA I expression. Furthermore, HLA II was upregulated less substantially by CL-MSCs and CB-MSCs after IFN-γ stimulation. Despite their lower IDO, HLA-G, and TGF-β1 expression, only CL-MSCs were able to reduce the release of IFN-γ by lymphocytes in a mixed lymphocyte reaction. They concluded that CL-MSCs showed the best characteristics for cell-based strategies, as they are hypo-immunogenic and show high proliferation and migration rates [18]. Zhu et al. investigated the differences in human placental MSCs (P-MSCs) of fetal and maternal origins in the aspects of clinical importance. Although all P-MSCs express typical MSCs phenotype, fetal but not maternal P-MSCs express high levels of CD200 and HGF. Compared with HGF- and CD200-negative P-MSCs, HGF- and CD200-positive cells demonstrated significantly higher potentials in promoting angiogenesis in vitro and increasing immunosuppressive function in vivo [19]. In 2017, we reported that placental chorionic villi (CV)-derived MSCs exhibited superior activities of immunomodulation and pro-angiogenesis compared to MSCs derived from the bone marrow (BM), adipose tissue, and umbilical cord (UC). Furthermore, we identified a subpopulation of CD106 (VCAM-1)$^+$ MSCs, which are present richly in placental CV, moderately in BM, and lowly in adipose tissue and UC. The CD106$^+$ MSCs possess significantly increased immunomodulatory and pro-angiogenic activities compared to CD106$^-$MSCs. Analysis of gene expression and cytokine secretion revealed that CD106$^+$MSCs highly expressed several immunomodulatory and pro-angiogenic cytokines. Our data offer new insights on the identification and selection of suitable source or population of MSCs for clinical applications [20].

Heterogeneity of Cell Surface Markers

Stro-1

Stro-1 is the best-known MSCs marker. However, Stro-1 is not expressed on MSCs populations derived from all kinds of tissues. So far, Stro-1 is reported to be expressed on MSCs derived from dental tissues [21], synovial membranes [22], and choriodecidua [23] but barely or at low level expressed on MSCs derived from adipose tissue [24], human umbilical cord blood [25], human umbilical cord [26], etc. Hongxiu Ning et al. suggested that Stro-1 is intrinsically an endothelial antigen and its expression on MSCs is probably an induced event [27]. Immunoselection with monoclonal antibodies against Stro-1 and CD106 prior to expansion resulted in a 1000-fold enrichment of mesenchymal precursors compared to standard isolation techniques. Moreover, intramyocardial injection of human Stro-1-selected precursors in an athymic rat model of acute myocardial infarction resulted in induction of vascular network formation and arteriogenesis coupled with global functional cardiac recovery [28]. Stro-1^+ cells may rather be used for gene delivery in tissues due to their stronger homing capabilities, while Stro-1^- cells may rather be used to support hematopoietic engraftment [29]. Compared to plastic adherence-isolated MSC (PA-MSCs), Stro-1-MSCs displayed greater clonogenicity, proliferative capacity, multilineage differentiation potential, and mRNA expression of MSC-related transcripts. In vitro assays demonstrated that conditioned medium from Stro-1-MSC had greater paracrine activity than PA-MSCs, with respect to cardiac cell proliferation and migration and endothelial cell migration and tube formation [30].

Thus, Stro-1 may get involved in MSCs colony forming, homing, and angiogenesis.

CD271

CD271, also called the low-affinity nerve growth factor receptor (LNGFR), is one of the two receptor types for the neurotrophins, a family of protein growth factors that stimulate neuronal cells to survive and differentiate. In vivo studies showed that CD271$^+$ MSCs promoted significantly greater lymphoid engraftment than did plastic adherence MSCs when co-transplanted with CD133$^+$ hematopoietic stem cells at a ratio of 8:1 in immunodeficient NOD/SCID-IL2Rgamma(null) mice. Therefore, CD271 antigen provides a versatile marker for prospective isolation and expansion of a subset of MSCs with immunosuppressive and lymphohematopoietic engraftment-promoting properties [31]. Hermida-Gómez et al. revealed that synovial membranes from human osteoarthritic patients contain more cells expressing CD271 antigen than those from healthy joints, and the cell subset CD271$^+$ MSCs provide higher-quality chondral repair than the CD271$^-$ subset [32]. CD271 is highly expressed on MSCs derived from the bone marrow [33], adipose tissue [34],

and periodontal ligament [35], lowly expressed on placental MSC [36, 37], and not expressed on MSCs derived from the synovial membrane [38], umbilical cord [39], and umbilical cord blood [40].

CD146

CD146, also known as the melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is a cell adhesion molecule which gets involved in the process of angiogenesis. CD146 is extensively expressed by MSCs derived from a variety of sources, such as the bone marrow [41], adipose tissue [42], umbilical cord [43], synovium [38], umbilical cord blood [40], placenta [37], dermis [44], etc. Human endometrial stromal $CD146^{+}PDGF\text{-}R\beta^{+}$ cells were enriched for colony-forming cells compared with $CD146^{-}PDGF\text{-}R\beta^{-}$ cells and also underwent differentiation into adipogenic, osteogenic, myogenic, and chondrogenic lineages [45]. Sorrentino et al. found that the $CD146^{+}$ MSCs represent a subset of stromal cells supporting hematopoiesis and secrete a complex combination of growth factors controlling hematopoietic stem cells (HSCs) function while providing a >2-log increase in the long-term culture (LTC) colony output in 8-week LTC over conventional assays. Thus $CD146^{+}$ MSCs may represent a tool to explore the MSC-HSC cross talk in an in vitro surrogate model for HSC "niches" and for regenerative therapy studies [46]. Moreover, CD146 expressing, subendothelial cells in human bone marrow stroma are capable of transferring, upon transplantation, the hematopoietic microenvironment to heterotopic sites [47].

CD106

CD106, also known as vascular cell adhesion protein 1 or vascular cell adhesion molecule 1 (VCAM-1), is a protein that functions as a cell adhesion molecule. CD106 is critical for MSC-mediated immunosuppression [48] and for the binding of hematopoietic progenitor cells [49]. Martens et al. reported that immunoselection with monoclonal antibodies against Stro-1 and CD106 prior to expansion resulted in a 1000-fold enrichment of mesenchymal precursors compared to standard isolation techniques [28]. Moreover, the combination of three cell surface markers (LNGFR, THY-1, and CD106) allows for the selection of highly enriched clonogenic cells (one out of three isolated cells) [50]. Fukiage et al. showed that the CD106-positive fraction contained less osteogenic and more adipogenic cells than the CD106-negative fraction, indicating the usefulness of CD106 as a differentiation-predicting marker of bone marrow stromal cells [51]. Our research team compared the phenotype and biological properties among different MSCs isolated from human placental chorionic villi (CV), umbilical cord (UC), adult bone marrow (BM), and adipose (AD) tissue. We found that CD106 (VCAM-1) was expressed highest on

the CV-MSCs, moderately on BM-MSCs, lightly on UC-MSCs, and absent on AD-MSCs. CV-MSCs also showed unique immune-associated gene expression and immunomodulation. We thus separated CD106$^+$ cells and CD106$^-$ cells from CV-MSCs and compared their biological activities. Both two subpopulations were capable of osteogenic and adipogenic differentiation, while CD106$^+$ CV-MSCs were more effective to modulate T-helper subsets but possessed decreased colony formation capacity. In addition, CD106$^+$ CV-MSCs expressed more cytokines than CD106$^-$ CV-MSCs. These data demonstrate that CD106 identifies a subpopulation of CV-MSCs with unique immunoregulatory activity and reveals a previously unrecognized mechanism underlying immunomodulation of MSCs [52]. Furthermore, we found that angiogenic genes, including HGF, ANG, IL8, IL6, VEGF-A, TGFβ, MMP2, and bFGF, were upregulated in CD106$^+$ CV-MSCs. Consistently, angiogenic cytokines especially HGF, IL8, angiogenin, angiopoitin-2, μPAR, CXCL1, IL-1β, IL-1α, CSF2, CSF3, MCP-3, CTACK, and OPG were found to be significantly increased in CD106$^+$ CV-MSCs. CD106$^+$ CV-MSCs showed remarkable vasculo-angiogenic abilities by angiogenesis analysis with Matrigel in vitro and in vivo, and the conditioned medium of CD106$^+$ CV-MSCs exerted markedly pro-proliferative and pro-migratory effects on endothelial cells compared to CD106$^-$ CV-MSCs. Finally, transplantation of CD106$^+$ CV-MSCs into the ischemic hind limb of BALB/c nude mice resulted in a significantly functional improvement in comparison with CD106$^-$ CV-MSCs transplantation. CD106$^+$ CV-MSCs possessed a favorable angiogenic paracrine activity and displayed therapeutic efficacy on hindlimb ischemia. Our results suggested that CD106$^+$ CV-MSCs may represent an important subpopulation of MSC for efficient therapeutic angiogenesis [53].

Nestin

Nestin (acronym for neuroectodermal stem cell marker) is a type VI intermediate filament protein expressed in the early stages of development [54]. Increasing studies show a particular association between Nestin and MSCs. Nestin could characterize a subset of bone marrow perivascular MSCs which contributed to bone development and closely contacted with hematopoietic stem cells (HSCs) [55]. Nestin$^+$ MSCs contain all the bone-marrow colony-forming-unit fibroblastic activity and can be propagated as non-adherent "mesenspheres" that can self-renew and expand in serial transplantations. Nestin$^+$ MSCs are spatially associated with HSCs and adrenergic nerve fibers and highly express HSCs maintenance genes. In addition, in vivo Nestin$^+$ cell depletion rapidly reduces HSCs content in the bone marrow and purified HSCs home near Nestin$^+$ MSCs in the bone marrow of lethally irradiated mice [56]. However, the intracellular location of Nestin prevents its use for prospective live cell isolation. The combination of surface markers PDGFRα and CD51 could be used for identifying Nestin$^+$ cells. PDGFRα$^+$ CD51$^+$ cells in the human fetal bone marrow represent a small subset of CD146$^+$ cells expressing

Nestin and enriched for MSCs and HSCs niche activities. Importantly, cultured human PDGFRα$^+$ CD51$^+$ non-adherent mesenspheres that could significantly expand multipotent hematopoietic progenitors were able to engraft immunodeficient mice [57].

Except for the above described specific markers for MSCs, there are some other surface molecules that have been found to be useful for identification of specific subset of MSCs, such as CD349 [58], CD49f [59], GD2 [60], 3G5 [61], SSEA-4 [62], etc.

Heterogeneity of Human MSCs Under Specific Conditions

Except for the individual donors and the tissue sources, the culture condition and microenvironment also contribute to the heterogeneity of MSC characteristics. For instance, if adipose-derived MSCs (ADMSCs) were cultured under hypoxic (1% O^2) conditions, ADMSCs proliferation and the expression of stemness genes, i.e., Nanog and Sox2, were significantly favored [63]. The heterogeneity of human umbilical cord MSCs (hUC-MSCs) cultured in serum-free medium (SFM) and serum-containing medium (SCM) was investigated by us. SFM-expanded hUC-MSCs were different from SCM-expanded hUC-MSCs in growth rate, telomerase, and gene expression profile. hUC-MSCs propagated more slowly and senesce ultimately in SFM. However, SFM-expanded hUC-MSCs maintained multipotency and the profile of surface antigen which were used to define human MSCs. Both SFM- and SCM-expanded hUC-MSCs gained copy number variation (CNV) in long-term in vitro culture [64]. Moreover, we found that bone marrow microenvironment of acquired aplastic anemia (AA) affects the heterogeneity of human bone marrow-derived MSCs (BM-MSCs). BM-MSCs from AA patients exhibited down-regulation of the CD106 gene and low expression of CD106 in vitro. The expression of NF-κB was decreased in AA MSCs, and NF-κB regulated the CD106 gene which supported hematopoiesis [65].

