Stem Cell Biology and Regenerative Medicine

Kursad Turksen *Editor*

Biology in Stem Cell Niche

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Stem Cell Biology and Regenerative Medicine

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Preface

While our notion of the existence of stem cells has been extant for more than 50 years, deciphering details of regulation of their self-renewal and maintenance potential has been an ongoing effort. The concept that a protected and nurturing location or environment was key to this regulation was first proposed by R. Scholfied in 1978. Since then, numerous—both physical and biological—potential components have been investigated, and a large number of so-called stem cell niche components have now been identified. As importantly, how systemic or epigenetic factors, or injury or disease states, or even normal aging can modulate functional aspects of the stem cell niche have become key questions over the last decade. Deconstruction of the stem cell niche and its reconstruction as biomimetic or engineered constructs in potential regenerative medicine applications are now also hot topics. Authors of chapters in this volume have tackled a range of these topics, summarizing advances made and challenges and opportunities lying ahead. I thank all the contributors for sharing their expertise and time in putting together their chapters and for making this a unique and state-of-the-art volume.

I thank Aleta Kalkstein for her support and help in getting this project off the ground.

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Kursad Turksen

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About the Editor

Kursad Turksen received his Ph.D. from the University of Toronto in the area of osteoprogenitor biology and cell selection methodologies. He then did postdoctoral training at the Howard Hughes Medical Institute, University of Chicago, studying epidermal biology through the use of genetically altered mouse models. He joined the Ottawa Hospital Research Institute (previously known as the Loeb Research Institute) where he rose to the position of Senior Scientist in the Division of Regenerative Medicine. His research interests focus on stem cell biology, with a particular interest in the Claudin family of tight junction proteins and their role in epidermal lineage commitment and progression during development in health and disease.

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The Hematopoietic Stem Cell Niche: Cell-Cell Interactions and Quiescence

Paul J. Childress, Marta B. Alvarez, Brahmananda R. Chitteti, Melissa A. Kacena and Edward F. Srour

Abbreviations

6C3	ENPEP glutamyl aminopeptidoase (aminopeptidase A)
Ang1	Angiopoietin 1
Arf	ADP-ribosylation factor
CAR	CXCL 12 abundant reticular cells
CD	Cluster of differentiation
CDI	Cyclin dependent kinase inhibitor
CFU-F	Colony forming unit fibroblastic
c-Kit	Identify hematopoietic progenitors, Kit oncogene
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
FACS	Fluorescent-activated cell sorting
FLT3	Fms-like tyrosine kinase 3
Fmi	Flamingo

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Fz8	Frizzled 8
G-CSF(R)	Granulocyte colony-stimulating factor (receptor)
GFAP	Glial fibrillary acid protein
GFP	Green Fluorescent Protein
GRP78	Glucose-regulated protein 78
HIF-1	Hypoxia-inducible factor
HPC(s)	Hematopoietic progenitor cell(s)
HSC(s)	Hematopoietic stem cell(s)
HSPC(s)	Hematopoietic stem and progenitor cell(s)
ICAM	Intercellular adhesion molecule
IL	Interleukin
INFγ	Interferon gamma
KitL	Kit ligand also stem cell factor or SCF
Lep-R	Leptin receptor (CD295)
Lin^-	Lineage negative
LSK	Lin^-Scal^+ c-Kit^+
LTBP	Latent TGFB1 binding protein
LT-HSCs	Long-term repopulating HSCs
MCAM	Melanoma cell adhesion molecule
MK(s)	Megakaryocyte(s)
MSC(s)	Mesenchymal stem/stromal cell(s)
MSPC(s)	Mesenchymal stem/progenitor cell(s)
mTOR	Mammalian target of rapamycin
Nestin	Intermediate filament protein
NFAT	Nuclear factor of activated T-cells
OM	Osteomacs
p57Kip2	Cyclin-dependent kinase inhibitor 1C
PDGFR	Platelet derived growth factor receptor
PF4	Platelet factor 4 (also CXCL4)
PIMO	Pimonidazole, hypoxia marker
PTH	Parathyroid hormone
RNA-seq	Ribonucleic acid sequencing
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
Satb1	Transcription factor/chromatin remodeling protein
Sca1	Stem cell antigen-1
SCF	Stem cell factor
SDF1	CXCL12
TCA	Tricarboxylic acid cycle
TGFB1	Transforming growth factor beta 1
TPO	Thrombopoietin
VCAM1	Vascular cell adhesion molecule 1

1 Introduction

Hematopoietic stem cells (HSCs) are perhaps the most studied population of stem/progenitor cells in mammals. Relative ease of collection, abundant historical data, readily available identifying cell surface markers, and availability of multiple informative in vitro and in vivo assays allow HSCs to be sorted and studied in fairly pure populations. HSCs reside in specialized niches in the bone marrow (BM). These niches are not simply passive structures, but a complex anatomical microenvironment composed of many cell types and extracellular matrix (ECM) proteins that directly or indirectly contribute to mitotic quiescence and therefore maintenance of HSC reconstitution potential. Postnatal HSCs are reported to be evenly distributed in the marrow, and concentrated near trabecular regions during hematopoietic recovery [51]. It is generally believed that quiescent HSCs are preferentially located near endosteal surfaces [42] and more rapidly dividing HSCs migrate toward small, fenestrated sinusoids in the marrow [44]. As might be expected, different anatomical niches contain different cells, which result in unique regulation of HSC maintenance and quiescence.

Organisms rely on a pool of HSCs to replenish blood cells of all lineages throughout life. This is achieved by maintaining a delicate balance between self-renewal of the stem cell and proliferation into progenitor cells destined to differentiate into more mature and specialized terminal cells. In addition, soluble and intrinsic factors impact functional properties of HSCs. The last several years have seen remarkable advances in our knowledge of cell signaling activities which maintain HSCs in the BM niche and control their quiescence. Quiescence is reversible to allow rapid HSC expansion and blood cell renewal following insult (e.g. blood loss or toxic exposure), followed by a return to homeostasis, which requires reestablishing a quiescent pool of HSCs. To achieve this plasticity, many cell-cell and cell-niche interactions as well as autonomous mechanisms have evolved which are the subject of this chapter.

2 Cell-Cell Interactions

2.1 Endothelial Cells

Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stem cell factor 1 (SDF1), is expressed in the membrane of several cell types important for maintaining HSCs in the niche. CXCL12 signals through its cognate receptor chemokine (C-X-C motif) receptor 4 (CXCR4) [58]. CXCL12-CXCR4 signaling is essential for normal hematopoiesis, and the role of CXCL12 in retention of HSCs in BM [2, 6, 66], as well as in the quiescence and engraftment of HSCs [60, 86], is well established. This axis will be discussed in the context of different cell types, beginning here with BM endothelium. Ding et al. [30] used a *Tie2*-Cre; *Cxcl12*$^{fl/fl}$

mouse model to delete CXCL12 conditionally in the endothelium. Specifically, at 8 weeks the *Tie2*-Cre; *Cxcl12*$^{fl/fl}$ mice had normal blood counts and normal levels of myeloid and lymphoid progenitors, but their BM HSCs were significantly lower and transplantation of BM HSCs resulted in lower reconstitution compared to controls. Together, these data suggest that ongoing expression of CXCL12 in the endothelium is required for HSC maintenance. This work was published simultaneously in *Nature* with a study by Greenbaum et al. [37] that reported similar finding using the same mouse model. These dynamics are also consistent with a recent report by Sun et al. [80] suggesting that steady state hematopoiesis is primarily maintained by a large pool of multipotent progenitors, with only a marginal contribution by BM HSCs. Collectively, these data suggest that endothelial cells of the niche (and in particular CXCL12 that they produce) are critical for the maintenance of primitive long-term HSCs (LT-HSCs, evidenced by loss of reconstituting potential of HSCs from *Tie2*-Cre; *Cxcl12*$^{fl/fl}$ mice [29]), but not more mature myeloid and lymphoid progenitors which continue to generate the required daily supply of circulating cells [76] in the absence of CXCL12.

Additional evidence for the role of endothelial cells in maintaining HSCs comes from an elegant study in zebrafish using a combination of live imaging and electron microscopy. In the caudal hematopoietic tissue (source of hematopoiesis in zebrafish) HSC extravasation adjacent to endothelium was shown to induce dynamic remodeling of the perivascular niche in a process termed "endothelial cuddling." The resulting structures were then confirmed in mouse fetal liver tissue (the major source of prenatal hematopoiesis in mice) [84]. Combined, these studies demonstrate the important endothelial cell contributions to HSC maintenance.

2.2 *Mesenchymal-Derived Cells of the Hematopoietic Stem Cell Niche*

Several studies have identified that quiescent HSCs are found in close association with OB lineage cells in the endosteal region. However, recent studies have highlighted the significance of perivascular mesenchymal stromal cells in the maintenance and regulation of hematopoietic stem and progenitor cells (HSPCs). Mesenchymal lineage cells expressing Nestin, platelet derived growth factor receptor—alpha (PDGFRα), CD51, CXCL12, Leptin receptor (Lep-R), CD146, and CD105 are reported to possess mesenchymal progenitor activity with self-renewal and multi-lineage differentiation potential [34, 53, 65, 67]. Here, we briefly describe the recent advances in the characterization of mesenchymal lineage cells and their impact on HSC quiescence. Because the repertoire of cell surface markers identifying mesenchymal stem cells and their progeny is not as thoroughly developed as their hematopoietic counterparts, there is significant overlap, and perhaps confusion, regarding the true phenotypic identity of these cells. Also, due to their multilineage differentiation potential, reports often refer to 'mesenchymal stem cells'

(MSCs) as a pure population of stem cells. However, MSCs are in reality a heterogeneous mixture of stem cells and multipotent progenitors. Rather than parse those differences, we will defer to the nomenclature found in the primary literature.

2.2.1 Chemokine Ligand 12 Expressing Cells

Most HSCs near the sinusoidal endothelium are found in contact with CXCL12 expressing reticular cells [78]. Conditional deletion of CXCL12 in adult mice leads to expansion of hematopoietic progenitor content and the reduction in long-term quiescent HSCs. Although CXCL12 abundant reticular (CAR) cells are found largely in perivascular regions, CXCL12 is produced by various mesenchymal lineage cells including OBs and endothelial cells [79]. Two simultaneously published articles in *Nature* in 2013 attempted to characterize the importance of cell type specific production of CXCL12 to HSC maintenance using a series of conditional knockouts [29, 37]. Specifically, they conditionally knocked out CXCL12 from mesenchymal lineage cells and systematically assessed the impact of loss of CXCL12 on both HSCs and lineage committed hematopoietic progenitor cells (HPCs). Selective deletion of CXCL12 from mineralizing mature OBs (using Col2.3 kb promoter) did not affect HSC number in the BM. However, both groups reported a small decrease in common lymphoid progenitors (CLPs). But the selective deletion of CXCL12 from osterix-expressing mesenchymal cells that include less mature OBs and CAR cells resulted in elevated HPC mobilization and a loss of B-lymphoid progenitors, although HSC function was found to be unaltered. Interestingly, selective deletion of CXCL12 from Nestin-expressing MSCs resulted in loss of LT-HSC repopulating activity, quiescence, and CLP content suggesting the role of Nestin-positive cells in the niche [37]. Further, decreased HSC quiescence in mice with CXCL12 ablated from Prx1-expressing mesenchymal cells which are early perivascular progenitors of the developing limb buds, was common to both groups [29, 37]. In other studies, using zebrafish, Tamplin et al. [84] demonstrated that HSCs become tethered with a CXCL12-expressing mesenchymal cell. This interaction oriented asymmetric cell division of HSCs during development such that the stem cell attached to the mesenchymal cell remained in the niche, while the more distal daughter cell was released and underwent differentiation. These findings need to be confirmed in higher order animals, but suggest an anatomical basis for asymmetric division of HSCs. Collectively, these studies demonstrate that the impact on HSC maintenance of OB-derived CXCL12 appears to be modest compared to that of endothelium and other mesenchymal lineage cells.

2.2.2 Nestin Expressing Cells

Nestin is a type VI intermediate filament protein, and is commonly expressed in nerve cells [54]. Nestin^{+} mesenchymal progenitors are identified as key players in the HSC niche. A recent study by Mendez-Ferrer et al. [53] reported that

Nestin$^+$ MSCs possess colony forming unit fibroblastic (CFU-F) activity, multi-lineage differentiation towards mesenchymal lineages, and can self-renew and expand in serial transplantations. These Nestin$^+$ cells are present mainly in perivascular regions of central BM, and at lower frequency closer to endosteum. Purified populations of LT-HSCs defined as Lineage-Sca1$^+$ cKit$^+$ CD48$^-$CD150$^+$ cells are found in close physical association with Nestin$^+$ cells within the BM. These Nestin$^+$ cells express higher levels of genes coding factors required for HSCs maintenance such as CXCL12, stem cell factor (SCF), angiopoietin-1 (Ang-1), interleukin-7 (IL-7), vascular cell adhesion molecule 1 (VCAM1), and osteopontin than mesenchymal cells in the BM that do not express Nestin. Upon administration of granulocyte colony stimulating factor (G-CSF), these genes are down regulated, except for osteopontin. Depletion of Nestin$^+$ cells results in a rapid reduction of HSCs in the BM. This study also found that transplanted HSCs home to locations near Nestin$^+$ cells in the BM of lethally irradiated mice. Moreover, HSCs found near Nestin$^+$ cells are relatively quiescent compared to cells found near Nestin$^-$ cells. Upon G-CSF administration, Nestin$^+$ MSCs undergo down regulation of osteoblastic differentiation genes, which promotes mobilization of HSCs. Whereas, parathyroid hormone (PTH) administration resulted in increasing the number of Nestin$^+$ cells that differentiated into Col2.3$^+$ OBs, and concomitant increases in HSC numbers [53].

Of importance, identification of Nestin$^+$ cells requires permeabilizing the cell membrane, which precludes prospective isolation of live cells. By evaluating putative cell surface markers, Pinho et al. [67] found that the expression of PDGFRα^+CD51$^+$ among CD45$^-$Ter119$^-$CD31$^-$ BM stromal cells identifies a large fraction of Nestin$^+$ cells, thus allowing for isolation of Nestin$^+$ live cells. In another recent study, it was determined that Nestin is expressed in endosteal OBs, endothelial cells, pericytes, along with perivascular stromal cells of developing bones. Thus, both endothelial and non-endothelial Nestin expressing cellular components could play an important role in the HSC niche [64].

2.2.3 Osteoblasts

A role for OB lineage cells in the maintenance of HPCs was first proposed by Taichman and Emerson in 1994 [81]. This initial study demonstrated the OB-produced cytokines regulate hematopoiesis—likely through intimate cell-cell contact. Since this groundbreaking work, the specific contribution of OBs to the niche has been an area of intense study. In 2003, two co-published papers by Calvi et al. and Zhang et al. in *Nature* [10, 95] provided more exquisite details as to how OB lineage cells support the HSC pool and stem cell function. The study by Calvi et al. [10] involved evaluating the role that PTH receptor-bearing cells play in HCS biology. This study demonstrated that constitutively active receptor (PTHR1) specifically in OBs (using the Col2.3 kb promoter) and exogenous intermittent PTH

result in increased ex vivo survival and in vivo HSC engraftment in irradiated hosts. Similarly, Zhang and coworkers [95] demonstrated that mice with increased numbers of N-cadherin^{+} CD45^{-} osteoblastic bone lining cells had increased HSC numbers. The increased HSCs were likely mediated by cell-cell contact in an N-cadherin and β-catenin dependent manner. In a complimentary study, Visnjic and coworkers [88] demonstrated that OB ablation decreased HSCs and HPCs. Ablation was achieved by expression of thymidine kinase (again using the Col2.3 kb promoter) followed by treatment with ganciclovir. Upon withdrawal of the drug, OB numbers increased in the BM as did HSCs and medullary hematopoiesis [88]. In other studies [26] that further explored the role of OBs on HSCs, investigators used an $Osx^{-/-}$ mouse model lacking OBs and examined HSCs in these mice. Specifically, they showed that BM HSCs would indeed develop without OBs, and were localized adjacent to the developing endothelium. HSCs from $Osx^{-/-}$ mice had greater proliferative capacity and less homing and engrafting ability. In control mice, the presence of OBs decreased proliferation and increased engraftment of HSCs. The results of this work suggest a role for OBs in promoting HSC quiescence and consequently establishing LT-HSCs [26].

In a series of studies, our group demonstrated that OB progenitors/early OBs play an important role in HSC maintenance [16, 18–21]. Furthermore, we established that other stromal constituents of the mesenchymal lineage, specifically adipocytes, repress HSC proliferation and engraftment efficiency [19]. In a specially designed co-culture system, we demonstrated that immature OB support hematopoiesis enhancing activity and in vivo engrafting potential [15, 18, 20]. Interestingly, using molecular and functional biomarkers of maturation, we also documented that more mature OB (whether collected fresh using an intricate phenotypic analysis schema designed by our group [18] or matured in culture [16]) are less effective in sustaining HSC function in vitro. Collectively, our data illustrate that OB lineage cells with high Runx2 expression, low alkaline phosphatase activity, reduced calcium deposition potential, and high levels of CD166 (Activated Leukocyte Cell Adhesion Molecule, ALCAM) expression identify immature OBs with high hematopoiesis enhancing activity [18]. Our more recent studies also demonstrated that CD166 is a critical functional marker that identifies repopulating HSC and confers niche competence when expressed on OBs in the niche, most likely due to its ability to mediate OB-HSCs hemophilic interactions [21].

In summary, these studies, and many others [4, 50, 72], demonstrate the role OBs have in HSC maintenance. However, there are outstanding questions that remain regarding the specific interactions that mediate OB involvement in HSC maintenance. For example, disorders which result in decreased bone mass and OB numbers, do not result in disruptions in HSC numbers or hematopoiesis [7, 74]. While more work is required to dissect the specific OB-mediated HSC contributions, one thing remains clear: as hematopoiesis shifts from fetal liver tissue to the postnatal skeleton, OBs play an important role in HSCs maintenance that prepares BM for a lifetime of blood production.

2.2.4 Leptin-Receptor Expressing Cells

Leptin hormone is secreted by adipose tissue and plays an important role in regulating adipose tissue mass by controlling energy homeostasis through the hypothalamus. Lep-R, also known as CD295, is a single transmembrane domain receptor of the cytokine receptor family, and functions as a receptor for the Leptin hormone. Lep-R polymorphism is associated with high plasma Leptin levels and increased obesity [49]. Lep-R is also expressed on various hematopoietic cells, including HSCs and HPCs, and can signal proliferation and differentiation to hematopoietic cells, though minor hematopoietic differences were found in Leptin or Lep-R lacking mice [36], reviewed in [35]. BM adipocytes express Lep-R and suppress the hematopoietic environment and marrow engraftment following irradiation [59]. Additionally, HSC function declines with age as marrow adiposity increases. Because adipocytes and OBs share a common progenitor, this shift away from HSC-maintaining osteoprogenitors toward HSC-suppressing adipocytes may explain the inverse relationship between HSCs and Lep-R expressing adipocytes [5]. Recently, it has been reported that Lep-R marks primitive perivascular MSC that can form CFU-F and can differentiate into bone, cartilage, and adipocytes upon transplantation. Phenotypically these cells express BM MSC markers such as Prx1-Cre, PDGFRα, and CD51 [97]. Conditional deletion of SCF, also referred to as KitL, from Leptin-expressing cells results in depletion of quiescent HSC where conditional deletion of SCF from hematopoietic cells, OBs, or Nestin expressing cells did not impact HSC frequency or function [30]. Similarly, conditional deletion of CXCL12 from leptin expressing cells results in increased HSC mobilization [29]. Thus, Lep-R$^+$ cells appear to be a major source of SCF and CXCL12, which are key components for HSC maintenance in the niche. Additionally, cells expressing both CXCL12 and Lep-R may be a population of cells which overlaps with CAR cells [97].

2.2.5 CD146 Expressing Cells

CD146, also known as the melanoma cell adhesion molecule (MCAM), is a cell adhesion molecule expressed on endothelial progenitors, endothelium, smooth muscle cells, as well as on MSCs [27]. Human BM analyses revealed that CD146 expressing BM stromal cells can form CFU-F with tri-lineage—osteogenic, chondrogenic, or adipogenic differentiation potential [71]. A subset of CD146$^+$ cells express PDGFRα, CD51, Lep-R, and Nestin [38, 67]. CD146$^+$ MSCs express HSC maintenance genes, SCF and CXCL12, and are found in close proximity to HSCs and adrenergic nerve fibers [67]. Corselli et al. [25] reported that the CD146$^+$ fraction of MSCs support the quiescence and long-term repopulating potential of cord blood derived CD34$^+$ cells in co-culture experiments through cell-to-cell contact and activation of Notch signaling. Whereas unfractionated MSCs or CD146$^-$ MSC induced CD34$^+$ cell differentiation. Sabine et al. [76] used both knock down and transgenic overexpression of CD146 to study the effect of this gene in MSCs on

HSPC differentiation. Knockdown of CD146 increased differentiation, conversely overexpression of CD146 in MSCs resulted in stronger adherence of HSC to stromal cells, enhanced their migration beneath the monolayer, and provided a conducive environment for HSPCs. Joan et al. [39] recently reported that human mesenspheres derived from $CD45^{-}CD31^{-}CD71^{-}CD146^{+}CD105^{+}Nestin^{+}$ cells display a relatively undifferentiated phenotype, with less adherence to plastic in culture conditions, and support the self-renewal, and expansion of cord blood derived $CD34^{+}$ cells. Further, these expanded HSCs sustained increased long-term engraftment in serial transplantation experiments. These studies show that mesenchymal lineage cells expressing CD146 constitute an important BM niche component in HSC maintenance and quiescence.

2.2.6 Bone Cartilage Stromal Progenitor Cells

Chan et al. recently reported on bone cartilage stromal progenitor (BCSP) cells within bones and BM of fetal, neonatal, and adult mice [12]. These bone cartilage stromal progenitor cells can differentiate towards OB lineage, chondrogenic, and at least three subsets of stromal cells differentially expressing CD105 (Endoglin), CD90 (Thy1), and 6C3 [ENPEP glutamyl aminopeptidase (aminopeptidase A)]. These three stromal subsets support HSC maintenance function to different degrees. While 6C3 expressing cells support HSC self-renewal and maintenance of primitive cells, both CD105 and CD90 expressing cells promoted HSC differentiation. Further Chan and coworkers [12] demonstrated that while $CD105^{+}$ $Thy1^{-}63C^{+}$ cells supported HSC self-renewal and their transplantability, $CD105^{+}Thy1^{+}63C^{-}$ cells gave rise to OB lineage cells that lack HSPC supportive function. Although more differentiated $CD105^{-}Thy1^{+}63C^{-}$ cells lack HSPC supportive ability, they supported B-lymphopoiesis. Finally, $CD105^{-}Thy1^{-}6C3^{-}$ expressing stromal cells provided cytokine signaling to HSCs and induced HSC differentiation [12]. Overall, this study demonstrates that HSC interactions with mesenchymal cells are specific and sensitive to differentiation state.

3 Other Niche Cells

3.1 Megakaryocytes

Megakaryocytes (MKs) are the largest and one of the rarest cells found in the BM [48]. These cells produce platelets in response to injury to stop bleeding and minimize blood vessel injury. MKs also play significant roles in the maintenance and quiescence of HSCs in the BM. Immature and mature MKs have been shown to stimulate immature OB proliferation [24] which support HSC maintenance. Additionally, MKs participate in restoring hematopoiesis following insult (such as

radiation) [31, 63]. Following irradiation, surviving MKs migrate to endosteal surfaces and stimulate a significant increase in OB number. These OBs in turn promote hematopoietic recovery.

Additional support for a role of MKs in the HSC niche comes from two recent studies [9, 96]. Bruns et al. [9] showed that HSCs lie in close proximity to MKs using three-dimensional whole mount immunofluorescence imaging. The distribution of HSCs followed a non-random pattern which implied a functional relationship. In an effort to determine the contribution of MKs to HSC function they generated a transgenic mouse that resulted in depletion of MKs by crossing the diphtheria toxin receptor (DTR) mice with *Cxcl4*(*PF4*)-*cre* mice [85]. Upon depletion of MKs, platelet numbers were decreased (as expected), and HSC ($Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD105^{+}CD150^{+}$) numbers were significantly expanded. Other progenitor cell populations were not similarly affected. The expanded population of HSCs showed greater bromodeoxyuridine incorporation as compared with the control mice, signifying increased cell proliferation. Gene expression in these HSCs showed increases of cyclin dependent kinase 2 and cyclin E1 both of which promote entry into S phase [56]. The authors determined by quantitative PCR analysis, the expression of various MK genes important in HSC proliferation and quiescence. The most highly expressed gene was CXCL4 (PF4), whose expression is limited to MKs and platelets. CXCL4 has been shown to inhibit proliferation of HSCs in vitro and in vivo [8]. Indeed, injection of WT mice with CXCL4 resulted in a decrease of HSCs due to increased quiescence. Conversely, in $CXCL4^{-/-}$ mice, HSC proliferation was increased in the BM, indicating that MKs through expression of CXCL4 act to mediate HSC quiescence.

In another recent study, Zhao et al. [96], using immunohistochemical techniques, identified 20.8 % of HSCs ($Lin^{-}CD41^{-}CD48^{-}CD150^{+}$) in direct contact with MKs (identified by the expression of von Willebrand factor). This agreed with the work by Bruns et al. [9] showing 20.3 % were touching MKs, which again alluded to crosstalk between MKs and HSCs. Zhao and co-workers [96] generated similar transgenic mice as Bruns et al. [9] to conditionally deplete MKs. They demonstrated increased short-term repopulating HSCs number and increases in LT-HSCs in the G1 and G2/M phase of the cell cycle, indicating an initiation of proliferation after MK depletion [9]. Zhao et al. [96] then used RNA-seq analysis of MKs, CAR, mature OBs, Nestin-GFP^{+} cells, and endothelial cells which identified transforming growth factor β1 (TGFB1) as the most highly expressed niche factor, with MKs generating the highest amount of TGFB1 [96]. Examination of the TGFB1/SMAD signaling pathway in these mice revealed a decrease in nuclear phosphorylated SMAD2/3 in HSCs from MK-depleted mice compared to controls. To confirm that TGFB1 from MKs regulates quiescence in the BM, a PF4-cre/$TGFB^{fl/fl}$ transgenic mouse was created, where TGFB1 expression was specifically deleted in MKs. The mice had higher overall HSC numbers with increases in LT-HSCs in G1 and G2/M phases, suggesting that conditional deletion of TGFB1 in MKs resulted in decreased HSC quiescence and increased proliferation. Although TGFB1 is implicated in regulation of HSC quiescence by MKs, additional studies are required to dissect its exact role. As an example, TGFB1 is secreted as latent complexes that

contain active TGFB1, latency associated protein (LAP), and latent TGFB1 binding protein (LTBP). This complex covalently binds to the ECM where it accumulates until active TGFB1 is cleaved from the complex [96]. The authors did not discuss how MKs activate TGFB1. It is possible that MKs express the majority of TGFB1 in the BM ECM and it is activated by non-myelinated Schwaan cells through its interaction with the cell surface integrin-B8 metalloproteinase [70]. Taken together, these studies suggest a significant role for MKs in maintaining HSC quiescence by expressing multiple factors that influence HSCs in the BM.

3.1.1 Non-myelinated Schwann Cells

Non-myelinated Schwann cells encase postganglionic sympathetic nerves which run along arterioles in the BM. Yamazaki et al. [93] have shown that these cells express glial fibrillary acidic protein (GFAP) as well as Nestin, and are negative for PDGFRα. These glial cells are in direct contact with approximately 23 % of HSCs ($Lin^{-}CD150^{+}CD41^{-}CD48^{-}$) and express stem cell niche genes such as CXCL12, SCF, Ang1, and thrombopoietin (TPO), but also express integrin beta-8. Integrin beta-8 has metalloproteinase activity that is thought to convert TGFB1 into its active form. Importantly, this work also showed that in vitro, active TGFB1, but not the latent form of TGFB1, induces HSC quiescence by inhibiting lipid raft clustering that assembles growth factor signaling microdomains. TGFB1 also induces SMAD2/3 signaling and $p57^{Kip2}$ expression, both negative regulators of cell proliferation in HSCs. Using an antibody to the active form of TGFB1, active TGFB1 was exclusively detected in non-myelinating Schwann cells that were in association with blood vessels. To further confirm the role of these cells in HSC quiescence, the lumbar sympathetic trunk was cut to denervate sympathetic nerves in the BM. This transection resulted in significant decreases in the number of GFAP and active TGFB1 positive cells, with concomitant increased HSC proliferation and decreased HSC frequency as compared to control animals. These data demonstrate the novel role of the non-myelinated Schwann cells as a major contributor to HSC quiescence by regulating the activation of TGFB1.

3.1.2 Monocytes/Macrophages

The role of monocytes and macrophages in the maintenance of HSC has been studied through the G-CSF receptor (G-CSFR), due to the clinical use of G-CSF treatment to mobilize HSCs. Liu et al. [47] have shown that G-CSFR signaling occurred in a subset of mature hematopoietic cells and was responsible for HPC mobilization. In a related report, a distinct fraction of macrophages called Osteomacs (OM) were described as trophic endosteal macrophages ($CD11b^{+}F4/80^{+}Ly6\text{-}G^{+}$) which form a canopy over mature OBs at the site of bone formation [90]. OMs support OB function in vitro, and are required for the maintenance of OBs in vivo [13]. Winkler et al. [91]

sought to determine the role of OMs in HSC retention in the BM, mice were treated with G-CSF that resulted in reduced OM numbers on the endosteal surfaces accompanied by the emergence of clusters of OMs at the sinusoids in the BM. Loss of OMs from the endosteal niche, by G-CSF stimulation, led to reduced expression in OB of HSC maintenance genes (SCF, CXCL12, Ang1), OB lineage-specific genes (osteocalcin, Runx2, osterix), and the concomitant mobilization of HSCs. In a subsequent study, Christopher et al. [23], found that G-CSFR was expressed in several different hematopoietic lineage cells, which included neutrophils, HSPCs, monocytes/macrophages, individual B cells, and natural killer cells. They used several mouse models in which the G-CSFR was deleted in these cell types and found that expression of G-CSFR in monocytes and macrophages was sufficient to induce normal HSC mobilization upon G-CSF treatment. Independently, Chow et al. [22] primarily focused on the monocyte/macrophage hematopoietic fraction in an effort to identify the cell type crucial for HSC mobilization. They generated several subsets of monocyte/macrophage cells by FACS analysis and found that $CD169^+$ macrophages were located in close association with rare $Nestin^+$ MSCs. When $CD169^+$ cells were deleted, the expression of HSC retention genes, CXCL12, Ang1, SCF, and VCAM1 from $Nestin^+$ MSCs were significantly down-regulated. Moreover, ablation of $CD169^+$ cells, using DTR under the control of the endogenous CD169 promoter, resulted in significant increases in circulating HSCs and a decrease in stromal cell production of CXCL12, confirming the importance of $CD169^+$ macrophages in HSC maintenance and retention. Taken together, these studies highlight the importance of a specific subset of macrophages in the retention of HSCs in the BM.

3.1.3 Sympathetic Nervous System

Mendez-Ferrer et al. [52] were investigating G-CSF induced HSC mobilization and coincidentally found their mice were being continuously exposed to light, which resulted in the alteration of the numbers of colony-forming units in culture and $Lin^-Sca1^+c\text{-}Kit^+$ (LSK, enriched for HSCs) cells in the peripheral blood. This observation led them to speculate that optic signals processed through the central nervous system may cause HSC egress from the BM. Further studies showed that HSC release was regulated by light/dark cycles, and also HSC mobilization was altered when these cycles were altered. Of importance, CXCL12 concentration, which regulates HSC movement to and from the BM, was lowest 5 h after the onset of light while HSC numbers were at their peak. CXCL12 levels were highest at 8 h later matching the lowest circulating HSC counts [52]. On the other hand, Katayama et al. [41] had previously shown that G-CSF-induced mobilization of HSCs is influenced by the sympathetic nervous system. Sympathectomy of the tibia resulted in CXCL12 expression that did not follow the normal circadian fluctuations that were found in the sham-operated tibia in the same mice. Specifically, this effect was mediated by the B2-adrenergic receptor on stromal cells but not OBs. It is

important to note that mature OBs express the B2-adrenergic receptor or the G-CSF receptor. Nestin$^+$ mesenchymal stem/progenitor cells (MSPCs) are in direct contact with sympathetic nerves in the BM and express the HSC maintenance genes, CXCL12, SCF, Ang1, and VCAM1. Therefore, Nestin$^+$ MSPCs are contributors to the regulation of HSC circadian fluctuation through sympathetic signaling and down-regulation of HSC maintenance genes.

4 Hematopoietic Stem Cell Niche Structure and Physiology

The BM hematopoietic niche is a complex setting that is influenced by various cell types, ECM components, and both local and systemic factors which contribute to the quiescence, maintenance, and mobilization of HSCs. Work in defining specific stimuli and their contribution to HSC homeostasis has progressed dramatically over the last decade, and readers are directed to several very good reviews for additional information [1, 17, 51, 57, 92]. It should also be mentioned that this chapter attempts to summarize current evidence regarding HSC quiescence and interactions with niche components; however, this field is changing rapidly—largely fueled by technological innovation which are improving resolution for lineage tracing and molecular profiling of stem cells [32].

4.1 Integrins

HSCs contain molecular cues which contribute to HSC quiescence and retention. Integrins, a prototypical cell adhesion molecule, tethers HSCs to the ECM (through collagens, fibronectin, and laminins, for example) and binds other cells through binding molecules such as intercellular adhesion molecule (ICAM) and VCAM1 [14]. Early studies using neutralizing antibodies to integrin α4β1 (VLA-4) demonstrated the importance of these adhesion molecules in maintaining HSCs in the niche. This adhesion molecule recognizes VCAM1 on endothelial cells, and thus contributes to HSC localization to vascular niches [28]. How adhesion impacts quiescence and/or mobilization is not completely understood, but is a complex process which can involve bidirectional integrin signaling. As an example, in HSCs TPO potentiated integrin β3-dependent transcription of chromatin modifying genes in an 'outside-in' manner with a net effect of decreasing HSC cycling and maintaining stem-like behavior. This effect was reported to be dependent on TPO concentration and was more apparent when the cells were undergoing rapid proliferation. Notably, HSC maintenance by integrin β3 was shown *not* to be dependent on SCF [87]. These results agree with previous studies demonstrating OB

derived TPO up regulates integrin 1 expression and maintains quiescent LT-HSCs. However, exogenously applied TPO resulted in a transient increase in these cells, consistent with the dose-response noted previously [94].

The leukocyte integrin CD18 is a β2 type integrin which restricts the population of HSCs in the BM [45]. In CD18 hypomorphic mice, reductions in the HSC population were not the result of differences in homing. The mutant mice had increased neutrophils in the peripheral blood, likely resulting from decreased extravasation of these cells. This impaired movement through vessel walls, has been shown to modulate the HSC niche as neutrophils egress and return to the BM daily [11]. However, infusion of WT neutrophils did not correct the phenotype in CD18 hypomorphic mice, suggesting a cell autonomous effect was not responsible for increased HSCs [45]. Clearly the ECM of the niche impacts HSC quiescence and trafficking, future studies will undoubtedly shed more light on these interactions and lead to potentially therapeutic interventions.

4.2 Wnts

Canonical and non-canonical Wingless-type MMTV integration site (WNT) signaling both play important roles in HSC maintenance. WNTs are secreted glycoproteins which have profound effects on many biological processes including regulation of HSCs. The cadherin Fmi (Flamingo) recognizes the non-canonical Wnt ligand Frizzled 8 (Fz8). In the BM niche, this receptor is localized primarily on LT-HSCs, which are in close proximity to N-cadherin^{+} pre-OBs and are important contributors to HSC quiescence. A two-pronged mechanism explains this action whereby Fmi-Fz8 signaling in HSCs prevents nuclear factor of activated T cells (NFAT) nuclear translocation (non-canonical) and simultaneously antagonizes canonical Wnt signaling—a traditional function of frizzled receptors [77]. Thus, Fmi/Fz8 signaling appears to attenuate the action of cytokines such as INFγ under homeostatic conditions, but becomes overwhelmed during periods of high IFNγ production which is a potent activator of HSC differentiation [68].

4.3 Hypoxia

The classically defined endosteal niche is believed to be relatively hypoxic and HSCs residing there rely on glycolytic metabolism more so than do their counterparts located near sinusoids. HSCs phenotypically defined as LSK $CD41^{-}CD48^{-}CD34^{-}Flt3^{-}CD150^{+}$ were located near areas of low perfusion and relatively low oxygen tension, and were found to have greater serial transplantation potential than these cells closer to areas of high perfusion, such as near sinusoids [90]. Consistent with this are reports that loss of hypoxia inducible factor 1α (HIF-1α) leads to decreased HCS quiescence and overall HSC numbers and

transplantation capacity. At the same time, stabilizing HIF-1α by disrupting the gene for the E3 ubiquitin ligase responsible for its degradation lead to increased HSC quiescence. Of note, in the latter scenario, transplantation capacity was not rescued, suggesting HIF-1α acts as a rheostat which regulates HSC quiescence in response to local O_2 levels [82]. Recent work identified the cell surface receptor Glucose-regulated protein 78 (GRP78) and secreted molecule Cripto as molecular mediators of glycolytic activity. Cripto caused an upregulation of thymoma viral proto-oncogene 1 (Akt) signaling, expression of glycolytic enzymes, and lower mitochondrial potential. Finally, HIF-1α binds the promoter region of *Cripto* and disabling HIF-1α lead to lower *Cripto* expression and fewer $GRP78^+$ HSCs in the BM. These results suggest a mechanism whereby a hypoxic niche can regulate HSC quiescence through the HIF-1α/Cripto/GRP78 axis [55]. The role of relative hypoxia and glycolytic metabolism is likely more complex than described. Indeed, recent work has demonstrated ubiquitous expression of HIF-1α and pimonidazole (PIMO) incorporation in BM HSPCs regardless of proximity to vasculature or endosteum, and regardless of cell cycle status (G0 vs. G1) [61]. Direct measurements of oxygen tension reveal that overall BM is more hypoxic than the arterial supply and that, in contrast to previous reports, the endosteal niche has a significantly higher oxygen tension than the more central sinusoidal-dense regions [75]. Taken together, control of HSC quiescence by regional oxygen tension is an evolving concept which will require further study.

4.4 Energetics

HSCs can be directed toward erythroid or myeloid fates based on availability of glutamine. Metabolism of this tricarboxylic acid cycle (TCA) intermediate is important for nucleotide synthesis, and it is this feature which controls HSC differentiation down the erythroid lineage—not generation of energy from TCA [62]. In contrast, LT-HSCs utilize energy production as a signal to maintain quiescence in the BM. HIF-1α controls expression of many glycolytic regulators, likely including Pdk1 which limits the amount of TCA cycle metabolites entering the mitochondria leading to low levels of mitochondrial activity and reactive oxygen species (ROS) generation—both characteristics of LT-HSCs [83]. Consistent with this idea, elevated ROS levels signal through p38 to increase LT-HSC proliferation and differentiation [3]. The mTOR/Akt pathway is one of the major cell sensors for energy state and nutrient availability. HSCs must maintain the proper balance between quiescence and proliferation which requires a balance of pathway activation and repression. Constitutively active signaling through this pathway or disabling the inhibitor PTEN both lead to accelerating the cell cycle and eventually exhausting the available HSC pool. Similarly, disabling tuberous sclerosis 1 (Tsc1), an inhibitor of mTOR signaling, leads to HCS exhaustion [40]. These findings suggest that HCSs are able to sense intermediates of energy production and use these queues to adjust the balance between HSC quiescence and mobilization.

5 Intrinsic Regulators of Cell Cycle

Dmtf1 is a tumor suppressor gene that suppresses proliferation and cell cycle progression of HSPCs. Mice with a genetic deletion of Dmtf1 have LT-HSCs with a higher self-renewal capability and more rapid recovery following myeloablation. At the molecular level, Dmtf1 acts by binding the promoter of the ADP-ribosylation factor (Arf) gene and activating its expression. Arf is part of the Arf-Mdm2-p53 complex that halts cell cycle progression, thus Dmtf1 acts to turn on expression of cell cycle inhibitors [43]. Cell cycle progression in HSCs is also regulated by degradation of cyclin dependent kinase inhibitors (CDIs) $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. This degradation is primarily controlled by the SCF E3 ubiquitin ligase, a complex whose target specificity for CDIs is conferred by the S-phase kinase–associated protein 2 (Skp2) subunit. Entry into the G1 to S-phase transition of the cell cycle is dependent on Skp2-mediated clearance of the CDIs, without which HSCs increase quiescence thereby increase overall HSC numbers. Skp2 activity is also responsible for supporting hematopoietic regeneration following stress, as seen following 5-fluorouracil treatment. Without Skp2 activity, HSCs have a significantly decreased ability to re-enter the cell cycle and sustain even short-term engraftment in myeloablated hosts [69]. The membrane protein CD81 (Tetraspanin) is important for the return of HSCs to quiescence following stress conditions such as transplantation into lethally irradiated hosts or recovery from myeloablation with 5-fluorouracil. HSCs lacking a functional copy of CD81 have severely impaired engraftment into secondarily irradiated mice due to a delayed return to quiescence following proliferation. The molecular mechanism responsible for this activity involved decreased Akt phosphorylation and subsequent nuclear translocation of FoxO1a to the nucleus [46].

In addition to transcriptional and cell cycle control, HSC quiescence is regulated by chromosomal structure. Satb1 is a transcription factor/chromatin remodeling protein that has been shown to maintain the genome in a ‘poised’ state where a cell can react quickly to stimuli, but remains in an uncommitted state. Poised chromatin is associated with lack of lineage commitment in progenitor cells [33]. Satb1 knockout mice have a shift away from quiescence and toward lineage commitment in HSCs, leading to exhaustion. This shift was caused by an increase in symmetric divisions in which both daughter cells become differentiated. Consistent with its role in maintaining quiescence, Satb1 represses transcription of both *Numb* and *Myc*, genes involved in lineage commitment [89].

6 Conclusions

Mechanisms leading to HSC quiescence and the impact of their interactions with niche constituents on maintenance of mitotic quiescence and functional potential are very complex. The field of hematopoiesis has experienced an explosion of new information which has greatly enhanced our understanding of the hematopoietic niche as it was first described by Schofield in 1978 [73]. We have highlighted many of the cellular and molecular interactions which contribute to HSC maintenance, with the caveat that the literature on this topic is much too vast for an inclusive review. A functional and competent HSC niche requires many cell types that communicate directly with HSCs and others that contribute indirectly by synthesizing specialized niche components or mediating their regulatory effects through other cell types. Better defining these dynamics is fundamental for a more comprehensive understanding of the complex biology of HSC-niche interactions and stem cell quiescence.

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The Mammalian Limbal Stem Cell Niche: A Complex Interaction Between Cells, Growth Factors and Extracellular Matrix

Federico Castro-Muñozledo

1 Introduction

After experiments by Till and McCulloch which led to the experimental evidence that supported—for the first time- the existence of stem cells [1, 2], the search for these cells and their site of residence became one of the major challenges in Cell Biology. At the beginning, people looked for cell populations that showed some of the theoretical characteristics expected for stem cells such as: (i) The expression of early differentiation markers previously identified either during embryonic development as well as in vitro [3, 4]. (ii) The use of assays to quantify the proliferative potential of cells directly isolated from tissues [5–9]; and (iii) the long-term engraftment of presumptive stem cells into injured tissues [10, 11].

After the evaluation of tissues that undergo a continuous replenishment, authors concluded that adult stem cells possess the self-renewal ability through mitotic cell division, a crucial property because of its participation in the creation of new tissues and in the maintenance of the stem cell pool [12, 13]. Moreover, it was predicted that this cell population should have an unlimited proliferative potential while its progeny undergoes differentiation into a wide range of specialized cell types.

On basis of the above properties and considering further studies which demonstrated that stem cells either remain in a quiescent state or slowly progress through cell cycle [14–16], in addition to results which suggested their presumptive localization [15, 16], researchers proposed that stem cells were found in specific anatomic sites. These sites were designated as “niches”.

The concept of a stem cell niche was first proposed by Schofield in 1978 [17], and its definition has evolved during the last forty years. In contrast to its initial meaning which corresponded to the site of residence of stem cell populations in

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animal tissues, the concept has changed following an equivalent evolution to its use in Ecology. Indeed, the niche was described as the role of stem cells in a developmental web and more recently as a restricted tissue microenvironment, in reminiscence of definitions of ecological niches by Grinnell [18] and Elton [19], respectively.

Currently, based on the different interactions that presumptive stem cells maintain with environment, stem cell niche definition is closer to the Hutchinsonian niche where environmental conditions and resources define the requirements of an individual or a species [20]. According to such characteristics, stem cell niche may be described as an anatomically defined and protected location that provides housing, positioning information and signaling inputs necessary to support normal stem cell activity [reviewed in 21].

By the use of different experimental approaches, such as retention of DNA precursor analogs [14, 16, 22, 23], the presence of specific surface antigens or the expression of specific adhesion molecules [3, 4, 24], and the lack of terminal differentiation markers [4], it has been possible to propose the location of presumptive stem cell niches for epithelial tissues as epidermis [4, 14, 16, 22–25], esophageal [26], urothelium [27, 28], bronchiole-associated [29], and conjunctival [30–32], among others.

In contrast with epidermis, the stem cell niche for corneal epithelium was not a clear entity. For a long time, corneal wound healing after injury was considered an outcome of conjunctival epithelial cell migration and transdifferentiation into corneal epithelial cells [33–37]. However, the incomplete and reversible conversion of conjunctival cells into corneal epithelium, the recurrent erosions observed in conjunctivalized corneas [30–38], and the discovery of the limbus as the supposed location of corneal epithelial stem cells, led to reject the conjunctiva as a source of cells for corneal epithelial renewal and healing.