Conclusion and Perspective

Though the minimal criteria to define MSCs were proposed by the Tissue Stem Cell Committee of International Society for Cellular Therapy in 2006, the isolation of MSCs produces heterogeneous, nonclonal cultures of stromal cells containing stem cells with different multipotential properties, committed progenitors, and differentiated cells. In addition to the common immunophenotypic markers of the isolated MSCs, there are some special surface molecules that may be used to define different functional MSC subgroups. Analysis of different subpopulations of MSC can enhance the understanding of MSCs' biological characteristics.

In the future, to establish stem cell banks based on the heterogeneity of MSC subpopulations is quite necessary. In addition, selective application of different MSCs subgroups with one or two unique advantage functions, such as osteogenesis, adipogenesis, chondrogenesis, immunomodulation, angiogenesis, and hematopoiesis support, for the treatment of differential diseases might be promising in the field of stem cell therapy. However, it remains elusive whether application of MSCs that show heterogeneity while being cultured in vitro will function differently in vivo. Therefore, the in vivo heterogeneity of MSCs warrants further investigation.

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Chapter 11
Mitochondrial Heterogeneity in Stem Cells

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Abstract Mitochondria are customarily acknowledged as the powerhouse of the cell by virtue of their indispensable role in cellular energy production. In addition, it plays an important role in pluripotency, differentiation, and reprogramming. This review describes variation in the stem cells and their mitochondrial heterogeneity. The mitochondrial variation can be described in terms of structure, function, and subcellular distribution. The mitochondria cristae development status and their localization patterns determine the oxygen consumption rate and ATP production which is a central controller of stem cell maintenance and differentiation. Generally, stem cells show spherical, immature mitochondria with perinuclear distribution. Such mitochondria are metabolically less energetic and low polarized. Moreover, mostly glycolytic energy production is found in pluripotent stem cells with a variation in naïve stem cells which perform oxidative phosphorylation (OXPHOS). This article also describes the structural and functional journey of mitochondria during development. Future insight into underlying mechanisms associated with such alternation in mitochondria of stem cells during embryonic stages could uncover mitochondrial adaptability on cellular demands. Moreover, investigating the importance of mitochondria in pluripotency maintenance might unravel the cause of mitochondrial diseases, aging, and regenerative therapies.

Keywords Mitochondria · Stem cells · Mitochondrial heterogeneity · Embryonic development

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Introduction

Stem cells are the initiating cells that give rise to all tissues during the development starting from embryonic stage to adult life in a multicellular organism. They are equipped with two fundamental propensities: the self-renewal ability and the hierarchical progeny differentiation. In broader terms, stem cells can be classified into embryonic stem cells (ESCs) and non-embryonic stem cells [1]. However, with respect to potency, stem cells can be further categorized into four different types: (a) totipotent stem cells (TSCs), (b) pluripotent stem cells (PSCs), (c) adult stem cells (ASCs), and (d) induced pluripotent stem cells (IPSCs). Totipotent cells are capable of giving rise to a complete multicellular individual (e.g., fertilized eggs or zygote till 4-day embryo). However, PSCs can get differentiated into cells and tissue of three primary germ layers but not the whole organisms, because of their inability to synthesize the extraembryonic tissues. For example, embryonic stem cells (ESCs) or the inner mass cells of the blastocyst. IPSCs are differentiated cells, ectopically reprogrammed back to a stem cell state through the process of "nuclear reprogramming." The non-embryonic stem cells are also known as adult or somatic or tissue-specific stem cells. They can be either multipotent or unipotent [2, 3]. The multipotent stem cells are derivatives of amniotic fluid and umbilical cord blood which have the limited ability to differentiate into cell types of their own lineage only (e.g., mesoderm). Again, the unipotent stem cells or the precursor cells are obligated to differentiate into a particular cell type only. The ESCs remain pluripotent inside the inner mass cells of blastocyst in preimplantation embryos, but during post-implantation development, they lose potency as they become committed to differentiate into specific somatic lineages [4]. Non-embryonic stem cells are reported to be more specialized than ESCs. Two phases of pluripotency, i.e., naïve and primed, are reported in ESCs [5]. The ESCs in the preimplantation epiblast of embryos represent "naïve" stem cells, and they become "primed" or relatively more mature during post-implantation development in the embryo [1]. Though the different types of stem cells share common properties, there exist significant differences in both structurally and functionally. Moreover, stem cells are widely used in the biological and biomedical fields such as cell replacement therapies, drug testing, genetic defect studies, differentiation, and early development studies.

The pivotal role of mitochondria in the stem cell biology is apparent. The mitochondria are double-membraned organelles evolved from endosymbiotic relationship between aerobic bacteria and primordial eukaryotic cells [6]. They are semiautonomous and equipped with their own circular genome of size 16.6 kb (appox) with 37 genes. Thirteen genes out of 37 genes of the mitochondrial DNA (mtDNA) encode list of protein subunits necessary for respiratory complexes I, III, IV, and V (not II), 22 tRNAs, and 2 rRNAs. Mitochondrial structure and function are regulated by the synergistic nuclear and mitochondrial genome. The nuclear transcription factors (NRF-1, NRF-2, and ERR1), nuclear transcription cofactors (PGC1-a, PGC1-b, and PPRC) and nuclear-encoded DNA polymerase (POLG and POLG2), and mitochondrial transcription factor A (TFAM), together control the

mitochondrial biogenesis, structure, mtDNA replication, transcription, and translation [7]. Moreover, mitochondria have the unique ability to modulate its function through biogenesis and selective degradation to meet different demands of distinct cell types and tissues. Again, mitochondria are highly specialized for energy metabolism, cellular homeostasis, and programmed cell death. Primarily, mitochondria provide a highly efficient route ATP production through oxidative phosphorylation (OXPHOS) and as a by-product generate endogenous reactive oxygen species (ROS) which in higher concentration may cause DNA damage, lipid peroxidation, and protein carbonylation but in low levels is essential for certain physiological activities [8]. It also plays a pivotal role in essential pathways like intermediate metabolism, fatty acid oxidation, steroid metabolism, amino acid biosynthesis [9], calcium homeostasis, redox regulation apoptosis, and cell fate determination [10]. Interestingly, mitochondrial structure and function vary in different cell and tissue types depending on cellular demand. In this article, we discuss the mitochondrial heterogeneity in different cellular system and their functions.

Mitochondrial Heterogeneity Within Cells

Structural Heterogeneity

Mitochondria have shown to adapt different morphological features on cellular demands. Various experimental studies involving the fluorescent probing that employs targeting mitochondria by the Rhodamine 123, JC-1, MitoTracker Red/Green, and green fluorescent proteins (GFPs), demonstrated the configurational changes in mitochondria. Moreover, the high-resolution 3D reconstitution imaging technology has revealed the tubular and interconnected network-like structure of mitochondria [11]. Studies found the existence of mitochondrial networks in rat hepatocytes [12], whereas in yeast cells, a single large mitochondrion is observed [13]. Again, the occurrence of interconnected mitochondrial network is also confirmed in HeLa cells [14]. That is why, it is a topic of intense discussion "whether mitochondria exist as a single network-like endoplasmic reticulum (ER) or independently" and "what is the nature of mitochondrial population distribution in a cell types?" However, a study in four primary cell types (hepatocytes, HUVEC, cortical neuronal cells, and cortical astrocytes) and three transformed cell lines (COS-7, HeLa, and PAEC cells) by Collins et al. demonstrated that mitochondria network are lumenally discontinuous and perform individually. Morphologically, mitochondria can be differentiated into small "grains" or larger branched and/or unbranched "threads." However, it was also reported that mitochondria within cells are not only physically interconnected but also functionally homogenous [15]. The presence of large electrically connected networks of mitochondria is shown in COS-7 cells [16] and cardiac myocytes [17]. Such network-like structure provides an efficient energy delivery system and calcium channeling inside cell. Studies involving mitochondria

staining with specific, membrane potential-sensitive fluorescent probes, depolarizing stimuli suggest physical connectivity within cells as evidenced by the fact that the membrane potential tend to downfall over the length of tens of micrometers within the network [11].

Distributional Heterogeneity

As a dynamic cell organelle, mitochondria are reported to alter the number, morphology, and subcellular distribution during cell cycle, development, stress, and cell death [18]. Such alteration in mitochondria inside cell is precisely governed through the process of fission and fusion. Moreover, it was found that the changes in the mitochondrial morphology and subcellular distribution are cell-/tissue-type specific. These cell-type specificity and variations in the mitochondrial dynamics are responsible for specific cellular functions and demands. Again, in some of the cell types like adult cardiomyocytes or skeletal muscles mitochondria are reported to behave in a fixed manner where the mitochondria could be seen organized in a specific pattern in between the myofibrils to offer bioenergetic basis for muscular contraction [19]. Moreover, adult cardiomyocytes are found to show no dislocation of mitochondria suggesting the cell-type-specific distinctions in the mitochondrial dynamics and spatial arrangement [17]. Mitochondrial localization patterns can be quantified by determining the fluorescence intensity of fluorophore-labeled mitochondria from the nuclear periphery to plasma membrane periphery. Adopting such approach, it has been discovered that mitochondrial population could be seen in three different forms such as homogeneous/random, aggregated/fused, or perinuclear. Reports also indicate that mitochondria of varying sizes were heterogeneously distributed. Moreover, mitochondria could be seen as separate entities and distributed throughout the cytosol with a tendency of perinuclear aggregation. It was also found that the mitochondria in stem cells have a perinuclear distribution. However, there needs a further elucidation of the functional implication of the perinuclear mitochondrial distribution in stem cells. Contrastingly, it is also shown that differentiated cells such as fibroblasts [20], pancreatic acinar cells [21, 22], astrocytes, and neurons [15] also display perinuclear arrangements of mitochondria. These facts lead to question the significance of perinuclear distribution of mitochondria. Few speculations are put forwarded to address the query. Firstly, it is proposed that if mitochondria are assembled near the nucleus, the transport of polymerase POLG and related transport into the mitochondria would be more efficient. The second important explanation for perinuclear clustering is effective energy transfer as energy-dependent Ran monomeric G-protein transport systems are involved in transport of macromolecules across the nuclear pores. Thirdly, it's believed that such perinuclear distribution might be responsible for buffering the nucleus during Ca^{2+} fluctuations in the cytoplasm [23].

Functional Heterogeneity

Functional heterogeneity of mitochondria was obvious in different cell types. Interestingly, early electron microscopy studies demonstrated that within single mitochondria, there exist mitochondrial populations with different matrix densities within single cells reflecting differences in metabolic states [19, 24]. A study by Collins et al. suggested that mitochondria have different $\Delta\psi_{mit}$ suggesting their functional heterogeneity with respect to bioenergetic status. Again, in cardiac [25] and skeletal muscle [26] cells, two distinctive mitochondria populations with different biochemical and respiratory properties are anticipated to be existent. More intriguingly, it was discovered that highly energized mitochondria are proportionately focused in the periphery of cells rather than the perinuclear position [11]. In the cleavage stages of development, mitochondria with high electron dense matrix are found, whereas in later stages of early embryogenesis, mitochondria with a matrix of low electron density and high inner mitochondrial membrane potential are reported to exist [27]. The association of mitochondrial membrane potential (ψ_m) and oxidative metabolism is described for the first time by Mitchell and Moyle [28]. High mitochondrial polarity is related to elevated respiratory activity during early development. Compartmentalization of mitochondria with different polarities inside oocyte is of specific importance as it proposes a mechanism by which domains of differential function could be established [29].