The following paragraphs will review the characteristics of the corneal stem cell niche, the cell populations comprised in its structure, as well as the extracellular matrix and signaling pathways that compose, regulate and maintain stemness in adult corneal epithelium.

2 Defining the Limbus as the Residence Site for Corneal Stem Cells

Adult stem cells reside in specialized, protected sites in many organs and differentiated tissues. The majority of adult stem cells are “tissue-specific”: they have the ability of self-renewal and they only differentiate into those cell types that compose the organ used as site of residence. So far, accumulated evidence suggests that nichesregulate the number and frequency of stem cell divisions, and the number of committed daughter cells; however, the homeostatic mechanisms involved in such regulation are still unclear and depend on the structure and regeneration needs of the tissue.

For a better understanding of the structural, functional and regulatory characteristics of stem cell niches, and their participation in tissue homeostasis and repair, the ocular surface epithelium provides an exceptional model, thanks to its accessibility, a defined anatomy and the presence of a well-defined stem cell niche.

As previously said, for many years conjunctival epithelium was considered the possible source of corneal epithelial cells, as suggested by results from different laboratories [34–38]. Nevertheless, Davanger and Evensen [39] studied the papillary structure located at the human limbus, and made wound-repair experiments in guinea pigs, leading them to speculate that corneal epithelial renewal depended on the limbus. Afterwards, immunostaining of eye surface and corneal cell cultures with monoclonal antibodies raised against the corneal-specific keratin K3 provided the first experimental evidence that suggested the specific location of corneal epithelial stem cells at the basal layer of the limbal epithelium [40]. This breakthrough rapidly led to a series of experiments that provided further support for the limbal location of corneal stem cells: mainly the lack of the K3/K12 keratin pair in limbal basal cells [40–42], and the existence of label-retaining cells at this location [43]. Later, such evidence was strengthened by studies showing that limbal basal cells have a higher proliferative potential than central corneal basal cells [31, 44–46], and they show a differential response to chronic stimulation with phorbol esters [47]. Moreover, limbal epithelial cells have a greater ability to grow in colony forming assays [46], serving as founders of holoclones similar to those described for epidermis [48] and hair follicle [49] (see Fig. 1).

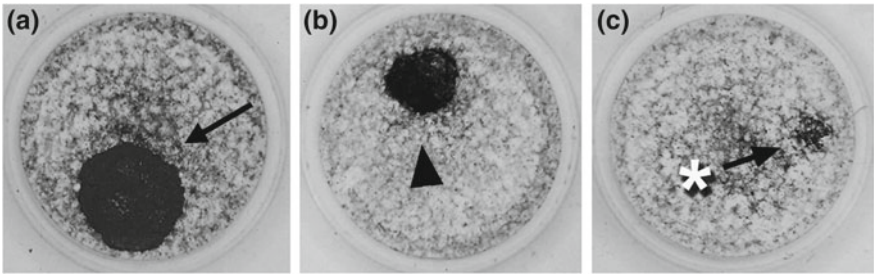

Fig. 1 Example of the three types of clonal growth shown by corneal epithelial cells. Beginning with a cell suspension obtained after dissociation of a primary culture, corneal epithelial cells were plated into 2.0 cm^2 wells (24 multi-well plates). For plating, cells were diluted up to a density of 1 cell/ml; by the use of a glass pipette one cell was placed into one culture well, previously inoculated with 2.2×10^4 cells/cm^2 mitomycin C treated 3T3 feeder cells. After 10 days, colonies were formalin-fixed and stained with Rhodamine B, as previously described [248]. The figure shows typical colonies formed by the isolated corneal epithelial cells. **a** Holoclone (*arrow*), which is a large colony with a smooth perimeter, formed by small cells and few differentiated cells [48]. After dissociation, holoclones generate more holoclones. **c** Paraclone (*asterisk*), which shows low or null proliferative potential. They are composed by differentiated cells [48]. Finally **b**, a Meroclone (*arrowhead*), which is a clone of mixed composition and gives rise to paraclones with high frequency [48]

Given that, differentiation-linked keratins are not useful to isolate stem cells because they are late intracellular markers associated to terminal phenotype [41, 42, 50, 51], the postulation of limbus as the presumptive site of residence of corneal stem cells prompted the quest for specific molecules useful to detect, separate and characterize the basal limbal epithelial cells. These studies allowed the recognition of molecular markers that revealed some of the regulatory, metabolic, proliferative and adhesive characteristics of corneal stem cells, besides their possible use as tools to isolate this cell population.

In many instances, researchers looked for molecules suggested as stem cell markers in other epithelia. For example, p63, a transcription factor previously proposed as a molecular marker of epidermal stem cells [52, 53], showed a restricted distribution to the limbal epithelium suggesting its participation in cell proliferation [54, 55]. More specifically, p63 isoforms (mainly ΔNp63α) seems to regulate the proliferative and migratory potential of limbal [55–59] and corneal epithelial cells [58, 59], establishing a corneal gradient in which ΔNp63α levels peak at the limbus [56–59]. A similar situation was described for TCF4, crucial for the Wnt signaling transduction pathway, which together with β-catenin participate in the maintenance and survival of stem cells [60, 61].

Among proteins that can be used to identify corneal stem cells, we find metabolic enzymes such as α-enolase [62, 63], and the cytoskeletal elements vimentin and K19 keratin [64]. Interestingly, vimentin intermediate filaments, considered typical of mesenchymal-derived cells [64–66], have been observed during epithelial-mesenchymal transition (EMT) [67] and might be involved in migration of epithelial cells that express some stem cell markers [68] as demonstrated for epidermal keratinocytes. Moreover, membrane proteins such as $\alpha_9\beta_1$ integrin which is a receptor for Extracellular Matrix (ECM) components involved in corneal epithelial cell adhesion and migration [69–71], shows a specific association with limbus and may contribute to the identification of corneal stem cells [70–73].

Other criteria used to identify the site of residence of stem cells depend on the physiological behavior of this cell population. Existing evidence suggesting that adult stem cells remain in a quiescent state, or that they slowly progress through the cell cycle, came from experiments in which corneal tissue was exposed to long labeling periods with DNA precursors. After a label-dilution period, label-retaining cells (LRC) exclusively found at the limbal basal layer, were considered corneal stem cells [43]. In addition, the use of the exclusion of vital DNA binding dye Hoechst 33342 to identify the Side Population (SP) [74] showed that limbal epithelial stem cells express high levels of the ATP binding cassette transporter protein ABCG2 and its corresponding mRNA [75–78], which could be playing a role in protection of stem cells [79].

The expression of these markers [for an exhaustive review see 80] in combination with the use of molecules associated to terminal phenotype, such as the high levels of CD71 [81], SSEA-4 [82, 83] or integrins β1, β4 and α3 [70, 72]; will allow the enrichment of corneal stem cell populations. This will establish a new perspective for the long-term maintenance of stem cells, their analysis, the study of the homing mechanism and their effective use for tissue regeneration in the clinic.

3 The Limbus: Its Structure and Composition

In addition of searching for molecular markers useful for the isolation and characterization of stem cells, different laboratories were involved in the analysis of the limbal microenvironment. Soon, it was proposed that the limbus possesses anatomical and functional dimensions that participate in the maintenance of "stemness". In contrast with the central corneal epithelium which lays on an extremely flat corneal stroma with no rete ridges, the limbus is covered by a stratified epithelium in which basal cells are smaller and more closely packed, showing a series of interdigitations with limbal stroma, and constituting a serrated basal side (Fig. 2) [84]. Moreover, the limbus is characterized by stromal invaginations known in humans as the Palisades of Vögt. These are papillae-like projections, which show a distinctive vasculature with radially oriented arterial and venous components [85]. So, the Palisades of Vögt were suggested as the reservoir which: (i) protects the stem cells from traumatic and environmental insults, (ii) allows epithelial-mesenchymal interactions, and (iii) provides access to chemical signals that diffuse from the rich underlying vascular network [39, 86–88].

Later studies showed that the limbus contains a specific anatomical structure which probably provides the microenvironmental characteristics that correspond to the stem cell niche. This structure was termed the Limbal Epithelial Crypt (LEC) [89] or Limbal Crypt (LC) [90], and consists of a cord or finger of cells that invaginates into the limbal stroma from the rete ridges located between the palisades, and extends radially into the conjunctival stroma [89, 90]. Some characteristics of this anatomic structure were part of the evidence that suggested that corneal stem cells reside in there: mainly the expression and content of cytokeratin K14, which showed a pattern similar to that observed in basal cells at the rest of the limbus, and the highest staining for ABCG2 [89] and p63 [90]. It is important to point out that, limbal crypts have not been found in other species besides humans and pigs [91].

As soon as the possible anatomical location of corneal stem cells was established, major questions emerged. How is the stem cell population regulated? Which nichecomponents maintain stemness? This interrogations are under active research, and attempts to understand limbal basal cell interactions with other cells, extracellular matrix (ECM) components and, growth factors and cytokines, associated to the limbal crypt. These components and interactions concurrently create the microenvironmental conditions equivalent to the Hutchinsonian multidimensional niche [20], that regulates and maintain stemness in corneal epithelium. These interactions might also be involved in the mechanisms that seem to be regulating stem cell homing and migration.

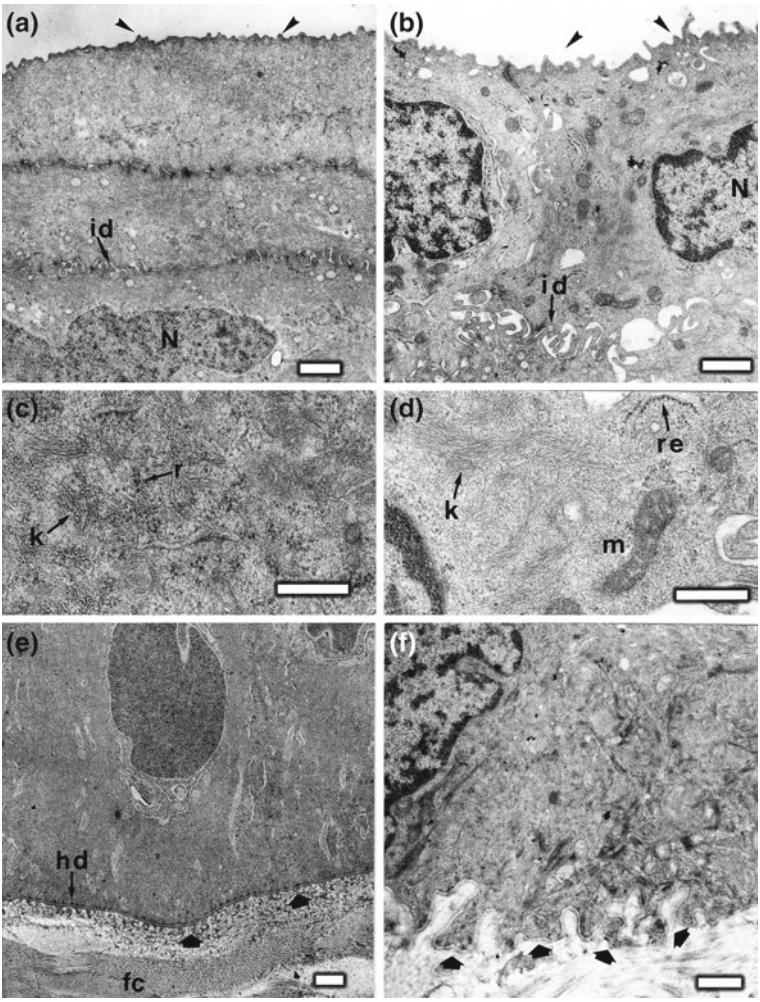

Fig. 2 Comparative ultrastructure of adult rabbit (**a**, **c**, **e**) corneal and (**b**, **d**, **f**) limbal epithelia. While basal cells of corneal epithelium (**e**) rest onto a flat basement membrane, and showed nuclei with a more condensed chromatin, basal cells at the limbal epithelium (**f**), had a lower nucleus/cytoplasm ratio, a less condensed chromatin, and showed a basal side highly interdigitated (*arrows*) with the corneal stroma. **a** Morphology of corneal epithelium superficial cells, with microvilli and microplicae (*arrowheads*) at their surface. **b** Limbal superficial cells were less keratinized a showed a higher number of mitochondria. Similarly, wing cells were more keratinized in cornea than at limbus (**c**, **d**). For (**a**–**c**, **e**–**f**), scale bar = 1.0 μm. In **d**, scale bar = 0.5 μm. *N* cell nucleus; *k* keratins; *id* interdigitations among cells; *r* ribosomes; rough endoplasmic reticulum; *m* mitochondria; *fc* collagen fibers at corneal stroma; *hd* hemidesmosomes

3.1 *Corneal Epithelial Stem Cells and Their Niche: Cells that Interact with the Limbal Stem Cell Reservoir*

Anatomically and physiologically, the limbusconsist in a complex structure proximally delimited by a line that intersects the peripheral extremes of Bowman's and Descemet's membrane and distally by a line that traverses from scleral spur to the tangent of the external surface of the ocular globe. Among its earlier described functions, limbus supplies nourishment for peripheral cornea and participates in maintenance of the intraocular pressure [84, 85].

As it normally occurs in other stem cell niches, the limbus houses different cell populations that participate in the conditioning of the microenvironment for nourishing, protecting, maintaining and regulating self-renewal and fate-decision of the resident epithelial cells. Different studies have recognized that, in addition to the epithelial cells, several other cell types such as stromal cells [92, 93], melanocytes [94, 95], dendritic or Langerhans cells [96] and telocytes [97], are present in the limbal niche. Moreover, at the Palisades of Vögt there is a rich, distinctive vasculature with narrow, radially oriented hairpin loops [98, 99], that participates in such functions, in a similar manner to the association between the vascular niche and the stem cell reservoirs in bone marrow and mouse germinal epithelium [92, 98–100].

3.1.1 Epithelial Cells

At the limbus, epithelial cells constitute a stratified epithelium similar to the one observed in peripheral and central cornea. However, the numbers of limbal cell layers increases to 10 or 15, and superficial cells have rough surfaces unlike the smooth superficial cells in central cornea [84]. In this region, basal cells are more closely packed and smaller, showing a large nucleus/cytoplasm (N/C) ratio [78, 101, 102]. They also express molecular markers associated to epithelial stem cells and showing high growth potential to generate holoclones [46, 103], as expected for stem cells. Nevertheless, basal limbal cells consist of a heterogeneous cell population composed by the stem cells and their progeny, which becomes committed to express the differentiation process after going through a limited number of cell divisions known as Transient Amplification [46, 104]. These transient amplifying cells exhibit intermediate features between stem and committed cells, until the expression of the differentiated phenotype leads to down regulation of stem cell markers [105, 106].

Despite the wide variety of molecular markers described for limbal epithelial cells (see Table 1), their use for the specific selection of the stem cell population has not been as successful as expected. This is a consequence of the persistence of stem cell markers in the transient amplifying cell population and in the early differentiating cells [105, 106]. Therefore, separation of cells by the use of techniques that take advantage of stem cell markers do not only assures the enrichment of stem

Table 1 Some molecular markers distinctive of limbal stem cells

Molecular marker	Limbal expression	Corneal expression	References
Cytoskeleton			
K3 keratin	Suprabasal	Basal and suprabasal cells	[40]
K12 keratin	Suprabasal	Basal and suprabasal cells	[41, 42]
K8 keratin	Basal, in clusters	Not detected	[249]
K15 keratin	Basal, in clusters	Not detected	[249]
K19 keratin	Basal	Not detected	[64]
Vimentin	Basal	Not detected	[64, 250]
Nestin	Suprabasal	Basal and suprabasal	[242]
Metabolism			
α enolase	Basal	Not detected	[62, 63]
LDH isoforms	Low, basal, high suprabasal	High both basal and suprabasal	[250]
$NADP^+$-dependent isocitrate dehydrogenase	Low levels or not detected	Overexpressed in corneal epithelium, except superficial cells	[251]
Adhesion			
Integrin α6	Basal (high levels)	Basal (low levels)	[70]
Integrin α9	basal	Not detected	[72, 73]
Integrin α3	Basal (low levels)	Basal (high levels)	[70, 72]
Integrin β1	Basal (low levels)	Basal (high levels)	[70, 72]
Integrin β4	Basal (low levels)	Basal (high levels)	[70, 72]
β-catenin	Nuclear localization, basal	Basal, membrane-linked	[61]
Nectin-3	Side population	Not detected	[71]
Transcription factors			
ΔNp63α	Basal (high levels)	Basal (low or null levels)	[55–59, 78]
ΔNp63β	Basal (high levels)	Basal (low or null levels)	[57–59]
ΔNp63γ	Basal (high levels)	Basal (low or null levels)	[57–59]
Pax-6	Entire epithelium, although probably lower expression in basal cells	Entire epithelium	[252, 253]
TCF-4	Basal (high levels)	Not detected	[60, 61, 80, 184]
Membrane proteins			
Notch-1 (contradictory information)	Basal (high levels)	Basal (low levels)	[206]
		High levels suprabasal	[171]
EGF receptor	Basal (higher than central cornea)	Basal (high levels)	[242]

(continued)

Table 1 (continued)

Molecular marker	Limbal expression	Corneal expression	References
CD71	Low levels	High levels	[81]
CD61	Side population	Not detected	[71]
ABCG2	High expression, strongly stained	Low expression	[75–79]
SSEA-4	Low or absent	High levels	[82, 83]
Wnt 2	Nucleus	Not detected	[61]
Wnt 6	Present	Not detected	[61]
Wnt 11	Present	Not detected	[61]
Dkk-1	Present	Not detected	[61]
WIF	Present	Not detected	[61]
FRZB	Present	Not detected	[61]
Fz1	Higher in limbus	Lower than limbus	[254]
Fz4	Higher in limbus	Lower than limbus	[254]
Fz10	Higher in limbus	Lower than limbus	[254]
Fz7	Preferentially at basal cells	Not detected	[254]
CLED (calcium-linked epithelial differentiation)	Not detected	Expressed in basal and intermediate cells	[248]
Connexin 43	Not detected in basal, only suprabasal	High expression	[78, 242]

In this table, are shown some of the molecular markers found at limbus, comparing their expression with the one observed in central cornea

cells [107], because the isolated population also includes committed cells that progress through the transient amplification period and generate a set of non-proliferative, terminally differentiated cells [104].

3.1.2 Melanocytes

One of the distinctive traits of the limbal region, besides the Palisades of Vögt, is the presence of melanocytes located at or close to the epithelial basement membrane [84, 103]. Depending on the species and the individual, these cells confer to epithelial limbal cells variable levels of pigmentation [39, 95, 108]. In humans, melanocytes interact with $K19^{+}$/N-cadherin^{+} basal limbal epithelial cells, which show melanin granules at their apical domain acting as a pigmented cap that faces the ocular surface [94, 109], with a distribution similar to that described previously in keratinocytes found at the deep rete-ridges of the skin [22, 23]. These melanocytes are found in a ratio of about 1 for each 10 limbal epithelial cells [94], and it is thought that they might have a role in the protection of the stem cell reservoir from UV radiation and oxidative stress [94, 109].

3.1.3 Langerhans' Cells

In the cornea, the permanent presence of Langerhans' or dendritic cells that represent the professional Antigen Presenting Cells (APCs) of the ocular surface, has been detected mainly at the limbus, peripheral and pericentral cornea [110, 111]. It was demonstrated amongst guinea pig, hamster, mouse, and human corneas [96, 110–112], the existence of a density gradient of mature dendritic cells which show the constitutive expression of the Major Histocompatibility Complex (MHC) class II antigens, and the expression of CD11c and CD45 [112, 113]. Under such density gradient, mature dendritic cells are highly abundant at the limbus and peripheral cornea, while immature cells are predominant at central cornea [96]. Interestingly, it was described that at the limbus, one fifth of the $ABCG2^{+}$ label retaining cells correspond to Langerhans cells [112]. The existence of this dendritic cell population has been related with the immunologic surveillance of the cornea, and with inflammatory processes [96]. In addition, the location of $ABCG2^{+}$/label-retaining dendritic cells at limbus also suggests that a specific subpopulation of Langerhans cells with stem cell characteristics or dendritic cell precursors [96] is maintained by the microenvironmental conditions prevalent at this anatomic site, that also support the stemness of limbal epithelial cells.

3.1.4 Stromal Cells

On the other hand, stromal cells adjacent to limbal stem cells could be crucial for the establishment and maintenance of the limbus as a niche. This proposal was supported by the description of the epithelial cell-filled crypts localized between the limbal palisades of Vogt [89, 90], where stromal cells closely underlie the epithelium [84] resembling the epidermal rete ridges [22, 23] and suggesting a cross-talk between epithelial and stromal cells.

The importance of the stromal cells was supported with experiments demonstrating that these cells could be grouped in two different cell types: limbal stromal cells (LSC) and limbal niche cells (LNC) [92]. After their cultivation and use to support the growth of corneal epithelial cells, it was concluded that LNC possess a higher ability than LSC to maintain stemness and to support the expression of factors that keep up the limbal epithelial stem/progenitor cells characteristics [93]. In addition, LNC enhanced the formation of stratified epithelial cell sheets and the growth of limbal stem/progenitor cells in colony forming assays [93, 113].

Further studies have shown that LNC interact with the basal epithelial cells at limbus [103, 113]. Such interaction seems to be established between adjacent cells [105] and depend on the stromal-derived factor 1 (SDF-1), also known as C-X-C motif chemokine 12 (CXCL12), and its receptor CXCR4 [114]. Since disruption of signaling mediated by SDF-1 leads to loss of holoclone-forming units as well as the disaggregation of cells with proteolytic enzymes, researchers have concluded that maintenance of stem cell phenotype depends upon physical contact between limbal epithelial cells and LNC [114].

The importance of this interaction is emphasized by results from different groups, which have highlighted the need of LNC in order to obtain a better growth and differentiation of the limbal epithelial cells [103, 113, 114]. These results have encouraged the search of methods to grow and expand LNC, oriented to their use for cultivation of limbal epithelial cells for clinical application [114, 115].

3.1.5 Corneal Innervation

An element that could also be essential for the establishment of niche microenvironment consists in the innervation of corneal tissue. Both cornea and limbus are the most densely innervated surface tissues. They are supplied by sensory nerve fibers derived from the ophthalmic division of the trigeminal nerve, via the long posterior and short ciliary nerves [84, 116, 117]. Besides the sensory nerves, corneal and limbal innervation also involves sympathetic branch derived from the superior cervical ganglion [84, 116, 117] and a parasympathetic network which originates from accessory ciliary ganglion neurons [118]. Within this network, epithelial nerve density and the number of nerve endings are higher at the center of the cornea, rather than the periphery [119].

Results from different laboratories have shown that the superficial network that surrounds the limbal area supplies the innervation at limbus and peripheral cornea, while nerves at central epithelium derive from branches of the stromal network [116–119]. Stromal nerves enter into central cornea establishing a radial pattern [119] which is reminiscent of the centripetal migratory pathway followed by corneal epithelial cells from limbus to central cornea which also shows a radial pattern [120, 121].

Therefore, in addition to their important sensory functions, the possible functional roles for innervation in the avascular cornea are not clear. It was reported that signals from the nervous system modulate localization and mobilization of hematopoietic stem cells into the endosteal bone [122], and the migration and proliferation of bulge stem cells in hair follicle [123]. In view of this evidence, it is tempting to speculate that innervation might regulate limbal stem cell/progenitor cell populations. Ueno et al. provided additional support for this hypothesis, in assays which showed that denervation depletes stem cell/progenitor compartment in cornea [124]. Among the possible candidates for neural regulation of epithelial cells, the combination of Substance P, which is abundant at corneal innervation [125–127] and stimulates cell proliferation [128], with IGF-I promotes migration [129]. In the next future, research should pay attention on the signals that mediate the interaction of nerves at the limbal crypt.

3.2 *Corneal Epithelial Stem Cells and Their Niche: Basal Membrane and Extracellular Matrix Components*

Since tissues with unique cellular propertiesmay synthesize different substrates to which the cells adhere, authors carried out the biochemical and immunological characterization of the Extracellular Matrix (ECM) components associated with corneal tissue. Before the description of the limbus as the possible location of corneal stem cells, it was known that corneal ECM constituents changed during development until adulthood in chick, mouse, bovine and human corneas. Authors described that corneas contained collagen types I–VI [130–133], glycosaminoglycans such as heparan, chondroitin, dermatan and keratan sulfates [134–138], fibronectin and laminin [139], and hyaluronic acid [140]. These initial evaluations also showed that limbal epithelial cells adhere to a more roughed surface, with a more complex arrangement of anchoring fibrils than the one observed in central cornea [141]. This suggested that limbal cells had a different adhesion capacity in comparison with the rest of the epithelium; a fact supported by the larger hemidesmosomal area detected in central corneal cells [141], which could also suggest differences in cell motility between both corneal regions.

To further understand the functional differences between the cornea and the limbus, and therefore, the interaction between epithelial cells and the niche, several authors made a careful analysis of the corneal basement membrane components. These studies lead first to recognize that the composition of basal membrane (BM) between conjunctival, limbal and corneal epithelia is heterogeneous [142]. An additional characterization of corneal BM provided controversial results, given that some authors reported that central cornea BM lacks of collagen IV [143], while others reported that collagen IV was found in both limbus and central cornea [142]. Such disagreement was later explained as a consequence of the shift in collagen IV chain isoforms between the limbus and the conjunctiva [144, 145]; collagen IV α1 (IV) and α2(IV) chains showed a more intense staining at the corneo-limbal border, whereas α3(IV) chain underwent an abrupt reduction at limbus [145, 146]. In contrast, collagen types IV (α3-α4 chains) and XII were present in central cornea [146], although collagen IV (α4 chain) was weakly expressed in such region [145, 147].

The differential composition of limbal BM was extended to other components. It was found that α2-α5, β1-β3, γ1-γ3 laminin chains, as well as nidogen-1 and -2, and agrin, were preferentially expressed in limbal BM [146]. In particular, limbal BM shows patches of components such as agrin, SPARC/BM-40, tenascin-C, laminin γ3 chain and versican, which co-localize with ABCG2/p63/K19-positive and K3/Cx43/desmoglein/integrin-α2-negative cell clusters, assumed to be formed by stem and early progenitor cells [147, 148]. On the other hand, researchers described that BM components such as type XVI collagen, fibulin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate, and versican, co-localized with vimentin-positive cell clusters containing putative late progenitor cells [144–146] at the corneal-limbal transition zone. In contrast, type V collagen, fibrillin-1 and -2,

thrombospondin-1, and endostatin were almost restricted to the corneal BM [145]; while others, such as type IV collagen α5 and α6 chains, collagen types VII, XV, XVII, and XVIII, laminin-111, laminin-332, laminin chains α3, β3, and γ2, fibronectin, matrilin-2 and -4, and perlecan, were uniformly expressed throughout all ocular surface epithelia [145, 146].

Together, these results suggest that BM at the LEC/LC has a specific ECM composition, different to that found in peripheral and central cornea; probably creating a specialized environment that regulates stem cells and their progeny. Such environment should support stemness, by inhibiting the expression of the differentiation process and preserving the proliferative abilities of limbal cells.

3.3 Corneal Epithelial Stem Cells and Their Niche: Growth Factors and Cytokines

Growth factors have an important role in epithelial maintenance and wound healing. Their role on corneal epithelial cell proliferation and regeneration has been studied either by in vivo assays [148–150], organ culture [148], or by cell culture [151–153].

Although different researchers have described the effect of different growth factors on corneal epithelial cells, the accumulated evidence suggests that Epidermal Growth Factor (EGF) [148, 151, 154–156] and Fibroblast Growth Factor (FGF) [149, 151, 153, 156] are the main proliferative and migratory regulators for corneal epithelial cells, as also seen in epidermal keratinocytes [157–161]. These in vitro results are supported by: (i) The immunolocalization of EGF receptors and aFGF protein at the corneal epithelium [162, 163]; (ii) experiments which show that the corneal epithelial basement membrane possesses a high capacity to bind FGF [164, 165, 255]; and (iii) in vivo assays that demonstrate the stimulation and improvement of ocular surface wound healing by EGF [165–168] or FGF [153, 169].

In spite of the abundant literature describing the effect of growth factors on corneal epithelial cell proliferation and migration, there are few studies dedicated to understand the regulation of limbal cell populations by these molecules. One of the earliest reports suggesting a differential susceptibility of basal limbal cells to growth factors showed that there are higher levels of EGF receptor in basal limbus than in basal central corneal cells mainly during early development [162]. Later, Tseng and collaborators found that bFGF, TGF-α/EGF receptor, IL-1 beta/IL1-receptor, and bFGF/FGF receptor-1 were more expressed by corneal than limbal epithelial cells [170]. These studies suggested that limbal cells have a different regulation, probably related with their less frequent progression through the cell cycle [43].

Further analyses suggested that regulation of limbal stem cells and progenitor cells involves genes that encode proteins that participate in signaling pathways, which control cell cycling and self-renewal such as WNT and Notch [171, 172]. These results also increase the interest in cell-cell interactions that implicate the

asymmetric signaling between neighboring cells [172], and the activation of genes transcribed by proteins activated by β-catenin [61]. Therefore, it was shown that Wnt/β-catenin signaling increases the proliferation and colony-forming efficiency of primary human LSCs, and at the same time preserves the high expression levels of putative corneal epithelial stem cell markers, and low expression of terminal differentiation markers [61]. On the other hand, the down regulation of Notch correlates with an increase in cell proliferation [172].

Thus far, the role of growth factors and cytokines as regulators of stem cells at the limbal niche is poorly understood. This is a consequence of the intricate network of signals that participate in stem cell regulation, as well as the result of the lack of adequate methods and molecular markers useful for stem cell isolation/purification. However, a more extensive discussion of the activities of the limbal niche components will be addressed in the next sections.

4 The Niche as Regulator of Limbal Stem Cells

Considering thedifferential composition between limbal and central corneal basement membranes, as well as the differential responsiveness of the limbal stem cells in relation to central corneal basal cells, it is clear that microenvironment has a tremendous, dramatic effect on corneal epithelial stem cells. The evidence that supports the role of the niche and provides the best examples of the influence of environment on epithelial differentiation was obtained from recombination experiments. In these studies, murine vibrissae hair follicle stem cells were induced to differentiate into corneal epithelial cells by cultivation in a limbus-specific like microenvironment [173]. Under such conditions that comprise laminin-5 as a major component and the addition of medium conditioned by limbal stromal fibroblasts, cells isolated from hair follicles formed stratified epithelia that expressed corneal-specific markers such as K12 keratin and transcription factor Pax6, both at mRNA and protein level, while showing a strong down-regulation of the epidermal specific K10 keratin [173]. Alternatively, in other experiments, central corneal epithelial cells from adult rabbit were recombined with mouse embryonic dermis, leading to the loss of the corneal-specific phenotype and a down-regulation of Pax6. The loss of expression of the corneal-specific K3/K12 keratin pair was accompanied by the induction of basal keratinocyte markers such as the K5/K14 keratins and the differentiation into epidermal keratinocytes, including cells with a hair follicle lineage phenotype [174]. Altogether, these experiments emphasize the effects of microenvironment on the programming of epithelial cells into specific lineages. Since cell fate may be regulated by specific signals arising from the basement membrane, including growth factors and cytokines, it is possible that in the cornea, the decision to leave the stem cell compartment could be dependent on ECM composition and structure at the limbus.

Under such circumstances, corneal epithelial stem cells could follow one of two alternative, different courses. The first establishes that stem cells and their progeny

proliferate by means of horizontal, symmetric divisions. This proliferative pattern would be prevalent at the basal layer of the cornea, including limbus, while stratification and expression of terminal phenotype would depend upon vertical asymmetric cell divisions. Such asymmetric divisions would result in daughter cells dissimilar in both morphology and proliferative potential; as a result of the division, those cells that enter into the suprabasal compartment would be bigger and suffer a severe restriction in their proliferative abilities to begin terminal differentiation [175, 176] (see Fig. 3). In this case, ECM would modulate proliferative abilities of basal cells according to their position along corneal surface (limbus vs. central cornea); and would control the orientation of the mitotic spindle, being decisive for terminal differentiation. So, cells that detach from the basement membrane would be irreversibly committed to express a differentiated phenotype. The possible participation of vertical asymmetric division as a mechanism to establish the expression of terminal phenotype is supported by the observation that most basal cells in the corneal epithelium express proteins involved in spindle orientation, such as Partner of inscuteable (Pins) [177].

In the other, alternative pathway, asymmetric cell division is restricted to the limbal stem cells, as proposed for most stem cells [178]. If this is true, the decision to leave the stem cell compartment would depend upon asymmetric divisions, that would be oriented either horizontally or vertically (Fig. 4). Consequently, symmetric cell divisions would be merely proliferative, and would not be essential for cell commitment. Consequently, the orientation of the mitotic spindle during asymmetric cell division would be defined by extrinsic mechanisms, i.e. the niche or microenvironment in which stem cells reside [179] (Fig. 4). To support this proposal, there are numerous BM components [141, 142, 144, 147, 180], as well as growth factors and cytokines such as keratinocyte growth factor (KGF) [181], IL-6 [182], EGF and, FGFβ [183], or molecules belonging to the Wnt family [61], among others, which show a differential composition or distribution at limbal, peripheral and central cornea. Together, they may be involved in the establishment of the corneal niche.

According to the second model, stem cells at the limbus undergo either vertical or horizontal asymmetric mitosis during corneal replenishment or during wound healing. After asymmetric cell division, one of the daughter cells loses contact with the limbal BM either by moving into the suprabasal cell layers or by moving and proliferating into the central cornea, and initiates the differentiation process [179] (Fig. 4). When such an event occurs, daughter cells also become regulated by the components of the central cornea basement membrane and growth factors such as IGF-I [183], or molecules of the Wnt family as Wnt3, Wnt7a, Wnt7b, and Wnt10a, which are up regulated in central cornea and limbus [61, 184, 185].

Bearing in mind the differences in basement membrane composition between limbus and central cornea, it is quite possible that the differential distribution of ECM components [144–147] regulates limbal epithelial stem cell character. This is supported by results that demonstrate the influence of ECM parameters such as stiffness and elasticity, on the differentiation, proliferative and migratory abilities of embryonic, mesenchymal, or adipose-derived stem cells [186–188].

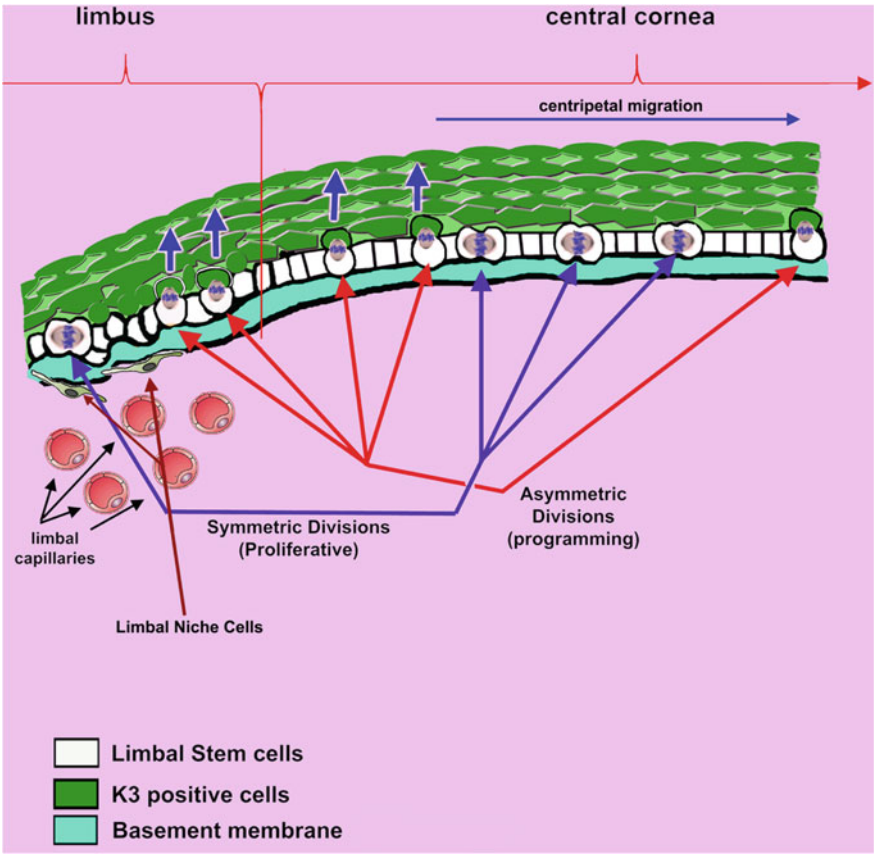

Fig. 3 Representation of corneal epithelial cell renewal dependent on proliferative symmetric mitosis of stem cells and their progeny. Stem cells and their progeny proliferate by means of horizontal, symmetric mitosis; on the contrary, asymmetric cell division only occurs in those cells that start stratification and the expression of terminal phenotype. In such case, basal cells that initiate the expression of terminal phenotype divide with a vertically oriented mitotic spindle; one of the daughter cells remains at the epithelial basal cell layer maintaining its proliferative abilities, and the other leaves the basal layer entering into the suprabasal compartment, becoming bigger and losing its proliferative abilities becoming terminally differentiated (*pink* cells). On this model, detachment from basement membrane (BM) would determine the programming of basal cells into terminal phenotype expression, modulating self-renewal and proliferative abilities of stem cells and their progeny on basis of its composition and structure. *Green* limbal BM. *Orange* peripheral and central cornea BM. *Yellow* conjunctival BM. *Blue arrows* stratification of terminally differentiating cells

Nevertheless, currently there is a debate about the role of stem cells regarding their interaction with the niche. Are they passive entities that respond to systemic or tissue signals by merely adapting their activity to tissue demands? Alternatively, do stem cells affect the surrounding tissue, having a more direct activity on the niche

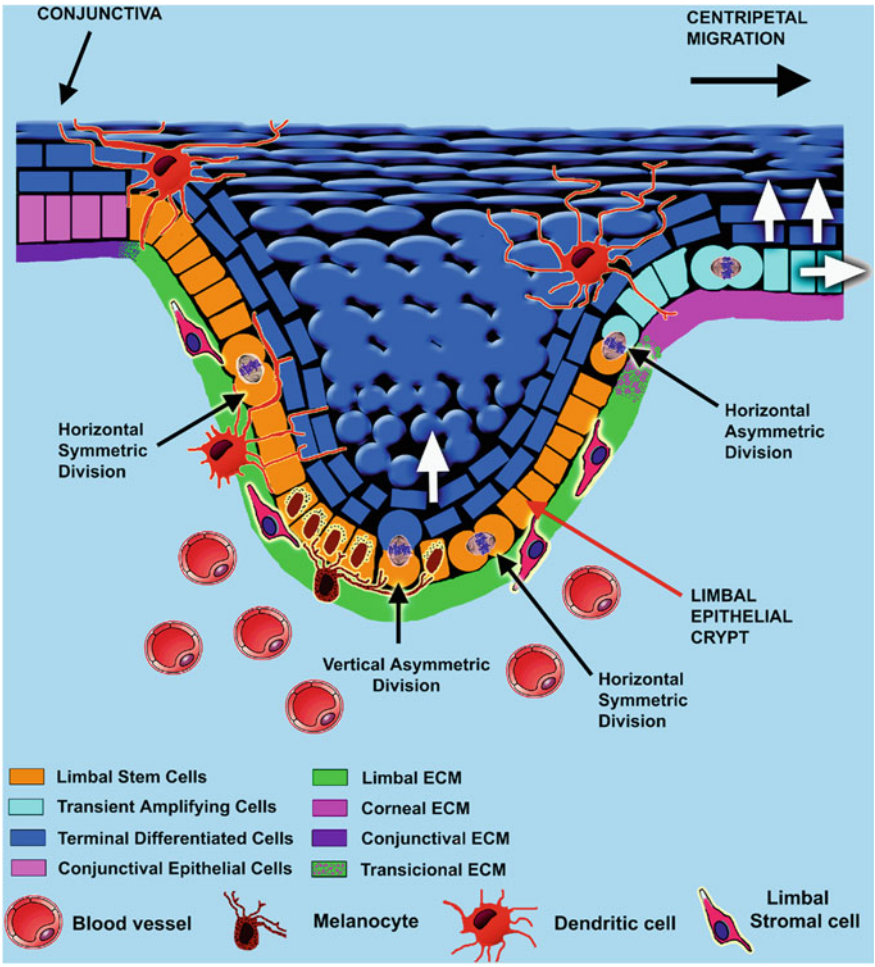

Fig. 4 Schematic representation of the limbal epithelial crypt. Here is shown the complex interrelationships between limbal epithelial cells with different factors that participate in the establishment of the niche. Accumulated evidence suggests that extracellular matrix composition and structure may regulate limbal stem cell fate providing information about their position. Depending on the position of cells at the limbal epithelial crypt, the orientation of mitotic axis during asymmetric cell division of limbal stem cells could be either vertical or horizontal. An asymmetrical dividing stem cell would give rise to another stem cell and a transient amplifying basal cell that would migrate to peripheral cornea when division occurs in a horizontal axis. Conversely, the stem cell could give rise to another stem cell and a limbal suprabasal differentiated cell when division takes place following a vertical axis; in such case, loss of contact between one of the daughter cells and basement membrane would determine the initiation of the differentiation process. *White arrows* indicate the movement of cells after commitment. Differentiation leads to the expression of terminal phenotype

where they reside? It is still unknown how limbal stem cells influence the surrounding cells, tissues, and organs, and therefore, the way in which they can modify their niche. Although there is some evidence regarding the participation of the family of Notch receptors and their associated signal transduction pathway in the regulation of corneal stem cells [172, 189–191], more knowledge is needed about how limbal cells interact with the niche to regulate and enhance responses involved in both tissue maintenance and repair.

5 Corneal Epithelial Stem Cells: Renewal and Wound Healing

Beginning with the discoveryof the centripetal cell migration that occurs in the cornea, early studies on epithelial cell renewal led to conclude that the proliferative source of the corneal epithelium resided at its basal cell layer and at the corneal periphery. In such experiments, authors showed that two separate processes participate in the renewal of the corneal epithelial cells: (i) the division of basal cells, mainly at the corneal periphery, with their successive movement into the suprabasal cell compartment; and (ii) the progression of cells across the limbus toward the center of the cornea, before desquamation of superficial cells [36, 192, 193].

Later, as previously discussed, the limbus was proposed as the presumptive location of corneal epithelial stem cells. Accordingly, corneal epithelium consists of a stratified tissue with a high self-renewal rate based on the regenerative capacities of the stem cells located at the basal layer of the limbus and the proliferation of basal cells from the central cornea [40, 43]. In such well-structured tissue, suprabasal cells both at the limbus and at the central cornea undergo terminal differentiation and lose their proliferative abilities. While basal cells located at the central cornea proliferate actively, basal cells at the limbus consist of a mixture of slow-cycling stem cells and cycling transient amplifying cells [43, 103].

As stated by this hypothesis, normal corneal epithelium remains in a steady state in which cell proliferation is necessary only for replacement of those cells lost by terminal differentiation and desquamation. Stem cells located at the LECs divide occasionally [40, 46, 104, 179], and subsequently, their progeny leaves the niche, while undergoes the transient amplification process, which occurs at the basal cell compartment of the peripheral and central cornea [40, 46, 104, 179]. Such transient amplification would imply a gradient or hierarchy of cells with a decreasing proliferative potential along the central cornea [46, 104], and comprises a still unknown number of cell divisions, mainly modulated by growth factors and cytokines [194–196] before cells become post-mitotic and begin to stratify.

This hypothesis recently received support from mosaic analysis of eyes in mice [256, 197, 198], from lineage tracing on eye surface [198, 199], and from chromosome in situ hybridization on human tissue [200]. These experiments documented centripetal movement of cells from limbus to central cornea. Moreover,

after a wound damage, trauma or exposure to tumor promoters such as TPA, the tissue's response consists of a rapid 8-9-fold rise in the proliferative activity at the limbus, which then is reduced to pre-trauma levels after 36–48 h, as well of a prolonged 2-fold increase in proliferation at peripheral/central cornea which returns to basal levels after wound closure [43, 47]. These results have been interpreted as a consequence of the recruitment and multiplication of the limbal stem cells, and the transient multiplication of the peripheral and central cornea basal cells, respectively [104, 201], and show that limbus contributes to corneal renewal while corneal progenitor cells possess the ability to maintain the corneal epithelium for several months [198, 199].

This possibility is supported by several lines of evidence which suggest that corneal stem cells reside at the limbus, mainly: (i) the lack of an adequate healing of wounds in corneas in which the limbus has been damaged or surgically removed [202–204], (ii) limbal transplantation to restore wound repair [205], or (iii) the presence of holoclone-forming cells in limbus but not in central cornea [31, 46], among others.

So, the reader may ask, which is the role of the niche in corneal wound healing? The answer is mostly unexplored. However, results from different groups suggest that niche rules stem cell behavior through regulation of cell division pattern, in part through an active role of basement membrane components at the limbus. Recent results strongly support the fact that in adult corneal epithelium asymmetrical divisions may only occur at the limbus [179], together with evidence that restricts the expression of specific markers and the expression of cell proliferation and cell fate regulators such as ΔNp63α [59] and Notch1 [206] to stem cells, suggest that asymmetrical cell division is part of the differentiation program in corneal epithelial cells [207]. Therefore, basement membrane would provide limbal stem cells with information about their position and fate. Hence, depending on the position of cells at the limbal epithelial crypt, the orientation of the mitotic axis during asymmetric cell division of limbal stem cells could be either vertical or horizontal. Consequently, an asymmetrical dividing stem cell would give rise to another stem cell and either a transient amplifying basal cell located at the peripheral cornea (when the division occurs in a horizontal axis), or a limbal suprabasal differentiated cell (when the division takes place following a vertical axis).

Accordingly, corneal wound healing should elicit a tissue response in which limbal stem cells undergo few cell cycles and give rise to numerous transient amplifying cells that constitute the migratory/proliferative edge of the wound. The size of the transient amplification of early precursors and committed cells, would then be modulated by changes in the ECM composition and its ECM receptors during corneal wound healing [208–210], and by changes in the expression of growth factors such as IGF-1 [183], Epiregulin [211] or Stem Cell Factor (c-kit ligand) [212].

6 Limbal Stem Cells and Therapy

The current expansion in research on the possible therapeutic use of stem cells also had an impact on the analysis of limbal stem cells and corneal epithelial differentiation. Most authors have approached the use of either limbal stem cells or embryonic stem cells to generate devices for the treatment of corneal damage associated to external agents such as burn injuries, or limbal stem cell deficiencies related with diverse pathologies and hereditary diseases [reviewed in 213].

Among the different strategies utilized by different groups, the transplantation of limbal epithelia whether surgically obtained from the contralateral limbus [205, 214], from donor tissue [215, 216], or bioengineered epithelia [217–220] are, by now, the most valuable tools for corneal surface reconstruction and the relief and cure of limbal stem cell deficiency (LSCD). Independently from variability in patient selection, the type of culture techniques, source of donor tissue, biocompatibility of materials and surgical technique, reconstruction of the corneal surface has shown a clinical success of about 60–80 % [214, 221–224]. In most cases, the outcome seems to be related to the presence of limbal stem cells in the tissue used as source for the manufacturing of the grafts, and supports the benefit of the use of limbal stem cells in ophthalmic therapy.

In view of the successful use of limbal stem cells for ocular surface reconstruction, different groups around the world focused on the use of other stem cell sources to engineer corneal substitutes useful to replace damaged tissue. Among these methods, it is important to emphasize on the generation of autologous corneal constructs by cultivation of hair follicle-derived holoclone-forming cells onto a fibrin carrier [225], as well as the use of human oral epithelia [218, 226, 227], mesenchymal stem cells [228, 229], or embryonic stem cells [230].

Based on the formation of multilayered epithelia expressing corneal differentiation markers [218, 220, 225] and its successful clinical application [231, 232], the use of stem cells different from limbal stem cells shows a high potential for corneal reconstruction. However, because stem cell markers persist in the transient amplifying cell population and in the early differentiating cells [105, 106], surgeons are not certain about the cell types transplanted onto the patients. Such unpredictability may lead to long-term graft failure [233].

Alternatively, the study of stem cell regulation by the niche may lead to develop therapies based on the interference or stimulation of the signaling pathways and microenvironmental components that control limbal stem cells. Since growth factors and ECM components regulate migration and proliferation of the transient amplifying cells, with the preceding proliferation of limbal stem cells, it is possible that growth factors and ECM can be used alone or combined, in order to accelerate and improve repair of corneal wounds, and reduce consequences associated with corneal damage. Examples of this approach consist in the application of growth factors to promote corneal wound healing such as EGF [234, 235], basic FGF [236], TNFα and Interleukin-1 [237]; or ECM components as Decorin [238].

Although some results have suggested that treatment of corneal wounds with growth factors or ECM components offers new opportunities for therapeutic intervention, accumulated evidence implies the need of a complex set of growth factors and ECM components, perhaps in a specific three-dimensional arrangement, to improve and accelerate corneal wound healing. This possibility is supported by the application of cultured epidermal sheets as temporary wound coverings on experimental excimer laser corneal ablations. These epidermal sheets increase at about 60 % the reepithelialization rate of wounds, besides reducing inflammation and scarring at the wound site [239]. Such corneal healing improvement has been explained through the synthesis and release of growth factors, cytokines and ECM onto the wound bed by the cultured epidermal sheets [240]. A similar mechanism for enhancement of wound healing could be occurring during treatment of corneal wounds with amniotic membranes [241].

7 Conclusion

So far, the study of limbal stem cells and their regulation by environmental signals, either cytokines, growth factors, and their interaction with other cell populations is almost unexplored. As mentioned, corneal epithelial stem cell niche constitutes one of the best examples in which niche can be envisioned as the result of a complex mixture of variables that interact and establish the microenvironment to enable the maintenance of stemness and the renewal and repair of the corneal epithelium. So far, we still ignore whether epithelial stem cells exert a reciprocal effect on the niche, although the existing indications do not support this possibility.

Despite researchers have identified a set of molecular markers that may be used for enrichment of stem cells in isolated populations, the results led to conclude that there is not a specific, unique marker for identification and isolation of limbal stem cells [171, 242–244]. The lack of such markers has become one major obstacle to develop therapies based on cell transplantation. Nevertheless, this collection of markers allowed the characterization of the stem cell niche, and demonstrated that the limbus shows special characteristics, both in composition and/or structure, which make it different from peripheral and central cornea [244].

The above evidence, together with cell culture and clonal assays, suggests that the corneal epithelial cells comprise two different populations: stem cells and transient amplifying cells. The latter corresponds to the progeny of the stem cells, and possesses a limited proliferative potential and it is probably committed to terminal differentiation. The number of cell cycles undergone by transient amplifying cells depends on stimuli from the environment [104, 194, 245].

Although numerous studies indicate that corneal epithelial stem cells reside preferentially at the basal layer of the limbal zone rather than uniformly in the entire corneal epithelium, recent results suggest that corneal stem cells may also be at the central cornea [246]. Moreover, other results suggest that corneal wound healing does not necessarily depend on limbal cells [247]. In spite of the controversial

nature of these results, they bring up many questions about the possible function of corneal stem cells during tissue renewal or their migratory potential from the limbus. In either of these cases, a major question involves the possible conditioning effect of stem cells upon environment: Can stem cells modify their surroundings in order to form new niches? The possible location of epithelial stem cells in the central cornea could help to explain the transdifferentiation of adult corneal epithelium when it receives signals from embryonic dermis [174], unless researchers could demonstrate that expression of corneal epithelial phenotype is reversible by stimulation of the appropriate signaling pathways.

Understanding of the niche'sbiological activity on stem cells, may lead us to develop new therapies to accelerate and improve corneal wound healing.

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Emerging Engineering Strategies for Studying the Stem Cell Niche

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Abbreviations

3D	Three-dimensional
ASC	Adult stem cell
BDNF	Brain-derived neurotrophic factor
Bmp	Bone morphogenic protein
DEP	Dielectrophoresis
DLL4	Delta-like ligand 4
DP	Dermal papilla
DTT	Dithiothreitol
ECM	Extracellular matrix
EPC	Endothelial progenitor cell

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Ephs	Ephrin receptor tyrosine kinases
ESC	Embryonic stem cell
GSC	Germline stem cell
hMSC	Human mesenchymal stem cell
HA	Hyaluronan
HSC	Hematopoietic stem cell
HSPG	Heparan sulphate proteoglycan
HUVEC	Human umbilical vein endothelial cell
IKVAV	Ile-Lys-Val-Ala-Val
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cell
MARC	Multi-ARChitecture
mESC	Mouse embryonic stem cell
MSC	Mesenchymal stem cell
NPC	Neural progenitor cell
NSC	Neural stem cell
PEDF	Pigment epithelium-derived factor
PEG	Polyethylene glycol
PDMS	Polydimethylsiloxane
RGD	Arg-Gly-Asp
RGDS	Arg-Gly-Asp-Ser
RGDSP	Arg-Gly-Asp-Ser-Pro
SDF-1	Stromal cell-derived factor-1
SVZ	Subventricular zone
TiO_2	Titanium dioxide
UV	Ultraviolet
UVO	Ultraviolet/ozone

1 Introduction

Stem cells have drawn great attention from the biomedical community as diverse players that assume central roles in development, tissue homeostasis, and tissue regeneration [1]. Defined by their ability to self-renew and differentiate into mature cell lineages, stem cells can be generally categorized into three main subtypes: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs). ESCs and iPSCs share similarities in their morphology, proliferation, and ability to differentiate into cell types from any of the three germ layers: endoderm, ectoderm, and mesoderm. However, ESCs and iPSCs differ in their point of origin. While ESCs are derived from the inner cell mass of mammalian blastocysts, iPSCs are generated via reprogramming of somatic cells through the retroviral introduction of key factors, such as the four Yamanaka factors

Oct3/4, Sox2, c-Myc, Klf4 [2]. ASCs, in contrast, generate a more limited or restricted number of cell lineages that help mediate cell turnover within adult tissues. ASCs populations, which by convention and contrary to their name can be derived from adult or fetal tissue, include, but are not limited to, hematopoietic stem cells (HSCs), neural stem cells (NSCs), satellite muscle stem cells, epidermal stem cells, and intestinal stem cells (ISCs).

Collectively, stem cells offer exciting therapeutic potential for replacing diseased and injured cell populations through regenerative medicine and tissue engineering strategies. These approaches include transplantation of stem cells and their differentiated progeny as well as stimulation of endogenous stem cell populations (i.e. ASCs). The clinical success of both these approaches hinges on the ability to control stem cell behavior, in particular through precise regulation of stem cell expansion and differentiation. For ex vivo stem cell therapies, a major challenge is producing cells of high purity, yield, and quality. In the case of endogenous cell stimulation, the ability to target specific stem cell niches to support endogenous repair represents another major hurdle [3, 4]. To date, considerable progress has been made in developing therapies based on stem and progenitor cells in the hematopoietic system. The use of HSCs has found encouraging success in treating conditions such as autoimmune diseases and blood defects [5, 6]. The primary challenge in stem cell research is to extend this clinical success to other stem cell systems. Therefore, it has become clear that, before stem cells can become a viable therapeutic agent, the complex mechanisms regulating their behavior must be deconstructed.

2 Stem Cells and Their Niches

Efforts within the past few decades have demonstrated that stem cells localize within physiological domains referred to as "niches"—a concept that Schofield first formulated in 1978 to describe the bone-marrow microenvironment of HSCs [7–9]. Since this time, a multitude of studies have confirmed the existence of a variety of microenvironments that house stem cells. For instance, NSCs have been found within the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus of the adult mammalian brain [10–12]. Epidermal stem cells have been shown to reside in a distinct anatomical location called the hair follicle bulge [13–16], muscle stem cells localize between basal lamina and the periphery of myofiber plasma membrane [17–19], and ISCs have been suggested to reside at the +4 position of the crypt base as well as the crypt base itself [20–22]. In addition to being described by their anatomical locations, stem cell niches are also defined by their functional properties [7, 23]. In response to physiological or pathological circumstances or demands, niches play an integral role in coordinating stem cell behavior to maintain homeostasis and stimulate repair [23].

The niche's regulatory role is the result of a dynamic interplay of signaling components that include soluble cues, surrounding extracellular matrix

(ECM)-associated cues, and neighboring niche constituent cells [4]. These signals manifest in various ways, including biophysical signals in the form of the stiffness and topography of imposing ECM in addition to biochemical cues, such as secreted paracrine factors as well as ECM-sequestered growth factors and cytokines [24–27]. Understanding the mechanisms by which these signals modulate stem cell behavior is an essential step in clinically translating stem cell therapies. Specifically, exploring the length and time scales over which individual signals and combinations of signals modulate stem cell behavior has increasingly become a research thrust within the field. In vitro models that mimic aspects of in vivo niche microenvironments have facilitated this investigation and have been made possible through an extensive breadth of novel engineering strategies. In this review, we examine the various strategies employed for recapitulating stem cell-ECM and stem cell-niche cell interactions, with a particular focus on more recent engineering strategies that have progressed in parallel with the field's growing knowledge of stem cell behavior.

3 Stem Cell-ECM Interactions

The ECM is an intrinsically complex, heterogeneous physical structure that plays key roles within stem cell niches. In addition to supporting cellular adhesion, the ECM presents biophysical cues related to the material's physical properties as well as biochemical cues in the form of insoluble ligands. Stem cells actively and dynamically probe this matrix by applying traction forces to "sense" these instructive inputs and subsequently respond by altering their cytoskeleton, adjusting focal adhesions, and remodeling the ECM via degradation and deformation [28–30]. This bidirectional communication is a major topic of interest, as studies have collectively demonstrated that the niche's ECM directly and indirectly regulates key stem cell behaviors, such as adhesion, proliferation, differentiation, and migration [28, 31–33].

3.1 Stem Cell Adhesion to Niche ECM via Integrins

The ECM is an intricate three-dimensional (3D) architecture comprised of diverse biomolecules, including proteins, polysaccharides, proteoglycans, morphogens, cytokines, and growth factors [34]. The composition of this ECM is unique to a given stem cell niche but, despite their considerable structural diversity, similarities among niches have been noted. One common feature is stem cell localization adjacent to basal lamina or basement membranes, which have specialized ECM structures rich in laminins, collagens, proteoglycans, and other important adhesive proteins (tenascin, fibronectin, nidogen, etc.) [35, 36]. For example, NSCs within the SVZ contact finger-like extensions of basal lamina (termed "fractones"), which

extend from surrounding vasculature [37, 38]. Similarly, ISCs inhabit the crypts of intestinal villi, where they share an interaction with the gut epithelial basement membrane [35], a physical fusion of basal and reticular laminas. Likewise, muscle satellite stem cells reside under the basal lamina of myofibers, and interfollicular epidermal stem cells lie adjunct to the encasing basal lamina in the hair follicle bulge [15, 18].

Integrins are a well-characterized family of heterodimeric cell surface receptors that mediate stem cell adhesion to this common interface [28, 39]. These receptors consist of two transmembrane chains (18 α- and 8 β-subunits), which combine to form more than 24 different integrins (excluding splice variants) [40]. Examples of integrins in stem cell niches include α5β1 integrin, a laminin receptor expressed by some NSCs, and α8β1, which mediates hair follicle stem cell binding to the ECM protein nephronectin. Many integrins also possess the capability to recognize the Arg-Gly-Asp (RGD) tripeptide motif within their ligands. Stem cells assemble these nanoscale integrin complexes into macroscale focal adhesions [41]. These adhesions are proposed to play a large role in translating extracellular ECM protein stimuli into intracellular biochemical signals (a process referred to as mechanotransduction), ultimately leading to global changes in cell morphology as well as regulating gene expression to modulate cellular behavior [42]. This complex cascade of signaling events, initiated from the binding of ECM ligands to focal adhesions, exerts tension onto the cell's cytoskeleton and induces stress on the nucleus, as the cytoskeleton is connected to the nuclear envelope [43]. As a result, nuclear remodeling occurs, which asserts force back onto the cytoskeleton and alters focal adhesions. The subsequent "inside-out" signaling allows cells to manipulate the clustering of integrins to their membrane, increasing or decreasing binding of their integrin receptors [44]. Therefore, focal adhesions represent a key mediator of dynamic spatial and temporal interactions between the environment and intracellular signaling [42]. Disruption to this integrin-based interaction can result in stem cells exiting their niche via differentiation or apoptosis [45]. Some integrin signaling pathways under investigation are the Ras/MAPK, RhoA/ROCK, and P13K/Akt pathways. YAP and TAZ have also recently been identified as key downstream transcription factors sensitive to mechanical cues [28, 43, 46].

Integrin signaling has also been shown to interface with growth factor-initiated pathways [39]. In neural progenitor cells (NPCs), for example, the addition of fibroblast growth factor upregulated the expression of β1 integrins, which is believed to enhance cell responsiveness to its ECM [39, 44]. Another example of growth factor-integrin interplay was suggested for mesenchymal stem cells (MSCs)—multipotent adult stromal cells of a mesodermal lineage. The activation of MSC α5β1 integrins on stretched fibronectin fibers promoted osteogenesis; however, inhibition of the epidermal growth factor receptor on the same stretched fibers decreased osteogenesis from 41 to 27 % [35]. As an example in ESCs, it is hypothesized that platelet-derived growth factor receptor coordinates with collagen IV-integrin α1/β1/αv to induce differentiation toward smooth muscle cells [40].

3.2 Cadherins, Another Class of Adhesion Receptors

While adhesion via integrins is a recurring theme in a majority of the stem cell niches, HSCs and likely other stem cells rely on another adhesion protein to interface indirectly with their physical microenvironment. Specifically, HSCs interact with an intermediate cell type, osteoblasts, to anchor themselves to the inner surface of the trabecular bone [47]. This physical cell coupling relies on the recruitment of cadherins and catenins, proteins that assemble to form intercellular adheren-junction complexes [38, 48]. Cadherins have been demonstrated to regulate stem cell behavior in a manner similar to that of integrins. For instance, in the testis stem cell niche of *Drosophila melanogaster*, N-cadherin assists in orienting stem cells for asymmetric division within the niche [49]. In the *Drosophila* ovary niche, loss of N-cadherin results in the retreat of stem cells from the niche [49]. In mammalian systems, N-cadherin-mediated anchoring of NSCs to ependymal cells lining the ventricle has been implicated in regulating the quiescence of NSCs within the SVZ niche. Upon the degradation of this cell-cell adhesion, NSCs translocate from the ependymal cells towards the blood vessels, enhancing their interaction with ECM and initiating their activation [49].

3.3 Molecular Sequestering of Growth Factors and Cytokines by ECM

In addition to mediating stem cell adhesion, the ECM acts as a reservoir for growth factors and cytokines [50]. Immobilization is achieved through non-covalent binding to ECM proteins, proteoglycans, and glycosaminoglycans [51, 52]. Specifically, ECM proteins possess intrinsic binding domains that facilitate the spatial localization of these regulatory factors [52, 53]. Collagen II binds through its von Willebrand domain to transforming growth factor β1 and bone morphogenetic protein 2 [54]. Similarly, fibronectin harbors a heparin II domain that binds molecules such as vascular endothelial growth factor and platelet-derived growth factor [54]. These factors can either be released to establish local morphogen gradients or instigate signaling from a bound state [50]. Liberation of these molecules occurs by either proteolytic degradation of the ECM or cell-generated forces.

While some growth factors directly bind ECM proteins, many others harbor domains that bind to heparan sulfate, a glycosaminoglycan consisting of a linear polysaccharide that attaches to core proteins to form heparan sulfate proteoglycans (HSPGs) [55, 56]. In addition to organizing the presentation of these ligands, HSPGs play a functional role in modulating signaling. They assist in bridging growth factors with their receptors and can serve as co-receptors, influencing growth factor activity by biasing activation thresholds and binding specificities [53, 55, 57]. HSPGs also assist in extending signaling duration through the inhibition of receptor-mediated endocytosis [54, 58, 59].

4 Seminal Engineering Strategies—Establishing a Foundation

An increased understanding of the regulatory role that native ECM plays within stem cell niches has been achieved through the synergistic efforts of biologists, materials scientists, engineers, chemists, and physicists [34]. Early investigations clearly established the importance and the associated mechanisms by which ECM composition, matrix rigidity, topography (both nano- and micro-), porosity, ligand presentation, and control of cell geometry regulate stem cell behavior [51, 60]. These findings were realized with the aid of engineering techniques that re-created static representations of stem cell-ECM interfaces. Materials with pre-defined topographies, patterned peptide sequences, and fixed mechanical properties represent only a few of these early approaches, and these initial studies were critical advances that stimulated interest in dissecting the surrounding physical microenvironment within the stem cell niche. The following sections highlight a variety of early, landmark engineering strategies pursued for studying the role that ECM elements play within the niche.

4.1 Micro/Nanofabrication Techniques for Generating Pre-Printed Topographies

Topography is an inherent characteristic of ECM that has been investigated as an instructive cue that guides the formation of focal adhesions and cytoskeletal tension [41]. The complex, heterogeneous composition of the niche's ECM contributes to an intricate blend of structural features, including pores, protrusions, ridges, and grooves [61]. Deconstructing the biophysical responses to these physiological topographies has required a reductionist approach due to the complexity of the dynamic bi-directional interactions between stem cells and ECM. Thus, many efforts have focused on recapitulating single-feature architectures in vitro and observing how these static systems affect stem cell behavior.

Studying the effects of static topographies requires a platform that must be precise and reproducible on the micro- and nanoscale. A wide spectrum of fabrication methods—including photolithography, soft lithography, dip-pen nanolithography, and electron-beam lithography—have been used in these platforms [61, 62]. Posts and grooves are two examples of structures that have been heavily investigated. Studies have not only manipulated the overall scale of these features (macro vs. micro vs. nano) but also varied the physical aspect ratios of these structures. Ahn and colleagues, for instance, employed ultraviolet (UV)-assisted capillary-force lithography to generate polyurethane nanoposts (Fig. 1a) [63]. They then investigated how varying post-to-post distances (i.e. post densities) at the micron scale influenced human mesenchymal stem cell (hMSC) fate and subsequently discovered that certain topographies biased the process of hMSC

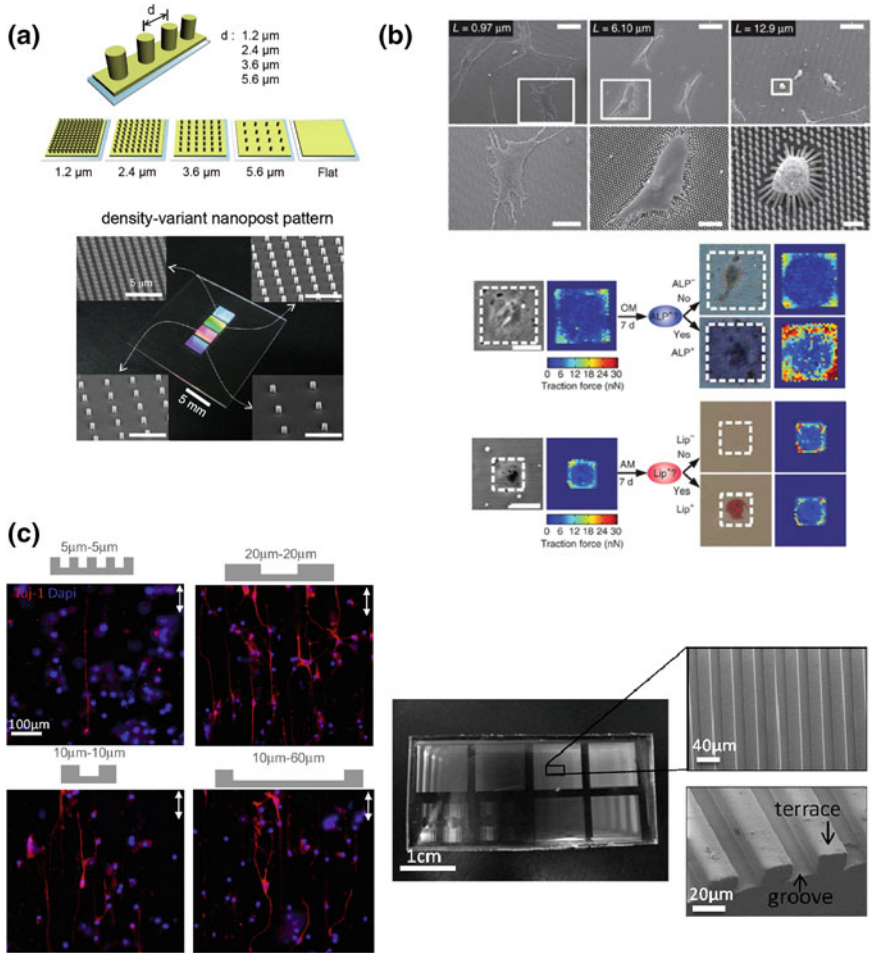


Fig. 1 Engineering strategies for generating static, pre-printed topographies. Panel **a** polyurethane nanoposts of varying densities fabricated using UV-assisted capillary force lithography [63]. Panel **b** SEM images of hMSCs cultured on islands of different PDMS micropost height arrays (*top*); brightfield micrographs and traction force maps of hMSCs exposed to osteogenic or adipogenic medium (*bottom*) [65]. Panel **c** Micropatterned PDMS grooves applied towards influencing NSC differentiation; cells stained for neuronal marker Tuj-1 (*red*) and nuclei (*blue*) [66]

differentiation. In particular, a greater nanopost separation (i.e. a post-to-post distance of 5.6 μm) favored osteogenic differentiation, whereas adipogenesis was maximized at a smaller post-to-post separation (2.4 μm) [63]. Motemani et al. [64] also investigated the effect of nano-columnar surfaces, created using glancing angle deposition, on hMSCs. Nanoscale columns were fabricated in vertical, slanted, and chevron geometries from titanium dioxide (TiO_2), a common implant material, by sputtering titanium at an oblique angle and using substrate rotation to bias the columnar growth direction before annealing to oxidize the films. Following plating

of MSCs on these surfaces, unique nano-sized pseudopodia extensions were observed and suggested to cause cytoskeletal tension and trigger mechanotransduction, though additional studies would be required to confirm these assumptions [64]. While the focus was not on hMSC differentiation but rather on cell morphology and cytocompatibility, this work does yield a promising technique for future studies in exploring the effects of nanoscale topographies on stem cell behavior [64]. In contrast, Fu et al. engineered elastomeric micropost arrays of varying post heights (0.97, 6.1, and 12.9 μm) for generating different mechanical substrate rigidities (1556 nN/μm, 18.16 nM/μm, 1.90 nN/μm) (Fig. 1b) [65]. Single hMSCs were adhered to islands of different post heights, and cell traction forces were tracked over a 7-day period. A strong correlation between osteogenic and adipogenic lineage commitment and traction forces suggested that MSC contractile state could be used as a noninvasive predictor of hMSC differentiation [65].

In addition to posts, considerable work has explored the effects of grooves on stem cell behavior, and in particular the effects of groove depth, groove pitch, and terrace widths. For example, Béduer and colleagues used conventional soft-lithography techniques to assess how adult NSCs responded to imposed micro-patterned polydimethylsiloxane (PDMS) surfaces with varying terrace and groove widths (5–5, 10–10, 20–20, 10–60 μm, respectively) (Fig. 1c) [66]. They found that smaller groove separations lowered differentiation rates and hindered the number of neurite extensions from differentiated neurons, despite promoting a high degree of cellular alignment [66]. Recknor et al. [67] also examined the effects of a micro-patterned polystyrene groove topography as a guidance cue for NPCs. Rather than modulating the physical dimensions of the grooves, however, Recknor et al. [67] studied the synergistic effects of a 16 × 13 × 4 μm (width/mesa width/groove) groove depth pattern in conjunction with a chemical and a biological cue. Specifically, NPCs were co-cultured on a confluent monolayer of cortical astrocytes, which resided on top of a laminin-coated, micro-patterned polystyrene substrate. The resulting microenvironment was found to enhance NPC neuronal differentiation selectively [67].

Many other creative approaches, including techniques for constructing 3D structures, have also been pursued in engineering models of ECM topology. To start, Christopherson et al. [68] revealed that modulations to nanofiber diameters were sufficient for biasing NSC proliferation and differentiation (Fig. 2a). Specifically, they fabricated laminin-coated polyethersulfone fiber mesh matrices exhibiting a range of average fiber diameters (283 ± 45 nm, 749 ± 153 nm, and 1452 ± 312 nm). An increase in fiber diameter in the presence of fibroblast growth factor-2, a mitogen that promotes stem cell maintenance, induced a decrease in NSC proliferation rate and migratory activity [68]. When cultured in differentiation conditions, on the other hand, NSCs tended toward a glial lineage on the 283-nm fibers as cells displayed a better ability to spread randomly along the nanofiber matrix. For the larger fiber diameters, NSCs were restricted to extending along single fibers, promoting a neuronal lineage [68]. Though correlations have been observed between topographies and cell behavior, the mechanisms of shape regulation remain elusive. Another such innovative study involves preparing porous

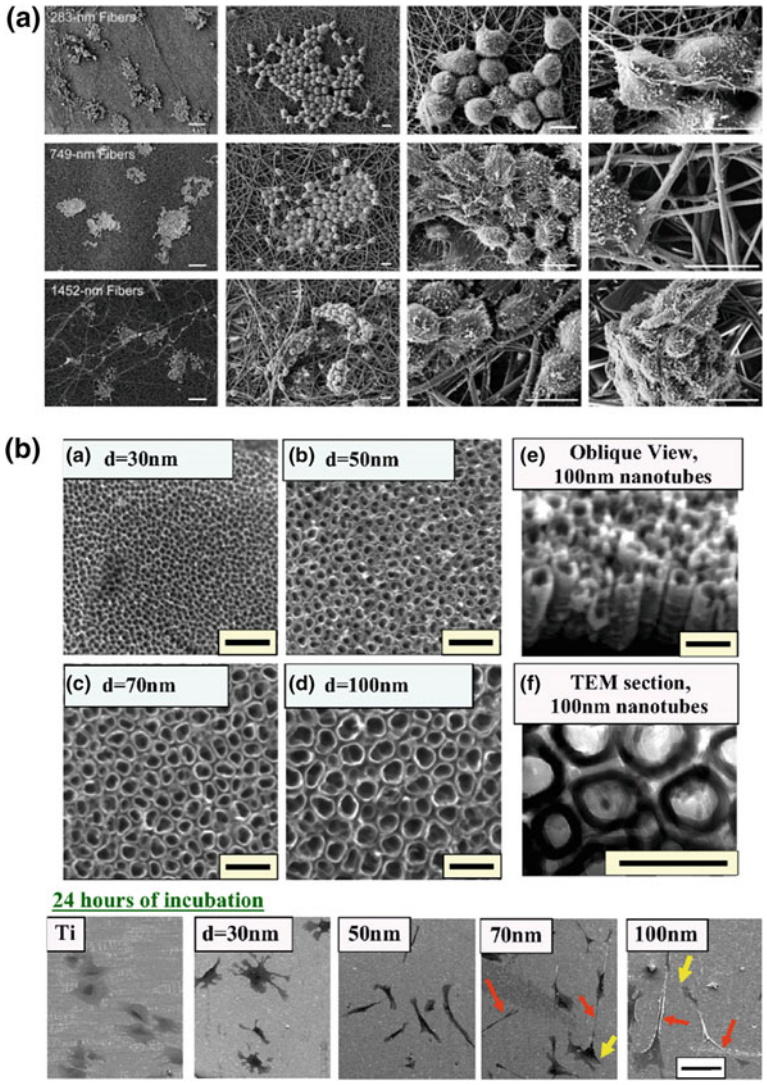


Fig. 2 Engineering strategies for generating 3D static topographies. Panel **a** SEM images of NPCs cultured on nanofibers of varying diameter [68]. Panel **b** SEM images of TiO_2 nanotubes of different pore diameters and hMSCs cultured on nanotube surfaces [71]. Panel **c** SEM images of silica-RGD nanoribbons with twisted and helical morphologies (*top*); SEM images of hMSCs cultured on grafted helical nanoribbon substrate, exhibiting extended filopodia-like structures (*bottom*) [70]

honeycomb polystyrene scaffolds by casting the polymer under humid conditions to form hexagonally arranged pores [69]. Kawano et al. [69] used this system to dissect the influence that cellular- and subcellular-scaled pore sizes have on hMSC behavior. For pore sizes smaller than the cell (1.6 μm), osteospecific differentiation

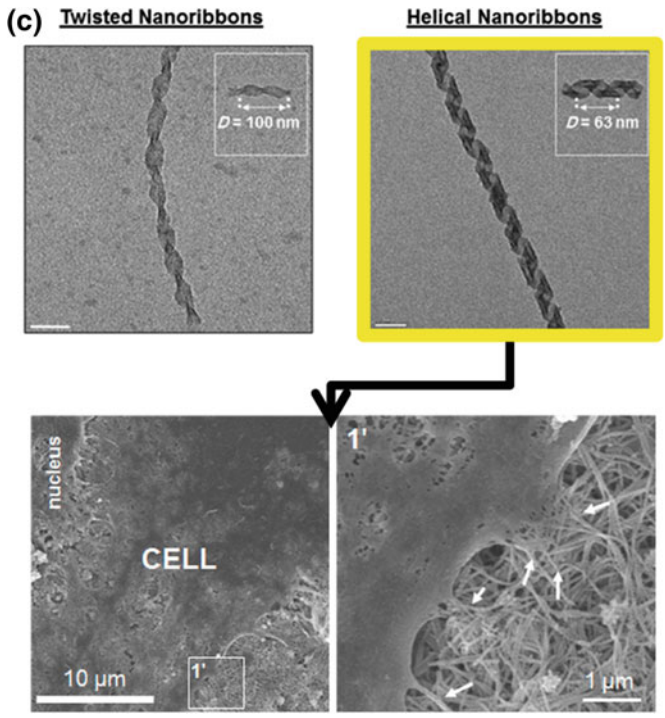


Fig. 2 (continued)

was prominent. In contrast, myospecific differentiation was associated with larger pore sizes (3.8 μm) [69]. Along the same lines, hMSCs were cultured on TiO_2 nanotubes of different pore diameters—30, 50, 70, and 100 nm [70]. The self-assembled, highly-ordered nanotube arrays were created by anodization, where different diameters were a result of manipulating anodizing potentials (5–20 V) (Fig. 2b). With this platform, Oh et al. [70] demonstrated that hMSC elongation increased with nanotube diameter and correlated with differentiation into an osteogenic lineage. Moreover, a saturation effect of hMSC differentiation was observed as diameters approach 100 nm. Finally, Das et al. [71] drew inspiration from collagen by engineering helical, silica nanoribbons covalently modified with RGD to mimic collagen fibril structures (Fig. 2c). They probed the role that different periodicities (63.5 ± 5 vs. 110 ± 15 nm) had in directing the lineage commitment of hMSCs and found that helical nanoribbons with smaller periodicity induced a strong commitment to the osteoblast lineage [71].

To increase the throughput of topographical investigations, novel on-chip systems that encompass various dimensions and architectural complexities within a single platform have been developed. Yim et al. [72] fabricated one such system, which they termed the Multi-ARChitecture (MARC) chip (Fig. 3). By utilizing

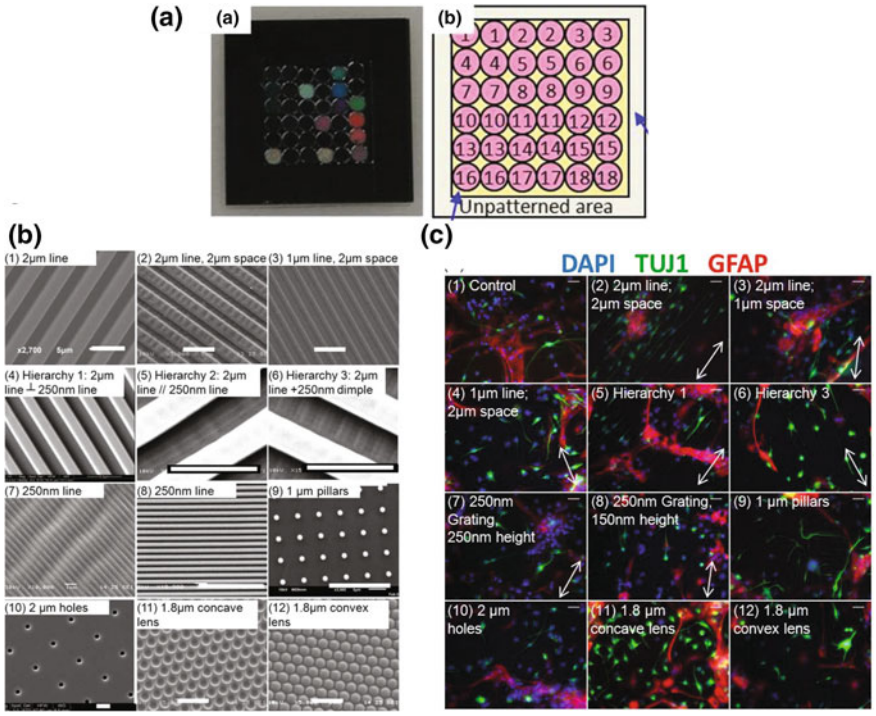


Fig. 3 MARC chip for high-throughput topographical investigation of hESC neural differentiation. Panel **a** Schematic overview of chip design [72]. Panel **b** SEM images of single and multi-architectural PDMS patterns [72]. Panel **c** Immunostaining of hESCs for neuronal (Tuj-1 *green*) and astrocytic (GFAP, *red*) lineages on the different topographies [72]

nanoimprinting lithography, they generated not only a variety of isotropic (1 µm pillars, 2 µm holes, 1.8 µm concave and convex lenses) and anisotropic (2 µm and 250 nm gratings) features but also hierarchical, composite structures of 2 µm lines and 250 nm dimples on top of 2 µm gratings [72]. Neuronal differentiation of human embryonic stem cells (hESCs) was studied with this system. When hESCs grew on laminin-coated PDMS replicas of these MARC chips, grating topographies favored neuronal differentiation, whereas isotopic patterns favored the glial lineage [72].

4.2 *Micropatterning Techniques to Relate Stem Cell Shape to Behavior*

Micropatterning techniques have been developed to control cell shape on a single-cell level to understand better how cytoskeletal state orchestrates stem cell behavior. The pioneering works of Ingber and Whitesides paved the way for the

development of a multitude of chemical patterning techniques, important tools for dissecting the relationship between stem cell shape and response [73–75]. These two groups demonstrated the ability to engineer cellular geometry through microcontact printing, a technique in which an elastomeric stamp is used to transfer, for example, square or rectangular patterns (2–80 μm) of self-assembled monolayers of alkanethiols onto a gold substrate [75]. An ECM component, such as laminin, can then be deposited onto the alkanethiol micro-islands and thereby be selectively adsorbed onto the printed regions, while the gold substrate remains adhesion-resistant. Though this platform was initially explored with hepatocytes, analogous efforts have extended into the stem cell field. A seminal effort by McBeath and colleagues helped elucidate the molecular basis of cell shape-mediated effects on hMSC commitment to an adipogenic or osteogenic fate [76]. Microcontact-printed fibronectin islands of 1024 and 10,000 μm^2 areas were used to control cell shape. The smaller islands promoted more rounded morphologies in contrast to the larger islands, which stimulated well-spread morphologies. Using this system, they discovered that hMSC differentiation was mediated by RhoA signaling with lineage specification occurring through the RhoA effector, ROCK [76]. RhoA activity, though capable of displacing soluble factor signaling, was found to be dependent on cell shape. A rounded morphology was necessary for adipogenesis and, similarly, a spread-out morphology was needed for osteogenesis. ROCK, on the other hand, was found to be downstream of these instructive signals. hMSCs with constitutively-active ROCK become osteoblasts, regardless of cell shape [76]. This landmark study highlights the importance of cell mechanics as an inductive cue for stem cell differentiation.

More recent efforts have focused on further dissecting the relationship between stem cell shape and behavior, resulting in the development of additional innovative materials. For example, Peng et al. [77] patterned a polyethylene glycol (PEG) hydrogel with gold micro-islands conjugated with RGD peptides. They investigated the effect that different anisotropic patterns (circle, square, triangle, and star) and rectangles of varying aspect ratios (1, 1.5, 2, 4, 8, and 16) had on single rat MSC differentiation (Fig. 4a). They found that cell-shape perimeter could be used as a simple parameter for predicting stem cell differentiation in the case of anisotropic patterns; however, isotropic patterns exhibited a non-monotonic osteospecific differentiation as a function of aspect ratio [77]. A similar study investigating the influence of cell shape on lineage commitment was conducted by Kilian et al., who also harnessed microcontact printing [78]. MSCs were cultured on three shapes with pentagonal symmetry but different curvatures: (1) flower shape with large convex curves; (2) pentagon with straight edge lines; and (3) star shape with concave edges and sharp vertices (Fig. 4b) [78]. The subtle geometric differences were sufficient to generate strikingly different differentiation profiles through varying degrees of actin-myosin contractility [78]. In general, pointed features between concave regions resulted in enhanced stress filaments and increased myosin contractility. Additionally, these local shape cues were associated with pathways promoting osteogenesis [78].

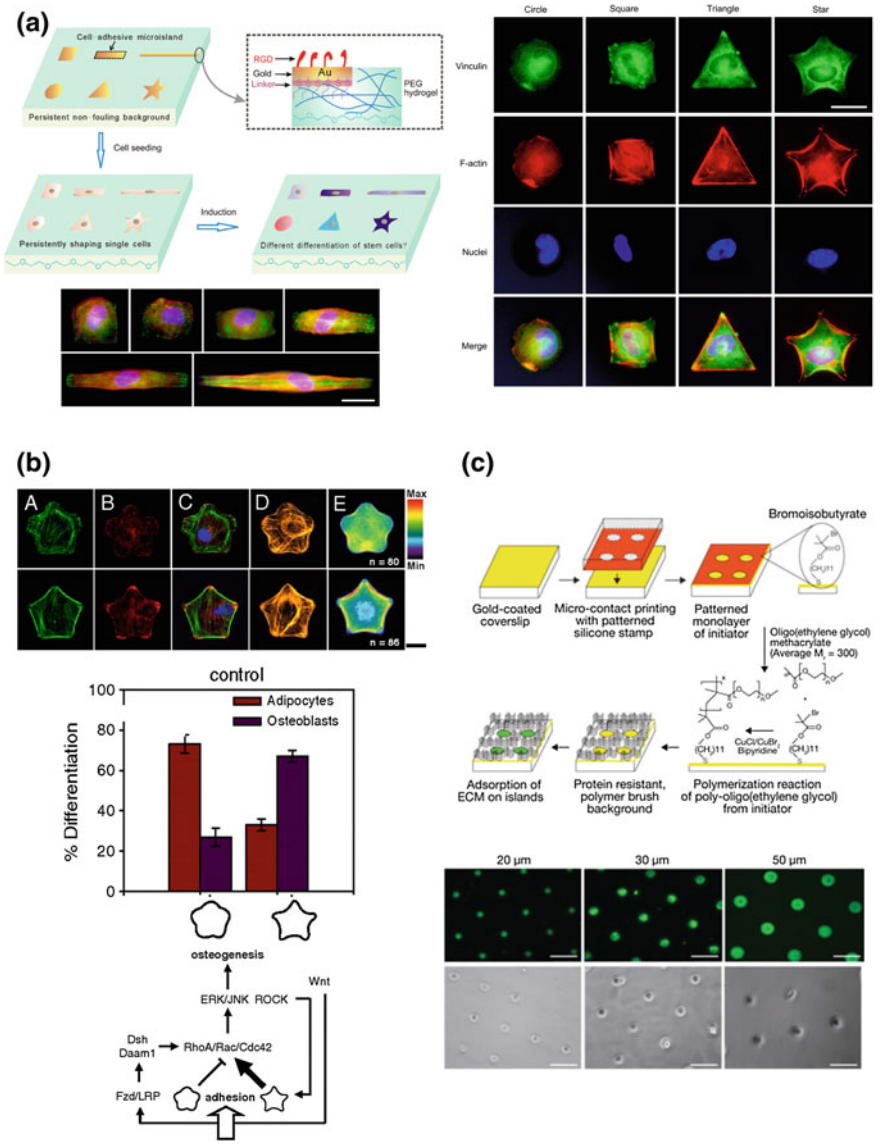


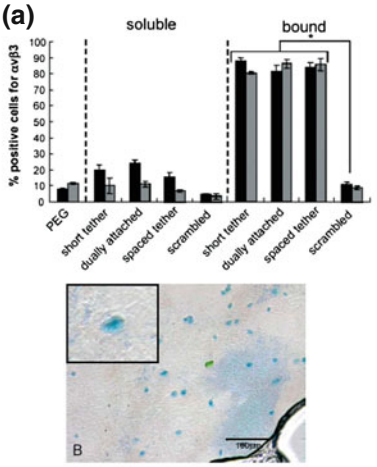
Fig. 4 Strategies for engineering stem cell shape. Panel **a** RGD-conjugated gold microislands of different anisotropic geometries patterned onto PEG hydrogels (*left*); immunostaining of single rat MSCs under different geometrical shape constraints [77]. Panel **b** Immunofluorescent images of single MSCs stained for F-actin (*green*), vinculin (*red*), and nuclei (*blue*) on *flower* and *star shape* patterns created by microcontact printing [78]. Panel **c** Microcontact printing schema for generating circular collagen microislands of different diameters applied towards studying single primary human keratinocytes [79]

Connelly et al. [79] also utilized microcontact printing in their system to generate patterned, polymer-brush surfaces for investigating the role of cell-ECM interactions in regulating human epidermal stem cell differentiation. Circular micro-islands of collagen were prepared with diameters ranging from 20 to 50 μm, thereby enabling the capture of single epidermal stem cells and control over cell spreading (Fig. 4c) [79]. More importantly, this platform enabled the researchers to dissect how changes to cytoskeletal organization influenced differentiation. This was achieved by altering individual parameters of the microenvironment systematically through the addition of actin-disrupting agents, such as latrunculin A, ROCK inhibitor Y27632, blebbistatin, and cytochalasin D. Connelly and colleagues thereby demonstrated that cell shape guides the initiation of differentiation more strongly than other factors, such as adhesive area, ECM composition, or ECM density [79].

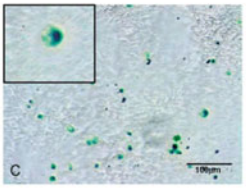
4.3 Soft Matter Hydrogel Systems with Predefined Characteristics

Great strides have also been made in the development of biomimetic hydrogel systems—both naturally-derived and synthetic—that recapitulate biofunctionality as well as key mechanical properties of the stem cell niche [80–83]. Hydrogel matrices have been utilized as a platform for presenting specific biological moieties to stem cells in vitro, such as cell adhesion ligands and growth factors (in both soluble and tethered fashions) [81, 84–86]. Strategies to explore the effects of tethered ligand type, ligand density, ligand flexibility, and ligand spatial patterns have been at the forefront of these recent studies. The RGD peptide motif (arginine-glycine-aspartic acid), a major binding site of fibronectin and other ECM proteins, is one integrin-binding ligand that has been frequently studied, tethered to many hydrogel matrices, and applied to a wide spectrum of stem cell systems. For example, Salinas and Anseth investigated hMSC attachment and viability when RGD peptides conjugated to PEG hydrogels were presented via two covalent mobilization schemas: pendant tethering with a spacer arm sequence (aka mono-functionalization) or dually attached with a loop-like structure (i.e. di-functionalization) (Fig. 5a) [87]. In short, they found that hMSCs demonstrated lower viability in the dually-tethered gel in addition to a lower expression level of αvβ3 integrins, most likely due to steric hindrance from the two links that prevented hMSCs from binding to the RGD motif through their integrins [87]. The use of a spacer arm sequence for immobilizing RGD was offered as a solution for overcoming integrin inaccessibility.