Mitochondria in Stem Cells

The structural analysis indicated that undifferentiated human and mouse ESCs have spherical mitochondria with poorly developed cristae, fewer copies of mtDNA, and perinuclear distribution compared to their long, tubular-shaped, branched, and cristae-rich somatic cell counterparts (Fig. 11.1) [30]. However, there appears an escalation in the mtDNA copy number, mitochondrial mass, oxygen consumption, respiratory reserve capacity, and reactive oxygen species (ROS) along with the morphological and ultrastructural changes during differentiation. However, glycolysis and lactate production rate are diminished in mouse and human ESCs and iPSCs [30–34]. Structurally immature mitochondria are seen in oocytes, and early embryos are in mammals where they appear as ovoid, spherical, rounded, or with few cristae and may contain vacuoles [35]. Report indicated that the human ES cell line HSF6 has few mitochondria in the perinuclear region [36]. However, under differentiating conditions, the same HSF6 cells showed a large number of elongated mitochondria with distinctive cristae. Again, it was shown that ESCs possess lower mtDNA content and mitochondrial mass in human HSF6 cell line, and mtDNA copies increased during differentiation [36]. An in vivo study on spermatogonia, inner cell mass, and early embryos indicates a prospective features of “stemness” in terms of the presence of relatively immature perinuclear mitochondrial cluster and comparatively

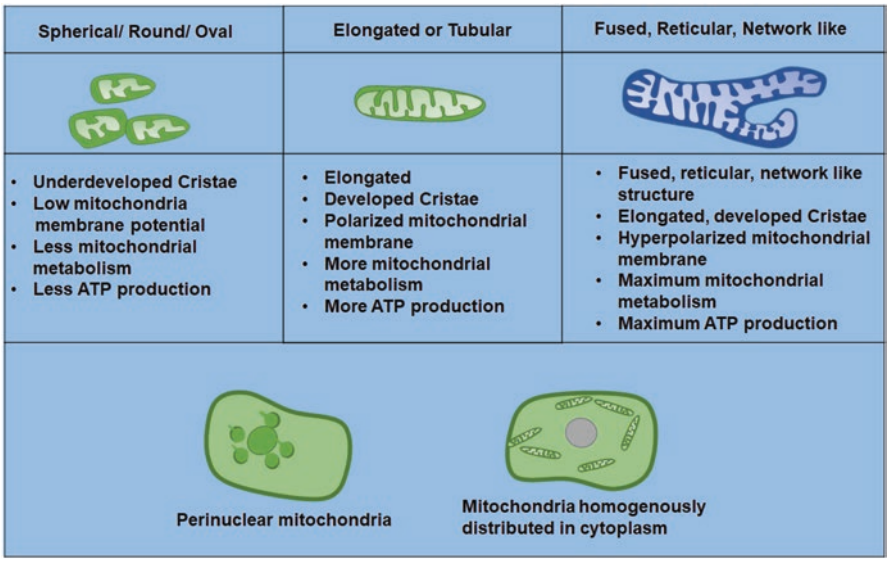

Fig. 11.1 Mitochondrial heterogeneity. It describes the structural, distributional variation in mitochondria in cells. Mitochondria can be oval/rounded/spherical or elongated/tubular or hyperfused and reticular. Mitochondria can also be seen with immature or underdeveloped cristae or with mature and developed cristae. Finally, mitochondria can be arranged in cluster or freely in perinuclear position, or it remain dispersed homogenously in the cytoplasm

lower mitochondrial energetics [37]. This findings are corroborated in monkey [38], mouse [39], and human [40] where mitochondria are around the pronuclei of fertilized oocytes and nuclei of cleavage stage embryos. In cancer, a rare subpopulation of cells termed cancer stem cells exist which are also capable to self-renew and differentiate into any type of cell in cancer [41–43]. Such cancer stem cells are reported to contain spherical mitochondria with under developed cristae and perinuclear distribution [1].

The respiratory activity of mitochondria can regulate the differentiation status of the cell (Fig. 11.2). The human ESCs are functionally similar to the glycolytic phenotype in cancer (Warburg effect) [44, 45]. The human PSCs show lower OXPHOS with perinuclear mitochondria. Again, it can perform a bivalent metabolism which render them the dynamic ability to switch between glycolysis and OXPHOS upon demand [44]. Naïve or ground state stem cells perform OXPHOS. However, the "primed" stem cells display glycolysis [45]. The alteration in the functional energetics and structural integrity in mitochondria causes metabolic reconfiguration leading to a shift from naïve to "primed" states of pluripotency culminating in lineage-directed differentiation [45]. Tissue-specific stem like the multipotent mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) resides in hypoxic environments and is more reliant on glycolysis as compared to their differentiated equivalents [46, 47]. Hypoxic environment protects somatic stem cells from oxidative stress and sustains self-renewal ability. Mitochondrial oxidative

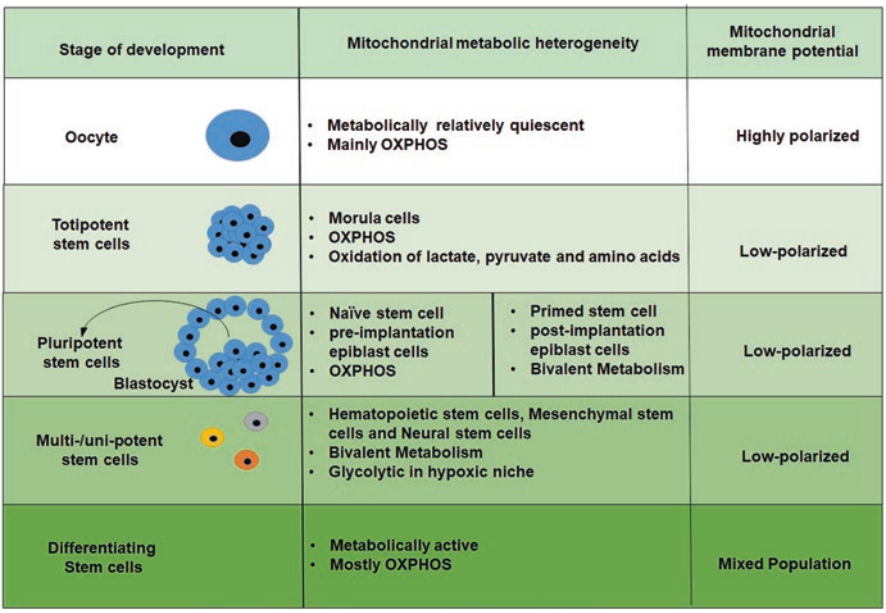

Fig. 11.2 Mitochondrial metabolic variations in stem cells during embryonic development. Metabolism is quiescent in oocytes, but it becomes active during cleavage. Generally, stem cell contains immature mitochondria thereby prefer glycolysis. However, naïve stem cells are OXPHOS phenotype, and adult stem cells are bivalent phenotypes (metabolism alters between glycolytic and OXPHOS depending on demand). Differentiated cells have OXPHOS phenotype

capacity in adult HSCs is diminished by HIF1-α expression and concomitant activation of CRIPTO, GRP78, and PDKs [48, 49]. But MSCs can adapt OXPHOS when cultured and expanded in normoxic conditions [50, 51]. Neural stem cells (NSCs) derived from the PSCs are comparatively more mature and contain mitochondria having densely folded and compact cristae structures [52]. NSCs are glycolytic than neuronal progeny via induction of Hexokinase-2 (HK2) [40, 53, 54]. But the mitochondrial copy number remains low in PSC-derived NSC till terminal neuronal differentiation [52], indicating quiescent phenotype of NSCs [55]. Moreover, the cancer stem cells are more glycolytic as compared to cancer cells and perform less mitochondrial oxidation [1].

Mitochondrial Structural and Distributional Heterogeneity During Embryonic Development

There occurs a significant remodeling of mitochondria during the cell fate transition in terms of subcellular content, configuration, and distribution of mitochondria (Fig. 11.3). In the time of early development, the manifestation mitochondrial

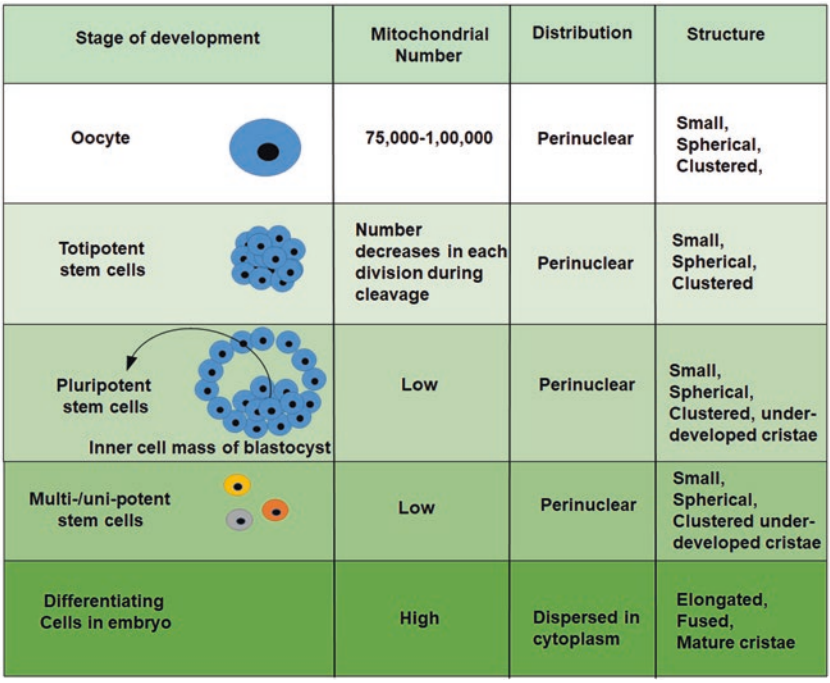

Fig. 11.3 Mitochondrial structural and distributional variations in stem cells during embryonic development. Unfertilized oocytes and preimplantation stage embryos contain immature mitochondria with perinuclear distribution. The mitochondrial mass and number are low in stem cells as compared to differentiated cells

spatial remodeling is considered as a customary characteristic where stage-specific changes in mitochondrial allocation during oocyte maturation and early embryogenesis could be seen in several mammalian species. Between the 4- and 16-cell stages of embryogenesis, the round/oval immature mitochondria gradually become more elongated by the intensification of transverse cristae in mouse [33], sheep [34], pig [35], and cattle [36]. During the pig embryonic development starting from oocyte maturation, fertilization, and early embryo development, the mitochondrial alteration was examined exercising MitoTracker Green fluorescent staining coupled with confocal laser scanning microscopy. The presence of active mitochondria was witnessed in the cortex of oocytes collected from small follicles. However, when fully grown oocytes taken large follicles were analyzed, they displayed active mitochondria in the peripheral cytoplasm. As the meiosis progresses from germinal vesicle breakdown (GVBD) to anaphase-I, the mitochondria tend to cluster in the perinuclear region of the cell.

In the course of embryogenesis, from zygote to two-cell stage of mouse embryos dumbbell-shaped mitochondria with concentrical cristae were found. However, when the embryo reaches from four cell to the morula stage, extensive remodeling occurs that incurs the elongation of mitochondria, transverse relocation of cristae,

and vacuole formation [56]. In fertilized eggs and early cleavage, active mitochondria are found to be aggregated in the pronuclear region. From this study, it can be inferred that alterations in distribution of active mitochondria are key event during oocyte maturation, fertilization, and early embryo development. Such kind of spatial remodeling could permit higher ATP level which facilitates energy-dependent stage-specific activities in the cytoplasm [29, 57]. In blastomeres of uncompacted embryos, there exists a random allocation of mitochondria all over the cytoplasm and around nuclei. But during the process of compaction, there occur stage-specific mitochondrial redistributions which involves the dynamic alteration of mitochondria from diffuse or cortical arrangement at the eight-cell stage to tight perinuclear distribution in the trophectoderm (TE) of expanded blastocysts [33]. Similar events were observed in case of mouse development in vitro. It observed that mitochondria often translocate to the perinuclear area during in vitro mouse oocyte maturation [58]. Moreover, in case of mouse [29], hamster [59], and human [60], mitochondria migrate to the perinuclear region surrounding pronuclei after fertilization. Early passage stem cells (P11) of ATSC line showed an approximate 85% of perinuclear mitochondrial localization, but in later passage (P17), it decreased to 18% cells as cells start to differentiate into adipocytes. In the early and mid-passages, mitochondria are appeared as threadlike, while the later phases predominantly exhibit a fused mitochondrial pattern. The cells displaying aggregated pattern of mitochondria were identified as presumptive adipocytes [4]. Interestingly, it was discovered that the geometry and symmetry of the mitochondrial accumulation in the perinuclear position at the 1-cell stage is related to mitochondrial inheritance pattern between the 2- and 12-cell stages [60]. During gastrulation, the mitochondria ultrastructure and distribution alters and is characterized by enlargement of mitochondrial size, enhanced cristae formation [36], and loss of perinuclear aggregation [61].