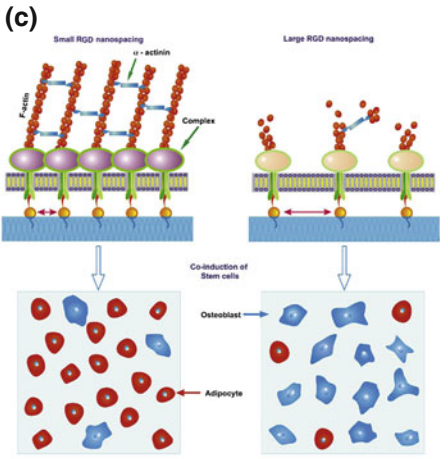
Building on earlier work with fibroblasts [88], Lam and Segura [89] investigated another mode of RGD presentation by exploring the effects of RGD clustering on guiding the behavior of encapsulated mouse MHCs within 3D hyaluronic acid hydrogels (Fig. 5b). While it did not play a significant role in altering MHC

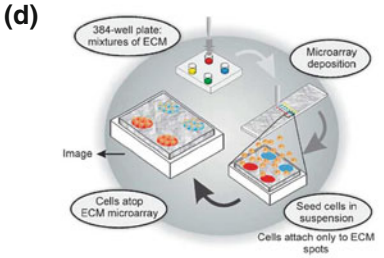


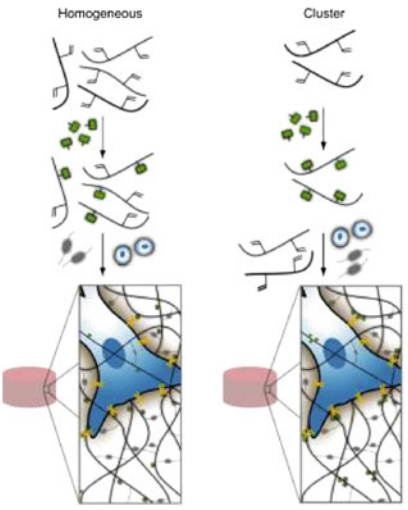
Pure PEG System

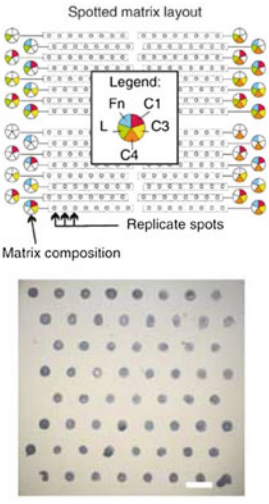


Di-Functionalized RGD System









◀ **Fig. 5** Engineering ligand presentation in hydrogel systems. Panel **a** Investigating the effects of RGD tethering via two mobilization schemas, i.e. mono- versus di-functionalization, on hMSC attachment. hMSCs stained for nuclei (*blue*) and αvβ3 cell surface integrin (*green*) [87]. Panel **b** Schematic of RGD clustering within hyaluronic acid hydrogels [89]. Panel **c** The effect of small vs. large RGD nanospacing on MSC differentiation [90]. Panel **d** ESCs cultured on an ECM microarray platform consisting of varying ECM compositions [92]

proliferation, varying the distribution of the bioactive signals did have an effect on cell spreading and integrin expression. Homogenous gels (i.e. gels that display the lowest level of RGD clustering) induced a low degree of spreading. As signal clustering increased, so did the degree of MHC spreading. Furthermore, the expression of cell integrins also varied. For example, the number of cells that expressed α2 and β1 integrins was significantly higher in gels with the lowest amount of clustering and, conversely, α3 integrins were more prominent in the highly-clustered gels [89]. Along similar lines, Wang and colleagues explored the effect of five RGD nanospacings from 37 to 124 nm on PEG hydrogels on MSCs lineage commitment (Fig. 5c) [90]. These underlying nanopatterns were obtained by grafting RGD peptides onto patterned gold nanodots, enabling single nanodot-integrin interactions. With this platform, the authors observed that cell circularity (i.e. area multiplied by 4π and divided by square of perimeter) increased in response to increases in RGD nanospacing [90]. Furthermore, under solely osteogenic or adipogenic differentiation conditions, increases in RGD nanospacings translated to an increase in the extent of respective osteogenic and adipogenic differentiation of MSCs. In the case of co-induction conditions, however, osteogenesis was found to be more sensitive to RGD nanospacings, as more MSCs pursued an osteogenic fate as nanospacings increased [90].

Elucidating the effect of ECM composition has also been a recent interest in the field. Battista et al. [91] dissected the role that material structure and molecular-binding domain density have in controlling embryoid body growth, cavitation, and differentiation of mESCs. Semi-interpenetrating polymer networks consisting of collagen type I fibers, fibronectin, and laminin were modulated to produce scaffolds of varying physical properties and compositions. Cellular adhesion cues from laminin in the 3D scaffold were found to guide EB differentiation into cardiac-tissue lineages, while the addition of fibronectin cues induced dose-dependent differentiation into epithelial lineage without the addition of soluble factors [91]. In addition, high-throughput microarray systems have been developed to allow for the simultaneous screening of ECM factors, both individually and combinatorially, to better investigate the complexity of the stem cell niche's ECM. Jongpaiboonkit et al. generated 3D PEG hydrogel arrays to screen for both individual and combinatorial effects of various ECM features: cell-adhesion ligand type, ligand density, and ECM degradability [85]. This group focused primarily on the fibronectin-derived Arg-Gly-Asp-Ser-Pro (RGDSP) and laminin-derived Ile-Lys-Val-Ala-Val (IKVAV) sequences. Additionally, degradability was induced by photocrosslinking PEG-diacrylate chains with varying concentrations of dithiothreitol (DTT), resulting in "DTT bridge" with ester bonds prone to hydrolytic

degradation [85]. Other high-throughput techniques have involved adopting robotic spotting printing technologies. For example, Flaim et al. presented an ECM microarray platform that deposits an array of ECM molecule mixtures [92]. 32 combinations were investigated with varying collagen I, collagen III, collagen IV, laminin, and fibronectin compositions (Fig. 5d) [92]. This method can be expanded to include a vast range of insoluble and soluble ECM cues.

Biochemical information within the ECM has thus been a focus of numerous studies. However, hydrogels have also enabled major strides in the field's understanding of how mechanical properties regulate and affect stem cell function. In particular, the elastic modulus (or stiffness) of the substrate has been widely explored. The initial landmark study utilized a collagen-coated polyacrylamide gel with tunable cross-linking properties, correlating to varying matrix stiffnesses as low as 0.1–40 kPa [33]. With this system, the physiological stiffnesses characteristic of brain, muscle, and bone were recapitulated in vitro and presented to naïve MSCs. The resulting differentiation of MSCs into tissue-specific cell types along with corresponding altered gene expression patterns demonstrated the significance that matrix mechanical properties have in the stem cell niche [33].

Gilbert and colleagues extended this initial strategy to illustrate the potency that substrate elasticity has on muscle stem cell self-renewal and cell fate [93]. In doing so, they engineered a tunable PEG hydrogel system covalently cross-linked with laminin in which stiffness could be controlled by varying the PEG polymer percentage in the precursor solution. Muscle stem cells cultured on soft PEG gels with an elastic modulus that mimicked adult murine skeletal muscle ($\sim$12 kPa) was found to enhance muscle stem cell survival when compared to cultures on traditional, stiff polystyrene surfaces ($\sim 10^6$ kPa) [93]. Substrate rigidity also influenced Myogenin expression (a transcription factor expressed by differentiated muscle stem cells). Soft substrates demonstrated a 3-fold decrease in Myogenin-positive cells. Additionally, muscle stem cells cultured on PEG substrates most closely tuned to their native muscle niche stiffness (as opposed to brain or cartilage) were found to retain the greatest stemness [93].

While many studies that investigate the effects of substrate stiffness on stem cell behavior (including the aforementioned studies) employ model systems that yield thin layers of tunable hydrogels coated on a rigid substrate, Saha et al. [94] highlighted one potential problem with this approach. Soft polyacrylamide hydrogels are prone to equi-biaxial compressive stress when exposed to an aqueous environment due to osmotic pressure difference. The ensuing instability causes the formation of sharp folds (i.e. creases) as a result of induced buckling of the polyacrylamide surfaces. The authors emphasized that these surface creases must be characterized and controlled as they influence stem cell behavior [94]. NSCs were demonstrated to migrate towards the folds and adopted mature neuronal and astrocytic phenotypes when compared to NSCs that were uniformly attached and differentiated when cultured on smooth and stable polyacrylamide surfaces [94]. Therefore, instable surface creasing of polyacrylamide substrates (and potentially other soft hydrogel systems) may bias stem cell mechanotransduction studies [94]. This highlights the need for well-characterized and tightly controlled synthesis of

soft-matter substrates. An overview of other key studies investigating the importance of matrix elasticity in stem cell biology are described in a number of extensive reviews [45, 51, 60, 95].

5 Second Generation Engineering Strategies—Increased Complexity with a Focus on Spatiotemporal Control

Engineered microenvironments are thus clearly valuable tools for dissecting how the ECM affects stem cell fate decisions, and there have been increasing advances in elucidating how these extrinsic cues modulate core transcriptional networks [79]. As demonstrated in the above section, initial engineering strategies in the stem cell field focused primarily on recapitulating *static* representations of the niche ECM. More recent engineering strategies, however, have evolved to emulate the dynamic interaction between stem cells and their physical environment. The creation of platforms with increasingly sophisticated structural and functional complexity is helping to bridge a gap between in vitro systems and what are likely highly dynamic in vivo physiological environments. In particular, the ability to engineer and incorporate tightly-coupled spatial and temporal control into these platforms has become a key objective of the field. The following section provides an overview of these emerging second-generation engineering strategies.

5.1 Biomaterials with Tunable Properties

An increased interest in mimicking the dynamic properties of the stem cell niche's ECM has spurred the development of smart biomaterials—ones whose properties can be manipulated by external stimuli [96]. Light, temperature, pH, electric fields, small molecules, and shear stress represent a variety of "triggers" that have been employed to induce changes in stiffness, topography, and adhesion [96]. These in situ perturbations are powerful tools because they allow for the investigation of spatial and temporal ECM cues, providing a deeper insight into stem cell behavior.

5.2 Spatiotemporal Control over Topography

To complement landmark studies with pre-printed substrates, in recent years, topographic presentation has evolved toward materials with active and tunable topographies. Shape-memory polymers represent one class of active materials that have been employed for probing stem cell response to localized topographical changes, and studies involved with such polymer systems have provided insights

into the dynamics of cytoskeletal organization and mechanotransductive signaling events [97]. These systems have relied primarily on the use of temperature as a temporal control for switching topography from a primary temporary pattern to a secondary permanent pattern. Davis et al. was one of the first groups to harness this effect [98]. They utilized a thermally-responsive polyurethane polymer substrate with end-linked thiol-ene crosslinks that was programmed to change from a lamellar surface to a flat surface upon a temperature transition from 30 to 37 °C [98]. More recent techniques have extended this strategy a step further by demonstrating the capability to switch between two distinct patterns. Le et al. [97] established this dual-shape capability by developing a poly(ε-caprolactone) surface in which the primary pattern was formed with replica molding, while the secondary pattern was generated by mechanically deforming the substrate at 130 °C using a second replica mold and, subsequently, cooling it to 78 °C. With this technique, a combination of pattern transformations was introduced to hMSCs: micron-sized cube arrays to hexnuts, cylinders to boomerangs, and channels to planar surface (Fig. 6a) [97]. Though pattern versatility was evident, there were significant challenges, including a lack of pattern reversibility and a high transition temperature of 40 °C (resulting in cell toxicity).

Gong et al. [99] illustrated another approach for utilizing shape-memory systems. They engineered a four-stage shape memory platform with tunable microgrooves (Fig. 6b). To start, poly(ε-caprolactone) was modified with A allyl alcohol as a plasticizer, shifting the shape-memory recovery function to within the physiological range of 32–41 °C. Two different dynamic surfaces were then pursued. The first modulated microgroove depth, increasing from 0 to 1.7, 3.5, and 4.9 μm at 32, 35, 38, and 41 °C, respectively. The other surface transitioned from a temporal microgroove with a width of 9 μm at 32 °C to 7, 4.5, and 3.1 μm at the same increasing temperature set-points. The changes in the first surface induced parallel upward forces, which had little to no effect on cultured rat bone marrow MSCs [99]. The latter, convergent force from the second surface, however, greatly affected cytoskeletal arrangement and biased differentiation fate towards a myogenic lineage [99]. In a final example, Tseng et al. [100] translated shape-memory polymers into 3D by utilizing an electrospun scaffold whose fibrous architecture transitioned from a strain-aligned state to its original random fiber arrangement upon thermal activation (Fig. 6c). This controllable change in scaffold architecture exhibited desirable shape recovery properties as well as cytocompatibility for human adipose-derived stem cells. Moreover, the recovery rate of the scaffold could be controlled by modulating the chemical composition of the polyurethane scaffold, which comprised of hard segments of polyhedral oligomeric silsesquioxane and soft segments of polylactide/caprolactone copolymer (resulting in an increase in the glass transition temperature or decrease in hydrophilicity) [100]. These shape-memory-actuated materials, while still in the early stages of development, offer exciting potential for supporting further in-depth studies of stem cell regulation.

While thermally-induced shape-memory polymers offer considerable advances, another means of creating quasi-static topography was demonstrated through a technique that combined strain-induced buckling of PDMS substrates with plasma

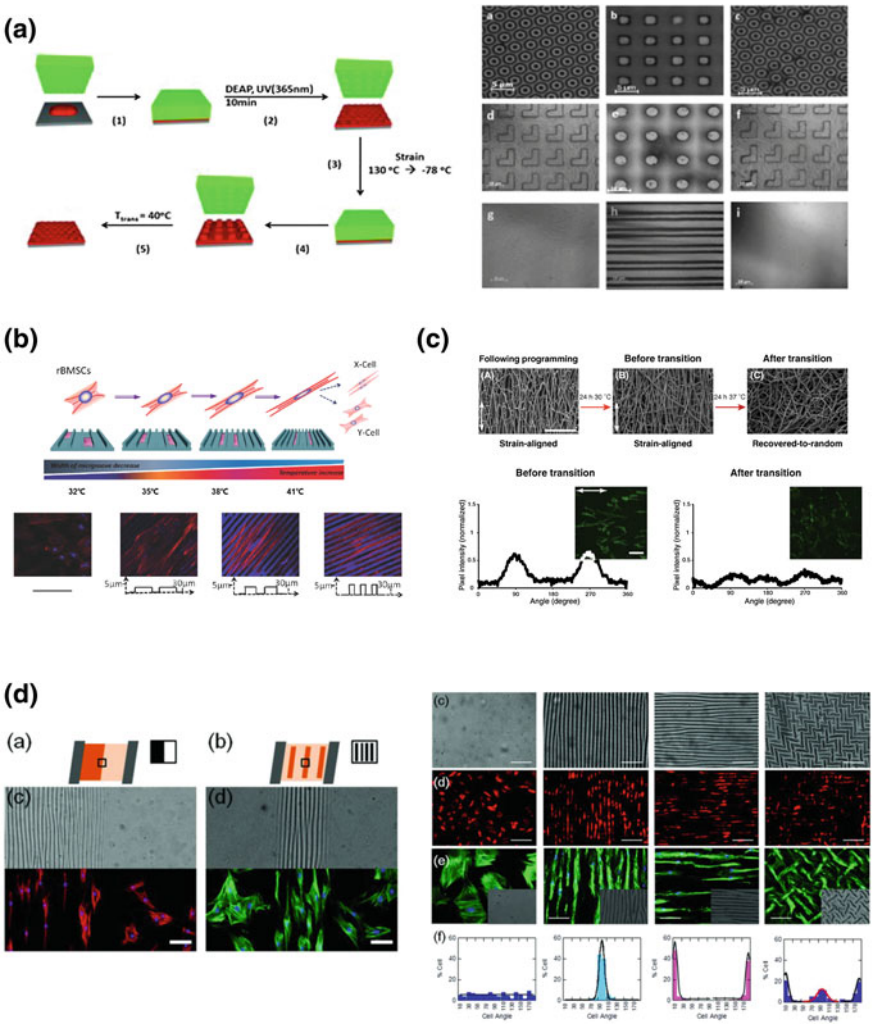


Fig. 6 Engineering dynamic topographies with spatiotemporal control. Panel **a** Schematic for fabricating thermally-responsive poly(ε-caprolactone) topographies [97]. Panel **b** Four-stage shape memory platform with tunable microgrooves applied towards studying MSC behavior; cells immunostained for F-actin (*red*) and nuclei (*blue*) [99]. Panel **c** Dynamic switching from fiber-aligned state to random fiber orientation via a cytocompatible temperature increase (*top*); cells stained with phalloidin (*green*) to visualize actin (*bottom*) [100]. Panel **d** Spatial control of lamellar patterns dictated by mask applied during UVO treatment (*right*); illustration of quadruple topographical switching from flat to lamellar patterns at 90° to lamellar at 180° to zigzag patterns (*right*); live hMSCs labeled with CellTracker red, and fixed cells stained for F-actin (*green*) and nuclei (*blue*) [101]

oxidation. Guvendiren and Burdick [101] introduced a strategy for fabricating versatile, high-fidelity, and reversible lamellar wrinkling patterns (Fig. 6d). To obtain this, PDMS sheets were stretched uniaxially, followed by exposure to ultraviolet/ozone (UVO). This exposure created stiff regions that resulted in perpendicular buckling when the strain was released. With this system, hMSCs were exposed to four changing patterns, starting with a flat, unpatterned surface to lamellar with 90-degree patterns to lamellar with 180-degree patterns and, finally, to zigzag patterns. hMSCs responded to these in situ dynamic patterning switches through changes in cell orientation angle [101]. Key advantages of this system include the ability to modulate pattern amplitude and periodicity by altering the degree of strain release. Moreover, spatial control of topographies could be regulated by selectively exposing the surface to UVO with different shadow-mask patterns. One disadvantage, however, is that high hMSC proliferation could lead to "masking" of the triggered topographical change. In other words, as culture time and cell division increases, cellular alignment to induced topographies diminishes [101].

Photo-induced manipulation of surface topography is another powerful approach that enables high spatial and temporal control. In comparison to its shape-memory polymer counterparts, light-responsive materials can be operated at standard physiological temperature (37 °C) as well as undergo countless sequential alterations that are not pre-determined, as long as phototoxicity does not occur. Kirschner and Anseth [102] engineered one such system—a photodegradable PEG-based hydrogel platform in which topographical cues can be formed in situ by user-controlled spatial erosion. Specifically, photolithographic techniques were used to pattern features (such as anisotropic channels and isotropic square patterns) on a photolabile gel, where pattern depths could be controlled by modulating the time of UV exposure (10 mW/cm^2). Moreover, sequential patterning steps could be applied to alter surface topography concurrently during cell culture. hMSCs were cultured on this tunable surface and demonstrated reversible changes in cell morphology and alignment [102]. Similar to the shape-memory materials, only initial studies have been conducted with this system. Future perspectives involve using this system for better understanding how stem cells respond to real-time changes of ECM topographical cues within their niches.

5.3 Spatiotemporal Control over Matrix Stiffness

In addition to modulating topography, light has also been used as a tool for creating dynamic cultures of switchable substrate stiffnesses. Yang et al. [103] synthesized a phototunable hydrogel that incorporates a poly(ethylene glycol) di-photodegradable acrylate crosslinker. Upon controlled exposure to UV light, the initially stiff hydrogel (Young's modulus of 10 kPa) transitioned into a soft hydrogel with a modulus of 2 kPa (Fig. 7a). With this system, they investigated the effects of mechanical dosing and mechanical memory on hMSCs [103]. In statically soft gels, hMSCs retained the capability to differentiate into both adipogenic and osteogenic

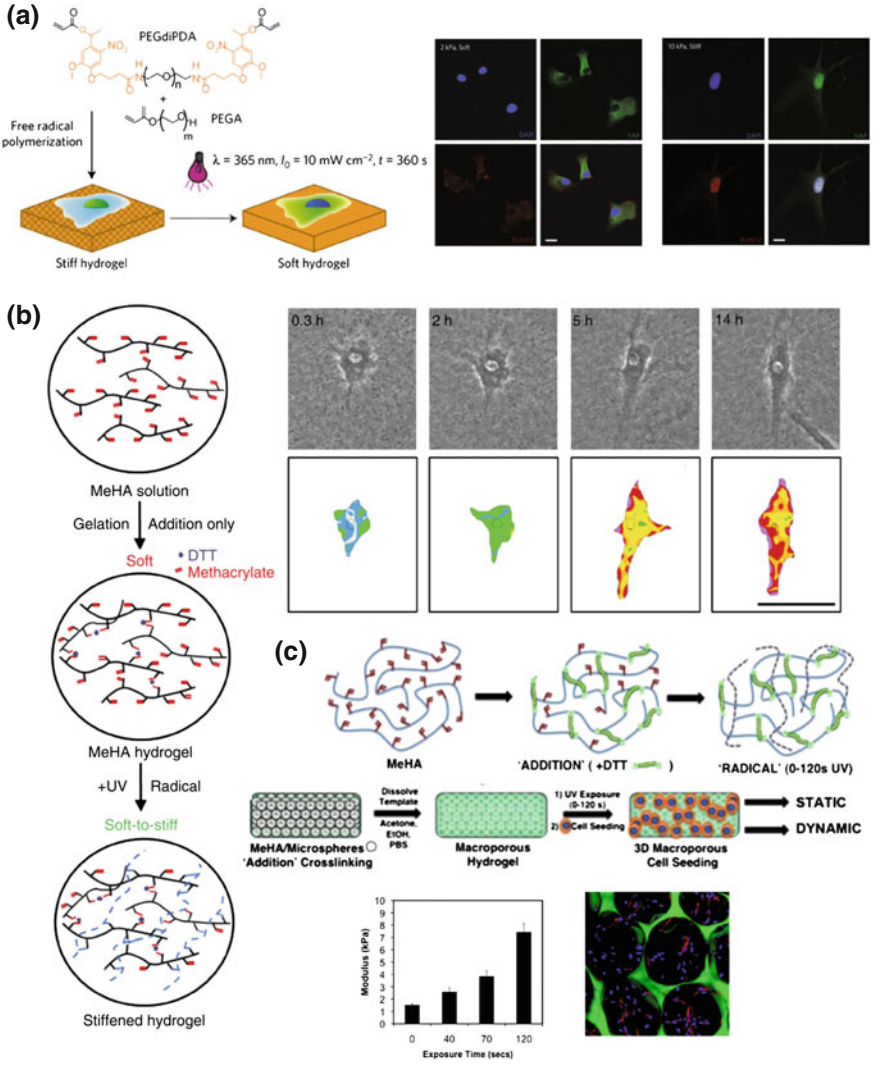


Fig. 7 Engineering strategies for dynamic control over substrate stiffness. Panel **a** Illustration of photodegradable hydrogel system (*top*); immunostaining of hMSCs for YAP (*green*) and RUNX (*blue*) localization (*bottom*) [103]. Panel **b** Crosslinking schematic (*left*) and traction stress maps of single hMSCs during in situ stiffening (*right*) [104]. Panel **c** Crosslinking schematic for generating porous hydrogel architectures; changes in bulk compressive moduli in responsive to UV crosslinking exposure times; hMSCs stained for actin (*red*) and nuclei (*blue*); porous hydrogels stained with FITC (*green*) [86]

lineages. However, upon mechanical dosing (i.e. culturing the cells on stiff substrates at variable time frames before in situ softening of the hydrogel), differentiation became biased towards osteogenic lineages. Specifically, cells were cultured from 1 to 10 days on stiff substrates prior to transitioning to soft hydrogels. The longer hMSCs were cultured on the stiff substrate, the more biased the cells became towards osteogenesis [103]. Transcriptional coactivators that play a key role in mechanotransduction, YAP and TAZ, were found also to persist in the nucleus (i.e. mechanical memory) even after cells were transitioned to soft substrates, suggesting that hMSCs retain information about past ECM states [103]. This system helped uncover a temporal aspect of stem cell mechanotransduction, where brief periods of mechanical dosing resulted in reversible activation of YAP and longer periods resulted in constitutive YAP nuclear localization [103].

In an analogous fashion, Guvendiren and Burdick [104] engineered a complementary strategy for in situ hydrogel stiffening in the presence of hMSCs—a system characterized by fast kinetics, long-term stability, and structural uniformity (Fig. 7b). This approach is potentially biologically relevant since matrix stiffening has been generally associated with key biological phenomena, such as disease and tissue development. To develop this dynamic substrate, hyaluronic acid macromers were functionalized with methacrylates, which react with thiols and radicals for crosslinking. Gelation was obtained through the addition of DTT, providing an initial stiffness of 3 kPa. Further, secondary crosslinking was achieved through a photoinitiator and subsequent UV light exposure for 2 min at 10 mW/cm^2, increasing the matrix modulus to 30 kPa. This temporal stiffening not only can be tuned by exposure time but also can be achieved via sequential exposures during cell culture [104]. The use of DTT, however, poses a potential caveat for this hydrogel system as it may impact hMSC redox state.

Marklein et al. [86] extended this photoactivated crosslinking approach to study hMSC behavior in 3D porous hydrogels, investigating the importance of the magnitude, context, and timing of presented stiffness stimuli. In their work, Marklein et al. generated a macroporous architecture by initially crosslinking methylated hyaluronic acid around a hexagonally-organized template of microspheres (Fig. 7c). These hydrogels were triggered to stiffen from 2.6 to 12.4 kPa, either on Day 2 or 7 of a 14-day culture. These variable mechanics were controlled by UV exposure (10 mW/cm^2) and found to affect the secretion profiles of cytokine and angiogenic factors [86]. In particular, hMSCs cultured on hydrogels that were stiffened on Day 2 (i.e. transitioned to the stiffer substrate sooner) displayed a greater reduction in key angiogenic factors and cytokine molecules compared to samples stiffened on Day 7. In contrast, morphology, proliferation, and differentiation did not exhibit significant dependence on stiffness dynamics [86].

Yoshikawa et al. [105] explored a different approach to achieving a dynamically tunable hydrogel platform. In lieu of using light as a stimulus, changes in viscoelastic properties were achieved through subtle pH changes and subsequently manipulating hydrophobic and interchain interactions. In this study, the pH-responsive polymer films consisted of a triblock ABA-type hydrogel, where A represented poly-(2-(diisopropylamino)ethyl methacrylate) and B represented

poly(2-(methacryloyloxy)ethyl phosphorylcholine) [105]. By narrowly adjusting the pH range between 7 and 8—a range that does have the potential to affect cellular function—the stiffness of the copolymer could be reversibly transitioned between 1.4 and 40 kPa. Mouse myoblasts were used as a model system for this study, where morphological changes and cell adhesion strength were evaluated in relation to dynamic modulations of substrate stiffness [105]. While recent efforts have demonstrated the capability of either dynamic stiffening or softening of gels, a significant advance within the field would be a system that allows for reversible switching with cues that are inert to cells. This level of control would enable more complex investigations of the effects of stiffness pulses at different temporal onsets and durations.

5.4 Dynamic Control of Integrin-Based Focal Adhesions

Achieving precise control over the spatiotemporal presentation of ECM bioactive ligands has warranted the development of additional sophisticated engineering strategies. As illustrated in the previous sections, cell-adhesive ligands are key mediators of cell-matrix interactions and, thus, stem cell function. While previous strategies investigated the influence of pre-patterned peptides that mimic the active domains of key ECM components in a static fashion, several groups have recently fabricated smart biointerfaces that control the activation and de-activation of these integrin-based signals.

Photolabile protecting groups are an attractive approach for achieving dynamic control over the formation of stem cell focal adhesions, which activate downstream signaling cascades. Weis et al. pursued this approach by anchoring "caged" RGD peptides to self-assembled monolayers of alkanethiols on a gold substrate (Fig. 8a) [106]. To ensure only specific cell attachment to RGD-anchored SAMs, oligo (ethylene glycol) groups were conjugated to the SAMs lacking tethered peptides, providing a non-biofouling background. This system was applied to study how RGD peptide density influenced the differentiation of myoblasts (myofiber precursors) [106]. With an initial surface RGD density $\sim$17 %, few cells attached to the substrate. However, upon a 3-min light exposure, the maximum surface RGD density was unmasked, and integrin-mediated myoblast interaction with the substrate was thus enabled. Light exposure for 3 min was applied at different time points during the culture timeframe: 1, 6, 24, and 48 h. Myogenic differentiation—analyzed via sarcomeric myosin expression and the formation of multi-nucleated myotubes—was more prominent when cells were exposed to high-density RGD peptides during earlier culture times [106]. This discovery highlights the importance of temporal presentation of ECM ligands, motivating additional exploration of their relationship to dynamic mechanical cues.

Another approach for achieving dynamic ligand manipulation during stem cell culture was demonstrated by Kloxin et al. [107]. Photolabile tethers consisting of a photodegradable acrylate monomer were conjugated to the fibronectin epitope

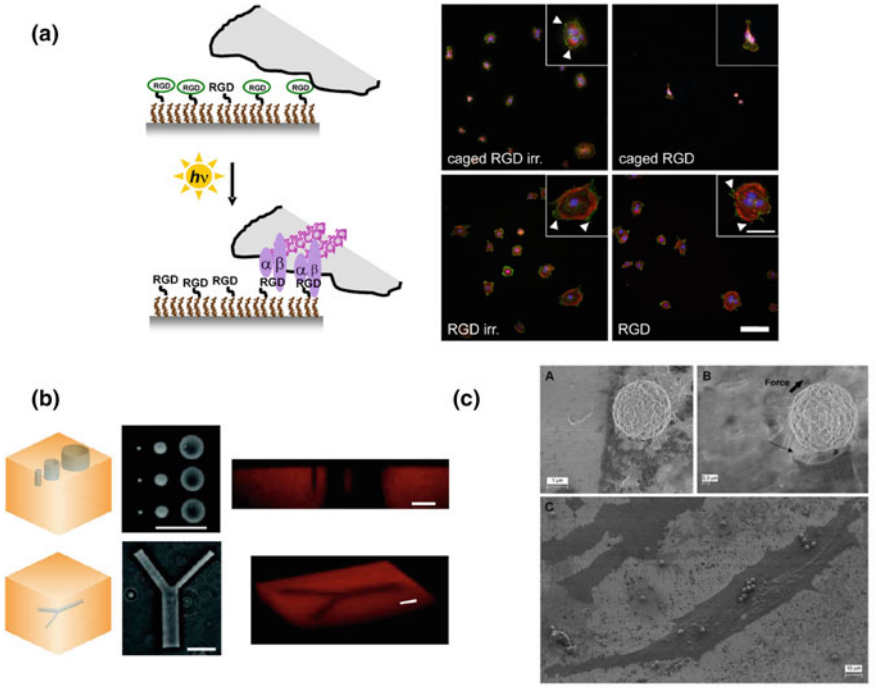


Fig. 8 Engineering strategies for in situ modulation of ligand presentation and hydrogel degradation. Panel **a** Schematic illustrating ligand tethering and UV irradiation to release caged RGD molecules; myoblasts stained for actin (*red*), vinculin (*green*), and nuclei (*blue*) [106]. Panel **b** 3D photopatterning of surface features, such as various sizes of microwells and a bifurcation channel, within a photodegradable hydrogel [107]. Panel **c** SEM images of paramagnetic beads attached to MSC β1 integrin subunit [108]

Arg-Gly-Asp-Ser (RGDS) within a nondegradable PEG-based hydrogel. Upon irradiation, the photolytic removal of RGDS moieties locally modified peptide presentation within the 3D microenvironment (Fig. 8b) [107]. The importance of persistent RGDS signaling on hMSC viability and differentiation was investigated by photolytically removing RGDS on Day 10 of a 21-day culture. In response to the temporal changes, hMSCs were found to downregulate the expression of αvβ3 integrins, while increasing the production of glycosaminoglycans as well as type II collagen, both of which are key markers of chondrogenic differentiation [107].

While photoresponsive materials have proved very effective for achieving spatiotemporal control over ligand presentation, Kasten et al. [108] demonstrated an alternate technique for probing stem cell mechanotransduction: the use of magnetic forces to induce integrin response. This strategy drew inspiration from earlier efforts, which utilized ferromagnetic microbeads coated with synthetic RGD peptides. These materials were employed for applying controlled mechanical loads to fibronectin receptors without inducing global changes to cell shape [109]. In this

particular application, however, Kasten et al. [108] coupled paramagnetic microbeads to hMSC integrins by coating beads with an antibody specific for the β1 integrin subunit (Fig. 8c). A custom magnetic device with an average magnetic field strength of 0.015 T was then applied to the culture system, thereby inducing the displacement of the magnetic beads, which subsequently applied a drag force on stem cell integrin receptors, created mechanical stress, and temporarily distorted the cell membrane. This study was also tested in conjunction with three different types of substrates: polystyrene, RGD-functionalized, and fibronectin-coated surfaces [108]. Differentiation markers associated with adipogenic (i.e. PPARγ), osteogenic (i.e. ALP), and chondrogenic lineages (i.e. Sox9) were investigated in addition to released soluble factors relating to angiogenesis (i.e. VEGF) and osteogenesis (i.e. collagen I). Kasten et al. [108] observed that VEGF expression increased in response to short-term integrin stress stimulated by the magnetic field when hMSCs were cultured on RGD peptides and fibronectin but not on polystyrene. Collagen I expression, in contrast, was upregulated when hMSCs were cultured on polystyrene but not the other two surfaces [108]. These initial results not only highlighted the dynamic ability to control integrin stress through a magnetic field but also emphasize the importance of multifactor interactions of ECM-niche components.

6 Dissecting Cell-Cell Interactions within the Stem Cell Niche

Cellular components within the stem cell niche serve as another key source of instructive inputs for regulating stem cell quiescence, proliferation, and cell-fate determination [4, 48, 110, 111]. The spectrum of intercellular communication that takes place within these niches encompasses a stem cell's interactions with other stem cells, stem cell progeny, and neighboring niche cells. Cell-cell signaling among these parties is achieved through various means: release of secreted soluble factors between neighboring cells (paracrine signaling), release of factors back to the same cell (autocrine signaling), cell-surface ligand-receptor binding between cells in direct contact (juxtacrine signaling), the transmembrane flux of signals through intimate gap junctions, and potentially mechanical interactions between cells.

The importance of cellular interactions and organization within stem cell niches was first demonstrated in early studies involving *Drosophila* germline stem cells (GSCs). Investigations of the ovary and testes niches showed that stemness and differentiation are balanced by critical communication between stem cells and their non-stem cell niche neighbors [112–114]. In the female fly, for instance, GSCs populate the anterior end of the ovariole and interact with three somatic cell types. GCSs indirectly adhere to the niche by intimately associating with cap cells via adherens junctions, cell-cell connections that form via homotypic cadherin binding [26]. During asymmetric division, the daughter cell that maintains this adhesion also retains its stem cell identity, whereas the daughter cell lacking adhesion

differentiates into a cystoblast [115]. Terminal filament cells and inner germarium sheath cells (also referred to as escort cells) augment this maintenance of stem cell phenotype by repressing the key differentiation gene bag-of-marbles (bam). This repression is achieved through the secretion of cytokines by terminal filament cells, which signal the cap and escort cells to produce bone morphogenic protein (Bmp) ligands that bind with receptors that act to downregulate bam in GSCs [115]. The *Drosophila* testis, though a more complex microenvironment, shares similar hallmarks with the ovary niche. Only GSCs that contact adjacent hub cells within the apex of the testis self-renew. Hub cells also secrete Upd, which stimulates GSC adhesiveness and prevents surrounding cells from outcompeting GSCs for niche contact [115]. Moreover, somatic cyst progenitor cells indirectly activate the Bmp pathway by secreting Gbb and Dpp, repressing differentiation yet again. These examples illustrate the balance of communication between stem cells and non-stem cell niche neighbors.

The degree of interaction between stem cells and other cellular players is particular to the stem cell niche under investigation. For instance, muscle satellite stem cells remain relatively isolated and quiescent as they reside near basal lamina of muscle fibers [48]. Not until activation do they proliferate and fuse with one other to form differentiated myotubes. HSCs, on the other hand, tightly associate with not only osteoblasts that line the endosteal surface of the trabecular bone but also endothelial cells that line blood vessels [26]. Similarly, NSCs closely associate with endothelial cells of surrounding vasculature, neighboring astrocytes, microglia, and in some cases ependymal cells [11, 110]. Epithelial stem cells that reside in a specialized "bulge" structure within hair follicles, in contrast, encounter periodic stimuli from specialized mesenchymal cells, referred to as dermal papilla (DP). Specifically, the regeneration of hair follicles exposes resident stem cells to dynamic, perpetual cycles of growth (anagen), regression (catagen), and rest (telogen). During the anagen stage, massive cell death occurs below the bulge area for all cells except DP. The basement membrane then shrinks and draws DP into close contact with stem cells within the bulge. This close association is believed to be necessary for re-activating hair follicle regeneration, thereby initiating a brief telogen phase followed by rapid anagen phase [116]. As a final example, intestinal stem cells populate the crypt base of intestinal villi and drive rapid cell turnover of the epithelial lining of the small intestine and colon [21]. Within this niche, stem cells receive a complex array of signals from neighboring epithelial and stromal cells—paneth cells, goblet cells, and transit-amplifying cells, to name but a few. Renewal of the epithelium is orchestrated by a complex array of cellular signals, which ultimately drive budding transit-amplifying cells to differentiate into mature lineages, such as enteroendocrine cells, tuft cells, and absorptive enterocytes. These committed cells migrate out of the crypt and up to the base of the villi [21].

While the well-studied *Drosophila* niches are not as complex as vertebrate niches, the insights obtained from these lower organism counterparts were essential in stimulating more rigorous investigations of key regulatory cellular signals. These

efforts have exposed a sophisticated interplay of signaling factors. Diffusible growth factors represent one class of secreted soluble signals that can positively or negatively regulate stem cell behavior within the niche—the effects of which are under strict spatial and temporal constraints [4]. For example, in the SVZ of the lateral ventricles, endothelial cells from surrounding vasculature produce a variety of paracrine factors that modulate key aspects of neurogenesis. The production of vascular endothelial growth factor, for instance, has been found to promote NSC self-renewal within the adult rat brain [117–119]. Also, the secretion of brain-derived neurotrophic factor (BDNF) has been suggested to direct NSC proliferation and balance the rates of neuroblast migration and differentiation in adult neurogenic niches [119–121]. In addition to these growth factor examples, endothelial cells are capable of secreting other types of short-range signals. For example, the chemokine stromal cell-derived factor-1 (SDF-1) is believed to regulate the migration and survival of SVZ NPCs. Additionally, the secreted glycoprotein pigment epithelium-derived factor (PEDF) promotes NSC self-renewal within the murine SVZ [119, 122]. These secreted factors have complex but essential functions in regulating stem cell behavior. Thus, engineering strategies for identifying and dissecting these paracrine signals is a key objective within the field.

Integral membrane proteins that mediate juxtacrine (i.e. cell-cell contact dependent) signaling are another important class of molecules. For example, Ephrin receptor tyrosine kinases (Ephs) and their membrane-bound ephrin ligands allow for bidirectional communication between ligand-expressing and ligand-receiving cells [123]. Several studies have investigated Eph-ephrin signaling within adult NSC and intestinal stem cell niches. A and B subclass ephrins and Eph receptors have, for example, been suggested to regulate proliferation negatively within the adult SVZ of the lateral ventricles [124]. In the adult hippocampal niche, the presentation of ephrin-B2 by hippocampal astrocytes induces neuronal differentiation of NSCs [125]. Eph-ephrin has also been implicated in coordinating migration and proliferation of stem cells within the intestinal epithelium [123]. Notch receptors and their Delta-like or Jagged family ligands represent another key signaling pathway active between juxtaposed cells in adult stem cell niches [126]. For instance, niche ependymal cells and astrocytes in the early postnatal SVZ express Jagged1, which activate Notch1 and inhibit differentiation of neural progenitors [126]. Specifically, forced Notch1 activation was found to increase NSC proliferation, whereas Notch1 repression promoted cell cycle exit [127]. Additionally, inactivation of the Notch/RBPJκ signaling pathway in adult hippocampal stem cells resulted in the depletion of Sox2-positive neural precursors and long-term suppression of hippocampal neurogenesis [128]. Therefore, Notch is viewed as a regulator of cell cycle progression that also prevents premature NSC depletion [129]. Recent in vivo studies also revealed that Notch also plays an instructive role in biasing NSCs towards an astrocytic fate within the hippocampus [130]. While Notch signaling has been demonstrated to play a crucial role in NSC maintenance in the adult dentate gyrus, it also been shown to participate in regeneration of muscle.

Notch is active in quiescent muscle satellite cells; however, upon injury, muscle stem cells experience a downregulation of Notch signaling and accordingly exit their quiescent state [126, 131].

7 Early Approaches for Studying Stem Cell-Niche Cell Interactions In Vitro

A diverse spectrum of engineering strategies has emerged in the stem cell field for modeling and dissecting heterotypic cellular interactions within stem cell niches. Early efforts focused primarily on the use of bulk co-culture studies for elucidating the effects of cell-cell juxtacrine signaling and soluble paracrine factors. To study juxtacrine signaling, co-culture systems have seeded two or more cell types onto the same monolayer culture, yielding random heterotypic interactions. To study soluble paracrine factors, permeable transwell inserts have often been employed to separate two cell populations while allowing for the diffusion of soluble factors between cells. Additionally, applying conditioned media—i.e. medium that has been cultured with one cell type that contains paracrine factors—to stem cell cultures can achieve a similar result to the transwell system, with the caveat that particularly labile factors can undergo decay in conditioned medium. In either case, the degree of cell-cell signaling can be controlled by adjusting the cell numbers for each population [132]. Often, both direct co-cultures and transwell co-cultures are conducted in parallel to isolate the paracrine from juxtacrine effects.

This two-pronged strategy has proved useful in a variety of studies. Ottone et al., for instance, employed this approach for investigating how cell-cell contact-dependent signaling of vascular epithelium governs NSC behavior [133]. In doing so, they pursued both co-cultures and transwell cultures of NSCs with three types of murine endothelial cells: primary brain microvascular endothelial cells, brain microvascular endothelial cell line, and conditionally immortalized pulmonary endothelial cells. Direct cell contact between NSCs and all three cell types through bulk co-culture studies was found to induce cell-cycle arrest in the G_0–G_1 phase and thereby promote quiescence [133]. To assess whether this outcome resulted from contact-dependent signaling, cell-cycle profiles of transwell cultures were conducted in parallel and compared with NSC monocultures. Similar results between these two culture systems indicated that the observed quiescence was, indeed, a result of juxtacrine signaling from endothelial cells [133]. In addition, this study showed that NPCs cultured in contact with epithelial cells as opposed to cultured in transwells failed to produce differentiated progeny, instead maintaining multipotent $GFAP^{+}Sox2^{+}$ markers [133]. Song et al. [134] also exploited the advantages of the two co-culture systems to study how niche cell types within the hippocampus affect neurogenesis. When NSCs were plated in primary neuron-enriched cultures, they observed an increase in oligodendrocyte production and a lack of neurogenesis. In contrast, NSCs cultured on a feeder layer

of primary hippocampal astrocytes displayed a 10-fold increase in the percentage of differentiated neurons compared to control laminin-coated surfaces. To elucidate whether hippocampal astrocytes instructed neuronal fate commitment via paracrine or membrane-bound factors, NSCs were cultured in medium conditioned by astrocytes and found to result in a lower level of neurons [134]. These parallel cultures indicated that hippocampal neurogenesis stems from a mixture of soluble and contact-dependent cues. Later work by Ashton et al. [125] revealed that the juxtacrine signal responsible for neurogenesis was ephrin-B2.

Dual co-culture approaches have also played an integral role in helping dissect the contributions of neighboring niche cell types in influencing the behavior of other adult stem cell types. For example, Loibl et al. [135] utilized this strategy for studying whether endothelial progenitor cells (EPCs) promoted angiogenesis through the induction of a pericyte-like phenotype in MSCs, which can be identified by an upregulation of CD146, NG2, αSMA, and PDGFR-β. In a method analogous to that of Ottone et al., cell-cell crosstalk was investigated by comparing direct co-cultures to transwell cultures and single-cell type control cultures. After 3 days in the different cultures, they reported an approximate 15-fold increase of CD146 expression for the direct co-culture versus only a three-fold and two-fold increase for single and transwell cultures, respectively [135]. A similar but less pronounced trend in gene expression was observed for NG2. Additionally, for αSMA and PDGFR-β, MSCs in direct co-cultures were better able to maintain expression while the other cultures demonstrated decreases in expression [135]. These findings suggest that EPCs play a key role in mediating differentiation of MSCs into pericytes through cell-cell juxtacrine interactions [135]. Moreover, these findings (along with those of Ottone and Song) highlight the major role that direct co-cultures and transwell co-cultures have in elucidating the effects of cellular interactions within stem cell niches.

7.1 Patterned Bulk Stem Cell Co-Cultures

While random bulk co-cultures are useful tools for studying cellular interactions that may occur within the stem cell niche, there has been significant work in developing patterned co-culture systems. These platforms are motivated by two key advantages. The first is the enhanced spatial control for more precise manipulation of heterotypic cellular interactions. The second is the high reproducibility of patterning techniques, which ensures consistent cellular localization across multiple experiments for statistical analysis [136]. These spatially-defined in vitro culture systems are also deemed by some as more accurate predictors of heterotypic cell-cell effects as they better mimic the inherently structured cellular organization of in vivo microenvironments [137].