Mitochondrial Functional Heterogeneity During Embryonic Development

Embryogenesis involves distinct conformational and metabolic changes in mitochondria (Fig. 11.2). After implantation, the relatively inactive egg at ovulation gets converted into actively metabolizing tissues. Mitochondria present in the inner cell mass of blastocyst are depolarized, spherical/round/oval, and low oxygen consuming in nature [3, 56, 62]. During the development from one-cell stage to the morula formation, autophagocytosis becomes the primary source of cellular energy production through protein catabolism [63]. However, the Krebs cycle and OXPHOS are the prime source of mitochondrial energetics during the first cleavage in the implantation embryo where pyruvate (mostly), lactate, triglyceride-derived fatty acids, and amino acids are used as the energy substrate (except porcine embryos) [64]. Glucose uptake and usage is limited until the morula formation [65, 66]. During blastulation, the metabolic activity increases via higher glucose uptake mediated by GLUT-1 and

GLUT-3 [67] and leads to increase oxygen consumption and OXPHOS within the trophectoderm [27]. As the development progresses to gastrulation, glycolysis decreases along with the elevation in the mitochondrial oxidation of glucose-derived pyruvates and fatty acids [68].

Mitochondrial Fission and Fusion Regulating Mitochondrial Heterogeneity

Mitochondrial morphology is an extremely dynamic parameter and highly subjective to alterations in their features. The mitochondrial heterogeneity in morphologies in various cell types starting from small spheres to tubular networks is the consequence of fusion and fission. It is noteworthy to mention that mitochondrial fission and fusion are instrumental in maintaining the mitochondrial dynamics [1]. Mitofusins (Mfn), the outer mitochondrial membrane GTPases, are essential for mitochondrial fusion and have two variants, i.e., Mfn1 and Mfn2. Along with Mfn, the dynamin family GTPase OPA1 is also responsible for the process of mitochondrial fusion. The mitochondrial fission is contrasting to the process of mitochondrial fusion. Moreover, the process of fission is critically dependent on another GTPase dynamin-related protein Drp1 which potentially spots marks the mitochondria for tentative fission [1]. Such localization of Drp1 onto mitochondrial membranes might induce their constriction, tubulation, vesiculation, and membrane scission through the GTP hydrolysis. [69]. Mitochondrial fragmentation can be activated by reducing the relative rate of fusion [70, 71]. The trophoblast giant cell layer of Mfn2 mutant mice shows fragmented mitochondrial population. Likewise, Mfn1 or Mfn2 lacking mouse embryonic fibroblasts had extreme mitochondrial fragmentation. Such fragmentation of mitochondria is the result of fission in the absence of fusion. On the other hand, there is an increase in the elongation and interconnectivity of the mitochondrial in absence of Drp1. In yeast, it has been found that if the process of fission is blocked with a dominant-negative version of Drp1, the mitochondrial tubules structures can be restored [72–75]. However, an obvious question pops up that whether the opposing function of mitochondrial fusion and fission is only responsible for governing mitochondrial morphology and distribution or it has any other role? In this scenario, blockade of fusion would lead to fragmentation of mitochondrial, but it would be able to do its normal function. However, deletion of Mfn1/2 and OPA1 in cells was shown to reduce the cell growth and activity of respiratory complexes [70]. Such type of relative inactivity of respiratory complexes is involved in driving mitochondrial heterogeneity indicting that mitochondrial fusion appears to be important not only maintains the mitochondrial structure but also mitochondrial function. Likewise, the disruption of mitochondrial fission through Drp1 depletion caused improper mitochondrial segregation during cell division [76]. In *Caenorhabditis elegans*, RNA interference of Drp1 could instill early embryonic lethality [73].

The fission/fusion cycle also governs mitochondrial elimination through mitophagy [77]. Mitophagy is the selective removal of mitochondria in an autophagy-dependent manner. Mitophagy can be (i) quality control-related mitophagy and (ii) developmentally induced mitophagy. Quality control-related mitophagy performs housekeeping functions, i.e., removal of superfluous mitochondria or damaged mitochondria. But the developmentally induced mitophagy performs cellular developmental processes like differentiation and dedifferentiation. Mitochondrial fission generates two daughter mitochondria that are structurally and functionally distinct, i.e., polarized and depolarized. The polarized phenotype might proceed to fusion and the depolarized phenotype undergo mitophagy [78, 79]. The fission process makes the fused and elongated mitochondrial network into manageable size that are easy to be removed by lysosomal degradation [18, 80, 81]. It is well established that stem cell contains fewer number of mitochondria as compared to differentiated cell. During dedifferentiation into stem-like state, mitochondrial fission and mitophagy might play crucial role in maintaining lower mitochondrial mass. However, little information is available regarding the reprogramming-associated reduction of mitochondria and activation of mitophagy. The activation of DRP1 is involved with the mitochondrial fission-associated pluripotency [82]. Furthermore, in another study, it was found that impairment in fission process could cause reduction in cellular reprogramming [83]. Contrastingly, mitochondrial fusion is reported to inhibit mitophagy [77]. Inhibition of mitochondrial fusion by depletion of Mfn1/2 and the maintenance of pluripotency [84] and the induction of mitochondrial fusion causes reduction of somatic cell reprogramming to pluripotency [85].

Conclusion and Future Perspectives

Cellular potency is the foundation for differentiation and development. Various cells are treasured with various level of potency. The pluripotent stem cells are able to self-renew and differentiate into any kind of cell. However, such criteria of stem cells are fulfilled by the variation in structural and functional aspects of mitochondria contained within which further creates the variation in the functional aspects of various kind of stem cells. Moreover, mitochondria show enormous functional diversity, which fulfills numerous environment-specific tasks. There remains a striking difference in the structure, function, and distribution of mitochondria in stem cells at different developmental stages. During different transitional phases, there occurs reconfiguration of mitochondria and related metabolic signature. Such reconfiguration helps mitochondria in fulfilling the demand during the course of differentiation, dedifferentiation, and early embryonic development. Moreover, there remains many unanswered questions that needs to be addressed. (1) What is the role of mitochondrial remodeling in terms of structure, distribution, and function in regulating the process of cellular reprogramming, i.e., differentiation and dedifferentiation? (2) What is the underlying molecular mechanism behind such remodeling? (3) Whether the process of cellular reprogramming is the cause or

consequence such remodeling? (4) How is the mitochondria function regulated by its structure? (5) Why do in the process of dedifferentiation the mitochondrial fragmentation does not culminate in the retention of fully functional mitochondria but immature mitochondria? (6) How is the mature mitochondria converted to an immature state and vice versa during dedifferentiation and differentiation, respectively? (7) What are the molecular events that govern the rearrangement of mitochondrial distribution during the process of differentiation (Homogenously distribution) and dedifferentiation (perinuclear)? (8) How do the mitochondrial remodeling associate with the alteration in metabolic preferences? (9) Is the metabolic switch the cause or consequence of mitochondrial remodeling? (10) Do mitophagy has a role in such remodeling in mitochondria then when and how? (11) How is the alteration in the mitochondrial structure, distribution, and function regulated during cellular reprogramming? Further work is needed to decipher the mechanisms underlying the restructuring of mitochondria structure, mass, distribution, metabolism, and redox balance to get introspect into the regulators of such kind of events which will be helpful in developing strategies protecting the stem cells for safe medical application and generation of iPSCs.

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Chapter 12
The Heterogeneity of Renal Stem Cells and Their Interaction with Bio- and Nano-materials

Fabio Sallustio, Loreto Gesualdo, and Dario Pisignano

Abstract For a long time, the kidney has been considered incapable of regeneration. Instead, in recent years, studies have supported the existence of heterogeneity of renal stem/progenitor cells with the ability to regenerate both glomerular and tubular epithelial cells. Indeed, several studies evidence that renal progenitor cells, releasing chemokines, growth factors, microvesicles, and transcription factors through paracrine mechanisms, can induce tissue regeneration and block pathological processes of the kidney. In this chapter the potentiality of the kidney regenerative processes is considered and reviewed, and the main classes of stem/progenitor cells that might contribute to the renal tissue renewal is analyzed. Moreover, we evaluate the role of biomaterials in the regulation of cellular functions, specifically addressing renal stem/progenitor cells. Materials can be synthesized and tailored in order to recreate a finely structured microenvironment (by nanostructures, nanofibers, bioactive compounds, etc.) with which the cells can interact actively. For instance, by patterning substrates in regions that alternately promote or prevent protein adsorption, cell adhesion and spreading processes can be controlled in space. We illustrate the potentiality of nanotechnologies and engineered biomaterials in affecting and enhancing the behavior of renal stem/progenitor cells. Although there

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are still many challenges for the translation of novel therapeutics, advances in biomaterials and nanomedicine have the potential to drastically change the clinical and therapeutic landscape, even in combination with stem cell biology.

Keywords Renal stem cells · Renal progenitor cells · Glomerular cells · Tubular cells · Kidney regeneration · Materials science · Biomaterials · Microvesicles · Soft lithography · Microcontact printing · Polymer nanofibers · Extracellular matrix

For a long time, the kidney has been considered an organ incapable of real regeneration. However, it is now well established that renal damage can at least partially regress and both the integrity and functionality of the damaged nephron can be recovered. In recent years, renal studies have supported the existence of heterogeneity of renal stem/progenitor cells with the ability to regenerate both glomerular and tubular epithelial cells and identified both in mice and rats and in humans. The existence of such cells in different evolutionary lines is a confirmation of the regenerative capacity of the kidney.

In this chapter the main classes of stem/progenitor cells that have been found to contribute to the kidney regeneration are reviewed and analyzed. In the first section, we will explore the regenerative capacity of the kidney. In the subsequent sections, we will consider adult renal progenitor cells in mice, rats, and humans. Then, we will explore how the potentiality of nanotechnologies and engineered biomaterials, applied to therapeutic and diagnostic fields, can be exploited to affect and enhance the behavior of progenitor cells. We will evaluate the role of biomaterials in the regulation of cellular functions, specifically addressing renal progenitors.

The Regenerative Capacity of the Kidney

Although the adult kidney does not exhibit the same ability as other organs (e.g., such as the liver) to regenerate parts after resection, it may be repopulated by new cells and, at least partially, to repair damaged tissue structures [1]. This happens although renal cell types, such as the podocytes, the main cells implicated in the glomerular filtration function, are terminally differentiated cells that, like neurons, have limited or nullified ability to proliferate and regenerate [2]. The cellular regeneration process, for example, following an acute tubular damage induced by an insult against resident cells involves the repopulation of damaged portions of the nephron and leads to structural and functional recovery through the replacement of damaged cells by new epithelial cells. This capability is inherited from the most ancestral developmental forms and is present in all animal species [3]. In contrast, the neogenesis of the nephron, or the ability to generate whole, new functional units, has been described in fish and other less evolved animals, but it does not occur in adult mammals, where it stops at birth [3–5]. It is intriguing to notice how the reduction of the regenerative capacity along evolution is a somehow generalized

phenomenon that involves all the tissues and the organs, more and more pronounced as the complexity of organism increases. When a large number of cell lines and increasingly specialized functions need to be managed, this might occur at the expense of regenerative potential. However, as reported in the next section, it has been recently demonstrated that renal stem/progenitor cells in humans may regenerate even whole tubular parts [6, 7].