Soft-lithography techniques are broadly utilized for fabricating such platforms, where success depends upon one cell type preferentially attaching to patterned regions comprised of a particular type of ECM and a second cell type preferring the

unpatterned regions [136, 138]. Rodriguez et al. [138] demonstrated this strategy by combining microcontact printing with avidin-biotin chemistry to generate hMSC and human umbilical vein endothelial cell (HUVEC) co-cultures of various geometrical interfaces at both the multicellular and single-cell level (Fig. 9a). This specific strategy relied on the patterning of three distinct regions: adhesive, non-adhesive, and dynamically adhesive. Microcontact printing was first utilized to pattern regions of fibronectin, a cell-adhesive material, followed by the printing of neutravidin, an initially non-adhesive material. Pluronic F127 was physisorbed onto the remaining non-patterned regions to produce a nonbiofouling background. For cell patterning, the first population was seeded onto the substrate and attached to the fibronectin areas. Neutravidin was then dynamically switched from non-adhesive to adhesive upon addition of biotinylated fibronectin, which allowed for the selective patterning of the second cell type [138]. Fukuda et al. [139] employed an analogous strategy by utilizing capillary force lithography and layer-by-layer assembly of polyelectrolytes to demonstrate the capacity to establish patterned co-cultures of ESCs and NIH-3T3 fibroblasts (Fig. 9b). Specifically, glass substrates were patterned with cell-resistive hyaluronan (HA) utilizing capillary force lithography. This was achieved by placing a PDMS mold on top of a spin-coated thin film of HA and subsequently allowing capillary action to create a positive replica of the PDMS mold. Fibronectin was then deposited onto the HA-patterned substrate and adsorbed to the bare glass-exposed regions. ES cells then selectively adhered to the fibronectin patterns. In order to accommodate the secondary cell type, fibroblasts, collagen was deposited onto the surface, adhered to the HA regions, and switched the regions to cell-adhesive [139]. Such patterned co-cultures offer useful platforms for studying fundamental stem cell biology and even exploring various tissue engineering strategies, though they rely upon selectivity of ECM proteins that may, in many other cases, be somewhat promiscuous in their cell adhesive properties.

Another engineering approach for controlling heterotypic cellular interactions involves the utilization of microfabricated elastomer stencils, which are advantageous because they do not rely on patterning of ECM components. In this approach, stencils with a distinct pattern are coupled to a substrate, thereby physically blocking cellular adhesion to specific regions upon seeding of the first cell type. The stencil is removed to expose the previously covered underlying substrate, and the second cell type is seeded. Wright et al. [140] employed this strategy for creating static and dynamic co-cultures of mouse ES cells with fibroblasts and/or hepatocytes. The static co-culture was achieved by attaching a reversibly sealed parylene-C stencil with hole patterns of diameters ranging from 40 to 200 μm to a fibronectin-coated PDMS substrate. Upon attachment of ES cells to the exposed hole regions, the stencil was gently peeled off. AML12 hepatocyte cells were subsequently seeded on the cell micropatterned surface, filling in the unpatterned regions. In the case of the dynamic co-culture, the authors demonstrated the capacity for temporal regulation of cell-cell interactions, though efficiencies of the process were not noted. Specifically, ES cells were cultured with fibroblasts and hepatocytes in a sequential manner, thereby exposing ES cells to two different cell types (Fig. 9c) [140]. Unlike the static platform that accommodated only two cell

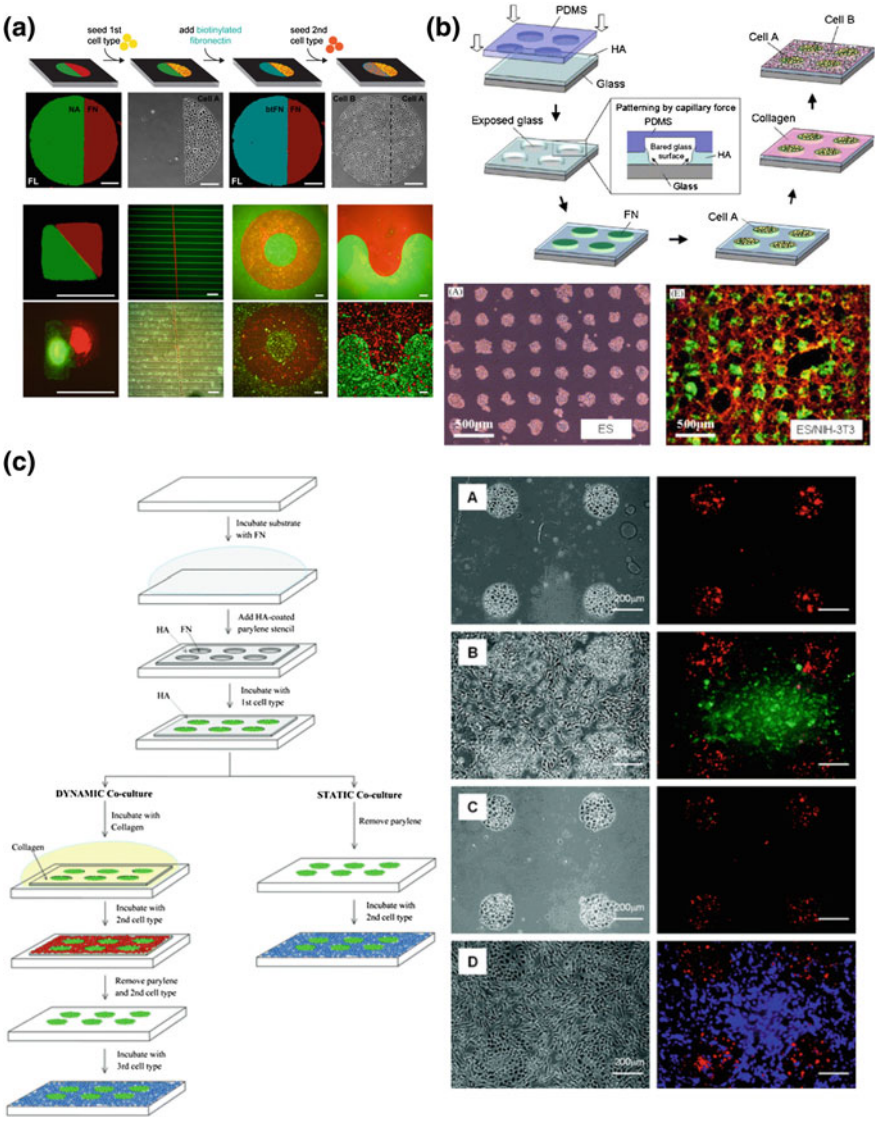


Fig. 9 Patterned bulk co-culture strategies. Panel **a** Patterning schematic for generating bulk and single-cell patterned co-culture systems; two MSC populations labeled with either CellTracker red or CellTracker green [138]. Panel **b** Schematic illustrating the use of capillary force lithography and layer-by-layer deposition for generating ESC (*green*) and NIH-3T3 (*red*) co-culture on a patterned HA/collagen surface [139]. Panel **c** Schematic for patterning static and dynamic co-cultures of mESCs (*red*), AML12 cells (*green*), and NIH-3T3 cells (*blue*) [140]

types, the dynamic platform utilized a parylene-C stencil initially treated with hyaluronic acid. ES cells were then seeded within the open hole patterns of the micro-stencil. To support the second cell type, collagen was absorbed onto the

HA-coated stencil, switching the non-patterned regions from cell repulsive to adhesive. Finally, ES cells were exposed to a secondary support cell by completely removing the stencil and seeding the third cell type [140]. This dynamic strategy has potential not only to elucidate how cues from other niche cells act independently but also for dissecting how these disparate cues may act in a combinatorial and hierarchical manner. Additionally, Wright et al. [140] claim that hole patterns on the parylene-C stencils could be fabricated down to a 3 μm diameter and can easily be adapted to support single-cell studies. These methods make elastomer stencils a powerful and unique engineering strategy for controlling heterotypic cellular interactions beyond two cell types.

7.2 Patterned 3D Stem Cell Co-Cultures

The push toward 3D patterned co-cultures has also been of recent interest within the stem cell field as they better emulate native cellular microenvironments within in vivo tissue niches. The drive from 2D to 3D has led to the development of many new engineering strategies. While micropatterning techniques generally manipulate cell-surface adhesion to obtain cellular patterns, this strategy cannot be applied for the formation of cell spheroids. Thus, additional approaches are required. Microfluidic methods encompass one such approach for generating patterned 3D co-cultures. Torisawa et al. [141] for instance, illustrated the ability to generate co-culture spheroids with various compositions and geometries (Fig. 10a). Their technique involved the fabrication of a two-layered PDMS device with two microchannels separated by a semi-porous membrane of polycarbonate. The top channel was dedicated to guiding the relative positions of the two cell types via laminar streams, thereby hydrodynamically focusing the cell populations into the bottom layer and ultimately controlling the geometry of the multicellular spheroids. Spatial control of these 3D co-cultures was achieved by changing the geometry of the bottom microchannel. With this system, Torisawa and colleagues patterned spheroids within a straight 200 μm channel, juxtaposing mouse ES cells with hepatocarcinoma HepG2 cells [141]. They demonstrated that ESC differentiation within the patterned co-culture spheroids revealed regional differentiation dependent upon initial cell-cell positioning. This microfluidic system was shown also to be compatible with other cell types and generated a variety of 3D co-culture spheroid patterns of breast cancer cells with HUVECs and monkey kidney cells. The capability of recapitulating more complex co-cultures was presented by patterning up to five distinct groups of cells (i.e. five alternating lines of cells with a total width of 1 mm that formed contacting spheroids after 3 days of culture) through the use of a five-inlet top channel [141].

Droplet microfluidics is another promising technique for generating high-throughput 3D cell co-cultures. Tumarkin et al. [142] utilized this technology to synthesize microgel emulsions that served as “micro-reactors”, in which discrete numbers of cells were compartmentalized to enhance heterotypic cellular

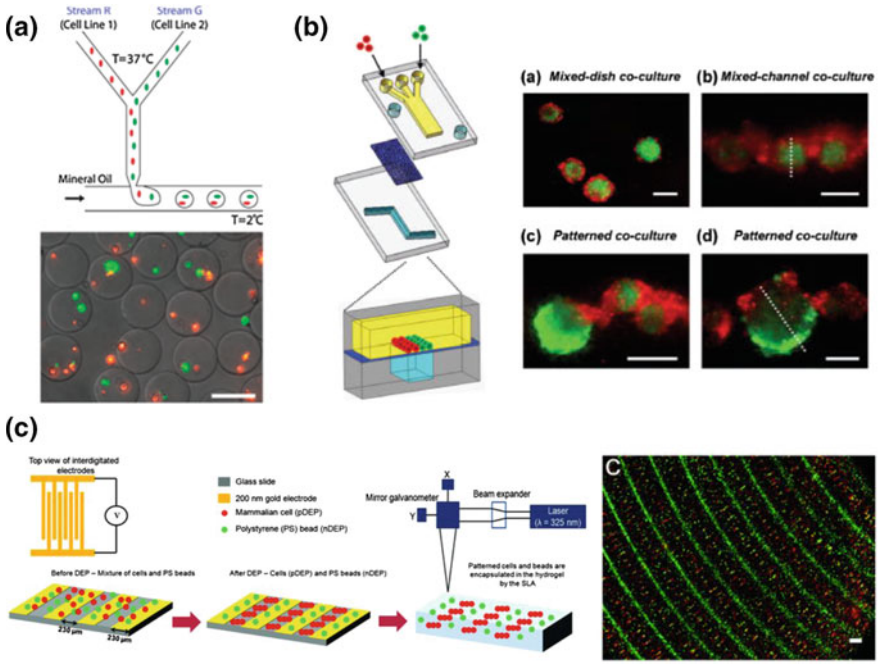


Fig. 10 Microfluidic strategies for generating patterned bulk co-cultures. Panel **a** Encapsulation of fluorescently labeled populations of mESCs into agarose microgels and formation of embryoid bodies after 4.5 days of culture [142]. Panel **b** Fluorescent images comparing 3D mixed dish versus patterned mESC spheroid co-cultures generated from a two-layered microfluidic device [141]. Panel **c** Patterning of mESCs (*red*) and polystyrene beads (*green*) using spiral electrodes [143]

interactions (Fig. 10b). The encapsulation of two different cell populations in agarose droplets was achieved using a T-junction microfluidic device. Co-encapsulation was tested on two populations of mESCs, where one was fluorescently labeled with a green cell tracker and the other labeled with a red cell tracker. Cells were suspended in agarose solution and supplied to the microfluidic device. Despite relying on random Poisson seeding, the relative cell numbers encapsulated from each population could be roughly controlled by tuning the ratio of flow rates for the cell suspensions. To generate droplets, a carrier phase of mineral oil containing 3 %(wt) of Span 80 surfactant was introduced perpendicular to the cell streams. Downstream of the junction, droplets were collected and cooled to induce gelation of the microgels, and analysis was conducted using optical microscopy and flow cytometry. Encapsulated cells not only demonstrated the ability to form embryoid bodies but also demonstrated viability approaching 80 % at the end of a 4.5-day culture [142]. These results are useful first steps, showing the viability of the technique for precisely encapsulating two different cell populations. Limitations of this strategy, however, include a practical restriction to two cell types due to Poisson statistics, an inability to control cell stoichiometry directly, and potential difficulties in extending the approach to adhesion-dependent cells.

The ability to control the assembly of heterotypic cellular interactions in 3D has also been demonstrated by Bajaj et al. utilizing a different microfluidic technique [143]. Dielectrophoresis (DEP), in combination with stereolithography and custom-made electrodes, was used to pattern and encapsulate two distinct populations of mouse ESCs within poly(ethylene glycol) diacrylate hydrogels of tunable stiffnesses (Fig. 10c) [143]. DEP refers to the induced motion of electrically polarizable entities (such as cells) when exposed to an electric field gradient [144]. Without dielectrophoretic forces, the two different cell populations exhibited minimal cell contact. However, upon inducing DEP by energizing the electrodes with an AC voltage, cell-cell contacts were stimulated and led to pearl chain geometries. In addition to patterning cells, Bajaj et al. extended this strategy to organize spheroids of cells spatially within hydrogels [143]. This method holds potential for enabling more robust investigations of stem cell-niche cell communication.

While microfluidics has been a key technology for generating in vitro platforms for studying juxtacrine signaling within stem cell niches, it has also played a pivotal role in elucidating the effects of paracrine signaling. Unlike standard cell-culture platforms, which are prone to unequal distributions of secreted factors, microfluidic devices utilize laminar flow to impose precise control of soluble factor profiles [145]. Microfluidic gradient generators, for example, have been employed for exogenous delivery of soluble factors (i.e. growth factors and cytokines) to stem cell cultures [146, 147]. Flow has also been used to modulate the distribution of secreted factors from niche cells to stem cells [148–150]. Moreover, another advantage of using microfluidics is the ability to isolate soluble factors for downstream analysis [145].

8 Shifting Focus to Single-Cell Resolution and Artificial Niches

The aforementioned bulk co-culture systems (both random and patterned) have yielded valuable insight into the effects of cellular signaling within stem cell niches. However, there are a number of additional features that would be advantageous to address. Micropatterned surfaces enable spatial control of cellular interactions yet can restrict cell motility and proliferation to chemically patterned regions [132]. Additionally, the ability to pattern more than two cell types remains a challenge. Microfluidic platforms, on the other hand, introduce shear forces, which may affect and bias stem cell behavior. Another significant concern with bulk co-culture systems is the difficulty in discerning each cell type's relative contribution to overall behavior [151]. In an attempt to address the latter issue, there is a growing focus within the stem cell field on developing engineering strategies that operate at the single-cell level. These types of systems allow for more focused and robust analyses of the effects of juxtacrine and paracrine signaling. Moreover, they hold potential for shedding insight onto the heterogeneity of intercellular interactions [145].

8.1 Microfluidic Approaches for Single-Cell Co-Cultures

The microfluidic field has fostered the development of a multitude of strategies to capture and pair different cell types at a single-cell resolution. Skelley et al. [152] presented a technique for individually pairing thousands of mouse ESCs with mouse embryonic fibroblasts at an efficiency approaching 70 %. Their microfluidic device consisted of a dense array of passive hydrodynamic traps, referred to as weirs, that operated via a three-step loading protocol (Fig. 11a) [152]. Each weir was comprised of a larger front-side cup optimized to accommodate two cells and a smaller back-side capture cup for temporary capture. mESCs were first flown toward the smaller back-side cups. Once cells fully occupied these cups, the flow direction was switched, and the captured mESCs were rapidly transferred to the large front-side cup. Fibroblasts were then flown in the same direction, trapped, and loaded adjacent to the captured mESCs [152]. Though these authors focused on applying the system to enhance cellular fusion, this platform also holds potential for

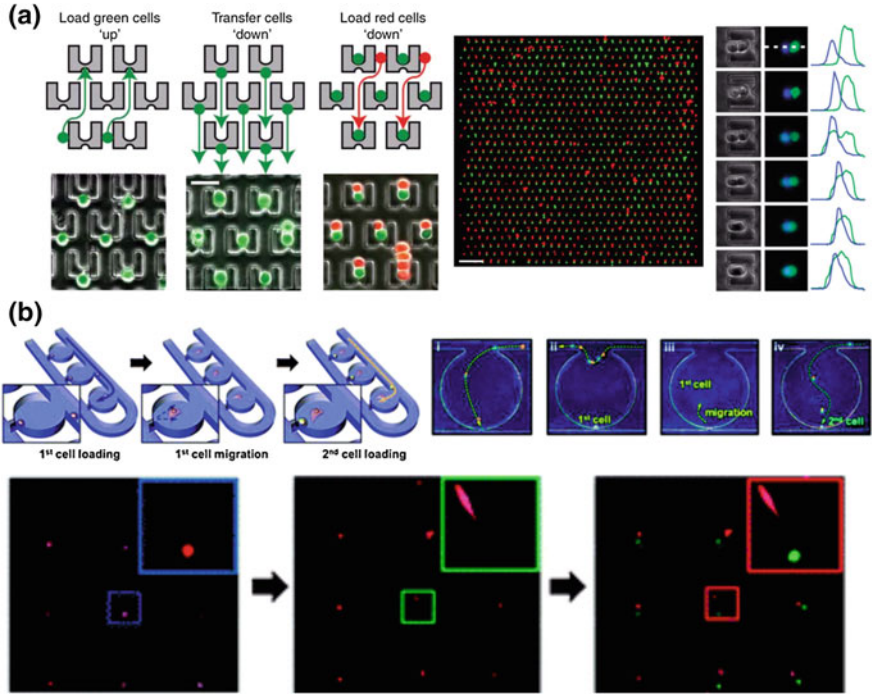


Fig. 11 Single-cell co-cultures using microfluidics. Panel **a** Cell-loading schematic for capturing cell tracker-labeled mouse 3T3s (*red* and *green*) (*left*); fusion of a paired green fluorescent protein-expressing mESC (*green*) and Hoerchst-stained mouse embryonic fibroblast (*blue*) (*right*) [152]. Panel **b** Overview of single-cell pairing protocol in which sequential trapping of mouse embryonic fibroblasts (*red*) and mESCs (*green*) is achieved [153]

elucidating the effects of heterotypic cellular interactions on dictating stem cell behavior, though attachment-dependent cells may pose a challenge.

Hong et al. [153] developed another microfluidic device that performed heterotypic cell pairing at a single-cell level and supported the culture and tracking of cell pairs over multiple generations. Rather than employing weir-based hydrodynamic traps, they relied on trapping junctions that implemented self-variable fluidic resistance to generate high-efficiency cell groupings (Fig. 11b) [153]. The basic principle of this approach is that, once cells enter the individual culture chamber and are trapped by small junctions located at the bottom of these chambers, fluidic flow resistance increases and blocks additional cells from infiltrating the chamber. Following capture of the first cell, cells are incubated to allow for migration away from the junction, resetting the traps to an "active" state and allowing for the capture of a second cell type [153]. Advantages of this device include high-throughput and minimized physical constraint to cell growth, allowing for multiple cell divisions and migration. Hong et al. [153] applied this system for the single-cell co-cultures of mouse embryonic fibroblasts and mESCs as a proof-of-concept.

Other microfluidic-based tools with considerable spatial control over sequential trapping and pairing of heterotypic single-cell pairs have been developed but not yet implemented within the stem cell field. The adoption of these emerging technologies offers potential for shedding light on the role of specific cellular interactions within stem cell niches. Dura et al. presented a deformability-based, cell-pairing device which utilized weir-based traps, similar to Skelley and colleagues [154]. However, upon capturing the first cell type, a transient increase in flow rate squeezed the arrested cells into the larger double-cell traps through constriction by flow-induced deformation (Fig. 12a) [154]. The second cell type was captured consecutively in a similar fashion. An advantage of this system is that paired cells were secured within the traps, allowing for the device to be disconnected and applied for other off-chip applications, while retaining cell pairing integrity. Dura et al. [154] also developed methods for pairing heterotypic cells of different sizes by tuning the geometry of the trapping structures. Finally, the ability to pair triplets of cells was illustrated, where one red fluorescently-labeled NIH3T3 fibroblast was sandwiched between two green fluorescently-labeled fibroblasts [154].

Frimat et al. [155] demonstrated another microfluidic approach for inducing single-cell co-culture contacts for studying the formation of gap junctions (Fig. 12b). To start, a microfluidic circuit based on differential fluidic resistance directed single cells into an array of trap structures within a superimposed serpentine channel. To capture a second single cell adjunct to the first, a second trapping structure was designed using a mirrored configuration. Despite the heterogeneous size characteristics of the cells employed (HT29 colon carcinoma cells, MCF-7 epithelial-like breast cancer cells, and SW480 epithelial cells), these cells were captured at an efficiency approaching 81 % with 96 % of cells retained within these traps during the first two days [155].

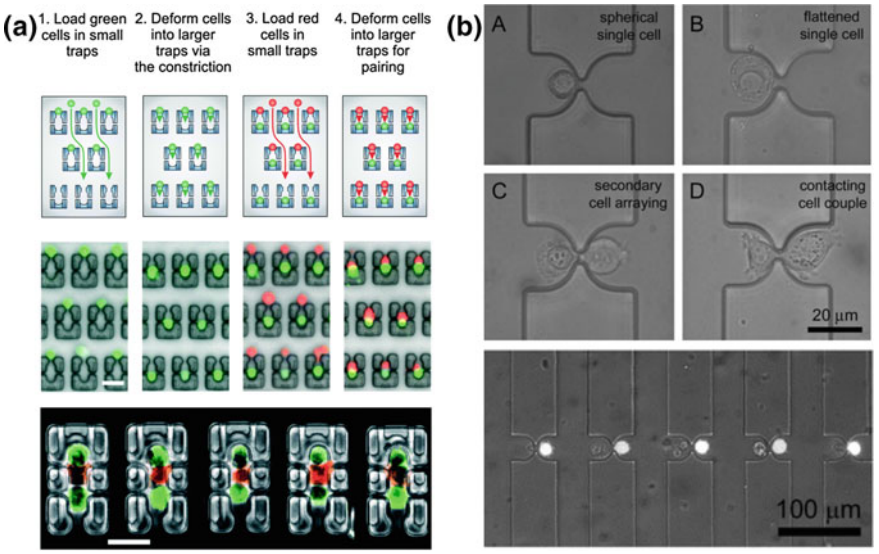


Fig. 12 Examples of additional microfluidic platforms with potential applications for studying stem cell-niche cell interactions. Panel **a** Loading protocol for pairing cells into traps possessing lock-in features (*top*); two-component and three-component pairings demonstrated (*bottom*) [154]. Panel **b** Heterotypic single-cell co-culture arrays, pairing one unlabeled SW480 cell with one fluorescently labeled with calcein AM [155]

8.2 Artificial Stem Cell-Niche Cell Signaling Approaches

The precise manipulation of different cell types remains an ongoing challenge within the field. While micropatterning and microfluidics enable more precise spatial control over the design of in vitro platforms, other approaches for dissecting cellular communication within the niche have been pursued. These approaches involve analyzing the natural complexity of cellular interactions and re-engineering more simplified versions in vitro. One notably powerful approach involves the immobilization of key cell surface ligands (cadherins, EpCAM, Delta-1, Jagged-1, and ephrins) to biomaterials as a means of mimicking communication from a secondary cell type.

Roccio et al. [156] demonstrated the fabrication of a microarrayed artificial niche platform dedicated to better understanding the role that the Notch ligand, Jagged-1, has on regulating single NSC behavior (Fig. 13a). A robotic spotter was utilized to immobilize the protein of interest to the bottom of PEG-based hydrogel microwells. Tethered Jagged-1 was found to increase survival and neurosphere-forming efficiency of single NSCs. They also assessed the potential synergistic effects of Jagged-1 in combination with Laminin-1 (though no additive effect was observed) [156]. In another system developed by the same group, a 3D-niche microarray system was presented that expanded the cell ligand repertoire to include E-cadherin

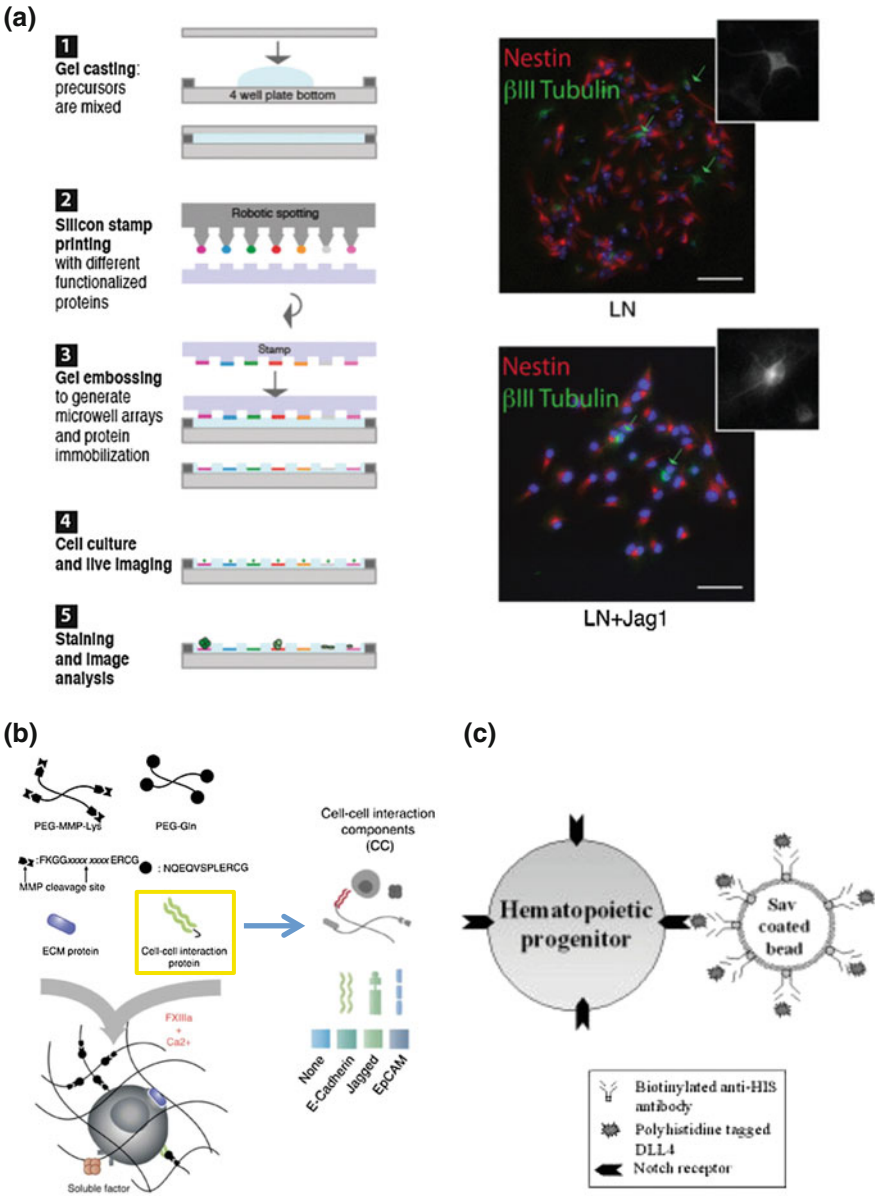


Fig. 13 Strategies for engineering artificial niche microenvironments that mimic cell-cell interactions. Panel **a** Overview of steps for fabricating microarrayed artificial niches (*left*); representative images of NSC cultures immunostained for Nestin (*red*) and βIII-tubulin (*green*) on hydrogels co-functionalized with Laminin-1 alone or Jagged-1 and Laminin-1 (*right*) [156]. Panel **b** Cell-cell interaction components incorporated into 3D microarray platform in combination with other factors (i.e. matrix elasticity, proteolytic degradability, cell density, ECM components, and soluble factors) for studying mESC behavior [157]. Panel **c** Illustration of DLL4-coated microbead interacting with Notch receptor on HSCs [158]

and EpCAM—not to mention a plethora of other key niche factors, including control over ECM stiffness, ECM components, soluble factors, cell density, and ECM degradability (Fig. 13b) [157].

An additional approach for developing functionalized biomaterials for mimicking cellular interactions was demonstrated by Taqvi et al. [158]. Magnetic microbeads were functionalized with the notch ligand, Delta-like ligand 4 (DLL4), thus creating a synthetic alternative to niche stromal cells that communicate with HSCs. Functionalization was achieved by first coating magnetic polystyrene microbeads with streptavidin. These beads were then washed and incubated with a biotinylated histidine tag antibody and, again, with the histidine-tagged DLL4 protein (schematic illustrated in Fig. 13c) [158]. This biomaterial-based artificial Notch-signaling system was utilized for investigating the induction of T-cell differentiation in HSCs [158]. This approach offers a simplified alternative to modifying niche stromal cells genetically to express Notch ligands followed by co-culture. More importantly, this system enables more thorough investigation of the effects of Notch ligand-receptor interaction. Quantitative and temporal studies are enabled by, respectively, tuning the ligand-cell ratio and duration of signaling. For instance, Taqvi et al. [158] found that a 1:1 bead-to-cell ratio generated a significantly higher T-cell differentiation efficiency when compared to a 5:1 functionalized bead-to-cell ratio.

9 Conclusions and Future Directions

Understanding the complexity of stem cell behavioral regulation remains a formidable challenge, and insights into the underlying mechanisms will greatly enable the development of stem cell-based therapies. The successful control of stem cell expansion and differentiation ex vivo in addition to the targeted activation of endogenous stem cell populations demands a comprehensive understanding of the regulatory role of environmental (i.e. niche) signals. Accordingly, the development of innovative engineering strategies for recapitulating key facets of stem cell-ECM interactions and stem cell-niche interactions has been instrumental in providing deeper insights into how stem cells respond to extrinsic cues at a molecular level.

Within the past few decades alone, the stem cell field has made tremendous progress in understanding these niche principles through initial strategies that focused primarily on fabricating static representations of niche ECM features (i.e. topography, matrix elasticity, ligand presentation, etc.) and bulk co-culture studies of heterotypic cellular interactions (i.e. paracrine and juxtacrine signaling). However, the desire to mimic dynamic in vivo niche phenomena has spurred the evolution of more sophisticated second-generation engineering tools. The push to incorporate spatiotemporal control into biomaterial systems has enabled an unprecedented ability for probing stem cell response to dynamic changes in the duration or intensity of presented ECM cues. In the case of studying niche cellular interactions, the robust isolation of single-cell co-cultures and the development of

artificial cell-signaling platforms allows for more controlled and reproducible study of cell-cell interactions.

As our knowledge of stem cell biology continues to expand, we anticipate that engineering strategies will also progress. Biomaterials with not only tunable but also reversible properties will be key for dissecting how stem cells respond to ECM-related signaling dynamics. For instance, biomaterials engineered to allow reversible stiffening *and* softening will be a significant advancement within the field. Additionally, platforms that allow for the ability to investigate combinations of ECM cues simultaneously and at different temporal onsets will be valuable for obtaining a more comprehensive understanding of stem cell niches that can ultimately be applied to accelerate the development of clinical applications. For studying the role of intercellular communication within stem cell niches, high-throughput strategies for creating precise cellular communities of more than two cell types at a single-cell resolution will reveal potential juxtacrine/paracrine signaling hierarchies. Another important advance would include engineering strategies that control the timing of cellular interactions to understand the duration of contact that is necessary to bias stem cell behavior towards a desired fate. With these advanced strategies in hand, the stem cell field will be better positioned to make stem cell therapies a clinical reality.

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Bioreactors and the Design of the Stem Cell Niche

Yongjia Fan, Donghui Jing and Emmanuel S. Tzanakakis

1 Introduction

Stem cells—especially human pluripotent stem cells (hPSCs)—are a promising inexhaustible source of cellular material for treating ailments such as cardiovascular, diabetes and Parkinson. Greater understanding of the stem cell niche, which is the specific microenvironment where stem cells reside and function, is critical for their study and applications. Mimicking the niche in vitro is essential for the propagation of stem cells using traditional static dish cultures and scalable bioreactors [1–5].

Signals from the surrounding milieu include soluble factors (salts, steroids, amino acids, growth factors, etc.), dissolved oxygen, extracellular matrix (ECM) for cell attachment, cell–cell interactions, mechanical forces and the scaffold or microenvironment conformation (i.e. two- (2D) or three-dimensional (3D) architecture) (Fig. 1). In this chapter, these factors are discussed in connection with their effects on hPSC proliferation and differentiation. Such discussion is particularly pertinent to processes for the culture of hPSCs intended for clinical uses. Motivated by the economical production of large quantities of stem cell derivatives, various

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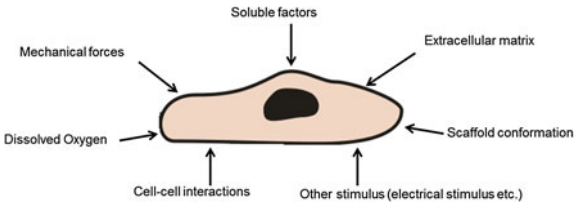

Fig. 1 Schematic representation of the microenvironment encountered by stem cells

platforms have been employed [6–8], for example, stirred suspension bioreactors [3], roller bottle systems [9] and rotating wall bioreactors [10].

2 Effects of Dissolved Oxygen

Oxygen tension is a critical factor of the physiological profile of the stem cell niche directly affecting the growth, viability and differentiation propensity of stem cells. During development, the embryo experiences a hypoxic environment in vivo [11–13] with the O_2 tension for human embryonic stem cells (hESCs) falling in the range of 1–9 % O_2, i.e. significantly lower than the ambient air O_2 fraction (21 %) [14] designated as the normoxic pO_2. Moreover, hESCs cultured under hypoxia (2–5 %) rather than normoxia exhibit reduced spontaneous differentiation and chromosomal abnormalities [15, 16].

Although sensing of O_2 tension and its influence on embryonic development and lineage specification are mediated through various processes, the family of hypoxia-inducible transcriptional factors (HIFs) is considered central to cellular responses based on the microenvironmental O_2 levels. In mouse ESCs (mESCs), HIF-2alpha (but not HIF-1alpha) binds to the promoter region of pluripotency marker gene *Pou5f1* (Oct4) inducing its expression [17]. HIF-1alpha, however, modulates Wnt/β-catenin signaling in mESCs and mouse embryonic carcinoma P19 cells by activating the expression of β-catenin and its downstream effectors LEF-1 and TCF-1 [18]. It should be noted that the effects of pO_2 on stem cell pluripotency or differentiation are species- and context-dependent. For example, hypoxia promotes the undifferentiated state of progenitor cells by blocking neuronal (in mouse neural stem cells) and myogenic (in mouse C2C12 cells) differentiation programs depending on the interaction between HIF-1alpha and the Notch-intracellular domain as shown in P19 cells [19]. Unlike the results in mouse cells, low O_2 tension (1–5 %) favors the expression of genes associated with endothelial differentiation and negative regulation of apoptosis in cultured H9 hESCs [20]. Cells grown at high O_2 tension (21 %) display more changes in genes related to division and O_2-based ATP production. Combined with media inducing differentiation, hypoxia (2 % O_2) promotes the chondrogenic differentiation of hESCs upregulating the production of collagens I & II and glucosaminoglycans [21]. Similarly, the culture of ESCs in 4 % O_2 yields an increased number of cardiac myocytes

(CMs) (3.77 ± 0.13 CMs/ESC) compared to normoxic cultures (2.56 ± 0.11 CMs/ESC) in the presence of appropriate differentiation-inducingfactors [22, 23].

Hypoxia also impacts mesenchymal stem cell (MSC) maintenance and differentiation. The self-renewal capacity of MSC populations is enhanced in hypoxic (rather than normoxic) cultures [24] concomitantly with increases in the expression of growth factors and their respective receptors. These findings suggest that the enhanced growth potential and preserved undifferentiated status can be attributed largely to the O_2-dependent gene expression in MSCs. Consequently, a lower pO_2 environment may facilitate overcoming issues including poor growth kinetics, genetic instability and poor engraftment after transplantation of hMSCs.

Thus, effective control of the pO_2 level in bioreactors becomes critical for stem cell expansion. When cells are cultured at low concentrations, the transfer of O_2 through the liquid surface (termed surface or overlay aeration) is sufficient to match the total cellular uptake rate of O_2. However, aeration through the air-liquid interface can be enhanced by O_2 enrichment of the overhead gas phase or headspace pressurization. Alternatively, the culture medium can be oxygenated via direct sparging, i.e. introducing air bubbles directly into the liquid phase. Sparging provides higher rates of O_2 mass transfer compared to the overlay aeration but may cause foaming or cell damage. This issue can be mitigated with microsparging in which a hydrophobic gas-permeable membrane is employed to provide bubble-free aeration [25]. Overall, overlay or headspace aeration is the most economical and least intrusive method generally used for low cell density/low working volume cultures or for cells exhibiting low O_2 uptake rates. Direct or open-tube sparging is generally preferable when stripping of system CO_2 is desired, whereas microsparging is most effective for cultivation at high cell densities. A schematic illustration of the aforementioned methods for supplying O_2 to cultured cells is shown in Fig. 2.

Different designs of bioreactors, which have been utilized for the culture of stem cells, offer alternatives for the large-scale culture of hPSC products [6–8]. Stirred suspension bioreactors are an appealing choice for large-scale cultures due to the

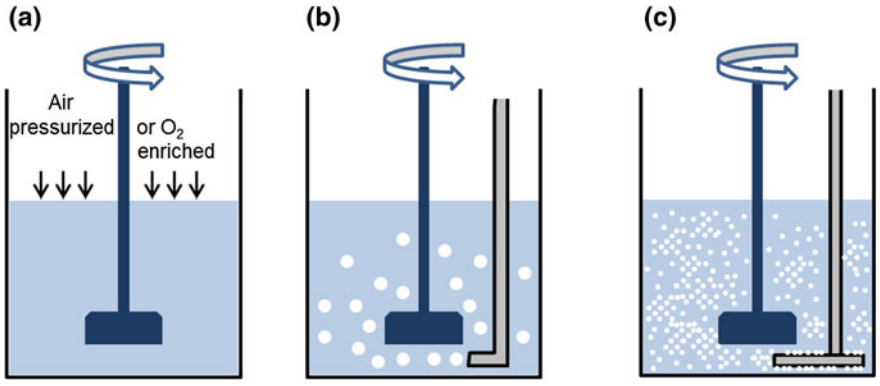

Fig. 2 Supply of O_2 to stirred-suspension bioreactors. **a** Overlay or surface aeration, **b** direct sparging or open-tube sparging, and **c** micro-sparging

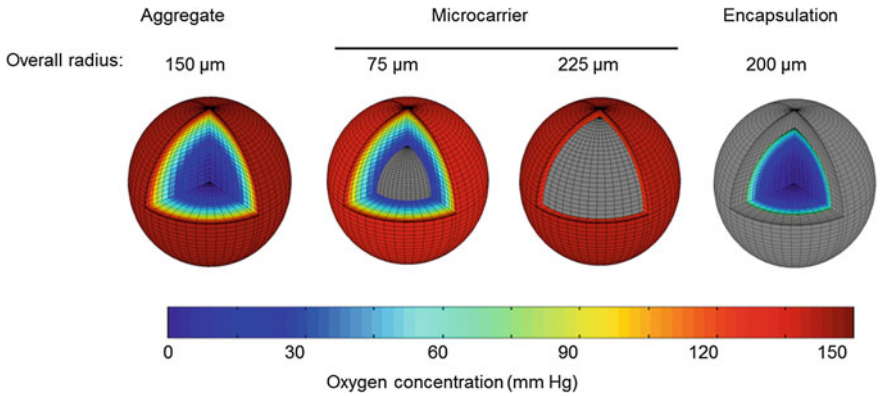

Fig. 3 Comparison of the O_2 diffusion profile within 3D cultures of hPSCs under different modes: aggregate, microcarrier and encapsulation in alginate beads. All models were run with the same volume/number of cells. Color regions represent the O_2 profile of hPSCs and *grey* regions represent different biomaterial positions (microcarriers and alginate capsule). For aggregate cultures, 150 μm hPSC clustered together to form a spherical model. For cell-loaded microcarriers, hPSC grew on the surface of microcarrier and two profiles were shown with microcarrier radii of 75 and 200 μm, respectively. For alginate encapsulation, a 200 μm bead of 1 % (w/v) alginate is shown encapsulating a 150 μm hPSC aggregate. Oxygen diffusion of 1 % alginate and hPSC were taken from Ref. [148]

homogenous environment and ease of operation and monitoring of culture. These bioreactors afford multiple culture modes including the cultivation of cells encapsulated, on microcarriers or as aggregates.

Using mathematical and computational models, the distribution of O_2 can be analyzed and predicted from experimental data. In a recent study, such data were collected from mouse and human ESC aggregates cultured in spinner flasks under different agitation rates [26]. At different time points and ultrastructural attributes (porosity and tortuosity) of aggregates, the effective diffusivity and the specific consumption rate of O_2 were calculated using a transient diffusion-reaction model coupled to a population balance equation (PBE) capturing the dynamics of cell aggregation. The model facilitated the calculation of the O_2 distribution in the medium and within the aggregates in spinner flasks. As a result, not only the fraction of cells experiencing hypoxia was predicted but also the 'residence time', i.e. the duration the cells experience O_2 concentrations within a particular range.

The availability of O_2 and nutrients to stem cells also varies depending on the culture mode (Fig. 3). Stem cells residing near the center of aggregates may experience hypoxia directly affecting their viability. The spatial gradient of O_2 can impact the proliferation and differentiation propensity of stem cells [27, 28] and their encapsulation (e.g. in alginate beads) poses an additional barrier to O_2 transport [29] reducing proliferation beyond the effect of scaffold rigidity. In contrast, cells grown on microcarriers experience O_2 and nutrient levels close to those in the medium bulk. Among different size microcarriers [30–32], those with a diameter of $\sim$200 μm expose cells to higher O_2 levels compared to those with a

size of 75 μm according to model prediction [26]. This is because stem cells on the microcarrier surface are assumed to be configured akin to cells in monolayers. In practice, cell-laden microcarriers form agglomerates posing additional restrictions to the exchange with the medium of O_2, nutrients and secreted molecules. Therefore, the culture configuration is a critical factor determining stem cell fate in addition to the chemical and biological properties of the scaffolds employed.

In conclusion, dissolved O_2 is a culture parameter affecting both stem cell growth and fate decision. Insufficient O_2 transfer can be detrimental by resulting in delayed growth rate and apoptosis. On the other hand, increased or uncontrolled O_2 supply may lead to commitment along undesirable lineages making obvious the need for fine tuning and monitoring oxygenation throughout the entire culture process.

3 Soluble Factors and the Stem Cell Niche

Soluble factors including proteins, salts, lipids, vitamins, cytokines and other small molecules play critical roles in maintaining the undifferentiated state of stem cells as well as guiding their lineage commitment. Soluble factors trigger cellular responses through multiple signaling pathways targeting gene networks which regulate the fate of stem cells [33, 34]. The transforming growth factor-beta (TGF-β) super family-activated cascades, receptor tyrosine kinase (RTK) signaling, canonical Wnt signaling [35, 36], and pathways activated by insulin or insulin-like growth factors (IGFs) [37, 38] participate in directing stem cell fate. Targeted gene networks include transcriptional factors [35, 39, 40], such as Nanog, Oct4 and Sox2 [39, 41, 42]. There is also a divergence in the cascades maintaining the pluripotency of mESCs and hESCs. Bone morphogenetic proteins (e.g. BMP4) and the JAK/STAT signaling activator, leukemia inhibitory factor (LIF), are sufficient to sustain the pluripotent state of mESCs but not of hESCs in vitro [43–46]. Instead, TGFβ signaling is important for preserving hPSC pluripotency [47–49].

Besides TGFβ signaling, basic fibroblast growth factor (bFGF) (RTK-type) signaling is another important pathway for hESC self-renewal. Basic FGF is a universal supplement in media for routine maintenance of hPSCs regardless of the use of feeder cells or serum [50, 51]. For hPSCs cultured on mouse embryonic fibroblasts (mEFs) [52] or in mEF-conditioned medium [53], the bFGF requirement (4 ng/ml) is lower than for feeder-free cultures (40–100 ng/ml) [50, 54, 55].

The roles of Wnt/β-catenin and BMP signaling have also been studied in sustaining hPSC self-renewal [56, 57]. Recombinant Wnt3a does not appear to suffice for the maintenance of undifferentiated hESCs without feeder cells [58] although caution should be exercised about the requirement for Wnt ligand supplementation given the disparate levels of endogenous canonical Wnt signaling among hPSC lines. The BMP antagonist noggin on the other hand, supports the uncommitted hESCs in non-conditioned medium containing 40 ng/ml bFGF but this effect is abolished when bFGF is supplemented at 100 ng/ml [59].

In the early days of hESC culture, those critical factors were supplemented with the addition of fetal bovine serum (FBS) or knockout serum replacer (KSR) to the medium [60]. However, the presence of undefined, non-human components (e.g. Neu5Gc [61]) in these supplements is not desirable for clinical applications and has motivated efforts toward the design of xeno-free systems for the culture of hPSCs and their products. The development of chemically defined media requires scrutiny of the stem cell niche for the identification of core elements stimulating and maintaining the propagation of stem cells in vitro [33, 62–64]. Basal media (e.g. DMEM or DMEM/F12) serve as sources of glucose, vitamins and salts at appropriate osmolarity for cell survival and proliferation. Growth factors specific for stem cell self-renewal are typically supplemented separately to the basal medium. For example, defined media consisting of DMEM/F12, 100 ng/ml bFGF and components such as TGF-β1, LiCl, insulin, gamma-aminobutyric acid (GABA) and BSA or human serum albumin (HSA) are routinely used for hPSC maintenance in vitro both in dishes and scalable stirred-suspension vessels [65, 66].

Despite the significant advances in designing and developing fully defined xeno-free media for stem cell cultivation, significant issues still remain. Almost all media for hPSC culture currently in use require daily exchanges which are costly and labor intensive. Even with frequent replacement, fluctuation of growth factor levels is unavoidable especially given the half-life of ligands in cultures. For instance, the human or zebrafish bFGF loses more than 40 % of its activity within 24 h [67]. This introduces variability to the culture impacting adversely stem cell proliferation and performance. A proposed solution to this problem is the incorporation of controlled release vehicles in the culture system facilitating the extension of growth factor or cytokine availability (and degradation) in the culture. Basic FGF-loaded PLGA microspheres can be added to the hPSC cultures reducing the frequency of medium changes from daily to every three days or biweekly [68]. Moreover, to deal with the labile nature of stem cell medium supplements and their high cost, researchers have turned to small molecules displaying similar bioactivity to native or recombinant proteins. Trimipramine and ethopropazine are two examples of small molecules with longer degradation times than bFGF and supporting hESC self-renewal in lieu of exogenously added bFGF [69, 70].

4 Extracellular Matrices for Stem Cell Cultivation

Extracellular matrix (ECM) proteins such as laminin, fibronectin, vitronectin, entactin, tenascin and collagen are critical for cell adhesion, survival, growth and differentiation [71]. Distinct domains on these molecules interact with cell surface receptors (e.g. integrins) mediating adhesion and triggering signaling cascades linked to cell fate adoption processes [72, 73].

Since first isolated, hESCs have been co-cultured with layers of mEFs which secrete various (mostly undefined) factors supporting the pluripotency of hESCs. Those cells include human fetal foreskin fibroblasts [74–77], adult epithelial cells

[78], bone marrow cells [79, 80] and placenta-derived feeder cells [81, 82]. Matrigel, which is an ECM mixture produced by Engelbreth-Holm-Swarm mouse sarcoma cells, was introduced and served as an alternative for the feeder-free maintenance of stem cells. Matrigel contains various ECMs such as laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, and nidogen [45, 83], and its use in hPSC cultures is straightforward compared to feeders. However, its composition remains undefined and variable between batches paralleling issues plaguing the use of mEFs.

Whether particular ECM molecules support cultured stem cells has been the focus of multiple published studies. The arginine-glycine-aspartic acid ('RGD') motif featured in various ECM proteins (e.g. laminin, vitronectin, fibronectin [84–86]) is a binding domain for cellular integrins. A mixture of vitronectin, fibronectin laminin and recombinant human collagen IV was demonstrated to promote the growth of hESCs over multiple passages [65]. However, the capacity of individual ECM proteins to support hPSC adhesion and growth is variable and highly dependent on the culture medium utilized. For instance, laminin binds to at least 8 integrin heterodimers including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, $\alpha7\beta1$, $\alpha9\beta1$, and $\alpha v\beta3$ [87]. Among those, $\alpha6\beta1$ is expressed in hESCs and is significant for their adhesion [83]. Indeed, natural or recombinant laminin maintains the growth and pluripotency of hESCs in mEF-conditioned medium [83, 88]. However, human placenta-derived laminin does not support hESC self-renewal beyond 3 passages in medium without serum or serum replacer [89]. Over a longer term (>10 passages), laminin failed to maintain the undifferentiated state of hESCs, which displayed reduced proliferation, widespread spontaneous differentiation and poor adhesion [90]. Similar to laminin, vitronectin and fibronectin mediate cell adhesion through the binding of integrins such as $\alpha_v\beta_5$, $\alpha_5\beta_1$ $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ [90–92]. Vitronectin from human plasma supports the growth and self-renewal of hESCs for over 20 passages without compromising their differentiation potential [93]. A chimeric glycoprotein based on vitronectin was shown to support the growth of undifferentiated hESCs in defined medium [73]. Conversely, hESCs could not be maintained on vitronectin for more than 7 days in defined medium [94]. Fibronectin isolated from human plasma promotes hESC proliferation and pluripotency in defined medium for more than 10 passages [91, 94] but others were unable to grow hESCs on fibronectin-coated surfaces in the absence of mEF-conditioned medium [90]. The discrepancies in the reported results may be due to the differences in cell lines, culture media and the length of culture (e.g. number of passages, time between cell splittings etc.). These differences also highlight the complexity of individual ECM components and their roles in supporting hPSCs in culture. They further emphasize the importance of considering multiple aspects of the culture system including the medium used.

Due to the varied performance of natural or recombinant ECM proteins as hPSC culture matrices and their high cost, efforts have been directed toward the development of synthetic ECMs. One strategy is to synthesize substrate peptides featuring known binding domain motifs such as the RGD sequence [95, 96]. Synthetic peptide sequences derived from natural ECMs like fibronectin, bone sialoprotein and vitronectin have been covalently attached onto acrylate-coated surfaces for

stem cell attachment in both feeder cell-conditioned (10 passages) and defined media (at least 5 day) [97, 98]. Synthetic peptides with binding domains require additional optimization and testing since not all resulting sequences are suitable for stem cell culture.

Besides synthetic peptides, synthetic polymers have also been investigated for hPSC maintenance due to their lower cost and higher availability compared to other alternatives [99, 100]. For example, poly(methyl vinyl ether-*alt*-maleic anhydride) [PMVE-*alt*-MA] was shown to support the long-term propagation without differentiation of three hPSC lines for five passages. Screening of 91 different polyacrylamide polymers yielded 16 candidates supporting hESC proliferation in 5-day cultures [100]. Polymers with ester ions and cyclic polymer ions were also demonstrated to promote hPSC adhesion [101].

Flat (2D) stem cell cultures afford convenience but 3D configurations mimic more closely the natural niches of stem/progenitor cells. To that end substrates which can be used with 3D hPSC culture systems are highly desirable. Hydrogels are commonly used to create 3D microenvironments in vitro. For example, scaffolds of 2.4 % (w/v) alginate and 2.4 % (w/v) chitosan prepared by lyophilization can be utilized to maintain hESCs over 21 days [102]. Indeed, scaffolds of alginate and chitin support various ESC lines for more than 10 passages [103] and alginate alone has been used for creating 3D niches for stem cell differentiation [104, 105]. Additionally, incorporation of poly(γ-glutamic acid) [γ-PGA] in alginate capsules promotes neural differentiation [106].

Hydrogels based on poly(N-isopropylacrylamide-co-acrylic acid) [p(NIPAA-co-AAc)] and Gln-Pro-Gln-Gly-Leu-Ala-Lys also support hESC growth [107]. This polymer can be degraded by collagenase facilitating downstream separation of the cells from the matrix. Hyaluronic acid, which is present during early embryo development, is used to prepare hydrogels for hESC proliferation and differentiation [108].

In addition to the composition, scaffold ultrastructure affects stem cell proliferation and differentiation. Beside gel matrices, fibrous scaffolds have also been reported to support the proliferation of stem cells. A fibrous scaffolds made from poly(desaminotyrosyl tyrosine ethyl ester carbonate) [pDTEc] and coated with poly-D-lysine was suitable for maintaining cultured hESCs for 14 days [109]. Poly (methacrylic acid)-coated carbon nanotubes, which are similar in scale to collagen and laminin moieties, also reportedly promote hESC proliferation and neuronal differentiation [110, 111].

5 Mechanotransduction and Stem Cells

Cells in the human body are constantly exposed and respond to mechanical forces and during development mechanotransduction influences differentiation and tissue morphogenesis. Similar observations are noted in vitro with external mechanical and electrical stimuli modulating the morphology, proliferation and specification of stem cells [112–114]. For example, cyclic strain not only inhibits the proliferation

of bone-marrow derived progenitor cells but induces the alignment of the F-actin cytoskeleton perpendicularly to the strain direction [113]. Under 10 % continuous cyclic strain (0.5 Hz) for 7 and 14 days, human intraoral mesenchymal stem and progenitor cells undergo osteogenic differentiation expressing markers such as type-I collagen (Col1A1), osteonectin (SPARC), bone morphogenetic protein 2 (BMP2), osteopontin (SPP1), and osteocalcin (BGLAP). Furthermore, significantly higher amounts of calcium and alkaline phosphatase (ALP) are observed in mechanically stimulated groups of cultured cells [115]. In contrast, commitment of MSCs under mechanical compressive force toward adipose cells is inhibited [116].

Apparently, the pattern of exerted mechanical forces is also important for stem cell differentiation as demonstrated by Park and co-workers [117]. The differentiation of MSCs toward vascular smooth muscle cells (SMCs) was reportedly promoted by cyclic uniaxial strain but inhibited by equiaxial strain based on the expression level of smooth muscle alpha-actin (SM α-actin) and SM 22α (a calponin-related protein). Moreover, transient expression of collagen I increased under uniaxial but not equiaxial strain. Cyclic strain also induces vascular smooth muscle cell differentiation of mESCs with the resulting cells orienting perpendicularly to the direction of strain. The authors attributed the differentiation to the activation by cyclic strain of the beta-type PDGF receptor (PDGFRB) in a ligand-independent manner. In hESCs, the tendon-specific transcription factor scleraxis (SCXA and SCXB) and mechanical stimulation synergistically promote commitment to tenocytes. This is achieved by inhibition of the osteogenic differentiation of hESC-derived MSCs through the antagonizing BMP signaling pathway [118].

The above reports illustrate that the effects of mechanical stimulation on the differentiation and proliferation of stem cells are not universal but depend on the lineage, environment, duration and magnitude of the strain and even the direction of the causative force(s). For example, in a bioreactorsystem, which was customized to study cell responses upon cyclic compressive strain in a hydrogel scaffold [119], bone marrow hMSCs expressed chondrocytic genes within 3 weeks of culture. However, under the same conditions, chondrocytic gene expression of hESC-derived cells in EBs was significantly down-regulated. After initiation of the chondrogenic differentiation, this reduction in gene expression was reversed with the addition of TGF-β1.

Moreover, Nanog in mouse ESCs is significantly downregulated while endoderm markers emerge after 2 days of exposure to cyclic stretch [120]. Unlike for mESCs however, cyclic strain appears to support the pluripotent state of hESCs. The percentage of SSEA-4^+ cells at 10 % strain of 30 cycles per minute (0.5 Hz) is reduced from 85 to 36 % with the reduction of strain to 8 % at 0.167 Hz [121]. Similarly, the fraction of $Oct4^+$ cells went from 21 % without strain to 67 % after almost 2 weeks of strain [122]. More recently, iPSCs subjected to cyclic strain showed enhanced formation of stress fibers and downregulation of Nanog, Oct4 and Sox2 [123]. These findings support the notion that hPSC self-renewal and differentiation are influenced by both mechanical forces and chemical signals. From a signal transduction viewpoint, the Rho/ROCK is a primary transducer of the effects of mechanical forces in pluripotent stem cells and acts as an upstream regulator in

pluripotency-related signaling pathways as suggested by the activation of small GTPase Rho and decreased AKT phosphorylation. The pluripotency of stem cells seems to be maintained by extended cyclic strain and this effect is reversed with the short application of higher-magnitude strain.

Application of mechanical forces to induce stem cells differentiation, for example to cardiovascular cells, has been reported. When sheep bone-marrow derived MSCs seeded onto a novel flex-stretch-flow (FSF) bioreactor, more extensive heart valve tissue formation was observed under flex-flow conditions (combined cyclic flexure and laminar flow) compared to cells subjected to cyclic flexure, laminar flow or typical static culture [124]. Others also showed that human MSCs in biaxial rotating bioreactors (BXR) have higher cellularity, confluence and more robust osteogenic differentiation than cells in spinner flasks, perfusion or rotating wall bioreactors [125].

Mechanical stimulation is caused by fluid shear stress due to agitation in stirred tank bioreactors and can potentially affect stem cell fate decisions. Although agitation is critical for ensuring a homogenous environment, a high stirring rate may result in greater shear stress to the cells. A window of acceptable agitation speeds for an operating bioreactor can be calculated based on the specific energy dissipation rate:

$$\tau\gamma = \frac{P}{V} \tag{1}$$

where γ is the average shear rate (s^{-1}) and τ is the shear stress (Pa), P is the power input (W) and V is the volume of the fluid in the vessel (m^3). The power input P is the amount of energy provided to the impeller for the rotational mixing.

By introducing the fluid's viscosity, μ ($=\frac{\tau}{\gamma}$; Pa s), the above equation yields

$$\gamma = \left(\frac{1}{\mu} \times \frac{P}{V}\right)^{0.5} \tag{2}$$

Theoretically, the average shear rate depends on the working volume in bioreactors, the viscosity of the fluid, and the power input. Several empirical equations to calculate the shear rate γ and its maximum, γ_{max}, in the impeller zone of stirred tank bioreactors are summarized by Sánchez Pérez et al. [126]. The average shear rate in Newtonian and non-Newtonian media in a stirred tank is proportional to the impeller speed N for laminar flow or $N^{3/2}$ for turbulent flow.

For bioreactor scale-up, the power input per volume (P/V) is kept fixed and calculated as:

$$\frac{P}{V} = q\rho N^3 D^5 / V \tag{3}$$

where q represents the power number. This is a property of the impeller and is generally supplied by the manufacturer. The density ρ of the fluid and the diameter

D of the impeller are also utilized in the calculations. The agitation rate after scaling up the volume of a bioreactorcan be calculated while keeping P/V constant. For example, when the working volume within one bioreactor is increased from V_1 to V_2, then agitation rate can be increased from N_1 to N_2:

$$N_2 = \left(N_1^3 V_2 / V_1\right)^{1/3} \tag{4}$$

Even if bioreactors with different impeller designs are used within a particular process, the media within both vessels can experience the same P/V. As long as the power numbers q of both bioreactors are known, the agitation rates can be determined based on Eq. 3.

In a fluid flow bioreactor, Wolfe et al. [127] demonstrated the application of steady laminar shear force in the range of 1.5–15 dynes/cm^2 to mESCs. Specification to ectodermal and mesodermal lineages depended on the magnitude of the applied force. The upregulated expression of Brachyury (T) and corresponding reduction of alpha-fetoprotein (AFP) corresponded to the increase of shear stress for 1–4 days. These changes transpired concurrently with the activation of Wnt and estrogen signaling pathways. The same group later reported that during early mESC differentiation fluid shear similarly promotes endothelial and hematopoietic differentiation for cells seeded on collagen-, fibronectin- or laminin-coated surfaces. The induction of endothelial differentiation was apparent by increasing the duration of culture under stress but hematopoiesis was less efficient at later stages. It was suggested that the membrane protein FLK1 (a VEGF receptor) is a critical regulator of fluid shear stress-induced differentiation to endothelial and hematopoietic linages [128].

6 Effects of Electrical Stimulation on Differentiating Stem Cells

Electrical stimulation has been shown to have beneficial effects for progenitor cells differentiating toward electrically active cell types including neurons and heart cells. To that end, culture (mainly 2D) of human cardiomyocyte progenitor cells has been combined with electrical stimulation leading to a significant increase in the expression of markers such as GATA4, MEF2A, structural protein genes and those related to Ca^{+2} handling [129]. Electrically stimulated adult neural stem progenitor cells give rise to neurites which are five times longer (up to 600 μm) compared to those from non-stimulated cells. Moreover, the cells display mature neuronal morphologies, expression of β-III tubulin, NeuN, organized filamentous actin (F-actin) and intracellular Ca^{2+} signaling akin to native cells [130].

Electrical stimulation in bioreactors combined with nutrient perfusion and unconstrained tissue contraction was also reported [131]. In this study, neonatal rat cardiac cells were seeded in a scaffold placed in a bioreactor with the simultaneous application of electrical stimulation and perfusion. The stimulated culture showed

improved function, expression of cardiac proteins, cell distribution and overall tissue organization over control (unstimulated) groups. Similar improvement through electrical and mechanical stimulations was also reported by Miklas et al. [132] using a microfluidic bioreactor with neonatal rat cardiomyocytes. These results indicate that elevated amplitude of contraction and improved sarcomere structure are observed in cells exposed to electrical and mechanical stimulations concurrently versus cells subjected to electrical or mechanical stimulation alone.

7 Microfluidic Technologies for Studying the Stem Cell Niche

The emergence of microfluidic technologies has opened new avenues for studying the stem cell niche. Microfluidic devices featuring patterns of tens to hundreds of micro-scale channels on customizable substrates and accessible for observation, for example, by fluorescence microscopy, allow the culture and real-time monitoring of stem cells as they proliferate and differentiate. Moreover, laminar flow profiles with well-defined and controlled dynamics can be achieved in conjunction with diffusive mixing for studying stem cell interactions in the niche [133, 134].

The miniaturized size and minute amounts of reagents required have made microfluidic platforms the tool of choice for high-throughput assays, including those for investigation of the stem cell microenvironment. Beyond screening a wide range of conditions, microfluidic devices afford greater flexibility versus traditional dish cultures as perfusion and 3D culture conditions can be incorporated [135–140]. A high-throughput microfluidic device featuring 1600 culture chambers of 4.1 nL each, was utilized for investigating the heterogeneity exhibited by hematopoietic stem cell (HSC) populations. For this purpose, proliferation was tracked at a single-cell level with dynamic medium exchange [141]. Furthermore, Steel factor (SF) was shown to regulate the survival of cytokine-activated HSCs within 16 and 24 h of being placed in vitro without an effect on the early division kinetics of the surviving cells. These results point to a regulatory role of SF when HSCs exit the G_0 phase to enter G_1. Beyond its suitability for high-throughput experimentation, the system allows medium replacement without disturbing cultured cells and makes possible the rapid and accurate generation of colony growth curves. Similarly, a microfluidic system with 96 chambers was engineered to study the effects of various combinations of cell seeding density, medium composition and feeding schedule on hMSC proliferation and osteogenic differentiation [142]. Cells treated with differentiation medium for 18 h or longer displayed significantly different motility compared to nonstimulated cells. In fact, cells stimulated for less than 96 h progressively adopted the same level of motility after stimulation ended as control cells indicating that the effects of exposure to osteogenic medium were reversible. Such studies would have been impractical to carry out in dishes or regular-scale bioreactors.

Microfluidic environments can also mimic aspects of the complex microenvironment of stem cells. Based on an Y-channel geometry, two streams of different media were combined directed toward single mouse embryoid bodies (EBs) located at the point of convergence of the channels. Cells on the EB side facing the combined streams were coaxed to neural fates but the other half of the EB remained undifferentiated [143] demonstrating that cell commitment may be controlled by simple changes in the flow of media containing differentiation cues.

In fact, microfluidic devices can be used for generating flows with a high Péclet number at low Reynolds regimes forming continuous gradients of soluble factors by diffusive mixing [144]. For example, human neural stem cell (hNSC) differentiation was studied in a microfluidic device in which concentration gradients were produced of three growth factors: epidermal growth factor (EGF), bFGF and PDGF. Cells were exposed to these gradients under continuous flow for more than 1 week. Astrocyte differentiation of the hNSCs was proportional to growth factor gradients without any threshold effects. The setup can be used to quantify the graded responses of cells to multiple concentration gradients of differentiation cues within a single chamber [145].

Given their small scale and high degree of control, microfluidic platforms are important tools for detailed studies of the stem cellniche. Outcomes from these studies can contribute to the development of strategies for the economical and effective culture of large quantities of stem cells. However, translation of findings from nano- or micro-liter scale systems to bioreactors with at least 6 orders of magnitude greater working volumes should be done with caution.

8 Conclusions

The stem cell niche comprises a complex assortment of cues driving proliferation and fate selection processes. Mimicking this microenvironment in different culture systems is a challenging task hampered not only by the large variety of growth factors and cytokines involved (with multiple remaining still elusive) but also by the combinatorial effects of their synergistic activities. Moreover, there are fewer stem cell cultivation platforms affording dynamic (as opposed to static) environments akin to those which the cells experience during embryonic development. To that end, continuous flow microbioreactor arrays may be a promising candidate system as these afford the application of statistical factorial multiplexing of multiple input factors involved in stem cell signaling hierarchies through high throughput culture chambers [146, 147]. The design and optimization of a relatively complex 3D culture system will require the systematic synthesis of the parameters and factors many of which have been summarized here. The progress noted to date in this area is deemed very promising for the development of fully automated, robust and scalable processes for the production either in the laboratory or commercially of stem cell products for regenerative medicine, tissue engineering, and drug discovery.

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Stem Cell Niche-Radiobiological Response

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Abbreviations

α-SMA	α-smooth muscle actin
Akt	Serine/threonine-protein kinase
ALP	Alkaline phosphatase
Ang1	Angiopoietin-1
ARE	Anti-oxidant response element
Bak	BCL-2-antagonist/killer 1
Bax	BCL-2-like protein 4
bFGF	Basic fibroblast growth factor
BFU-E	Burst forming unit-erythroid

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BM	Bone marrow
CAR	CXCL12-abundant reticular cells
CD	Cluster of differentiation
CFU-E	Colony forming unit-erythroid
CFU-F	Colony-forming unit-fibroblast
CFU-GEMM	Colony forming unit of granulocytes, erythrocytes, monocyte/macrophages, and megakaryocytes
CFU-GM	Colony forming units of granulocytes and monocyte
c-Kit	Cellular receptor-type tyrosine kinase
COX2	Cyclooxygenase-2
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	C-X-C chemokine receptor type 4
DEARE	Delayed effects of acute radiation exposure
DNA	Deoxyribonucleic acid
DSB	Double strand break
EPCs	Endothelial progenitor cells
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage CSF
GSR	Gluthathione reductase
Gy	Gray
H-ARS	Hematopoietic acute radiation syndrome
hFOB	Human immortalized osteoblast
HO1	Heme oxygenase-1
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem progenitor cell
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
IR	Ionizing radiation
KSL	c-Kit + Sca-1 + Lin-
LD	Lethal Dose
Lin-	Lineage-negative
M-CSF	Macrophage colony stimulating factor
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NFκβ	Nuclear factor kappa β
NK	Natural killer cell
Nrf2	Nuclear factor erythroid-2–related factor 2
Notch-IC	Notch intercellular domain
OPG	Osteoprotegerin
PDGF	Platelet derived growth factor
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase

PK	Protein kinase
PRR	Pattern recognition receptor
RANKL	Receptor activator of nuclear kappa-B ligand
RBC	Red blood cell
RBMD	Residual bone marrow damage
RBP-Jk	Recombination signal binding protein for immunoglobulin kappa J
REDD1	Regulated in development and DNA damage response 1
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
Sca-1	Stem cell antigen-1 positive
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SSB	Single strand break
TBI	Total body irradiation
Tie2	Tunica internal endothelial cell kinase 2
TLR	Toll like receptor
TMC	Trifluoromethyl-2'-methocychalone
TPO	Thrombopoietin
TRAP	Tartrate-resistant acid phosphatase
TXNRD1	Thiordoxin reductase 1
VCAM-1	Vascular cell adhesion molecule-1
VE-Cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor 2

1 Stem Cells

Stem cells are rare tissue cells that possess the ability to both make more stem cells, i.e., self-renew, as well as proliferate and differentiate giving rise to the various cell types of the mature tissue. These two characteristics are the defining features of stem cells. Stem cells reside in defined microenvironments that regulate their number and function. These microenvironments or niches exist in many tissues, e.g. hair follicles [5], gut crypts [6], and BM [7]. In this chapter, we will focus on HSC that produce all the formed elements of the blood, and their supportive niche(s)/microenvironment in the BM. Under homeostatic conditions most HSC are quiescent with only a small fraction contributing to maintenance of mature blood cell numbers. However, they can respond to environmental stressors/niche signals that threaten tissue integrity and the need to enhance cell production. HSC respond to stress by initiation of proliferation and differentiation into multipotent and lineage-committed hematopoietic progenitor cells (HPC), which continue to proliferate and differentiate into mature blood elements [8].

1.1 Acute Effects of Irradiation on Hematopoietic Stem and Progenitor Cells

Owing to its highly proliferative nature to produce the billions of blood cells needed daily throughout life, BM is the most radiosensitive tissue in the body. Total body exposures of 2–10 Gray (Gy) in mice and 1.5–7.5 Gy in humans result in the hematopoietic acute radiation syndrome (H-ARS), with rather benign symptoms resulting from low exposure, but probable death at higher exposures from loss of white blood cells and platelets, resulting in opportunistic infection and hemorrhage [9]. The relative radiosensitivity of HSC has been debated [10–14], but the prevailing belief is that radiosensitivity correlates with proliferative status, such that the most primitive HSC are more radioresistant than more mature proliferating HPC. However, initiation of HSC proliferation in response to radiation-induced loss of more mature proliferating cells and the need to replenish dead and dying blood cells renders HSC susceptible to the lethal effects of residual radiation and to genotoxic stress from the oxidative and inflammatory hematopoietic microenvironment following radiation exposure.

Among HPC exposed to sublethal radiation (1.5–3 Gy), burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) were found to be the most radiosensitive, while CFU-granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte (CFU-GEMM), and CFU-granulocyte, monocyte (CFU-GM) were more resistant [15]. Higher doses of radiation (4 Gy) resulted in complete depletion of BFU-Es and CFU-Es progenitor cells within two days post radiation [16]. Mouse models of H-ARS demonstrate a dose dependent loss of total BM cells including the HSC/HPC phenotype, cellular receptor-type tyrosine kinase (c-Kit) + Stem cell antigen-1 (Sca-1) + Lineage-negative (Lin-) (KSL) cells, after 3–6 Gy exposures partly due to radiation-induced decreases in c-Kit expression [17]. Despite being mostly quiescent, lymphocytes undergo rapid apoptosis within 2 days following exposure to the LD50/30 in mice, with cluster differentiation (CD) $CD19^+$ B cells being the most radiosensitive lymphocyte subset, followed by $CD3^+$ T cells, and natural killer cell (NK)1.1^+ natural killer cells being most radioresistant [18]. Neutrophil nadir occurs by day 5–6 post irradiation [19] while erythrocyte and platelet nadirs occur between days 17–22 [19].

1.2 Late Effects of Radiation/Residual Bone Marrow Damage

It is becoming clear that in addition to the acute effects, survivors of H-ARS are plagued later in life by the delayed effects of acute radiation exposure (DEARE), a myriad of chronic illnesses affecting multiple organ systems and believed to be partly due to chronic inflammation. DEARE effects on the hematopoietic system are known as RBMD, a latent condition characterized functionally by compromised

HSC repopulating potential, increased cell cycling [20, 21], overexpression of cell cycle related genes in KSL cells [22] and myeloid skewing [20, 21, 23], with overall depressed hematopoiesis lasting months to years [20, 21, 24–30]. These findings suggest that increased cycling in HSC from H-ARS survivors leads to exhaustion and loss of self-renewal potential in HSC [20, 21]. Most studies on RBMD have investigated isolated HSC in sophisticated phenotypic and/or functional assays, or by studies examining hematopoietic output following stressors. However, more recently, the influence of the microenvironmental niche as a contributor to RBMD is being considered.

1.3 Mechanisms of Radiation-Induced Damage in HSC

After irradiation, HSC, HPC and BM stromal cells are exposed to tremendous stress resulting in acute and long-term defects [25, 31]. These effects are mediated intrinsically and through paracrine/extrinsic mechanisms by stromal cell interactions [32]. Irradiation-induced oxidative stress plays a central role in the pathogenesis and recovery from injury, resulting in the elimination of damaged cells from the stem cell pool. For instance, the production of reactive oxygen species (ROS) in HSCs results in detachment of HSCs from the BM niche [33]. HSC and HPC counterbalance the effect of ROS by enhancing expression of antioxidant enzymes including heme oxygenase-1 (HO1), gluthathione reductase (GSR), and thiordoxin reductase 1 (TXNRD1) through the nuclear factor erythroid-2–related factor 2 (NRF2)/anti-oxidant response element (ARE) pathway [34–36]. In addition, while irradiation depletes HPC, it also increases inflammatory cytokines and the possibility of bacterial infections, both of which further stimulate HSC to replenish hematopoiesis. [37].

Recent evidence shows that endogenous molecules or danger signals released from cells damaged by radiation directly act on HSC which in turn produce more mature cells to recover radiation-induced BM damage [38]. These effects are mediated by pattern recognition receptors (PRR) such as Toll like receptors (TLRs) expressed on HSC and HPC [39, 40]. Activation of TLR signaling results in mobilization of HSC, enhanced differentiation, and lineage-biased output, especially with respect to myeloid skewing and cytokine production [39, 41, 42]. Similar findings have also been observed in auto-immune diseases and bacterial infections [43, 44]. In summary, radiation-induced HSC damage may be partly mediated by oxidative stress and endogenous molecules released by damaged cells.

2 Hematopoietic Stem Cell Niche and Irradiation

HSC proliferation, differentiation, and fate decisions are tightly regulated by functionally specialized stromal cells that comprise hematopoietic microenvironments/niches in the BM [7, 45, 46]. Recent advances in imaging and use of genetic mouse models have identified mesenchymal stem cells (MSCs), perivascular cells, endothelial cells, osteoblasts, macrophages, megakaryocytes, and sympathetic nerve fibers as important cellular constituents of the HSC niche, see Fig. 1a [47–54]. High-dose irradiation used for myeloablation in cancer patients, radiation exposure in acts of terrorism, and catastrophic nuclear incidences damage the BM microenvironment (see Fig. 1b) and limit its regenerative potential and ability to support HSC function [55]. The contribution of molecules and pathways in stromal supportive cells following stress/injury and their effects on HSC function are not well understood. The diversity of the HSC niche cell types essential for HSC functions through radiobiological stress will be the focus of this section.

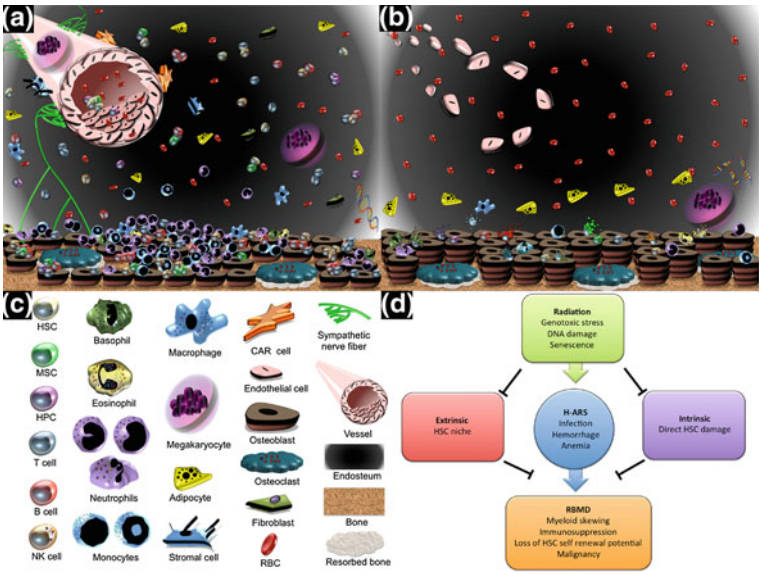

Fig. 1 **a** Schematic representing normal homeostatic hematopoiesis flourishing in the interactive bone marrow stem cell microenvironment. **b** Acute radiobiological effects on the hematopoietic stem cell microenvironment illustrating sinusoid deterioration, red blood cell (RBC) leakage, adipocyte infiltration, osteoblast proliferation, mature megakaryocyte homing to the endosteal surface, and complete obliteration of HSC, MSC, progenitor, and white blood cell (WBC) cells. **c** Legend key. **d** Chart outlining the extrinsic and intrinsic effects of radiation on stem and niche cells of the bone marrow microenvironment resulting in H-ARS

2.1 Mesenchymal Stem Cells

MSCs are self-renewing cells that can differentiate into bone, fat, cartilage, muscle, fibroblasts, and BM stromal cells [56]. MSCs expressing Nestin are found co-localized with HSC in the BM and constitute an essential component of the HSC niche through production of soluble factors, e.g., stromal cell-derived factor 1-alpha (SDF-1), stem cell factor (SCF), osteopontin, angiopoietin-1 (Ang1), and vascular cell adhesion molecule-1 (VCAM-1) that maintain HSC number and localization in BM [47]. Exposure to ionizing radiation severely and irreversibly damages MSC function. In BM transplant recipients, MSC colony forming cells defined by in vitro colony forming unit-fibroblast (CFU-F) clonogenic assays were reduced by 60–90 %, and the numbers did not recover up to 12 years after transplant [57, 58]. Recovery of CFU-F was delayed up to 4–8 months post-exposure in young mice irradiated at 4 weeks old with sublethal doses (5–7 Gy) [28]. Interestingly, transplantation of MSC in irradiated recipients results in faster hematopoietic recovery and long-term survival [59, 60]. Irradiation also impairs MSC differentiation into osteolineage cells, suggesting a possible cause of bone injury and fractures observed in patients receiving curative doses of ionizing radiation [58].

2.2 Osteolineage Cells in the Niche

The endosteal niche is a complex membranous structure lining the interiors of cortical bone and trabecular bone surfaces that contains several mesenchymal lineage cells. Endosteal osteoblasts are derived from mesenchymal precursors and are involved in the regulation of bone formation and HSC function [7, 61, 62]. Osteoblasts produce calcium and phosphate-based mineralized osteoid to replenish cortical and trabecular bones. Osteoblasts expressing angiopoietin, thrombopoietin, Wnt, Notch, N-cadherin, and osteopontin support HSC survival and differentiation [45], primarily through secretion of cytokines that regulate HSC function, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and interleukin-6 (IL-6) [45, 48, 63]. Effects of radiation on osteoblasts and bone structures include cell cycle arrest, reduced proliferation and differentiation, collagen and vascular suppression, increased sensitivity to apoptotic agents, osteoradionecrosis, bone demineralization, loss of trabecular connections, sclerosis, and disruption of the stem cell niche architecture [4]. Irradiated osteoblasts also play a role in radiation-induced bone loss by promoting osteoclast differ-entiation and proliferation mediated by RANKL/OPG and macrophage colony stimulating factor (M-CSF). Osteoblasts produce M-CSF that is required for survival of cells in the macrophage-osteoclast lineage, and proliferation and differentiation of osteoclast progeni-tors. Osteoblasts exposed to 2 and 4 Gy at the terminal differentia-tion stage stimulate osteoclast differentiation and proliferation by increased receptor activator of nuclear kappa-β ligand (RANKL) and macrophage colony stimulating

factor (M-CSF) expression and decreased osteoprotegerin (OPG) expression [64]. There was a greater radiation effect on the early stage of osteoblast differentiation compared to the late stage.

Interestingly, we have observed that following irradiation, the primary function of osteoblast lineage cells appears to switch from bone matrix production to supporting hematopoiesis and therefore organism survival (unpublished observations). Additionally, it is known that skeletal homeostasis requires a delicate balance between osteoblasts and adipocytes and alterations in this balance impact hematopoiesis. Following irradiation, osteoblast and adipocyte number expand in the BM. Lipid content in adipocytes increases and suppresses HPC proliferation and differentiation resulting in myelosuppression and yellow marrow [65, 66].

Within 48 h following TBI, there is a marked expansion of osteolineage cells lining the endosteal surface, including endosteal osteoblasts and mesenchymal origin cells, transforming from a normal single layer to a multilayered arrangement of cells expressing increased levels of collagen I and osteocalcin [67, 68]. This expansion in the endosteal niche is prerequisite for the initial engraftment of HSC and recovery of hematopoiesis in a BM transplant setting and subsequently returns to the single cell orientation within days post-transplant.

2.3 *Megakaryocytes in the Endosteal Niche Following Radiation*

Megakaryocytes are HSC derived multinucleated cells that produce platelets needed for blood clotting. Within the BM, megakaryocytes are primarily located centrally, near sinusoidal endothelial cells, where they release platelets into the circulation. Recent whole-mount imaging and computational studies in mice show that HSC are frequently located adjacent to megakaryocytes, which in turn regulate HSC quiescence and pool size [69]. Megakaryocytes regulate HSC directly through platelet factor 4 and transforming growth factor β secretion and indirectly through stromal cell production of fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) [53, 67, 70]. These studies define a HSC niche provided by a differentiated hematopoietic derived cell.

Megakaryocytes are resistant to irradiation and remain functional for 7–10 days post-exposure. It has been established that megakaryocytes provide a regulatory support necessary for proliferation of osteoblast lineage cells and bone formation [71–77]. After irradiation megakaryocytes are observed in close proximity to endosteal osteoblasts [67]. In the BM transplant setting, HSC preferentially lodge within 2 cell diameters of mature megakaryocytes [70]. The reciprocal regulatory interaction between megakaryocytes and osteoblasts creates a specialized niche for the successful engraftment of HSCs after transplantation. Expanding osteoblasts secrete SDF-1, which serves as a pro-survival and homing factor for C-X-C chemokine receptor type 4 (CXCR4) expressing megakaryocytes [78]. SDF-1 regulates

megakaryocyte spatial distribution in the BM following radiation injury and during normal physiological states [79].

Mature megakaryocytes that localize near the trabecular surface after irradiation, produce growth factors that stimulate increased cycling of CD45-Nestin-expressing MSCs, leading to their differentiation into pre-osteoblasts potentially increasing HSC number [67, 80]. Cell surface receptor expression of c-MPL and CXCR4 by megakaryocytes and HSCs allow response of these cell types to thrombopoietin (TPO) and SDF-1, respectively. Osteoblasts, BM stromal cells, perivascular mesenchymal stromal cells, sinusoidal endothelial cells, and endothelial progenitor cells produce these mediators [78–81].

2.4 Signaling Pathways of the Irradiated Osteoblast and Regeneration of the Niche

Osteoblast lineage cells constitute an instrumental component of the niche(s) that orchestrate HSC function; however, little is known about the effects of irradiation on the pathways and mechanisms involved. In human immortalized osteoblast (hFOB) cells regulated in development and DNA damage response 1 (REDD1), a stress response gene, was highly expressed after exposure to 4 or 8 Gy, via nuclear factor kappa β (NFκβ) and p53 regulation [82]. REDD1 inhibits mammalian target of rapamycin (mTOR) and cyclin-dependent kinase inhibitor of p21, preventing radiation-induced premature senescence [82]. Further elucidation of the molecular mechanisms and pathways governing osteoblasts may provide greater insight of their function in response to HSC niche regeneration after irradiation.

Emerging studies point to the involvement of osteoblasts in radiation-induced bone loss. Irradiation of osteoblasts with 2 or 4 Gy radiation resulted in altered expression of Notch-1, Jagged1, Jagged 2, Delta 1, Hes1, alkaline phosphatase (ALP), M-CSF, RANKL, and OPG [64]. A target of Notch canonical signaling, Hes1, plays an important role in osteoblast differentiation and osteoblast-osteoclast interaction, binding to the osteocalcin promoter and suppressing transactivation of osteocalcin. Hes1 expression was increased 2.2-fold following irradiation-induced osteoblast proliferation and differentiation. Irradiation inhibited terminal-stage osteoblast differentiation and ALP expression indicating greater Notch signaling activity. A second target of Notch signaling, Jagged1 is capable of enhancing bone mineral deposition, and several studies have shown that osteoblasts expressing Jagged1 are part of the HSC niche [83]. Elevated levels of Notch1 are known to inhibit Wnt/β-catenin signaling and decrease osteoblast differentiation and osteoblastogenesis. The Wnt/β-catenin signaling pathway plays critical roles in chondrogenesis, hematopoiesis, MSC commitment of osteoblast lineage cells, and is required for osteoblastogenesis. Notch signaling may play a role in communication between osteoblasts and osteoclasts during and after irradiation considering the effects of irradiation on Notch signaling and its important role in osteoblast regulation.

During normal osteoblast differentiation, Runx2, an essential transactivator for osteoblast differentiation and bone formation, interacts with Notch1-1C and disrupts the Notch intercellular domain (Notch1-1C)- recombination signal binding protein for immunoglobulin kappa J (RBP-Jk) transcriptional complex, thereby inhibiting Notch [84]. Exposure to 2 and 4 Gy of irradiation represses Runx2 and ALP expression in mature osteoblast differentiation. Interestingly, our group and others have demonstrated that less mature osteoblast lineage cells are better able to support hematopoiesis than are more mature osteoblast lineage cells [64, 85–87]. Thus, the reduction in Runt-related transcription factor 2 (Runx2) and ALP from these cells following irradiation maybe an important regulatory pathway to allow the cells to better support hematopoiesis.

2.5 Endothelial and Perivascular Cells

Recent studies on the BM vasculature indicate that most cycling HSCs are localized adjacent to sinusoidal endothelium, near CXC chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells and Nestin^{dim} leptin receptor^{+} perivascular cells, whereas quiescent HSC localize to arterioles in the endosteal region [51, 88]. Emerging evidence suggests that the endothelium not only bridges BM tissues to circulation, but also supports hematopoiesis. Conditional deletion of genes that encode the gp130 cytokine receptor in endothelial cells leads to BM hypocellularity and a reduction in HSC numbers [89]. Endothelial cells release angiocrine factors such as jagged-1, jagged-2, angiopoietin, FGF, delta-like 1, pleotropin, SCF, and cell surface expressed E-selectin that affect HSC function [90–93]. Of note, angiocrine factor production in endothelial cells is regulated through mTOR mediated serine/threonine-protein kinase (Akt) activation [94].

Radiation exposure not only affects HSCs but also destroys the BM vasculature [92]. Unlike arterioles, sinusoids lack a regular vessel wall sensitizing them to the severe effects of ionizing radiation resulting in ultrastructural signs of necrosis, marked dilation, and overt breakdown with plasma and blood cell leakage. Radiation-induced endothelial cell necrosis/apoptosis is mediated through BCL-2-antagonist/killer 1 (Bak) and BCL-2-like protein 4 (Bax) expression. Targeted deletions of these proapoptotic proteins in tunica internal endothelial cell kinase 2 (Tie2)$^{+}$/vascular endothelial-cadherin (VE-cadherin)$^{+}$ endothelial cells confer radioprotection to BM vasculatures and augment the regeneration of the hematopoietic system [95]. Vascular endothelial growth factor (VEGF) signaling through vascular endothelial growth factor 2 (VEGFR2) promotes endothelial cell recovery. Antibody inhibition of VEGFR2 signaling in irradiated mice impairs the regeneration of sinusoidal endothelial cells and prevents HSC recovery [92]. Radiation exposure also induces senescent growth arrest and functional defects in endothelial progenitor cells (EPCs), which leads to attenuated vascular regeneration. The growth arrests and the senescent and functional defects in EPCs are

dependent on p53 transcriptional activity after ionization irradiation exposure, which leads to p21Cip1 upregulation and VEGF down regulation [96].

2.6 Macrophages

Macrophages have recently emerged as regulators of HSCs. BM macrophages are critical in hematopoietic stem progenitor cell (HSPC) retention within the niche, as genetic or pharmacological deletion of macrophages increase HSPC egress from the BM [52, 97, 98]. Although high-dose irradiation treatment substantially depletes macrophages in the BM, rare activated BM monocytes and macrophages with high expression of α-smooth muscle actin (α-SMA) are shown to be radioresistant and produce higher level of Cyclooxygenase-2 (COX-2) expression under stress conditions. COX-2-derived prostaglandin E_2 (PGE_2) prevents HSC exhaustion by limiting ROS production via Akt kinase inhibition and higher stromal-cell expression of the chemokine CXCL12 [52, 99].

Bone loss is a consequence of exposure to high-dose radiation. Radiation exposure results in altered HSC function and myeloid skewing. Since macrophage/monocyte cells are the source of mature, bone resorbing osteoclasts, this may explain the observed bone loss following radiation. Indeed, increased osteoclast activity confirmed by a 14 % increase in osteoclast activity marker, tartrate-resistant acid phosphatase (TRAP)-5b in serum chemistry analyses, and TRAP staining of histological tibial metaphysis sections was observed in mice exposed to a whole-body dose of 2 Gy of radiation [100]. Similarly, exposure of the osteoclast-like cell line (RAW264.7 cells) to 2 Gy enhanced calcitonin receptor expression, a marker of mature osteoclasts [101].

3 Therapeutic Approach to HSC Niche Protection and Regeneration

The efficiency of hematopoietic reconstitution after radiation exposure would likely benefit from the presence of an intact, supportive marrow microenvironment. While extensive efforts have been made to uncover the factors and mechanisms that regulate HSC niche maintenance during homeostasis, understanding of mechanisms that provide HSC niche radioprotection and regeneration remains far from complete. Factors that protect or promote regeneration of HSC niche components after irradiation could improve hematopoietic regeneration when provided along with known medical countermeasures that stimulate HSPC proliferation and differentiation.

3.1 Antioxidants, Growth Factors, Hormones, and Lipids

Ionizing radiation exposure and resulting elevated levels of free radicals and ROS can disrupt the redox status of HSC supporting niche cells by inducing single strand (SSB) and double strand breaks (DSB), DNA damage, protein oxidation, and lipid peroxidation [55, 102]. Oxidative BM cellular damage is associated with marked reduction in levels of vitamins C and E [103, 104]. Accordingly, dietary supplements consisting of vitamin C, vitamin E, succinate, α-lipoic acid, L-selenomethionine, and N-acetyl cysteine may improve hematopoietic regeneration and reduce mortality. Anti-oxidant treatment has been shown to improve vasculature retention and recovery as demonstrated by angiographic imaging [105]. Therefore, antioxidant repletion may be a putative therapeutic target for radioprotection or recovery of the hematopoietic system after exposure to ionizing radiation.