While the kidney morphology and architectural organization differ among the species, the nephron, the renal functional unit, is highly conserved in its structure from more ancient organisms up to humans. In all animal species, kidney development begins with the mesenchyme metanephric, a group of cells of the intermediate mesoderm, through a mutual interaction with Wolff's duct, an epithelial structure that induces condensation around the ureteral gem and the subsequent mesenchymal-epithelial transition. These primitive epithelial cells thus form a spherical cyst, which is called renal vesicle [8, 9]. A series of invaginations and elongations then transform the renal vesicle in "comma-shaped bodies" and then into "S-shaped bodies," whose proximal end is invaded by the blood vessels, giving rise to the capillary glomerulus. At the same time, the middle and distal portions of the S-shaped body, which remain in relationship with the ureteral gem, merge, thus giving rise to a structure covered by a single cell type that starts to express tubular epithelium features [8, 9].

Until a few years ago, it was believed that regenerative strategies were only partially shared among the various animal species. Recent studies suggest instead that stem cells and renal progenitors play a major role in renal regeneration throughout the evolutionary pathway (Fig. 12.1) [10]. Studies on transgenic mice by the Six2 gene have demonstrated the existence of a population of multipotent progenitors at the level of the metanephric mesenchyme, capable of self-renewal and capable of generating each portion of the entire cortical nephron [11]. In the human embryonic kidney, renal stem cells have been identified by evaluating the expression of staminal markers, such as (a) CD133, marker of hematopoietic stem cells and other types of adult stem cells [11]; (b) CD24, a surface molecule widely used for characterizing different types of stem cells in humans [12, 13]; (c) Bmi-1, a critical transcription factor for the maintenance of the self-generating capacity of stem cells [14, 15]; (d) Oct-4, a transcription factor specific of stem cell [16]; and (e) Pax2, a transcription factor that is active in the undifferentiated mesenchyme and in proliferating tubular epithelial cells in response to a necrotic damage [17]. The surface expression of CD133 and CD24 allowed a cellular subpopulation to be identified and isolated that exhibits self-regenerative and multi-differentiative potential. Upon injection into mice with acute renal damage, these cells can regenerate the damaged cells in different parts of the nephron, reduce tissue necrosis and fibrosis, and lead to an improvement in renal function [18]. These progenitor cells are located in the primordial structures derived from the metanephric mesenchymal, i.e., in primitive vesicles, in the comma-shaped bodies, and in the S-shaped bodies, from which both the globular and the tubular structures originate to shape the adult kidney [18]. At the beginning of the third month of pregnancy, once metanephros is formed, the most of renal progenitors are still present. Afterward, their amount progressively

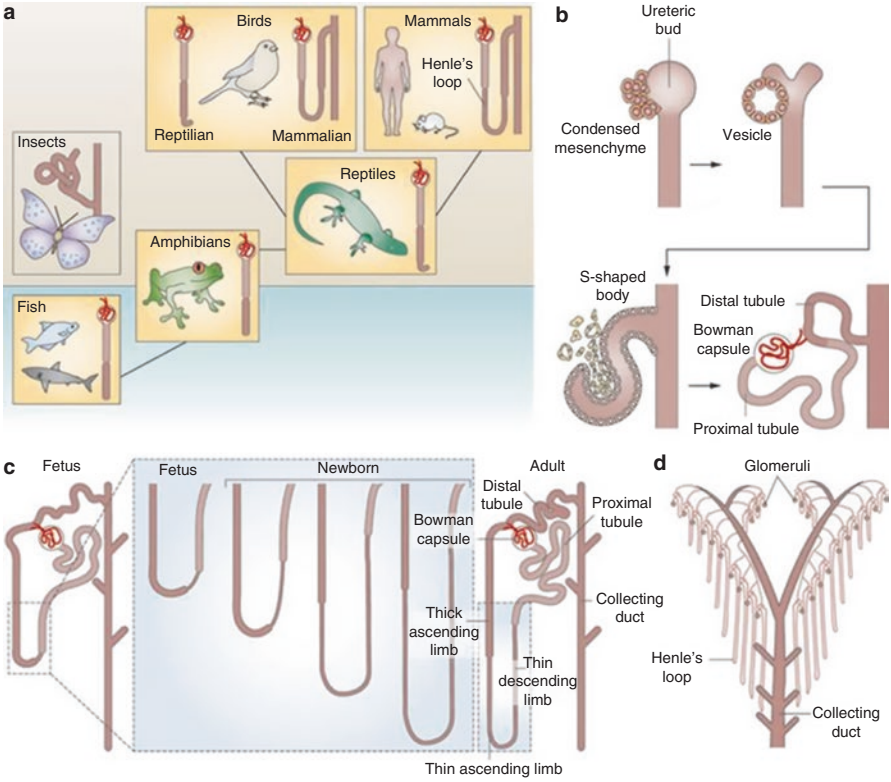

Fig. 12.1 (**a**) Nephrons as fundamental functional components of the kidney are rather comparable in organisms of different phyla. In vertebrates, they comprise a filtering unit (corpuscle), which is joined by means of a neck to a tubule subdivided in a proximal tubule, an intermediate segment, and a distal tubule connecting to an excretion unit. In avian and mammalian kidneys, the Henle's loop concentrates urine. Bird kidneys contain two kinds of nephrons: nephrons with Henle's loop ("mammalian type") and nephrons without Henle's loop ("reptilian type"). (**b**) In all animals the kidney develops through similar phases. The metanephric mesenchyme condenses around the ureteric bud, converts into the epithelium, and generates a vesicle and then an S-shaped body. Subsequently, the S-shaped body is reached by blood vessels at one extremity and elongates and segmentates at the other, generating the whole nephron. (**c**) In birds and mammals, after the end of nephron development and simultaneously with the renal papilla development in the newborn, the S3 segment of the proximal tubule and the distal tubule anlage of the nephron generates, as an outgrowth, the thin ascending limb of Henle's loops. (**d**) Pattern of nephron distribution along the collecting ducts in adult mammalian kidney, with characteristic pyramid shape. Reprinted by permission from Springer Nature, Nat. Rev. Nephrol. 9, 137–146 (2013). "Renal progenitors: An evolutionary conserved strategy for kidney generation", P. Romagnani, L. Lasagni and G. Remuzzi.

decreases up to ~2% of cells constituting the adult kidney. Interestingly, at the stage of S-shaped bodies, the renal progenitors already show a phenotype commissioned to glomerular or tubular lines. One can figure out that that renal stem cells, initially capable of generating all renal cells, progressively lead to progenitors committed to

an increasingly specialized type of cells [19–21]. This progressive differentiation hierarchy reflects the location of the different progenitor lines in the developing kidney and then in the adult kidney.

Adult Renal Progenitors

In the last years, some evidence showed that in mammals, kidney damage can, at least in part, regress and that both the integrity and functionality of the damaged portion of the nephron can be recovered, strengthening ideas about the existence of a population of resident progenitors. In fact, several studies performed in animal models of diabetic and other nephropathies have found that chronic treatment with ACE inhibitors and sartans not only prevents the progression of renal damage but also promotes regression of glomerulosclerosis, through the remodeling of glomerular architecture and the regeneration of podocytes [22–26]. It was, also, found that the adult human kidney can undergo regeneration, with regression of glomerular and tubular lesions, in patients with diabetic nephropathy. In patients with type 1 diabetes mellitus and diabetic nephropathy, who underwent pancreas transplantation, there was a regression of renal lesions after 10 years of normoglycemia [27]. On the basis of this evidence, numerous studies have been carried out to identify a population of progenitors that would be responsible for these regenerative processes in the kidney. It should be pointed out that while anatomically and functionally the kidney seems to be extraordinarily complex, such complexity is reducible to quite simple terms. Each kidney is made up of just over a million microscopic units, the nephrons, all substantially the same. These millions of units are anatomically and functionally independent of each other suggesting that each structure may accommodate its alleged niche of stem cells able to repair lesions.

Adult Renal Progenitors in Rats and Mice

Renal progenitors exist in the adult kidneys of some animal species, as in fish, in which there is neogenesis of the nephrons following a damage [3]. In mammal models, attempts to identify adult stem cells were made on the basis of the general principles of stem cell biology. One method for identifying stem cells exploits the property of these cells to feature a very slow replicative cycle. For this reason, they are called slow cycling cells. When the DNA of these cells is labeled with substances such as bromodeoxyuridine (analog of thymine, incorporated together with the other nucleotides and detectable), the same cells retain the marker for a long period of time. This retention, if quantified, may be the key for identifying a putative stem cell population [28]. These cells were firstly identified in adult rat kidneys. They are cells that actively proliferate and contribute to the process of tubular regeneration in an ischemia-reperfusion model. Following the damage induction, they

were found not only in the tubules but also in the renal interstitium. The increase in the number of label-retaining tubular cells, the change in regional distribution, and the expression of fibroblast markers have suggested that this population is able to proliferate, migrate, and transdifferentiate, potentially contributing to the fibrotic process in this model [29]. In addition, through the retention of bromodeoxyuridine, one of the most important renal niches has been characterized in mice: the papilla, a region known for its critical role as an osmotic regulator and for its marked hypoxia [29]. Here, the highest percentage of cells with a slow replication cycle has been found. In addition to the renal papilla, other niches have been identified. Among the most supported are the medulla and the cortical [30].

Adult Renal Progenitors in Humans

The phylogenetic observation that during human organogenesis the nephrons are generated repetitively from a single population of multipotent progenitors suggests that a small number of such progenitors is still present in the human adult renal tissue. The human kidney has presented many difficulties for the identification of a pool of resident stem cells/progenitors, despite its well-established architecture suggesting that each single nephron unit could have its own regenerative nucleus that would allow it to self-repair a damage [31].

In 2006 a population of progenitor cells, residing at the level of the Bowman capsule urinary pole, was first identified in the adult human kidney [20, 32]. These cells were identified through the evaluation of the co-expression of two surface protein markers: CD133 and CD24. Moreover, they express also the marker Pax2 [20, 33]. Once isolated, these cells showed the ability to differentiate both to the glomerular and tubular cell line, through a sequence of commissioned progenitors [19, 34]. Once transplanted into a murine model of focal and segmental glomerulosclerosis (GSFS), these cells proved to be able to integrate into the damaged renal tissue and subsequently to proliferate and differentiate by generating new podocytes and new proximal and distal tubular cells [35]. If injected intravenously into SCID mice suffering from acute renal failure, the $CD133^{+}/CD24^{+}$ progenitors were not only able to regenerate structures in different portions of the nephron but also to reduce the damage at the morphological and functional level. The $CD133^{+}/CD24^{+}$ cells represent the same population of common progenitors of podocytes and tubular cells during the phases of renal development [36]. At full development, a cluster of these cells remains localized to the urinary pole of the Bowman capsule, site of connection between the glomerular epithelium and the tubular epithelium, while in part they go to localize as scattered cells in the elongating nephron [18]. The cells located at the urinary pole are therefore able to differentiate both toward the glomerular and the tubular cell line [19, 36]. Progenitors commissioned to the cellular podocyte line are hierarchically organized in the Bowman capsule between the urinary pole and the vascular pole, whereas progenitors commissioned to the tubular

cell line are distributed as scattered cells along the proximal tubule and the distal convoluted tubule/connector segment.

In 2010, renal progenitor cells were isolated for the first time also starting from tubules [19, 36]. Likewise glomerular progenitor cells were characterized by the expression of marker CD133, CD24, and Pax2 [19, 37]. Overall, these cells do not express markers such as CD34 (hematopoietic stem cell antigen), CD45 (blood lineage marker), and CD105 (mesenchymal stem cell express) and erythropoietin receptor, highly expressed by hematopoietic stem cells. The stem cell markers Bmi and CD44 were instead found to be expressed on the cell membrane of renal stem cells. The CD44 antigen appears to be involved in the homing of mesenchymal stem cells in the damaged tubular areas. A positivity was also found for the interleukin-1-beta receptor CD29 and for the blastocyst stem-transcription factor Oct-4 [19, 38].

Tubular renal stem/progenitor cells were able to differentiate toward epithelial-, endothelial-, osteogenic-, and adipogenetic-like cells [19, 38]. As mentioned above, cells with similar characteristics to those of human progenitors and with a similar anatomical localization have also been identified in mice and rats, but they only partially share markers with human cells [39]. Sophisticated transplantation and genetic labeling experiments in mouse models have shown that renal progenitors located along the Bowman capsule are able to generate progenitors commuted to the podocyte cell line, which in turn can differentiate into mature podocytes [39]. Recent studies have also suggested the possibility that renal progenitors migrate from the Bowman capsule to capillary tuft in regions far away from the vascular pole [36].

Overall, there are many pieces of evidence about the presence of a population of adult stem cells in the Bowman capsule, able to replace lost podocytes through multiple mechanisms of glomerular regeneration [19, 36, 39]. Tubular renal progenitor cells may be distinct from progenitors of the Bowman capsule because they do not express the surface marker CD106 (vascular adhesion molecule 1, VCAM-1) [40]. Renal progenitor cells expressing CD106, located in the urine pole of the Bowman capsule, show the ability to differentiate both podocytes and tubular cells, while renal progenitors that do not express CD106 probably have a phenotype mainly commissioned toward tubular cells [40]. Although both of these progenitor populations show the ability to regenerate tubular structures and reduce morphological and functional renal damage following acute kidney injury (AKI), their contribution to tubular regeneration may be different. In fact, $CD133^{+}/CD24^{+}/CD106^{+}$ cells increase in the kidney of patients affected by both acute and chronic renal damage, while during AKI, epithelial cell repair mostly depends on tubular renal progenitor cells directly derived from the adjacent tubular epithelium. After tubular damage, renal progenitors become able to migrate and proliferate and/or to secrete reparative factors to regenerate the renal tissue [19, 41, 42]. In fact, studies on rodents have shown that, in chronic renal and renal aging, there is a numerical increase in the glomerulo-tubular junction of the cells expressing a mixed phenotype between the parietal cells and tubular cells [43, 44]. Studies in humans and in mouse have also recently shown that tubular progenitors are resistant to apoptosis,

and their percentage among surviving cells increases proportionally to the severity of the lesions [42, 45]. In humans, these tubular progenitor cells are predominantly located in the proximal tubule, namely, in segment S3, which is highly susceptible to ischemia and toxic insults, but show a remarkable ability to repair its structure and function [19, 42] as well as in the distal tubule and in the connector segment.

In summary, the recent discovery of this complex system of progenitor cells dedicated to the renewal of epithelial renal cells has allowed scientists to better understand how the regenerative process can occur in the adult kidney. Gene expression experiments on renal progenitor cells, obtained from both tubular and glomerular fractions, have shown a high similarity of expression profiles [19], which suggests that these are two very similar cell populations. Furthermore, the same study has shown that some genes are overexpressed by stem cells compared to renal proximal tubular cells. Among these genes, the one that encodes the TLR-2, the transmembrane receptor belonging to the Toll-like receptors (TLRs) family, stands out. It has a role, especially in case of damage, on the resident stem population. The TLR-2 can function as a sensor of the damage, and its activation can produce different effects such as stem cell proliferation and differentiation. This would be useful for maintaining the stem pool in order to prevent any depletion associated with the extent of the damage; moreover, it could also be the result of an activation signal of the multipotent progenitor cells that would lead to a retrieval of the cell cycle. In the second case, the possible differentiation toward mature cells may be effective for a more immediate tissue regeneration. In renal multipotent progenitor cells, following stimulation of TLR-2, activation and subsequent translocation of the NF-kB transcription factor from the cytoplasm to the nucleus is considered the key event of the TLR-2 signal transduction pathway. Moreover, it has been observed that, following appropriate Toll-like receptor stimulation, there is a release of cytokines and inflammatory chemokines such as C-3 and MCP-1, IL-6, and IL-8 [19]. Additionally, the TLR2 stimulation improves the differentiative capacity of renal progenitors.

Renal progenitors can repair both a physical damage, such as a wound in the epithelial tissue, and damage produced by a chemical agent such as cisplatin (CisPt), a widely used chemotherapeutic agent that can have nephrotoxicity side effects [42]. The mechanisms by which the progenitors carry out this repair can be either direct or paracrine, through the secretion of various kinds of reparative factors. These cells can, in fact, differentiate and, through the activation of the WNT pathway, go directly to regenerate and replace the irremediably damaged cells. This is now proven by sophisticated studies of cell fate tracking, in which cells are labeled in vivo with appropriate fluorescent dyes able to reveal their origin [6, 7]. It has been recently shown that these renal stem cells can reconstitute entire portions of renal tubules [6]. However, the second mechanism, the paracrine one, is also fundamental because it allows the progression of renal damage to be blocked and repaired. Indeed, it has been shown that proximal renal tubule cell damage induced by CisPt may be repaired by renal progenitors [42]. This recovering effect on differentiated cells was due to progenitors of tubular origin and not those of glomerular origin, through the secretion of chemokines, such as inhibin-A and decorin, that block the apoptotic process of the damaged cells and induce them to resume their cell

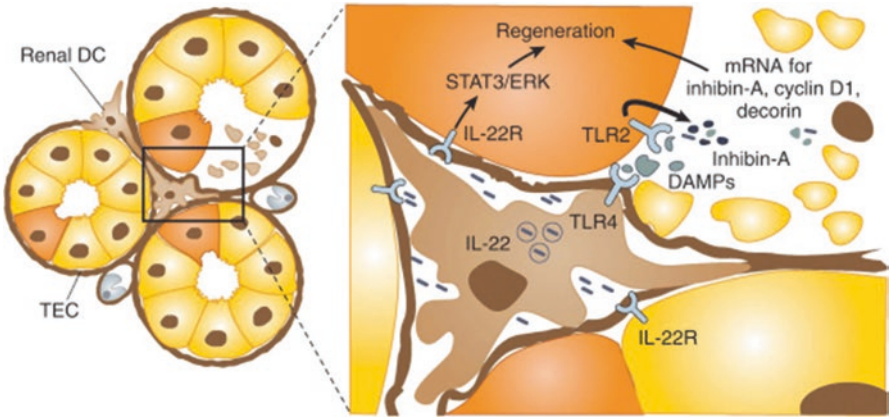

Fig. 12.2 Regenerative mechanism driven by Toll-like receptors on renal progenitors and dendritic cells. Acute kidney injury generally causes apoptosis of tubular epithelial cells (TECs, yellow). TEC death, in turn, leads to the relief of damage-associated molecular patterns (DAMPs) into the extracellular space, where they can activate Toll-like receptors (TLRs) on contiguous cells that survive the triggering insult. Renal tubular progenitors (orange) have a high capacity to survive injuries and can drive regeneration by TLR2 activation at their surface leading to the release of inhibin-A, cyclin D1, and decorin. Secondly, DAMPs capable of agonistic activity on TLR4 on the membrane of renal dendritic cells (DCs) in the interstitial compartment could induce the secretion of interleukin-22 (IL-22), which enhances tubular regeneration via the IL-22R/STAT3/ERK signaling pathway. Reprinted by permission from: Elsevier Inc: Elsevier Inc, Kidney International, 2013 Mar;83(3):351–3. "What can tubular progenitor cultures teach us about kidney regeneration?", P. Romagnani, HJ. Anders. © 2013 International Society of Nephrology

replication cycle (Fig. 12.2). This mechanism is TLR2 receptor dependent and acts as an antenna for the damage [42].

Renal Stem/Progenitor Cells Behavior in Response to Biomaterials

Nanotechnology and Nanomedicine

Today, micro- and nanotechnologies enable the assembling and processing of materials going from single atoms/molecules to micrometer-sized items. At this scales, artificial architectures match the ordinary size of natural functional units in living organisms, and they can communicate with the biology of living life forms through chemical/physical properties not shown by their larger counterparts [46]. Utilizing nanoscience and nanotechnology in biomedical sciences and pharmaceutics, a new and highly promising field of medicine was conceived: nanomedicine. It deals with, among others, the utilization of precisely built materials to design new therapeutic and diagnostic methods. The potential that nanotechnology brings in stem cell

biology is multifaceted, offering upgrades to current procedures as well as giving altogether new instruments and capacities [47].

The general objective of nanomedicine is to analyze, precisely and right on time, to treat as adequately as possible without side effects, and to assess the efficacy of treatments [48]. While early efforts were focused on enhancing the properties of already existing medical technologies, more recently new therapeutic and diagnostic tools (theranostics), made possible through the supramolecular assembly of parts through nanoscale design, have been addressed [49, 50]. Nowadays, nanomedicine platforms include, among other, polymeric nanoparticles (NPs), micelles and liposomes, dendrimers, nucleic acid-based constructs, magnetic NPs, NPs made of silica and other oxides, graphene and other two-dimensional materials, and quantum dots [51–53].

By nm-sized particles, the enhanced functional surface area per unit volume might provide more effective ways for drug administration, tailored therapeutic toxicity, and finally reduce health-care costs [54, 55]. In addition, in almost all cases, the chemico-physical properties and bioactivity of these materials can be improved through drugs, organic modifiers, and other functional compounds. Engineered nanosystems allow targeted delivery and controlled release to be achieved [56]. In diagnostic applications, nanosystems enable improved detection at molecular scale [57].

For these reasons, there have been a rapidly increasing number of nanotechnology platforms that have been developed to address therapeutic or diagnostic issues, and some of these are currently under clinical evaluation (Fig. 12.3) [58]. Although there are still many challenges facing the translation of novel therapeutics, advances in nanomedicine have the potential to drastically change the clinical and therapeutic landscape, even in combination with stem cell biology.