Several growth factors and cytokines such as basic fibroblast growth factor (bFGF), VEGF, and angiopoietin can prevent endothelial cell apoptosis, leading to regeneration of the hematopoietic system [92, 106–109]. Angiopoietin acting through the Phosphatidylinositol 3 kinase (PI3 K) pathway prevents endothelial cell apoptosis and promotes HSC functions [106]. Oral administration of 2-trifluoromethyl-2′-methoxychalone (TMC) in mice, which increases NRF2 signaling in HSCs by increasing jagged 1 expression in the BM niche enhances hematopoietic reconstitution and mitigates ionizing irradiation-induced myelosuppression and mortality in some models [110]. Similarly, insulin-like growth factor-1 (IGF-1)-receptor tyrosine kinase signaling stimulates rapid expansion of endosteal osteoblasts and donor HSC engraftment after TBI, possibly through the proliferation and differentiation of Nestin^{bright} MSCs [111]. In addition, recent studies demonstrate stromal cell-secreted TPO stimulates DNA-protein-kinase (PK)-dependent non-homologous end joining DNA repair, the primary repair pathway in HSC [107]. This suggests that niche factors can modulate the HSC DSB repair machinery and opens up new avenues for TPO agonists to minimize radiotherapy-induced HSC injury and mutagenesis.

4 Conclusions

In summary, we provide a glimpse into the complexity of the cellular interactions within the hematopoietic stem cell niches, the intrinsic and extrinsic, short-term and long-term effects of radiation, and the radiobiological response of these components. Two illustrations of the stem cell niche in normal hematopoiesis and in the aftermath of acute, high dose radiation exposure (H-ARS) are represented in Fig. 1. The H-ARS microenvironment is severely depleted of lymphocyte and HPC populations, with remnants of deteriorated and discontinuous sinusoids resulting in RBCs infiltration. Osteoblast lineage cells proliferate, adipocytes expand in the BM, and mature megakaryocytes move towards the endosteum. Each specialized cell

population contributes to the stem cell niche and influences hematopoiesis and BM regeneration.

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Impact of Radiation on Hematopoietic Niche

Setsuko Mise-Omata, Takahiro S. Doi, Kazuhiro Aoki and Yuichi Obata

Abbreviations

BM	Bone marrow
BMD	Bone mineral density
CFU-F	Colony-forming unit of fibroblasts
CFU-OB	Colony-forming unit of osteogenic progenitors
CXCL-12	CXC chemokine ligand 12
CAR cells	CXCL-12 abundant reticular cells
DAMP	Danger-associated molecular pattern molecule
E4PRF1	Early region 4 encoded open reading frame 1
EGF	Epidermal growth factor
FL	Fetal liver
G-SCF	Granulocyte-colony stimulating factor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
MSC	Mesenchymal stem cell
NO	Nitrogen oxide
PTN	Hairpin-binding growth factor pleiotrophin

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SCF	Stem cell factor
TPO	Thrombopoietin
VEGFR2	Vascular endothelial growth factor receptor 2

1 Hematopoietic Niche Cells and Radiosensitivity

Bone marrow (BM) consists of hematopoietic stem cells (HSC)-derived hematopoietic cells and mesenchymal stem cells (MSC)-derived osteoblasts, vascular endothelial cells, chondrocytes, and adipocytes. MSC-derived cells provide the microenvironment for the HSC niche to maintain the quiescent state of HSCs and to support their proliferation and differentiation [1–4]. The HSC niche consists of several cell types [3–5] and two main parts: the vascular and endosteal niches [2, 4].

The concept of the vascular niche emerged from studies using in vitro cultures of HSCs with BM endothelial cells, which support the proliferation and differentiation of HSCs through their attachment with HSCs and production of hematopoietic cytokines [6, 7]. Self-renewal of HSCs is supported by the generation of immortalized cells derived from human primary endothelial cells transduced with an adenovirus gene, early region 4 encoded open reading frame 1 (E4PRF1). This repopulation activity occurs through Jagged-1 and -2, which are ligands of Notch signaling [8]. E4PRF1-transduced cells exert niche ability by the activation of Akt and mTOR signaling [9]. In addition, to exert niche ability, endothelial cells produce stem cell factor (SCF) [10], E-selectin [11], angiopoietin-like protein 3 [12], and hairpin-binding growth factor pleiotrophin (PTN) [13]. Furthermore, the concept of a vascular niche is supported by the observation of many HSCs localized adjacent to sinusoidal blood vessels [14, 15].

In the perivascular region, cell types other than endothelial cells support the HSC niche, including CXC chemokine ligand 12 (CXCL-12)-abundant reticular (CAR) [16], Schwann [17], PαS [18] Nestin$^+$ [19], leptin receptor$^+$ cells [20]. These cells express important niche components, such as CXCL12 and SCF, to maintain HSCs [12].

On the BM endosteal surface, two types of cells mediate bone turnover: osteoclasts (which are derived from HSCs and resorb bone) and osteoblasts (which form bone matrices). Osteoblasts may also provide for HSC niche [1–4]. The concept of endosteal niche is supported by the evidences from mice having a significant increase in HSCs when their osteoblasts and trabecular bone content are increased through the activation of parathyroid hormone signaling [21] or inactivation of bone morphogenetic protein receptor 1A [22]. Likewise, in vivo depletion of osteoblasts by a transgenic herpesvirus thymidine kinase under the control of a collage alpha 1 type I promoter and administration of ganciclovir reduces the numbers of HSCs and their differentiated cells [23]. Thus, BM osteoblasts positively regulate the HSC pool in vivo; this is likely mediated by Jagged-1 and

N-cadherin [21, 22]. In addition, osteoblasts maintain HSC quiescence through angiopoietin 1, a ligand of Tie-2, which is expressed in HSCs [24].

Ionizing radiation is used in cancer therapy and the pretreatment of patients with acute leukemia, aplastic anemia, and hemophilia who undergo BM transplantation. Systemic, lethal irradiation of mice and humans causes rapid depletion of radiosensitive HSCs and their progenitors; however, MSCs are thought to be radioresistant, surviving after lethal irradiation to the hematopoietic system [25]. Here, we address whether cells of the HSC niche really are radioresistant: does radiation injure osteoblasts and endothelial cells, impairing the ability of the hematopoietic niche?

According to recent observations, the cells and activity of the HSC niche are damaged by radiation. For example, the sinusoidal vasculature of the BM is radiosensitive but is regenerated and reorganized within 3–4 weeks after sublethal exposure [5, 26, 27]. Long-term survivors of autologous and allogenic HSC transplantation have severe bony complications accompanied by loss of bone mineral density (BMD), osteoporosis, and bone fracture [28–31]. It is difficult to determine from these results in human patients whether radiation diminishes bone metabolism, because loss of BMD can be attributed to multiple factors, including cancer itself; steroid and other immunosuppressive treatments; reduced metabolism of calcium and vitamin D caused by impaired function of kidney, liver, and bowel; and graft-versus-host disease [29, 30]. However, radiotherapy in cancer patients also reduced BMD [32], and the irradiation of mice without cancer cells induces loss of trabecular bone volume [33]. Therefore, radiation may affect bone metabolism. In this review, we communicate recent findings regarding the effects of radiation on vascular and endosteal niches in mouse models and discuss how HSC niches may be repaired.

2 Impact of Radiation on the Vascular Niche

BM sinusoidal endothelial cells are radiosensitive [5, 26, 27]; they regress after irradiation and then regenerate (Fig. 1). For example, minor regression of the vasculature is induced by sublethal total body irradiation and by mild myeloablation

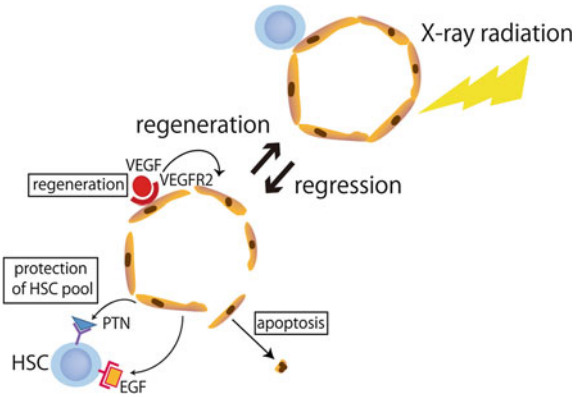

Fig. 1 Vascular endothelial cells regress after sublethal and lethal irradiation and then regenerate. Cells avoiding apoptosis produce EGF and PTN to protect HSC from radiation injury

treatment, such as 250 mg/kg of 5-fluorouracil; in mice, this regression is characterized by discontinuous or hemorrhagic vessels. In contrast, lethal irradiation induces severe regression and destruction of the vasculature, characterized by the presence of discontinuous and hemorrhagic vessels as well as denuded endothelial cells from vessel walls [26]. The HSC transplantation is required for the regeneration from severe regression of vasculature [26], and the regenerated endothelial cells produce cytokines and growth factors to protect the HSC pool from radiation injury. Specifically, regeneration after minor or severe regression is dependent on the expression of vascular endothelial growth factor receptor 2 (VEGFR2); a decrease in VEGFR2 by neutralizing antibody or conditional knockout methods abolishes regeneration [26]. Deficiency of VEGFR2 also prevents engraftment and reconstitution of hematopoietic stem and progenitor cells (HSPCs). Furthermore, total body irradiation induces apoptosis in endothelial cells, but the apoptosis can be avoided by the deletion of the proapoptotic proteins BAK1 and BAX [34]. The prevention of apoptosis not only preserves the BM vasculature but also protects BM HSCs, resulting in enhanced survival of mice. Therefore, HSC niche activity may parallel the integrity of the endothelial vasculature.

BM endothelial cells produce the cytokines (such as SCF, FMS-like tyrosine kinase 3 ligand, thrombopoietin [TPO], interleukin-3, and CXCL-12) that possess antiapoptotic activity for HSCs [5, 35–38]. Endothelial cells protected against apoptosis through the deletion of BAK1 and BAX produce epidermal growth factor (EGF), which mediates HSC regeneration after irradiation [39]. Radiation induces the expression of EGF receptor and its phosphorylation in HSCs [39]. Furthermore, in sublethally irradiated, wild-type mice, systemic administration of EGF increases hematopoietic and repopulation activities, whereas the administration of an EGF antagonist impairs hematopoietic activity. Specifically, EGF reduces the radiation-induced cell death of HSCs by repressing the expression of the proapoptotic protein PUMA, which is an essential mediator of radiation-induced hematopoietic toxicity [40]. Therefore, the production of EGF in endothelial cells is triggered by radiation and protects HSC pools from radiation injury.

Another factor that is produced by endothelial cells and protects HSCs is PTN [13]. PTN knockout mice have decreased numbers of HSCs; PTN regulates the maintenance of the BM HSC pool. Furthermore, the expression of PTN in endothelial cells is required for hematopoietic regeneration, homing, and survival after both sublethal and lethal irradiation and BM transplantation [13]. PTN activates RAS/MEK/MAPK signaling in HSCs through a transmembrane receptor, protein tyrosine phosphatase receptor ζ [41]. Notably, systemic administration of PTN increases HSC recovery in and the survival of mice exposed to sublethal and lethal irradiation and BM transplantation.

Therefore, BM endothelial cells that support the HSC niche undergo apoptosis and regression after radiation. Reorganization of these endothelial cells occurs through the VEGF/VGEFR2 pathway [26], and is indispensable for hematopoietic cells survival. Regardless of whether the activation of the VGEFR2 pathway in BM endothelial cells is autocrine or paracrine, hematopoietic cells may modulate the expression of VEGF. Furthermore, EGF and PTN, which are produced by irradiated endothelial cells,

support hematopoietic regeneration [13, 39, 41]. Thus, BM endothelial cells and HSCs may interact after irradiation, resulting in regeneration of the BM vasculature and hematopoietic cells. Additional investigation of how hematopoietic cells are involved in the regeneration of the BM vasculature is anticipated.

3 Impact of Radiation on Endosteal Niche

Radiation exposure may affect bone metabolism. Indeed, recipients of allogenic and autologous HSC transplants [28–31] and cancer patients undergoing radiation therapy [32] experience bone loss. Similarly, in mouse experiments, systemic and local irradiation induce significant bone loss in the absence of chemotherapy or exogenous glucocorticoid and in mice not receiving cancer cells [42–44]; thus, radiation itself may induce bone loss. Furthermore, in a murine model, both low and lethal doses of radiation following by BM transplantation increase the number of osteoclasts [44, 47, 48]. Irradiated mice and human have enhanced serum levels of markers of osteoclast activation, such as tartrate-resistant acid phosphatase 5b and β-crosslaps [29, 44, 47]. Furthermore, in irradiated patients and mice, excessive osteoclastogenesis may be treated with bisphosphates, which are antiresorptive drugs that decrease the activity of osteoclasts and induce their apoptosis, to ameliorate bone loss [42, 44–46]. Osteoclastogenesis may be enhanced due to increases in inflammatory cytokines, such as TNF-α, IL-6, and monocyte chemotactic protein 1 [44]. Therefore, radiation may induce osteoclastogenesis.

To address the effect of enhanced osteoclastic activity on the HSC niche, many mouse lines that lack functional osteoclasts are available, such as *op*/*op* (a loss-of-function mutation in M-CSF), *oc*/*oc* (a mutation in the Tcirg 1 gene encodes the a3 subunits of the vacuolar-ATPase), *c-fos*-deficient, and RANKL-deficient mice. In all of these osteopetrotic mice, the volume of the BM cavity and consequently the number of BM cells are reduced [49]. In addition, whereas HSC numbers and B lymphopoiesis are decreased, myelopoiesis is enhanced in these animals [49–51]. The lack of osteoclasts in *oc*/*oc* mice may be due to the impaired differentiation of osteoblasts from mesenchymal progenitors [51]. Protein tyrosine phosphatase ε-knockout mice [52] but not *op*/*op*, *c-foc*$^{-/-}$, and RANKL$^{-/-}$ mice have impaired mobilization of HSCs from the BM into the periphery [53]; this mobilization can be induced by the administration of granulocyte-colony stimulating factor (G-SCF). Because the sensitivity to G-SCF varies among the lines of mice lacking functional osteoclasts [52, 53], the role of osteoclasts in HSC mobilization is debated. Therefore, radiation enhances osteoclastogenesis, but it remains to be elucidated whether radiation impairs the ability of the HSC niche.

The effect of radiation on osteoblasts is complicated. There is evidence from murine models that radiation impairs osteoblastic function [27, 43, 44, 54]. In contrast, systemic lethal irradiation in the absence of subsequent HSC transplantation induces rapid and transient proliferation of osteoblasts [55]. The expansion of osteoblasts occurs within 48 h after irradiation, resulting in the formation of

multiple layers of flattened osteoblasts along the endosteal surface instead of the signal layer detected in non-irradiated mice [55]. The expansion of osteoblasts is induced by megakaryocytes, which remain functional at least 7–10 days after irradiation [55, 56]. Megakaryocytes migrate from parasinusoidal regions to endosteal surfaces, after TPO signaling through their c-MPL receptors and CD41 integrin-mediated adhesion [56]. Osteoblastic expansion is accompanied by the upregulated expression of growth factors for osteoblasts and HSC niche factors, such as CXCL-12, PDGF-BB, TGF-β, bFGF and IGF-1; thus, radiation may expand the endosteal niche [57]. In particular, IGF-1 may be important because the blockage of IGF-1 signaling with picropodophyllin, a potent and selective IGF-1 receptor tyrosine kinase inhibitor, disrupts both osteoblastic expansion and HSC homing to BM [57]. However, BM transplantation halts the postirradiation expansion of the endosteal niche. In particular, this arrest is induced by the Sca-1^+ HSC-containing fraction of BM cells but not Sca-1-depleted cells [57]. In addition, delaying the transplantation of BM cells until 24 h after irradiation increases HSC engraftment compared with that after immediate transplantation [58]. Therefore, although there is a mechanism for the restoration of osteoblasts after radioablation, HSC-derived cells may disturb it. The pathway by which HSC-derived cells halt the expansion of the endosteal niche is unknown as yet.

As mentioned previously, osteoblastic function is impaired after irradiation and BM transplantation. Specifically, osteoblastic function is decreased in mice that receive local [27] or systemic [31, 44, 54] irradiation, indicated as reduced bone formation and decreased in the numbers of colony-forming unit (CFUs) of osteogenic progenitors (CFU-OB) and CFU-fibroblast (CFU-F). CFU-OB and CFU-F indicate the frequency of osteoblastic progenitors and mesenchymal cell in the BM, respectively. Patients that undergo either autologous or allogenic HSC transplantation have fewer CFU-OB than do healthy control [31]. Like BM endothelial cells, BM osteoblasts may be damaged after irradiation and subsequently repaired by unknown mechanism.

In our studies, we use fetal liver (FL) cells deficient in RelA (p65), which is a subunit of the transcription factor NF-κB; lethally irradiated mice transplanted with RelA-deficient FL cells develop severe osteopenia with granulocytosis and reduced lymphopoiesis (Fig. 2) [54]. These mice do not develop osteopenia or abnormal hematopoiesis when wild-type BM and RelA-deficient FL cells are co-transplanted; these defects are likely not due to a dominant effect of RelA-deficient cells because wild-type BM cells are able to compensate for the loss of function. Therefore, the osteopenia of the mice transplanted with RelA-deficient FL cells only is likely not due to hyper-differentiation of osteoclasts; in fact, the number and function of osteoclasts in these mice are either unimpaired or increased. In contrast, mice transplanted with RelA-deficient FL cells only demonstrate markedly impaired osteoblastic function. The osteoblasts are derived from wild-type host mice; thus, RelA-deficient FL-derived cells may fail to support the function of osteoblasts.

We therefore wondered which cell type loses its supportive function and have focused on macrophages; BM macrophages not only support osteoblasts to form bone matrices [59–61] but also act as HCS niche cells [61–68], as described later.

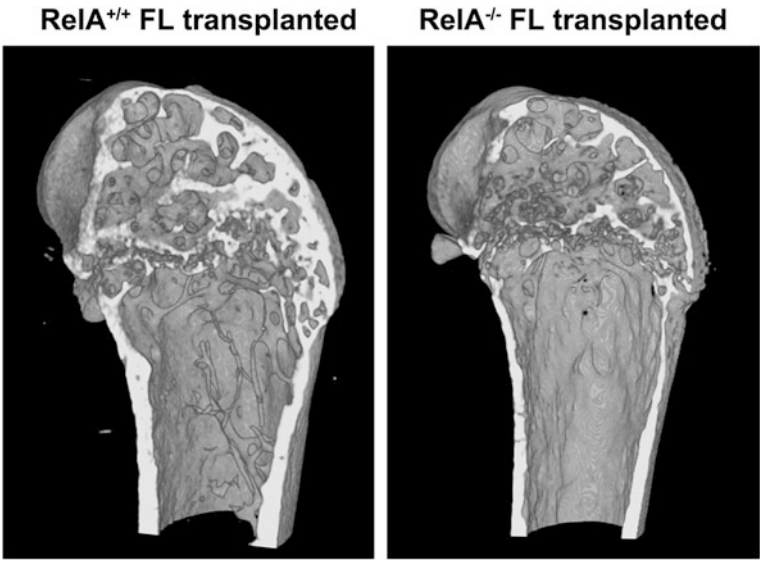

Fig. 2 MicroCT images of femurs between mice transplanted with wild-type compared with RelA-deficient FL cells. Mice transplanted with RelA-deficient FL cells have markedly decreased trabecular bone mass and thinner cortical bone

Co-transplantation of F4/80^{+} macrophages in our RelA-deficient FL transplantation model rescues osteogenesis and hematopoiesis. Therefore, the proliferation and function of osteoblasts, which are derived from MSCs, may be regulated by cells that derived from HSCs, such as megakaryocytes and macrophages, after irradiation.

4 Role of Macrophages in the Regeneration of the Microenvironment for the HSC Niche After Radiation

The macrophages that localize over the osteoblasts that line the endosteum and periosteum (that is, "osteomacs") support osteoblasts to form bone matrices [59–61]. Depletion of BM macrophages not only reduces osteoblast numbers and bone formation but also the expression of CXCL-12, kit ligand, and angiopoietin 1, resulting in the mobilization of HSCs into the peripheral circulation [65]. Osteomacs produce oncostatin M to induce the differentiation of osteoblasts and inhibit adipogenesis [61]. Furthermore, BM macrophages have roles in the HSC niche distinct from other types of niche cells, osteoblasts, and endothelial cells [64]. In this regard, macrophages are responsible for the mobilization of HSCs after G-CSF administration; expression of the G-CSF receptor is restricted to

macrophages and is sufficient for the mobilization of HSCs after the administration of G-CSF [62]. The depletion of $CD169^+$ macrophages from BM reduces the expression of CXCL-12 in $Nestin^+$ niche cells, resulting in the egress of HSCs to the peripheral bloodstream [63]. Furthermore, BM macrophages likely support erythropoiesis; in murine models, depletion of macrophages reduces the number of erythroblasts, impairs erythropoietic recovery from hemolytic anemia, and ameliorated polycythemia [67, 68]. BM macrophages expressing α-smooth muscle actin directly associate with HSCs and protect them from exhaustion under stress conditions [66].

Macrophages can be divided into M1 and M2 subtypes [69, 70]. M1 macrophages are considered classically activated macrophages, which produce high levels of pro-inflammatory cytokines and mediators; these cells are important for host defense but can cause tissue damage. In contrast, M2 macrophages are known for their wound-healing and regulatory properties. In addition, M2 macrophages produce arginase, which converts arginine to ornithine, a precursor of polyamines and collagen, thereby contributing to the production of the extracellular matrix [71].

Radiation activates macrophage and induces them to differentiate into the inflammatory M1 type [72]. This activation is not due to direct effects of radiation but is caused by bystander effects due to the tissue response to radiation [73, 74]. As a result of tissue damage by radiation, intracellular danger-associated molecular pattern molecules (DAMPs) are released into the surrounding tissue [74, 75]. DAMPs are recognized by Toll-like receptors to activated macrophages and produce inflammatory cytokines and free radicals, such as reactive oxygen species and nitrogen oxide (NO) [76–78]. After the elimination of dead cells, acute

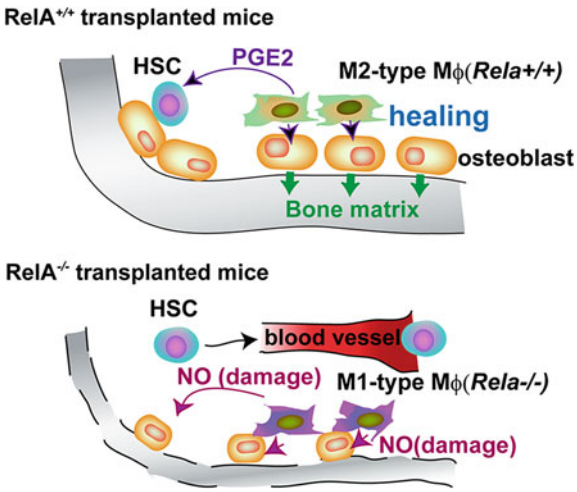

Fig. 3 In BM of mice transplanted with RelA-deficeint FL cells, prolonged activation of inflammatory macrophages fails to support osteoblasts to form bone matrices and impairs the microenvironment for hematopoiesis. In contrast, mice transplanted with wild-type FL cells have wound-healing macrophages

inflammation resolves and is converted into an anti-inflammatory response to repair tissue damage [74].

In mice transplanted with RelA-deficient FL cells (described earlier), BM macrophages continuously express high amounts of inducible NO synthase, in contrast, this expression is elevated only transiently and declined after irradiation in mice transplanted wild-type FL cells [54]. The expression of other inflammatory cytokines, such as TNF and IL-6, was intact. However, M2 type macrophages are reduced in mice transplanted with RelA-deficient FL cells. In these mice, BM macrophages may fail to switch from an inflammatory to a wound healing type and thus sustain a prolonged inflammatory state (Fig. 3). As a result, the BM microenvironment for bone metabolism and hematopoiesis may fail to be maintained.

Radiation damages not only hematopoietic cells but also HSC niche cells, such as osteoblasts and endothelial cells, and induces the disruption of microenvironment for homeostasis. Additional studies to elucidate the mechanisms that underlie the repair of are warranted.

5 Conclusion

Immunologists and HSC researchers have long thought that irradiation performed before BM transplantation induces the death of the hematopoietic cells but spares the cells derived from MSCs. However, according to current studies, radiation induces the regression of BM endothelial cells and decreased osteoblastic activity, thus preventing the formation of bone matrices. The mechanisms by which the vasculature and osteoblastic functions of the BM regenerate are not well understood. The regression of endothelial cells and osteoblasts directly correlates with the hematopoietic activity of HSCs, indicating that radiation impairs the activity of HSC niche cells. It remains to be elucidated whether radiation affects other niche cells in the perivascular region, such as CAR, PαS, Nestin$^+$, and leptin receptor$^+$ cells. Revealing which cell populations are damages by radiation and how the damage is repaired will increase our understanding of the side effects of BM transplantation and raidotherapy and improve these treatments.

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From Neurogenic Niche to Site of Injury: Stem Cell-Mediated Biobridge for Brain Repair

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Abbreviations

CC	Corpus callosum
DCX	Doublecortin
ECM	Extracellular matrix
MSC	Mesenchymal stromal cell
MMP	Matrix metalloproteinase
OEC	Olfactory ensheathing cell
PD	Parkinson's disease
SC	Schwann cell
SGZ	Subgranular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury

1 Introduction: A Bridge Over Troubled Water

Stem cells, which exist throughout adulthood, exhibit self-renewal and differentiation abilities [1]. The beneficial effects of stem cells are thought to be exerted both endogenously [2–5] and exogenously following their transplantation into injured organs, e.g., the brain [6–12]. Furthermore, stem cells are also known to help maintain homeostasis [13]. There are two major neurogenic niches in the mammalian adult brain: subgranular zone (SGZ) of the hippocampus and the subven-

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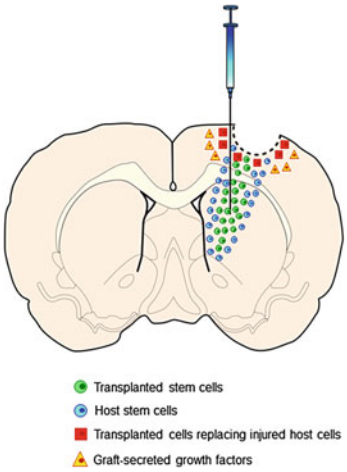

Fig. 1 Multi-pronged stem cell repair processes. The concept of "cell replacement" indicates that transplanted stem cells facilitate repair by replacing dead or dying host cells thereby requiring neuronal differentiation of stem cells and reconstruction of the damaged synaptic circuitry. Additionally, grafted stem cells may exert "bystander" effects, which entail secretion of neurotrophic factors, anti-inflammatory substances, and anti-oxidative stress molecules by the transplanted cells, which subsequently rescue viable host cells or stimulate neurogenesis. A third mechanism we propose involves stem cell-paved biobridges whereby transplanted cells form a biological pathway, enriched in MMPs, and ferry newly born host stem cells from the neurogenic niche SVZ to the injured host tissue. These mechanisms may altogether work in concert to unravel the therapeutic benefits of stem cell therapy

tricular zone (SVZ), producing new neurons that primarily use the rostral migratory stream from the olfactory bulb to reach the injured brain regions [14, 15]. Quiescent neural stem cells (NSCs) have also been found in other brain regions [16].

Recent studies showed activation of stem cells after brain insult or injury [2–12, 17–19]. This finding, which not only led to improved understanding of stem cell mechanism and biology, but also opened new research directions in regenerative medicine [2–12, 17–19], unraveled windows of opportunities for translational stem cell research for brain disorders [20–22]. Despite these scientific advances and clinical applications, much remains to be understood about the mechanisms underlying stem cell-mediated repair in brain injury. To date, there are two widely held views on how stem cells facilitate repair in brain damage caused by injury or neurodegenerative disorders [23, 24]. On one hand, stem cells implanted into the brain are assumed to directly replace dead or dying cells (the concept of cell replacement) (Fig. 1), while on the other, transplanted stem cells have been argued to secrete growth factors that indirectly rescue the injured tissue (the "bystander" effects of stem cells) [25, 26] (Fig. 1).

In a recent study, we observed improvement of traumatic brain injury (TBI)—induced motor and neurological deficits in rats transplanted intracerebrally with cultured Notch-induced human bone marrow-derived mesenchymal stromal cells

(referred to as SB623, supplied by SanBio Inc.) [27]. These exciting findings strengthen the putative therapeutic benefits of stem cell transplantation for TBI, and our research on the mechanism of action of SB623 revealed breakthrough findings that point to a novel stem-cell mediated brain repair mechanism. Based on our recent data, we advanced the concept that transplanted stem cells may exert therapeutic benefits by harnessing a "biobridge" between the neurogenic niche and injured sites, facilitating long-distance migration of host neurogenic cells and activation of endogenous repair mechanisms. Here, we discuss the properties and characteristics of stem cell paved-biobridges, and describe the unique mechanism by which these cells promote neural repair in a rat model of TBI. Moreover, we also discuss the clinical significance, issues and challenges involved in exploiting this novel stem cell-mediated brain repair concept for the treatment of other neurological disorders characterized by similar biological gaps between the neurogenic niches and the damaged brain tissues.

2 Stem Cell-Paved Biobridge: A Ferry to Aid Migration of Stem Cells Towards TBI Sites

Previously, we investigated the therapeutic value of intracerebrally transplanted SB623 (gene-modified human mesenchymal stromal cells) [21, 27, 28] in rats subjected to TBI [27]. Improvement of TBI-induced motor and neurological deficits was measured at 1, 2 and 3 months post TBI. Corresponding immunohistochemical studies were also performed to evaluate effects of SB623 transplantation at indicated time points. The behavioral studies revealed significant motor and neurological improvement in TBI rats which received SB623. Histological studies also showed profound reduction in TBI-induced damages to the cortical core and peri-injured cortical areas in SB623-transplanted subjects. Interestingly, the behavioral and histological improvements in SB623-transplanted TBI rats were achieved despite minimal graft survival—0.60 and 0.16 % at 1 and 3 months, respectively. This observation led us to explore the mechanism underlying functional recovery despite lack of graft persistence.

Parallel cellular investigations using immunohistochemistry, we observed notable increase in endogenous cellular proliferation (Ki67) as well as immature neural differentiation (nestin) in the peri-injured cortical areas and SVZ, along with a stream of migrating cells along the corpus callosum (CC) of SB623 transplanted TBI animals at 1 month after TBI. Furthermore, at 3 months post-TBI, we observed enhanced cellular proliferation and neural differentiation in the peri-injured cortical areas, accompanied by a solid stream of neuronally-labeled cells (nestin and doublecortin [DCX]) migrating not only along but also across the CC from the SVZ to the impacted cortex, of SB623-transplanted TBI animals. Indeed, these immunohistochemical images depict stem cell-facilitated formation of a "biobridge" which we initially thought as a structure that could facilitate migration of host cells from

neurogenic niche to the site of injury. In contrast, TBI rats which received vehicle infusion exhibited elevated cellular proliferation; however, the newly formed cells localized within the SVZ and cortex and did not migrate to the injured cortex.

We analyzed further the cellular and molecular components that constituted this biobridge by examining the characteristics of cells migrating from the SVZ and moving towards the site of injury. By laser capture assays, we observed presence of highly proliferative, neutrally committed, and migratory cells in the biobridge between the SVZ and the impacted cortex. Moreover, zymography assays showed 2- and 9-fold upregulation of the matrix metalloproteinase-9 (MMP) activity and expression, at 1 and 3 months post TBI, respectively, in rats transplanted with SB623. Further in vitro studies also showed the capacity of SB623 cells to enhance cell migration via MMP-rich signaling cues, which are crucial to the migration of endogenous cells that assist functional repair of damaged tissue. At just 1 month post TBI, a surge of proliferative Ki67 positive cells and neutrally immature nestin labeled cells in the peri-injured areas and SVZ were observed. The high level of MMP-9 in the biobridge indicates the importance of this neurovascular proteinase. Interestingly, this proteinase was upregulated in the vehicle group, but reverted back to control-sham levels at 3 months post-TBI. Together, the above findings illustrate the role of MMP in long-term recovery and add another dimension to mechanism by which stem cells aid in repairing the damaged tissue.

In view of the above findings, further in vitro studies were conducted to determine whether transplanted SB623 cells promoted cell migration via an extracellular matrix (ECM)-mediated mechanism. Primary rat cortical cells were grown both alone and co-cultured with SB623 cells. These cells were grown either in the presence or absence of the MMP-9 inhibitor Cyclosporin-A. Migratory cell assays revealed noticeable enhancement in the migration of primary rat cortical cells in the chamber containing SB623, which was then significantly suppressed by treatment with the MMP-9 inhibitor. In contrast, treatment with Cyclosporin-A alone, combined treatment with SB623 and the inhibitor, and absence of both SB623 and the inhibitor, did not alter migratory potential of primary cortical cells.

Endogenous repair mechanisms are initiated post-TBI, although these effects are typically limited to the neurogenic SVZ and quiescent neurogenic resident cells around the impacted cortex. Hence, endogenous repair mechanisms are not robust enough to counteract TBI or other disease-induced cell death cascades, necessitating introduction of exogenous cells which can aid migration of endogenous stem cells from the neurogenic niche to the site of injury. Stem cell transplantation into the peri-injured cortical areas purportedly created a biobridge comprised of a neurovascular matrix, which allowed newly-formed endogenous cells to migrate efficiently to injury sites. Moreover, once the biobridge has been established, exogenously transplanted cells slowly receded and were supplanted by newly-formed endogenous cells that resumed the task of repairing the brain even in the absence of transplanted stem cells.

3 An Analogous Biobridge in Stroke: Reconstruction of the Core and Peri-Infarct Areas

That transplanted SB623 aided the regenerative process after TBI via stem cell paved-biobridge between the SVZ and the peri-injured cortex (Fig. 1) indicates a novel mechanism of stem cell therapy and entails clinical significance of "creating" biobridges between neurogenic and non-neurogenic sites which could aid in injury-specific migration of cells across tissues that are barriers to cellular motility.

Recently, the US FDA has approved a limited clinical trial of SB623 transplantation in TBI patients. A phase I/IIa study of SB623 cell transplantation in chronic stroke patients has also been initiated. Furthermore, emerging in vitro and in vivo studies using animal models of brain disorders have also shown that SB623 cells attenuated histological and behavioral deficits associated with stroke, spinal cord injury, and Parkinson's disease (PD).

Understanding the role of SB623 in facilitating the migration of endogenous cells via a biobridge highlights the active role of MMPs and ECMs in stroke pathology [29, 30] and their increasingly prominent contribution as therapeutic targets for stroke. The function and levels of MMPs and ECMs have been shown to be altered by a variety of cells coming from variable sources such as umbilical cord blood, peripheral blood, and the adult brain [31–33]. This suggests potentiality of MMPs and ECMs to serve as biobridges akin to the currently described function of Notch-induced SB623 mesenchymal stromal cells.

The important roles of cells present in neurogenic niches such as the SVZ in the recovery after stroke are well known [34–38]. Nevertheless, a major limiting factor for endogenous repair is the limited migration of newly formed host cells to the site of injury. Our recent studies showed that SB623 cell transplantation boosts endogenous repair mechanisms by guiding the migration of new cells from the neurogenic SVZ across a non-neurogenic brain area to the injured area via stem cell-mediated formation of biobridges which contain MMPs and ECMs. Furthermore, after transplanted SB623 cells have pioneered the formation of biobridges, they appear to relinquish their task of reconstituting the brain after injury to endogenous stem cells. These findings in animal models of TBI have important ramifications for neural repair after stroke.

The precise mechanisms by which grafted cells integrate into the recipient brain tissue and how they interact with the host cells to afford functional restoration are not yet identified. Such essential interaction between the transplanted cell and host cell becomes even more obscure when graft survival is minimal. This indicates that the role of the SB623 is to initiate robust and stable therapeutic benefits, particularly by guiding and facilitating migration of host cells to sites of injury even across non-neurogenic and damaged tissues. The involvement of MMP in the recovery after chronic brain injury has been described through studies which involved inhibition of MMP activities. Accordingly, these studies found that MMP inhibition halted neurogenic migration from the SVZ into damaged tissues and also delayed neurovascular remodeling. Hence, the above observations support the concept that

exogenously added cells can express MMPs which in turn, reinforce the neurovascular unit aiding transplant-mediated host cell migration towards the site of injury.

4 Bridging "Biological Gaps" in Other Neurological Disorders

Most adult stem cells in the brain are found in the SVZ of the lateral ventricles and the SGZ of the hippocampus dentate gyrus. Moreover, the microenvironment of a stem cell niche is maintained by the signaling molecules, growth factors and receptors. In the adult brain, stem cells typically remain quiescent and in a non-dividing state unless activated by an insult (e.g. TBI). When an insult occurs, motility ensues although endogenous stem cells may not exert regenerative effects as they could be "trapped" and not reach the site of injury. The novel discovery that grafted cells can facilitate endogenous stem cell migration from the neurogenic niche to the impacted cortex is indeed a significant progress in the both stem cell and TBI research.

Aside from TBI, there are a number of other neurological disorders characterized by a "biological gap" between the site of injury and intact tissue (e.g., stroke and PD). Hence, the treatment of disorders like stroke and PD may benefit from a deeper understanding of the mechanisms and significance of stem cell-paved biobridges. Stroke entails an ischemic core and penumbra residing next to intact tissue. While cell damage in the ischemic core cannot be reversed, the potential for neural repair has been demonstrated by targeting the penumbra. Therefore, a biobridge between the penumbra and the intact tissue (i.e., neurogenic niche) could potentially aid in neural recovery after stroke. On the other hand, PD involves the degeneration of the nigrostriatal dopaminergic pathway, which could be mitigated with directed migration of host stem cells towards this region in the form of a biobridge. Indeed, further studies are warranted to determine how the concept of stem-cell paved biobridge could be exploited for the treatment of other neurological disorders.

5 The 3Rs: Replacement, Release, and Recruitment—a Multi-pronged Mechanism of Stem Cell Therapy

That stem cells are capable of harnessing biobridges to facilitate brain repair begs the question of whether these biobridges provide scaffold or trophic factors that promote stem cell migration [39]. Interestingly, recent studies have reported candidate ECMs serving as a scaffold or trophic factor-rich soluble molecules. For instance, a study showed that the limits of interstitial cell migration depended upon scaffold porosity and deformation of the nucleus, with pericellular collagenolysis and

mechanocoupling as modulators acting as scaffolds and assisting with biobridge formation (i.e., stem cell migration) [40]. Moreover, functional analysis of mesenchymal stem cell proliferation, migration, and adhesion to ECMs revealed that interleukin 1β did not affect proliferation but rather induced the secretion of trophic factors and adhesion to ECM components such as collagen and laminin [41].

Of note, although newborn cells are crucial for repair, neurogenesis per se, does not ensure successful integration of new neurons within the damaged area. Thus, physiological and functional assays (e.g., synaptic circuitry reconstruction, evoked potentials, long-term potentiation, etc.) of stem cells will be required to unequivocally prove the efficacy of newly formed cells. Depending on the target disease of stem cell therapy, it is likely that neuronal differentiation to specific disease-phenotype, as in the case of PD and Huntington's disease, may be required to fully establish their therapeutic potentials. However, we caution that such neuronal differentiation may occur in both exogenously transplanted cells and the mobilized endogenous stem cells. The concept of biobridge formation highlights the need of guiding the migration of stem cells to the site of injury in order to enhance therapeutic efficacy of newly formed cells that have committed to neuronal lineage. Nevertheless, even in the absence of neuronal differentiation, we propose that the biobridge may also exert bystander effects, in that with the directed migration of these stem cells towards the site of injury, it is capable secreting growth factors, anti-inflammatory substances, and/or anti-oxidative stress molecules.

The mechanism of stem cell-paved biobridge may resemble the actions of olfactory ensheathing glia in spinal cord injury. Preclinical and clinical studies have provided compelling evidence on the efficacy of transplantation of olfactory ensheathing cells (OECs), specialized glia in the olfactory system, in central nervous system (CNS) injuries and neurodegenerative diseases. The unique therapeutic effects of OECs have been attributed to enhanced production of cell adhesion molecules and secretion of growth factors, which supported neuron survival and neurite outgrowth [42]. Another study showed that transplantation of OEG and Schwann cells (SCs) in a sub-acute phase improved anatomical outcomes after a contusion injury to the spinal cord by increasing the number of spared/regenerated supraspinal fibers, reducing cavitation, and enhancing tissue integrity [43]. Most spinal cord injury models evaluating the therapeutic efficacy of OEC transplants have reported functional recovery via indirect and direct reparative pathways involving growth factor secretion, neuronal and axonal regeneration, and remyelination [44].

Although similarities exist between biobridge formation afforded by OECs in spinal cord injury and the one we reported in TBI, the biobridge seen in the former involves ensheathing features of OECs, fabrication of scaffolds (such as laminin and fibronectin) and seeding of stem cells onto these matrices to create a biobridge. Contrastingly, the biobridge in our TBI studies involves a natural process of the stem cells themselves serving as matrices that aid the migration of endogenous stem cells from the neurogenic niche towards the injured host tissue. Furthermore, a similar biobridge strategy was documented in PD whereby the transplanted dopamine-secreting cells were deposited along the nigrostriatal system (instead of

merely transplanting the cells into the striatum) to closely reconstruct the major dopaminergic afferent and efferent pathways [45–47]. But while the bridging graft in PD has been attributed to the artificial reconstruction of the dopaminergic system whereby micro-deposits of immature cells are undertaken along the nigrostriatal pathway, the biobridge formation in TBI reveals a natural process of the transplanted stem cells homing from the neurogenic niche and forming a bridge towards the injured site, subsequently attracting endogenous stem cells to populate the biobridge and to eventually continue the reparative process. Altogether, these observations suggest that while distinct mechanisms of action may be individually facilitating the neural repair of transplanted stem cells in the injured brain, overlapping regenerative processes involving cell replacement, by-stander effects, and biobridge formation may altogether work in concert to unravel the therapeutic benefits of stem cell therapy.

6 Conclusion: Follow the Stem Cell Brick Road

Our recent study advances the concept of the stem cell-mediated formation of biobridge as a mechanism of cell repair in experimental models of TBI, and opens new opportunities for translational applications of cell therapy in TBI and also in other brain disorders. The treatment of neurological disorders characterized by a biological gap between the site of injury and intact tissue can indeed benefit from an improved understanding of the mechanism of stem cell-paved biobridge for neural repair. Further studies are warranted to address issues related to graft-host interactions, which will be key to realizing the clinical significance of stem cell-paved biobridges. A closer examination of this biobridge reparative process, in relation to previously described mechanisms involved in stem cell-mediated repair (i.e., cell replacement and trophic factor secretion), will likely provide new therapeutic directions for TBI and other neurological disorders.

Assessment of stem cell behavior, fate, and migratory pattern following transplantation may reveal pivotal insights into the mechanisms of action underlying stem cell therapy for neurological disorders. Grafted cells which lodge in injured brain tissue can directly replace the cells (cell replacement). Grafted cells can also readily secrete growth factors, thereby stimulating neurogenesis, angiogenesis, synaptogenesis, among other developmental processes that recapitulate the "genesis of life" (i.e., trophic factor release). As described in this chapter and invoked as another mechanism of stem cell-induced recovery, grafted cells can form a biobridge between neurogenic niche and injured tissue to serve as pathway for the direct migration of endogenous stem cells towards the damaged brain area (recruitment). Altogether, these multi-pronged processes may coalesce both exogenous and endogenous stem cells to act in concert towards affording a much more improved brain repair outcome. Although exploiting the 3Rs of stem cell-mediated repair may offer promise for the treatment of a host of brain injury and disorders, there are gaps in knowledge and factors related with stem cell treatment that need to

be addressed before we can realize full therapeutic potentials of stem cells. For instance, as each disease may present unique features and clinical challenges, tailoring these mechanisms (3Rs) to address distinct or overlooked features of certain diseases will likely produce robust therapeutic outcomes. In TBI and stroke, white matter injury remains neglected [48–51], therefore honing the 3Rs to address white matter disruption in these disorders may provide an extensive therapeutic strategy. In PD, directing the 3Rs to nigrostriatal dopamine system as well as non-dopaminergic neurotransmitter systems will mitigate the motor and non-motor symptoms of the disease. Moreover, in spinal cord injury, where demyelination is a critical disease pathology feature [52, 53], directing the 3Rs to target this pathologic hallmark will likely provide more meaningful therapeutic outcomes. In the end, a close monitoring of the stem cell fate, stem cell-secreted growth factors and its matrix, will promote a better understanding of the mechanisms of stem cell therapy and offer insights into optimizing the therapeutic value of stem cells for treating TBI and other neurological diseases.

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Competing Interests

CVB is an inventor on a patent application related to the stem cell research reported here. CVB received research financial support from SanBio Inc. for this study. CVB is additionally supported by NIH NINDS R01NS071956-01, NIH NINDS R21 NS089851-01, and DOD W81XWH-11-1-0634.