Role of Biomaterials in the Regulation of Cellular Functions

In vivo, cells recognize the environment that surrounds them and interacts with them. The local microenvironment, in which the cells are immersed, is represented by the extracellular matrix (ECM), a highly complex system composed of numerous proteins and polysaccharides that aggregate in an organized network and accomplish structural and signaling functions. The interaction between cells and ECM promotes adhesion and triggers a cascade of intracellular signals that drive growth, proliferation, and differentiation. The ECM, therefore, does not only provide the tissue architecture with mechanical support but plays instead a key role in regulating some of the most important cellular functions [59, 60]. The variations in the relative quantity of the various types of macromolecules constituting the ECM and the way in which they are assembled originate a diversity of forms, each suited to the functional requirements of a different tissue. For instance, proteoglycans form a gelatinous substance in strongly hydrated connective tissues, in which fibrous proteins are immersed: the polysaccharide gel is resistant to compressive forces exerted

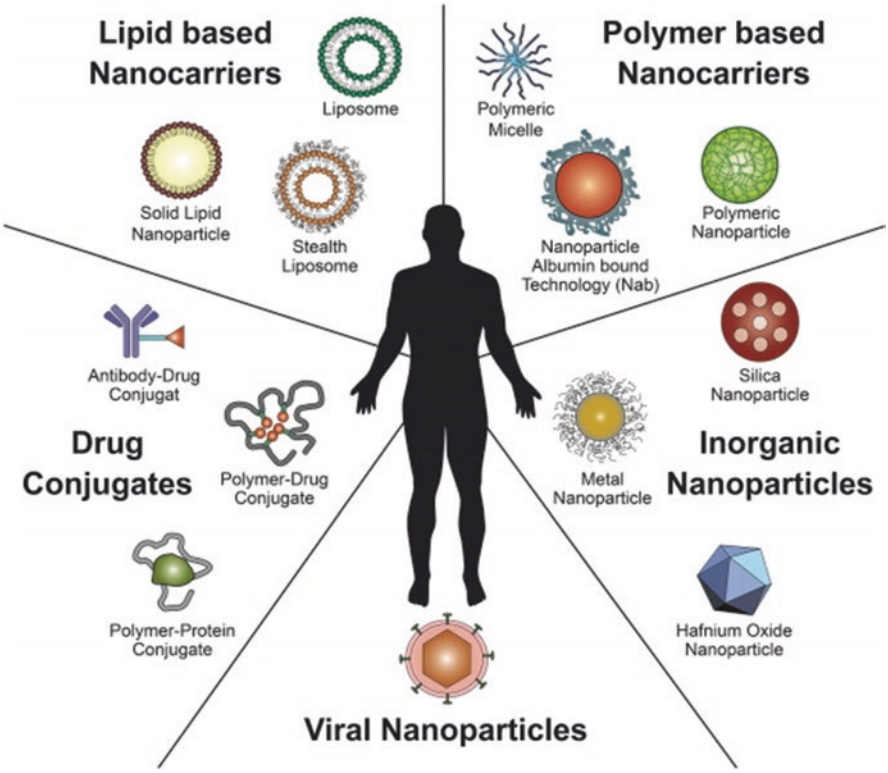

Fig. 12.3 Schematics showing some examples of nanotechnology platforms that have been developed to address therapeutic or diagnostic issues. Reprinted by permission from: Elsevier Inc: Elsevier Inc, Journal of Controlled Release, 2015 Feb 28;200:138–57. "Nanomedicine in cancer therapy: challenges, opportunities, and clinical applications", A. Wicki, D. Witzigmann, V. Balasubramanian, J. Huwyler, Copyright © 2014 Elsevier B.V

on the matrix, while collagen fibers ensure one tensile strength. Adhesion proteins, on the other hand, have their own task in organizing the matrix and in providing a stable interface with the cells that, due to this contact, have the possibility to move within the ECM, namely, migrating, remodeling the protein network, and carrying out their biosynthetic activities [61].

Main desired properties of biomaterials include of course high biocompatibility (the material should neither be cytotoxic nor immunogenic) and biodegradability (the material must be easily disposable once carried out its function). However, from what described above the importance of the cell-matrix interaction in the regulation of cellular functions should be fully clear. For this reason, the science of biomaterials is trying to properly engineer compounds and architectures to induce cells to respond to the most common needs of biomedical applications. In other words, currently developed biomaterials should also interface with the biological environment to modulate the cellular response in a specific way. The biomaterial,

therefore, becomes not only a support for the regeneration of a tissue or a vehicle for the transport of a drug but an active part in the regulation of cellular functions. In engineering steps, all the different parameters that can influence cell-material interaction should be taken into account, including both chemical (such as the nature of the material and the presence of biochemical signals) and physical aspects (mechanical properties, morphology, structure, crystalline/amorphous nature, etc.).

The materials used for biomedical applications can be natural or synthetic. The former are generally based on proteins as building blocks (e.g., collagen or fibrin) or polysaccharides (such as hyaluronic acid). The main advantage in the use of natural materials lies indeed in their amino acidic or polysaccharidic nature which facilitates their recognition by the cells, since they intrinsically show biochemical signals for activating given cellular responses. On the other hand, however, materials of natural origin have some drawbacks, such as the frequently varying composition, poor mechanical performance, limited residence time (i.e., fast degradation), and, especially in the case of animal-derived materials, issues related to immunogenicity [62]. Synthetic materials, on the other hand, have low production costs, excellent reproducibility, and high mechanical performance. The major disadvantage is that these materials are not recognized by the cells in a specific way and, therefore, are generally not able to drive specific cell response without proper functionalization (Fig. 12.4) [63]. In fact, materials are frequently modified in order to recreate a finely structured microenvironment (by nanostructures, nanofibers, bioactive compounds, etc.) with which the cells can interact actively. For instance, many polymers can be suitably modified and bioactivated, in order to act as permissive substrates

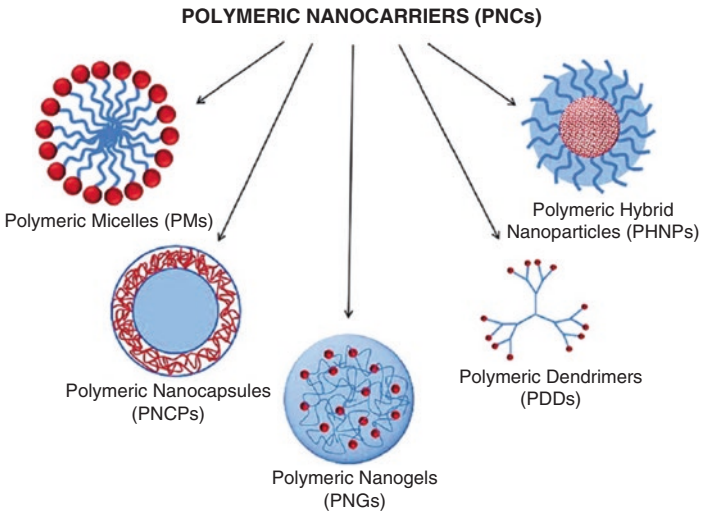

Fig. 12.4 Schematics showing different types of polymeric nanocarriers. Reprinted by permission from: Royal Society of Chemistry: RSC Advances, 2014, 4, 48,639. "Polymeric nanocarriers for expected nanomedicine: current challenges and future prospects", B. Daglar, E. Ozgur, M. E. Corman, L. Uzund and G. B. Demirel. © The Royal Society of Chemistry 2014

for cell growth and implantation [64–66]. Higher bioactivity can be promoted by superficial treatments or by the introduction of biologically active molecules (including growth factors, growth inhibitors, angiogenic factors, and/or immunosuppressive agents) in the material compounds. This allows for inducing a specific biological response in the cells. A material for tissue engineering can be bioactivated in order to promote cell adhesion or migration; in drug delivery or gene therapy, bioactivation aims at specifically favoring or inhibiting endocytic processes and others [67, 68].

The first stage of a synthetic material placed in contact with a biological sample (such as blood, plasma, or any cell culture medium) containing dissolved proteins of different types is generally protein adsorption on the biomaterial surface. Next processes are cell adhesion and reorganization in tissues, depending on the nature and quality of the adsorbed protein layers. Factors affecting the protein adsorption process in terms of quantities, composition, spatial conformation, flexibility, and accessibility to the integrins include both physical and chemical properties of the material (wettability, electrical charge, roughness, topography, mechanical properties, such as rigidity or flexibility, crystallinity, porosity, solubility, pH, etc.). These factors determine the way in which biological molecules are adsorbed to the material and, in particular, determine the orientation of the adsorbed molecules and, consequently, the behavior of cells in contact with it [69, 70]. Also, the spatial distribution and the density of the signals on the substrate are of paramount importance in the modulation of the cellular response. For example, cells migrate differently according to the distribution (homogeneous or clustered) of surface biochemical cues [71]. Furthermore, by patterning substrates in regions that alternately promote or prevent protein adsorption, cell adhesion and spreading processes can be controlled in space. In vivo, the processes of migration and differentiation depend on biochemical signal gradients. Also in vitro scientists tried to modulate the cellular response by creating materials in which the signals were distributed in a directed way (Fig. 12.5) [63]. Gradients have been created both by soluble factors and by factors covalently linked to a substrate, and it was found that cells orient themselves according to the gradient direction [72].

Role of Biomaterials in the Regulation of Renal Progenitor Cells

Micropatterning techniques have been used also to study and influence the behavior of renal progenitors. Linear patterns can influence the differentiation toward tubular epithelial lineages in vitro [73]. Experiments using fibronectin on tissue-culture polystyrene have been carried out with renal progenitor cells. This substrate was engineered with linear protein features of varied widths to provide anchorage points facilitating the cell adhesion and mimicking the renal tubule basement membrane through micro- and nanoscale topographic structures (Fig. 12.6). Data showed that

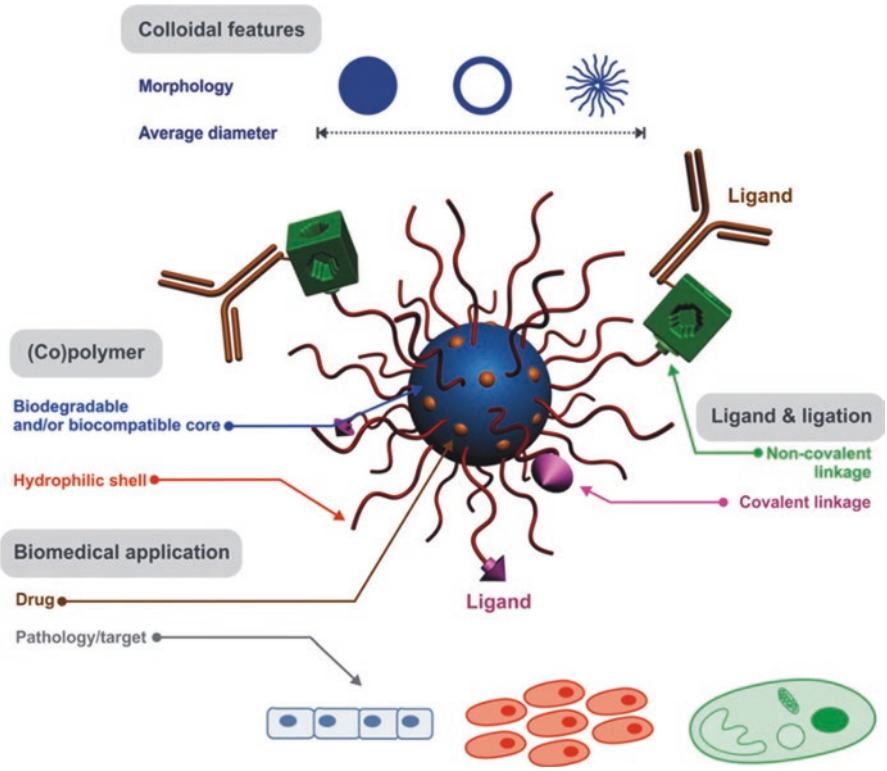

Fig. 12.5 Scheme showing how nanocarriers can be engineered to recognize target cells in a specific way and, through proper functionalization, to drive specific cell response. Reprinted by permission from: Royal Society of Chemistry: Chemical Society Reviews, 2013, 42, 1147. "Design, functionalization strategies and biomedical applications of targeted biodegradable/biocompatible polymer-based nanocarriers for drug delivery", Nicolas J, Mura S, Brambilla D, Mackiewicz N, Couvreur P., © The Royal Society of Chemistry 2013

this system gets a firm control over the ARPCs morphology leading to a stretched cell shape and to a reduced cell spreading area compared to cells cultured on a uniform substrate. Moreover, the cytoskeleton and the nuclei of cells become clearly elongated lengthwise the direction of the microfeatures [73]. Patterned samples, compared to normal substrates, were able to modulate the epithelial differentiation of ARPCs inducing tubular markers (CK19, ECAD, and GLUT1). On the contrary, the expression of the stem cell marker, CD133 and AQP1 (a water channel transporting protein) [38], decreased on bioengineered surfaces: the stemness profile was gradually lost on by decreasing the width of driving fibronectin structures from 50 to 5 μm (Fig. 12.7). Moreover, differentiated cells remained stable and viable for a relatively long time, a result that normally is hard to obtain. Therefore, the variation of cell shape by specific substrates has been confirmed as a crucial regulator and driving factor of cell fate also for renal stem/progenitor cells.

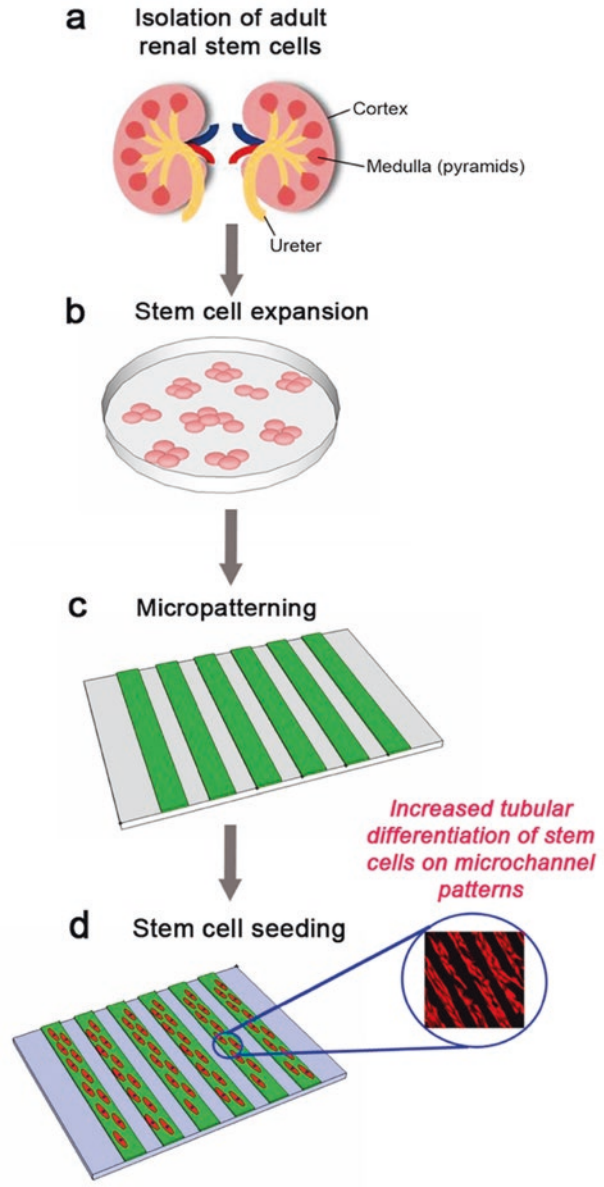

Fig. 12.6 Schematics of micropatterning procedure for ARPCs. Renal progenitors were obtained from the kidney (**a**), expanded in vitro (**b**), and plated on a substrate engineered with linear protein features (**c**). Linear patterns can influence the differentiation toward tubular epithelial lineages in vitro (**d**). Reprinted by permission from: Elsevier Inc: Elsevier Inc, Biomaterials, 2016 Jul;94:57–69. "Micropatterning control of tubular commitment in human adult renal stem cells", AG. Sciancalepore, A. Portone, M. Moffa, L. Persano, M. De Luca, A. Paiano, F. Sallustio, FP. Schena, C. Bucci, D. Pisignano. © 2016 Elsevier Ltd

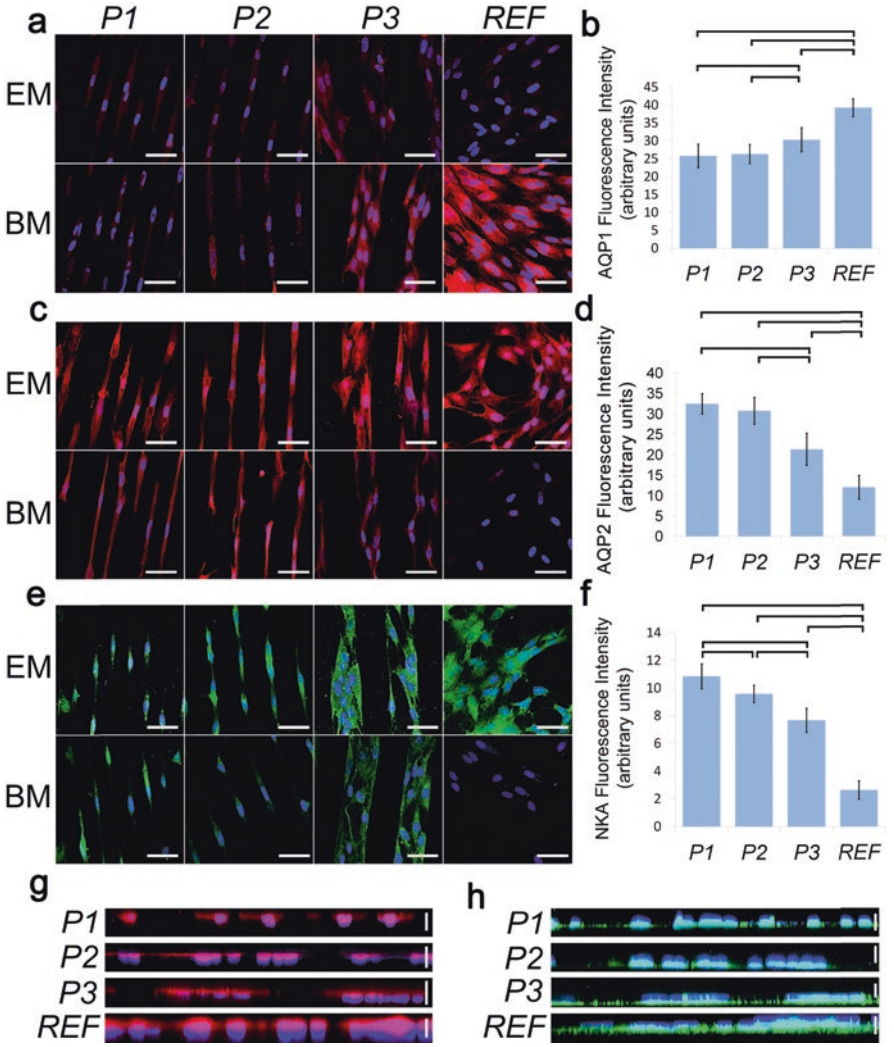

Fig. 12.7 Differentiation and polarization of ARPCs cultured on patterned samples. (**a**) Fluorescent-labeled Aquaporin 1 (AQP1, red) and nuclei (blue) in ARPCs cultured for 10 days in either differentiative (EM) or basal medium (BM) conditions on patterned substrates with linear stripes with a width of 5, 10, and 50 μm (P1, P2, P3, respectively) and unpatterned (REF) surfaces. (**b**) AQP1 expression in BM cultures. The same analysis is shown for Aquaporin 2 (AQP2, red) in (**c**, **d**) and for Na^+/K^+ ATPase (NKA, green) in (**e**, **f**). Scale bars = 50 μm. Bars in (**b**), (**d**), and (**f**) show statistically significant differences ($P < 0.01$). (**g**, **h**) *x–z* cross-sectional confocal micrographs of AQP2 (**g**) and NKA (**h**) for cultures under BM conditions on micropatterned and REF surfaces. Scale bars = 5 μm. Reprinted by permission from: Elsevier Inc: Elsevier Inc, Biomaterials, 2016 Jul;94:57–69. "Micropatterning control of tubular commitment in human adult renal stem cells", AG. Sciancalepore, A. Portone, M. Moffa, L. Persano, M. De Luca, A. Paiano, F. Sallustio, FP. Schena, C. Bucci, D. Pisignano. © 2016 Elsevier Ltd

Another application in which ARPCs showed a specific response to biomaterials was the construction of a multilayer microdevice, resembling a bioartificial proximal tubule-like structure, made up of two superimposed elastomeric sheets, embedding a porous polycarbonate membrane and renal progenitor cells [74]. The assembly was expressly engineered to imitate the in vivo configuration of a renal tubule, with a superior microchannel arranged for the lumen area (on which the apical part of the cells faces) and an inferior microchannel reproducing the interstitial zone that is in connection with the cell basal membranes. The polycarbonate membrane allowed water and solutes passage and worked as a framework for cell culture. The culture of ARPCs, used for the first time in a miniaturized chip, demonstrates that it is possible to induce a well-defined polarization exploiting the forced flow of the culture medium: the aquaporin-2 water transporter localized at the cell apical area and the Na^+/K^+ ATPase pump at the cell basal zone, similarly to in vivo renal tubular cells and at variance with stationary cultured cells [74]. Moreover, the device allowed a good regaining of urea and creatinine to be obtained. While these "micro-organoid-on-a-chip" devices will need further validation, they undoubtedly open a new route to recapitulate renal functions using embedded stem cells.

Biomaterials have been also used to synthesize natural polymer-based nanosystems for efficacious delivery of inhibin-A (INHB-A) and decorin, two chemokines secreted by ARPCs to recover renal proximal tubular epithelial cells (RPTECs) damaged by toxic agents [42]. To this aim alginate (AL) and chitosan (CS) were used, due to their promising properties for the development of drug delivery systems [75]. INHB-A-loaded polysaccharides synthetic vesicles (INHB-A-PSSV) were synthesized by a two-step method, i.e., ionotropic pre-gelation of AL core followed by CS polyelectrolyte complexation. A microfluidic device was fabricated to optimize the INHB-PSSV at the interface-assembly process, in terms of polymers and INHB-A working amount as well as vesicles size distribution. Cellular uptake and INHB-A-PSSV effectiveness were tested in an in vitro model of CisPt-induced cell toxicity. The addition of INHB-A-PSSV to CisPt-treated RPTECs led to a substantial increase in cell number and viability after 3 days of culture. Remarkably, a very low dosage of functional loaded protein was sufficient to induce cell regeneration, and the percentage of viable cells was similar to that of RPTECs without CisPt treatment [42]. These results may be very important in the advance of new precise therapeutic strategies for kidney damage recovery. In fact, synthetic vesicles can include proteins specifically targeting tubular or glomerular cells and therefore favoring a precise transport of the reparative molecules.

Finally, polymer nanofibers are being currently developed as powerful tool for growing and controlling renal stem cells. The proliferation rate of renal stem cells was studied on electrospun nanofibers made of blends of polymethyl methacrylate and a poly-*N*-isopropyl-acrylamide system (PNIPAM), and cell differentiation was investigated through a variety of methods including immunofluorescence and genetic analyses of specific podocyte markers (e.g., Wilms' tumor 1 gene, nephrin, and podocin) [76]. These markers were found to be overexpressed in renal stem cells when they were cultured on aligned fibers compared to cells cultured on

randomly oriented fibers, highlighting a precise role of the scaffold texture and morphology in driving differentiation. Cells on aligned fibers were found to differentiate toward podocyte precursors even in basal medium conditions, namely, without exogenous chemicals. Overall, electrospun polymer nanofibers organized in scaffold architectures are highly promising materials, capable to provide instructive cues for renal stem cell engineering.

Future Trends

Renal progenitor cells enable innovative concepts for renal regeneration. Evidence has emerged that renal progenitor cells, releasing chemokines, growth factors, microvesicles, and transcription factors through paracrine mechanisms, can induce tissue regeneration and block pathological processes of the kidney. The understanding of the mechanisms that control the fate and function of renal progenitor cells is therefore the crucial step toward the beginning of the regenerative nephrology era. A further improvement in this field could be obtained combining these regenerative biological processes with biomaterials that impact on the activation of various cellular functions, such as differentiation, proliferation, adhesion, migration, and endocytosis. For this reason, further efforts will be necessary to better understand the mechanisms that regulate the cell-material interaction and how the modulation of some characteristics of the material can influence the cellular response.

In the future, new polymeric matrices could simultaneously incorporate different kinds of molecules such as chemokines, messenger RNA, and microRNA, to provide an adequate "milieu" for the development of three-dimensional structures similar to tissues. In addition, synthetic materials may be designed to incorporate only specific peptides capable of directing cell differentiation or creating selective surfaces for specific single cell type.

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