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Using Stem Cells to Promote Wound Healing: An Emerging Solution for a Clinical Problem

Anthony D. Foster and Thomas A. Davis

Abbreviations

ASC	Adipose stromal cells
BM-MSC	Bone marrow derived mesenchymal stem cells
CCl_4	Carbon tetrachloride
CLI	Critical limb ischemia
ECM	Extracellular matrix
EGF	Epithelial growth factor
HSC	Hematopoietic stem cells
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin growth factor
IL	Interleukin
KGF	Keratinocyte growth factor

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MIP-1α	Macrophage inflammatory protein 1-α
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cells
PDGF	Platelet derived growth factor
TGF-β	Transforming growth factor
TNF-α	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

1 Wound Healing: *A Brief Review of a Dynamic Cellular Process*

Cutaneous wound healing is a well-regulated, multi-phased process beginning with acute injury and (usually) resulting in re-epithelialization and wound closure. Slowly healing or non-healing chronic wounds, which are a significant cause of morbidity and mortality [1, 2], present a clinical challenge with only limited treatment options. A common feature of slowly/non-healing wounds is a prolonged inflammatory phase that fails to progress to wound closure, often leading to excessive fibrosis. A variety of factors may act as impediments to wound healing such as comorbidities (e.g. age, diabetes, obesity, depression), the size of the wound, microbial colonization of the wound site, and the complexity of the wound [3]. The need for novel treatment options has led to a growing interest in evaluating the use of stem cell populations as potential adjunct therapeutic interventions for treating difficult-to-heal wounds, primarily aimed at enhancing wound epithelialization, and revascularization as well as attenuating chronic inflammation. To understand the role of stem cell treatments in improving the wound healing process it is first necessary to review the basic stages of wound healing with respect to the cellular mediators of that process. Importantly, the scope of this summary is not intended to include an exhaustive review of the mechanisms of wound healing. Rather, the goal here will be to examine the overall process, with an emphasis on the role of important cellular mediators of wound healing.

1.1 Phases of Wound Healing

The number of phases in the wound healing process is a subject of some debate. Beginning with acute injury to the skin, wound healing may go through three [4, 5], four [6], or even five [7] phases before achieving full resolution of the injury. Further, whether scar formation following wound closure should be considered a full resolution of injury or the histopathological outcome of incomplete wound

healing [8] is also a subject of discussion. Wound healing however, is better thought of as a fluid process that progresses through several overlapping stages, rather than a series of mutually exclusive steps, so as to achieve effective barrier formation. In this respect, the number of phases or extent of scar formation is less meaningful than the elucidated discreet highly integrated and overlapping cellular and molecular processes that must occur to achieve effective wound closure. Below we describe the three major stages that occur in optimal cutaneous wound healing, and the major cell types involved.

1. *Hemostasis and inflammation*: The first phase immediately following acute injury is typified by sensory perception (pain), the collection and adhesion of platelets at the site of injury followed by the coagulation cascade, clot formation and hemostasis, and the recruitment of important leukocytes [9]. Activated platelets bind together while also releasing pro-inflammatory mediators and growth factors. This in turn enables the recruitment of neutrophils and macrophages that participate actively in the inflammatory phase of wound healing. Neutrophils are the predominant cell type in the early stages after injury [9]. They are important in the phagocytosis of cellular debris and necrotic tissue as well as in limiting the infiltration of potentially infectious bacteria [10]. Mast cells participate through the release of granules containing histamine and other biologically active molecules that facilitate the classic features of wound inflammation: redness, pain, and swelling [11]. The early mediators of wound repair, including platelets and mast cells, also produce chemo-attractant molecules, such as platelet derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β), that recruit monocytes, which transform into macrophages, and other leukocytes to the site of injury. Importantly, macrophages play a key role in wound repair. Like neutrophils, macrophages actively engulf cellular debris, including dead neutrophils, and produce pro-inflammatory factors as well as growth factors [12]. Factors released by macrophages result in increased vascular permeability, which in turn facilitates the homing of other cell types to the site of injury [13]. Additionally, macrophages may produce factors that promote angiogenesis, the creation of granulation tissue, and production of extracellular matrix (ECM) proteins [13, 14]. Due to these roles, macrophages are thought to be critical to the progression of wound healing and contribute to the transition from the inflammatory phase of healing into the proliferative phase. As such, it is thought that normal vs. aberrant macrophage function is a critical event distinguishing normal from defective wound healing, respectively [12]. Indeed, macrophages have well characterized phenotypes that distinguish different phases of functionality that have been associated with healing versus non-healing wounds.
2. *Proliferation*: The initial inflammation, induced by proinflammatory cytokines and chemokines (Interleukin(IL)-1β, IL-6, IL-8, epithelial growth factor (EGF), fibroblast growth factor, insulin-like growth factor (IGF), monocyte chemoattractant protein-1, macrophage inflammatory protein 1-α (MIP-1α), PDGF,

TGF-β, TNFα, vascular endothelial growth factor (VEGF), and others) released from activated resident and infiltrating innate immune cells, and localized swelling at the wound site contribute to both the local and distant mobilization/recruitment of various reparative cell types such as fibroblasts, keratinocytes, endothelial cells, macrophages, lymphocytes and stem/progenitor cells [15, 16]. Mast cells promote the proliferation of fibroblasts, endothelial cells, and keratinocytes [17, 18]. Fibroblasts subsequently produce collagen that is a key component of the newly forming ECM. Type III collagen and fibronectin are produced as major components early in this process, to be replaced by stronger Type I collagen later on [6, 16]. Fibroblasts also contribute to the formation of granulation tissue that covers the wound bed after hemostasis and prior to re-epithelialization, and to the contraction of the wound site [13, 19, 20]. The activity of fibroblasts that results in the formation of granulation tissue is dependent on the availability of oxygen and nutrients, and thus perfusion. Concomitant with fibroblast activity is the process of angiogenesis within the wound bed. Localized macrophages in a hypoxic environment produce factors that activate endothelial cells and promote neoangiogenesis. Migrating endothelial cells interact with and also break down the initial fibrin network via degradation by matrix metalloproteinases (MMP), such as collagenase, gelatinase, and stromelysin [21]. Endothelial cells and stem cells migrate to the site of injury and proliferate [22]. Newly formed blood vessels perfuse local tissue, supporting the cellular activity within the wound bed. Finally, once granulation tissue is available, epithelial cells including keratinocytes from local healthy tissue migrate over the wound bed to form a new epithelial layer of tissue [23]. As they migrate, keratinocytes break down the previously formed clotted tissue and ECM components using MMPs as they go. During this process the previously formed eschar is dissolved, and replaced with living epithelial tissue. The resulting advancement of newly formed epithelial tissue starts at the edges of the wound, where surrounding healthy tissues act as a source of keratinocytes, and proceeds inward towards the center of the wound [13, 23]. Contraction of the wound represents the last portion of the proliferative phase of wound healing. However, the process is still not complete as the newly formed healthy tissue must still be remodeled in order to fully resemble the tissue that existed prior to injury. Importantly, a failure in any of the steps of this phase, such as a failure to produce new blood vessels to support re-epithelialization, may result in aberrations in the healing process such as fibrotic scarring. Additionally, hypertrophic and keloid scarring can occur due to excessive fibroblast proliferation and subsequent overproduction of ECM proteins [24, 25].

3. *Tissue remodeling*: The final phase of wound healing is necessary to reorganize the ECM to reflect that of normal tissue. During the remodeling phase, Type III collagen that was initially produced in a disorganized manner is broken down and replaced with Type I collagen. The resulting ECM is cross-linked to increase the tensile strength of the newly formed tissue, thus decreasing the likelihood of re-opening the wound [6, 16]. A failure in this phase of wound

healing may result in a chronic wound that re-opens frequently with new insults to the area that was previously injured. This phase may also take a great deal of time to resolve, depending on the size and severity of the wound as well as the health of the individual, possibly taking a year or longer [26]. However, for adults "normal" wound closure may retain some level of scarring and still be considered acceptable [27].

Scar formation occurs as a result of ineffective tissue regeneration. More specifically, scarring occurs when the rate of tissue regeneration is exceeded by that of cell death [28]. At a histological level, scarring involves the excessive buildup of fibrous tissues. Notably, scarless fetal wound healing is a well-observed phenomenon that serves as a model that may shed light on how to treat adult injury so as to achieve scar-less healing later in life [8, 29]. It should be noted that the closure of difficult-to-heal wounds, such as that commonly observed in diabetic foot ulcers, are not the only goals of wound healing research. Indeed, scar-less healing is a major goal given the complications (hypertrophic scars, keloids and contracture formation) that may arise from scar tissue formation even where effective closure is achieved [30].

2 Role of Endogenous Stem Cells in Wound Healing

The ultimate aim of stem cell-based therapy is to repair and/or regenerate injured, damaged, or diseased cells/tissues. Following injury, transplanted stem cells can serve as the source of new cells that will ultimately replace damaged tissue, and also their communication within the inflammatory microenvironment of the wound through paracrine signaling [17] and secretion of trophic factors can suppress inflammatory responses and support tissue regeneration processes. This dual function is important in different types of injury, including both cutaneous and internal injury as well in a given tissue such as skin, different stem cell populations have different roles in recovery. As interest grows for using exogenous stem cell treatments (even to include autologous derived stem cells that are re-introduced) it is important to consider the basic function of endogenous stem cells in the normal wound healing process.

2.1 Role of the Stem Cell Niche

Stem cell fate during regeneration is critically dependent upon the interaction between stem cells and their immediate microenvironment, also referred to as the niche. Under normal conditions the niche provides the stem cell with the necessary structure and signaling for maintenance of the population as well as for the normal

replacement of tissue as seen in the epidermis. Following injury, conditions within the niche may be altered such that stem cells are stimulated to proliferate at a higher rate or become mobilized and travel to the site of injury. In the case of cutaneous injury, epithelial stem cells proximal to the site of injury become stimulated to proliferate and provide cell populations necessary for re-epithelialization and wound closure. Interestingly, subtle divisions within stem cell niches of the skin play a critical role in determining the cellular fate of stem cell populations during the healing process [31]. This will be discussed in greater detail in a subsequent section.

Corneal epithelial stem cells are thought to reside in the limbus of the eye, within the limbal epithelial crypt [32]. The structure of the crypt provides the necessary microenvironment for the maintenance of this stem cell population. This should include control over the rate of proliferation of this stem cell population, although further work is necessary to confirm. Following injury, putative changes within the niche result in corresponding alterations in stem cell function. This most likely occurs through changes in the ECM and growth factor production. Consistent with this proposal are observations that, following injury, proliferation within the limbus increases 8- to 9-fold, and is subsequently reduced within 1–2 days [33]. Notably, surgical removal of the limbus impairs healing whereas limbal transplantation restores wound repair [34, 35]. These findings support a critical role for the stem cell niche in controlling cellular proliferation following injury in a manor critical to normal healing, though further research is necessary to confirm [32].

In addition to the influence of signaling molecules found in the niche, stem cells may even respond to more fundamental stimuli such as mechanical stress. Specifically, it has been proposed that in addition to responding to locally available signaling molecules as well as direct interactions with neighboring cells, stem cells may also receive instruction and in fact alter responses based on mechanical stresses within the niche [36]. For example, the shape of human MSCs, flattened versus rounded, was found to alter cell fate as cells differentiated into either osteoblasts or adipocytes, respectively [37]. Keratinocytes also respond to mechanical stress within their environment, which is significant to wound healing as keratinocytes most likely experience tensile force during wound closure [36, 38].

2.2 Tissue of Origin

Mesenchymal stem cells (MSC) are tissue-derived stromal/stem cells which have the capacity to both self-renew and differentiate into multiple mesenchyme cell lineages including bone, cartilage, fat, dermis, muscle, tendon/ligament, and other connective tissues. MSCs are negative for CD34 and lineage-specific cell surface markers, and are positive for CD73, CD90, and CD105 [39, 40]. MSCs have a role in all three phases of wound healing [9, 40]. MSCs are of particular interest here due to the ease with which they can be isolated from multiple tissues (including bone marrow, adipose, muscle, placenta, and others) and expanded ex vivo. This is, in part, why they are commonly used in studies exploring the therapeutic potential

of stem cell-based treatments in promoting wound healing. MSCs migrate to the site of injury via chemotaxis [41]. During the inflammatory phase, MSCs participate through immunoregulation, by suppressing inflammatory mediators and promoting transition to the proliferation phase [15, 42]. MSCs may participate in the clearance of microbes that infiltrate the site of injury, mitigating the risk of infection [43, 44]. In addition to these important roles, bone marrow derived MSCs (BM-MSCs) have been shown to play an important paracrine role in releasing signaling molecules that drive various portions of the healing process, including factors that promote the proliferation phase [22]. Additionally, MSCs direct the development and differentiation of other cellular populations that actively participate in wound healing. For example, BM-MSCs have been demonstrated to promote the differentiation of hematopoietic stem cells (HSC) into dendritic cells [45]. HSCs, also derived from the bone marrow, serve as the source for wound infiltrating leukocytes that mediate the inflammatory phase of healing [46]. Finally, MSCs regulate tissue remodeling phase including regulation of collagen deposition [47].

Epidermal stem cells (ESC) play an important role in the recovery from a cutaneous injury. ESCs may be found through the skin in various niches including hair follicles, sebaceous glands, and the interfollicular epidermis [48, 49]. Importantly, the role of ESCs in wound healing may vary based on the niche in which they reside. Local ESC, from both the hair follicle bulge and interfollicular epidermis, serve as a source for keratinocytes responsible for rebuilding the epidermis [50–52]. Following injury, ESCs from both hair follicles and from the interfollicular epidermis of tissues proximal to the site of injury, are involved in re-epithelialization [23]. However, hair follicle bulge stem cells contribute more to the early stages of healing by rapidly sending cells to the epidermis during re-epithelialization, but only participate transiently in wound closure as they are apparently short-lived in the epidermis after healing. In a "fate mapping" experiment that tracked the replacement of damaged epidermal tissue with stem cells originating from the epidermis, cells derived from hair follicle bulge stem cells were largely eliminated from the repaired epidermis several weeks after injury [50]. By extension, it has been suggested that hair follicle bulge stem cells be considered as a distinct population from other epidermal stem cells with respect to tissue regeneration [23, 50]. While wound healing has been shown to occur in the absence of hair follicle bulge stem cells, it occurs at a slower rate in genetically modified animals that are deficient for this source of stem cells [53]. Importantly, both ESC populations serve as a source of keratinocytes that mediate re-epithelialization [50, 52]. BM-MSCs are unlikely to contribute significantly to the keratinocytes responsible for re-epithelialization [54], though they may participate in the overall process through paracrine mechanisms [55]. This is consistent with a role for BM-MSCs in directing the differentiation of other cellular populations described above.

Restoration of a functional vascular system is critical to the restoration of healthy tissue as newly regenerated tissues require angiogenesis to avoid hypoxic stress. Localized endothelial precursor cells have been shown to possess angiogenic capability [56] that could contribute to angiogenesis in wound healing. Endothelial precursor cells derived from bone marrow may also participate in the

vascularization of injured tissue [57]. As in other phases of wound healing, BM-MSCs may contribute by paracrine production of VEGF and other angiogenic factors that recruit endothelial precursor cells to the site of injury where they can participate in the angiogenesis of newly formed tissue [58, 59].

In summary, the role for endogenous stem cells in the normal wound healing process varies widely between stem cell types and sources. Proximal to the site of injury, hair follicles appear to be a significant source of stem cells that contribute to wound healing. Consistent with this statement is the observation that a local abundance of hair follicles is associated with more rapid healing, whereas injured tissue that is deprived of hair follicles heals more slowly [17]. This highlights the importance of the hair follicle niche in the regeneration of epidermis following cutaneous injury. Peripheral stem cells derived from circulation and (ultimately) from the bone marrow compartment frequently participate through the production of paracrine signaling. That signaling can in turn direct the differentiation of other progenitor cells that ultimately reside in newly formed tissue after healing, while the former paracrine participant will be absent after healing. While subsequent portions of this chapter will now focus on the role of exogenous stem cells as a treatment modality for promoting healing in chronic or non-healing wounds, it should acknowledged that endogenous stem cells represent a highly relevant reservoir that is first of all vital to healing under normal conditions and may additionally be a therapeutic target to improve the wound healing process [60].

3 Potential Roles for Exogenous Stem Cells in Wound Treatment

Stem cells have outstanding potential as treatment options for wound care. In cases of slowly healing or non-healing chronic injury some level of biological treatment is necessary to restore the normal healing process. Whereas individual biological treatments, such as exogenous cytokine or monoclonal antibodies, target only a single pathway, stem cells have the capacity to respond to local stimuli and produce a wide range of biologically active molecules that could better facilitate the restoration of the healing process. For treatment purposes, autologous stem cells should always be considered as the first option, whether they are isolated from the patient and then re-introduced [61] or simply mobilized by pharmacological action [60]. With autologous cells, there is no potential for immunological rejection of those cells or of tissues subsequently formed from them. However, in many cases this may not be on option. With aging for example, stem cells remain present but lose much of their function [62, 63]. Likewise, co-morbidity, such as diabetes, may result in inherent dysfunction of autologous cells. As will be discussed below, allogeneic stem cells remain a viable option for the treatment of immunologically competent patients because of two important characteristics: they lack the expression of MHC Class II and so illicit a minimal host immune response, and because

they are widely recognized as having immunoregulatory properties that mitigate or even suppress allo-immune responses [64–66]. It should be noted that there are some reports that MSCs have the capability to upregulate MHC II under inflammatory conditions, which is a potential impediment to their use in an allogeneic transfer setting [47].

3.1 Differentiation into New Tissue Layers

The most direct role for stem cells in the wound healing process is as progenitors that provide cells to replace those lost due to injury. As described in the last section, endogenous stem cells serve in this capacity including ESCs that function in re-epithelialization or progenitor cells that differentiate into endothelial cells that make up new blood vessels during angiogenesis. Likewise, exogenous stem cells used as part of a treatment could potentially serve a similar role [67]. However, it is unlikely that allogeneic stem cells would serve in this capacity. Human MSCs have the potential for mis-matched transfer without host rejection in that they are weakly immunogenic as they express variable levels of MHC Class I and lack expression of MHC Class II [68]. However, cellular differentiation may possibly result in the upregulation of MHC Class I and to the eventual expression of MHC Class II on some resident cells, increasing the risk of cellular/tissue rejection.

3.2 Modulation of the Inflammatory Response

As described above, inflammation is necessary for the progression of normal wound healing. This is highlighted by the observation that drugs which severely suppress immune function (such as Sirolimus, a potent immunosuppressive drug used in graft-versus-host disease prophylaxis and therapy), may actually slow healing [69, 70]. However, high levels of prolonged inflammation, such as that resulting from severe or chronic injury, are also capable of impeding the wound healing process [71, 72]. In such cases the repair process fails to progress through the proliferation stage of healing. Rather, an excess of fibrotic material and additional pro-inflammatory factors prolong the inflammatory phase. Notably, treatments that mitigate inflammation have been shown to improve healing in models of slowly or non-healing wounds [73].

Macrophages, cellular components of the innate immune system that actively participate in inflammatory responses, are recognized as critical mediators in tissue repair and healing [74]. The phenotype of wound macrophage may play a critical role between healing and non-healing with respect to the inflammatory state. As described previously, inflammation results in the recruitment of macrophages as well as peripheral monocytes that differentiate into macrophages. However, macrophages themselves have a high degree of plasticity [14] and respond to

environmental cues with changes in phenotype that are also reflected in function. Specifically, exposure to lipopolysaccharide and interferon-γ orient a macrophage towards the M1 phenotype that is pro-inflammatory and has been associated with some autoimmune disorders including type I diabetes [12, 75]. Conversely, M2 macrophages result from exposure to IL-4, IL-13, and M-CSF. M2 (alternatively activated) macrophages are anti-inflammatory as noted by the production of high levels of IL-10 [12]. M2 macrophages in turn support angiogenesis and wound healing such that an imbalance in macrophage phenotype towards M1 is associated with chronic and non-healing wounds [12]. Because macrophages maintain the ability to alter phenotype even after activation due to their plasticity, anti-inflammatory factors may re-orient M1 to an M2 phenotype [14]. As such, changes in the inflammatory milieu resulting from the activity of MSCs may re-orient macrophages from an M1 phenotype to an M2 phenotype that supports the healing and resolution of a wound, as has been observed experimentally [76].

Exogenous stem cell treatment has been shown to downregulate inflammation in a variety of disease and allograft transplantation models as well as in clinical settings [77–79]. MSCs for example are capable of producing a variety of immunmodulatory factors including IL-10, TGF-β1, prostaglandin E2, and indoleamine 2,3-dioxygenase (IDO) [80]. In this scenario, the use of stem cells to treat severe or chronic injury would effect improved wound healing by restraining the inflammatory state such that progression through the proliferation and remodeling stages may be achieved. It is notable that intravenously injected MSCs have been shown to relocate to the lungs, rather than to sites of injury or inflammation [81]. Such trafficking, associated with the resolution of an inflammatory state, is consistent with a role for MSCs in immunomodulation and/or paracrine activity rather than direct differentiation into new healthy tissue at the site of injury. Indeed a variety of studies have investigated the use of MSCs and other stem cells for the treatment of inflammatory disorders including allograft tolerance, autoimmunity, and the prevention of graft versus host disease [77, 79, 82]. Therefore, it is reasonable to assume that the immuno-modulatory characteristics of various stem cell populations, including BM-MSCs, play an important role in controlling the wound healing process in a similar manner.

3.3 Production of Paracrine Growth Factors/Soluble Factors

A third potential mechanism for improved healing with stem cell transplantation is the production of soluble factors that promote the wound healing process. MSCs have been shown to produce factors that promote the proliferation and activation of key cellular populations. For example, VEGF produced by BM-MSCs supports angiogenesis by promoting the proliferation of microvascular endothelial cells [83, 84]. Soluble factors such as MIP1-α, keratinocyte growth factor (KGF), and

IGF are produced by MSCs in a paracrine fashion that recruits macrophages and endothelial cells into the wound site, promotes angiogenesis, and improves wound healing [22]. Adipose stromal cells (ASCs) have likewise been shown to enhance wound healing through mechanisms that include the paracrine activation of dermal fibroblasts that participate in re-epithelialization [85]. Similar to MSCs, ASCs produce VEGF and IGF that promote neoangiogenesis and mobilization/homing of endothelial progenitor cells from the marrow compartment [85, 86].

Currently available literature support a variety of potential mechanisms with respect to the improvement of wound healing through stem cell based treatments. Some of the differences in mechanism may reflect variation in the route of administration or the source of stem cells used. For example the application of autologous stem cells at the site of injury as part of an artificial matrix may be more likely to result in the differentiation of those stem cells into new tissue, whereas the intravenous injection of allogeneic stem cells may be more likely to support wound healing via paracrine or immuno-modulatory mechanisms. In this respect, the niche in which the transplanted stem participates in the wound healing process may play a significant role in determining the physiological outcome of the treatment. It is important however to recognize that these mechanisms, as well as others not described here, are not mutually exclusive but may work in concert with each other to improve the healing process. Stem cells transferred intravenously may produce growth factors that support angiogenesis as well as cytokines or other factors that limit inflammation, thus acting in a synergistic manner to advance the healing process through the inflammatory phase into the proliferative phase. What is consistent in the literature is that stem cell based treatments show promise in enhancing healing in a wide range of injuries, including both external cutaneous injury as well as the internal fibrotic scarring as will be discussed below. Having focused on the basic concepts and the current understanding surrounding the use of stem cell treatments that promote wound healing, subsequent sections of this chapter will focus on pre-clinical, as well as clinical, findings in this area of research.

3.4 Additional Considerations: The Wound Microenvironment

As mentioned above, stem cells are capable of sensing local stimuli and altering their function based on the micro-environmental conditions they encounter. For example, they have the capacity to home to the site of injury where they can interact directly with the wound environment [41, 47], although there is also evidence that BM-MSCs supplied exogenously traffic to the lungs where they remain for only a short period of time [81]. Whether a stem cell is of endogenous or exogenous origin, the niche in which it functions as well as its tissue of origin are of critical importance. In a manner that is reminiscent of Paget's "seed and soil" hypothesis governing the development of metastases, the type of stem cell (seed) and the local

tissue conditions under which it functions (soil) critically impact the manner in which that cell will respond [87]. As such the microenvironment conditions of the niche dictate cell fate, rather than the cellular outcome being purely determined by the stem cell phenotype. Practically speaking, this means that the physical characteristics of the wound, the dynamics of the inflammatory milieu at a given point within the "stem cell niche", the type of stem cell acting on that wound, and the location from which the stem cell responds to the wound in question will all intimately affect the nature of the response elicited from the transplanted stem cells.

This concept can be observed in patients with type II diabetes where endogenous stem cells show a diminished capacity to home to the site of injury, proliferate, and become incorporated into the vascular structures [88]. In such cases, physiological conditions resulting from diabetes critically alter the normal function of endogenous stem cells. Further, in healthy individuals there are observed differences within the epidermis between the role of stem cells from the hair follicle bulge and those from the interfollicular epidermis during normal wound healing [50]. Similarly for a given stem cell, the nature of the wound environment (or soil) will govern the nature of the stem cell response. Once present, stem cells produce a milieu of signaling molecules that are specific to that environment. The effect of those molecules produced may promote angiogenesis, the formation of granulation tissue, limit inflammation, or promote tissue remodeling [47]. Stem cells may even participate in the clearance of bacteria [43, 44]. The anatomical location of the injury is therefore also important. The niche found in the epidermis of the foot is intrinsically different from the epidermis of the scalp, and further different from the parenchyma of the liver. In each respective tissue, even within a single individual, a stem cell will have a different response due to the different local stimuli it senses. As such it is of critical importance to understand the specific molecular microenvironmental nature of a given wound if a rational treatment strategy involving stem cells is going to be considered. With this in mind we will next explore two examples of the types of chronic/non-healing wounds where stem cells are being considered as a treatment option. There are of course many other injury types for which stem cell treatments are actively being investigated. The focus here will be on the nature of the wound, the stem cells being used, and insights into the mechanisms by which stem cells are improving the wound healing process.

4 Wound Healing and Diabetes: *Chronic Non-healing Wounds*

Both type I and type II diabetes are recognized as having a wide range of primary and secondary physiological complications downstream of their respective pathology. Included are vascular complications that have been associated with chronic, non-healing ulcers, especially of the foot [2, 89]. The increased incidence of diabetes in recent years has thus resulted in an increased burden, both medical

and economic, of patient cases with risk factors predictive of tissue and wound complications. Difficult-to-treat and non-healable wounds are a significant cause of morbidity, mortality, and costs in patients with diabetes [2]. The etiology of this chronic condition is attributed to a lack of progress through the inflammatory phase of wound healing [90]. Diabetic foot ulcers fail to achieve the proliferative phase due to a series of failures in the healing process. These failures include, but are not limited to, defects in growth factor production, altered macrophage function, and abnormalities in the formation of granulation tissue. The ultimate result of these defects is an inability to achieve wound closure, and the aforementioned prolongation of a chronic inflammatory phase. As such, effective treatment of chronic diabetic wounds would need to promote progression through the proliferation phase as described above. To that end a variety of stem cell treatments have been tested in pre-clinical animal models of diabetes that show promise for clinical efficacy. Further, initial clinical studies have likewise supported the use of stem cells in diabetes patients with chronic wounds.

Using a rat model of slowly healing diabetic wounds, allogeneic BM-MSCs enhance the formation of granulation tissue, angiogenesis, and cellular proliferation ultimately resulting in improved wound closure [91]. These improvements were attributed to the increased production of VEGF, suggesting a paracrine mechanism of action for the stem cells used in treatment. However, it must be noted that similar studies of stem cells in diabetic wound healing models indicate a role for stem cell differentiation in the treatment mechanism [67]. In this study the use of allogeneic stem cells may have limited the potential of those stem cells due to host rejection of MHC Class I bearing cells. Likewise, the intravenous infusion of xenogeneic human MSCs into a rat model of diabetic foot ulcers improved wound healing through in vivo MSC trafficking to wounds and accumulation in the ulcerative tissue (following IV transfer) and stimulating keratinocyte activity [92]. Consistent with a paracrine role for stem cells in enhancing wound closure, the infusion of human MSCs that were cultured ex vivo with neurotrophin-3 (to increase the expression of VEGF) was found to enhance vascular density during wound healing in a murine model of diabetic foot ulcers [93]. While untreated MSCs improved wound healing versus control animals, ex vivo treatment of MSCs further improved the rate of closure. Finally, topical treatment with both ASCs and BM-MSCs appears to stimulate angiogenesis in a variety of diabetic wound models [94, 95]. Collectively, these data support a role for stem cell treatment in diabetic wound healing that is at least partially dependent on improvements in angiogenesis.

Similarly, another study using a rat model of diabetic foot ulcers showed restoration of normal epithelialization of the wound when rats were treated using BM-MSCs [96]. In this case the authors attribute the improvement in healing to the restoration of normal keratinocyte function following exposure to MSCs. In vitro exposure of human keratinocytes to BM-MSCs increased the expression of growth factors including EGF, IGF-1, MMP-2, and MMP-9. This is consistent with a paracrine role for exogenous MSCs where they improve the function of existing host cells. The increased expression of MMP-2 and MMP-9 is of particular interest as these proteolytic enzymes play an important role in degrading the fibrin matrix

formed during hemostasis. A failure to break down this matrix would result in prolonged fibrotic tissue accumulation, scarring, and a delayed regenerative response as is the case in diabetic foot ulcers. Conversely the restoration of MMP-2 and MMP-9 activity would be expected to enhance the healing process as described in these findings [96].

Wherever possible, autologous stem cell treatments are preferable to allogeneic as there is a lower risk of transplant complications such as host rejection. Autologous tissue may provide a valid treatment option for conditions in which the disease state is not thought to have altered the stem cell niche significantly. The local administration of autologous ASCs in a wound site, using a rat model of induced diabetes, resulted in improved wound healing. The authors attribute mechanism of improved healing to both paracrine production of growth factors as well as the differentiation of stem cells into endothelial and epithelial lineages [67]. Additional studies using autologous BM-MSCs highlight a role for TGF-β induction. TGF-β, which plays an important role in wound closure by downregulating previously activated fibroblasts in normal wound closure [97, 98], has been shown to be suppressed in chronic wounds [99]. Consistent with this role, TGF-β is notably increased in expression when autologous BM-MSCs were used to treat wounds in a diabetic rat model [100]. However, it should be pointed out that the source of autologous stem cells for each of these studies was normal syngeneic tissue from healthy rats that had not been treated with streptozotocin. In a clinical setting, one must choose between treating a patient with allogeneic tissue from a healthy individual, or autologous stem cells taken directly from the patient. In the latter case, stem cells derived from a location distal to the wound of a diabetic patient would still have been exposed to the systemic disease state observed in diabetes. Notably, ASCs derived from diabetic mice were less capable of improving wound healing than similar stem cells taken from healthy control animals [101]. Likewise, an assessment of endogenous ASCs in diabetic mice revealed specific defects in their ability to stimulate neovascularization [102]. The immediate conclusion of these findings is that, in the case of diabetic patients presenting with chronic ulcers, endogenous stem cell therapy may not be effective without ex vivo manipulation. Further, this study may also point to a defect in normal stem cell function, with respect to wound healing, which contributes to impaired wound healing in diabetic patients.

The clinical application of stem cells in the treatment of diabetic foot ulcers has shown significant improvements in wound healing as compared with standard methods of treatment. A recent study investigated the use of ASCs in diabetic patients with critical limb ischemia (CLI), some of whom had non-healing foot ulcers. While the population of the study was low, repeated localized injection of ASCs was shown not only to be safe but to significantly improve clinical features of disease in 66.7 % of the patients tested. Clinical improvements were attributed to enhanced localized vasculature, consistent with ASC induced angiogenesis. A similar study using multiple localized injections of either peripheral blood or bone marrow derived mononuclear autologous stem cells (with both cellular populations giving similar results) in patients with CLI and diabetic foot ulcers

demonstrated efficacy in stem cell treated individuals. Specifically, the stem cell treated group showed improvements in the clinical features of CLI versus that of control groups. Further, the study observed a significant improvement in the healing of diabetic foot ulcers in the stem cell treated group versus all other treatment groups [103]. The safety of a stem cell treatment was likewise confirmed with similar improvements in wound healing using autologous ASCs in patients with diabetic CLI [104].

5 Wound Healing and Liver Fibrosis

Internal tissue and/or organ injury, and the ensuing repair, may also become dysregulated resulting in complications similar to that of cutaneous non-healing wounds. Successful healing of injury to the liver results in the activation of liver progenitor cells that replace temporary fibrotic tissues with healthy regenerated tissue [105]. By contrast chronic liver disease, such as cirrhosis, results in the inability to fully heal damaged tissue in the liver, leading to a build-up of fibrotic tissue [106]. Similar to non-healing cutaneous injury, liver fibrosis includes features of prolonged inflammation. Importantly, wound healing in the liver follows a similar script to cutaneous wound healing. Under normal conditions, injury to the liver results in localized inflammation that coalesces in an anti-fibrinolytic coagulation cascade that triggers clotting and hemostasis. Leukocyte recruitment to the site of injury results in phagocytosis of dead or dying cells, and the promulgation of a pro-inflammatory cascade that includes IL-6, TNF-α, and IL-1β [107]. Growth factor production activates mesenchymal precursor cells that differentiate into myofibroblasts. These cells along with hepatic stellate cells induce fibrosis [108, 109]. Properly regulated fibrosis eventually resolves the injury with normal wound closure and healing. In such cases prolonged scarring and permanent fibrotic tissue in the liver are not observed. However, with chronic injury (or in some cases of severe acute injury) a dysregulated inflammatory response is associated with an excess of fibrosis and failure to heal properly [107, 109]. In such cases, hepatic stellate cells derived from myofibroblasts [28] mediate pathological liver fibrosis by contributing to scar formation through the production of excessive amounts of ECM [110]. The eventual result is the replacement of functional parenchyma with non-functional scar tissue [109]. With progression this condition will lead to liver failure, with a liver transplant as the only viable treatment option at present. Liver failure, whether due to viral infection, alcoholism, or other etiology, is a significant cause of morbidity and mortality. As such, alternative treatment options that preclude the need for transplantation are of great interest.

Presently, the use of various stem cell populations as potential therapeutic options for liver disease is being investigated in pre-clinical animal models. In addition to determining the efficacy of the treatment, a primary question being asked is of course the mechanism of action. As with cutaneous healing, the possible modes of action include differentiation into new tissue, paracrine production of

immuno-modulatory/growth factors [20], and fibrinolytic degradation of fibrotic scar tissue. Animal studies typically involve the introduction of an exogenous stem cell source proximal to induction of fibrosis in rodents using carbon tetrachloride (CCl_4) [106, 111]. In such a model of liver fibrosis, bone marrow-derived MSCs (BM-MSCs) labeled with green fluorescent protein were found to home from the blood to the site of injury resulting in improved healing [112]. CCR9, CXCR4, and c-MET were found to be necessary for BM-MSC homing to the site of injury. Likewise, C57BL/6 mice with CCl_4 induced liver fibrosis showed improvement in fibrosis when treated with bone marrow derived MSCs alone [110]. Interestingly, even when an autologous stem cell source was used, stem cell survival remains a limiting factor. While BM-MSC treatment alone improves the healing of induced chronic liver injury in mice, using a co-treatment of a nitric oxide producing substrate (sodium nitroprusside) appears to improve the survival of autologous BM-MSCs after transfer as well as the treatment effect. Supplementing stem cell treatment with a nitric oxide producing substrate led to apoptosis of hepatic stellate cells and prolonged survival of BM-MSCs, resulting in an even greater reduction in liver fibrosis than MSCs alone. This study suggests that stem cell survival may be a limiting factor in stem cell treatments that improve wound healing. While many studies have focused on the use of bone marrow derived mesenchymal stem cells, these are not the only stem cells being considered. For example, stem cells isolated from the amniotic fluid of pregnant mice were found to improve tissue repair in an inducible murine model of liver fibrosis as observed by lowered levels of liver enzymes in the blood and a reduction in fibrotic tissue [113].

An alternative mechanism for improvements in liver fibrosis is seen in a study that utilized unfractionated whole bone marrow in the treatment of liver injury in CCl_4 treated mice wherein MMP-9 expressing bone marrow cells were responsible for the direct hydrolytic degradation of liver fibers and reduction of scarring [114]. A similar study that replaced unfractionated bone marrow cells with a bone marrow mesenchymal cell line found reduced fibrosis that was associated with increased expression of MMP-2 and MMP-9 [115]. The authors also investigated the use of adipogenic and hepatogenic cells differentiated in vitro from the original mesenchymal stem cell line. While improvements in fibrosis were observed, along with the expression of MMP-2 and MMP-9, the results were not as effective as that observed with the parental undifferentiated stem cell line. These findings highlight a role for stem cells in wound healing other than the mere replacement of damaged or dead cells (through differentiation), otherwise it would be expected that the hepatogenic differentiated cells would likely have been as effective as the original stem cell line.

As with the treatment of cutaneous injury, when considering the possibility of exogenous stem cell treatment, one must also consider the potential role of endogenous stem cells in resolving liver fibrosis. Specifically, what does the stem cell used in treatment do differently than stem cells already present in the individual? Treatments that mobilize endogenous hematopoietic stem cells from the bone marrow, such as granulocyte colony stimulating factor, have been shown to reduce liver fibrosis and promote healing in animal models of cirrhosis [116].

Importantly, endogenous stem cell mobilization was found to decrease the expression of inflammatory markers while also increasing the hepatic expression of peroxisome proliferator-activated receptor gamma (PPAR-γ). This is significant as PPAR-γ is of great interest in the wound healing process with respect to chronic liver injury. As hepatic stellate cells are being activated in the process of fibrinogenesis, PPAR-γ expression is downregulated [117, 118]. Conversely PPAR-γ binding to its ligand is associated with the hepatic stellate cell deactivation and a reduction in fibrosis [118, 119]. As with the potential use of endogenous stem cells in treating diabetic foot ulcers, one must consider the efficacy of stem cells that have been exposed to a prolonged disease state. The relatively rapid induction of liver fibrosis in CCl_4 may be effective in replicating disease with respect to liver histology, but may not replicate the peripheral effects of a prolonged systemic disease that results in liver fibrosis in a patient. In line with this view point, prolonged injury to the liver leading to cirrhosis has been associated with functional changes in bone marrow endothelial cells, resulting in changes in the output of cytokines and matrix proteins produced by those cells. This in turn is thought to contribute to alterations in the bone marrow niche, and may contribute to the dysfunction of endogenous HSCs [120]. That said, endogenous stem cells may be less effective in resolving liver fibrosis than allogeneic stem cells from a normal healthy individual. Still, mobilization of endogenous stem cells represents an intriguing low risk approach to achieving a stem cell treatment while being minimally invasive and further research is necessary in this area.

In contrast to the above discussion, it has been suggested that BM-MSCs may not be entirely beneficial as a treatment option for liver fibrosis, and in fact may increase fibrosis of the liver or other organs. Russo et al. [121] find that donor bone marrow cells differentiated into functional myofibroblasts and hepatic stellate cells that were responsible for collagen Type I production, and actually contributed to liver fibrosis rather than resolving it as reported elsewhere. This is in sharp contrast to related animal studies that showed a reduction in liver fibrosis attributed to BM-MSC treatments. It suggests that caution be used when considering the bone marrow compartment as a potential therapeutic target for the treatment of liver fibrosis.

Given the overall efficacy of stem cell treatments in animal models of liver fibrosis there has been interest in applying this method clinically. Several studies of stem cell treatments in patients suffering from liver fibrosis have been completed recently, including testing that confirmed the safety of stem cell treatments in patients with cirrhosis [122]. Likewise limited testing of autologous bone marrow derived mesenchymal cells in patients with cirrhosis have shown both clinical [123] and histological [124] improvements in features of liver disease. There are several benefits for the use of stem cell treatment in liver disease. The most direct benefit is the potential to resolve a given pathology without major surgery or risk of graft rejection, and in a safe manor. Additionally, for those patients that will require transplantation due to liver failure, but for whom a viable donor is not yet available, stem cell treatment may improve disease features, including quality of life, and prolong survival until a viable transplant organ becomes available [123].

6 Conclusions

Recent advances in our understanding of stem cell biology, including the therapeutic application of stem cells, present an excellent opportunity for the development of novel modalities that may benefit a variety of scenarios involving non-healing or slowly healing chronic injury. The discussion above highlights those advances in these areas of study. However, many questions remain regarding the potential efficacy of stem cell based treatments in wound healing. While confirmation of the safety of such treatment remains paramount, much remains unknown with respect to treatment optimization. Variables including stem cell type, route of administration, timing, cell number, and inclusion of supporting drug treatments must be tailored to the type of wound in question. Optimization of these variables will likely be dependent on the elucidation of the cellular and molecular mechanisms by which stem cells treatments enhance the wound healing process. To that end, additional pre-clinical studies are necessary to determine the as yet unknown cellular and molecular mechanisms of action of stem cell treatments in improving the wound healing process.

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Cancer Stem Cell Niche

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Abbreviations

ABC	ATP-binding cassette
ALDH-1	Aldehyde dehydrogenase-1
BDNF	Brain-derived neurotrophic factor
Bmi-1	B Lymphoma Moloney murine leukemia virus (Mo-MLV) insertion region 1 homolog
CAF	Carcinoma-associated fibroblast
CD29	Also known as integrin-ß1
CRC	Colorectal cancer
CSC	Cancer stem-like cell
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAP	Fibroblast activation protein
FGF	Fibroblast growth factor
Her2	Human epidermal growth factor receptor-2
HGF	Hepatocyte growth factor
IL	Interleukin
Lgr-5	Leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49
MDR	Multidrug resistance
MSC	Mesenchymal stem cell
PDGFR	Platelet-derived growth factor receptor
SCs	Stem cells

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TAM	Tumor-associated macrophage
Tert	Telomerase reverse transcriptase
TGF	Transforming growth factor
TIC	Tumor-initiating of cancer cell
TNF	Tumor necrosis factor
TrkB	Tropomyosin-related kinases B
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless/Int

Core Tip

In colorectal cancer, one of the major hallmarks of carcinogenesis, the limitless replicative potential of cells, a property of stem cells, has been the basis of comprehensive of the failures of cancer-therapies. The cancer stem cells represent less than 2.5 % of the tumor mass, but are responsible for the resistance to therapies and the recurrences.

1 Introduction

Carcinogenesis is a multistep process reflecting a series of genetic and epigenetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. In 2000, Hanahan and Weinberg suggested that cancer results from six essential alterations in cell physiology that dictate malignant growth [1]: self-sufficiency in growth factors, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and ability to invade and metastasize [1]. Moreover, they propose that these six capabilities are shared in nearly all types of human tumors. In the last decade, the description of two new hallmarks, reprogramming of energy metabolism and evading immune destruction, contribute to a new concept, the tumor microenvironment [2]. This reconceptualization of cancer cell biology drove profound changes in studying cancer, and in its therapeutic approach. The study of angiogenesis and limitless replicative potential, a feature of stem cells and their microenvironment, has been the basis of the main progress in the treatment of cancer, especially colorectal cancer.

Formation of vasculature, named angiogenesis, is actively involved in tumor development, progression and metastasis. The initial step of tumor angiogenesis is yet not well understood. The recruitment of perivascular support cells is necessary for the formation of the blood vessels [3]. Diverse tissue-specific stem cell types contribute to create the tumor niche, such as tumor-associated stromal cells including carcinoma-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), lymphocytes, pericyte cells, inflammatory cells or normal epithelial cells,

and mesenchymal stem cells (MSC). Recently, MSCs were found to home to tumors and transit into CAFs [4–6]. These processes appear at the earliest stage of tumor development. In the tumor niche, cells and especially the less differentiated of them, find the best conditions to proliferate. These cells possess stem cell properties such as self-renewal and multi-potentiality [7]. They have been named cancer stem-like cells (CSC) or tumor initiating of cancer cells. CSCs are a minor population of tumors. They may be responsible for the resistance to cancer therapies and recurrence of tumors in numerous of solid cancers such as glioblastoma [7] and colorectal cancer (CRC) [8, 9]. Colon cancer as others solid cancers, is composed by a heterogeneous population of cells, even for CSCs including dormant (or quiescent) and active cells [10]. CSCs share many properties with normal adult stem cells, and are able to have intrinsic resistance to apoptosis, and to express several members of the ATP-binding cassette (ABC) family, a family of membrane transporters over expressed in the multidrug resistance (MDR) phenotype [11]. In the tumor niche, CSCs find local and systemic conditions for proliferation and protection against conventional therapies [12]. Moreover, microenvironment stimuli such as hypoxia, contribute to chemoresistance by inducing a stem-like-phenotype in cancer cells [13]. The relationship between cancer cells and CSCs on one hand, and the stimuli of microenvironment promoting angiogenesis, secondly, is fundamental to understand the driving force of tumor progression and therapeutic resistance.

1.1 Intestinal Stem Cells

Normal intestinal epithelia are in continuous renewal, with a lifetime of around 5 days. The replacement rate of these epithelia is regulated by stem cells (SC) and is under microenvironmental influence [14]. The intestinal SCs are involved in tissue homeostasis and repair. They are located at the crypt base. These cells divide mostly asymmetrically and give rise to two different daughter cells, one being identical to the original cell, the other has the potential to differentiate and migrate to the top of the crypt, reproducing the fully differentiated intestinal cells. Intestinal crypts contain two pools of SCs. The first pool is located at the lowest part of the crypt base and is characterized by the expression of Lgr-5 (leucine-rich repeat containing G protein-coupled receptor 5). The second resides at the +4 position, i.e. the fourth cell above the lowermost cell of the intestinal crypt, and expresses Bmi-1 and Tert (telomerase reverse transcriptase). Lgr-5 + cells could be the active population, whereas Bmi + SCs or Tert + cells are quiescent SCs and represent a reserve pool of SCs with the ability to replace Lgr-5 cells [15] (for review see Vaiopoulos et al. [16]).

Identification and isolation of SCs remain an issue of debate. Many molecules have been proposed as putative stem markers, but none are widely accepted as specific molecular markers. Mostly are located on the cell surface. The most are Lgr-5, ALDH-1 and CD29 (Table 1).

Table 1 Markers used to identify normal colonic stem cells and colonic cancer stem-like cells

	Marker	Function
Normal stem cell	Integrin ß1 (CD29)	Cell surface receptor—Cell adhesion molecules
	Hes-1	Transcriptional repressor—transactivated by Msi-1
	Msi-1	RNA binding protein—Maintenance of undifferentiated state
	Bmi-1	Polycomb receptor—Maintenance of chromatin silencing
	Lgr-5	Wnt target gene, potential of self renewal
	ALDH-1	Detoxifying enzyme
	DCAMKL-1	Kinase—Radioresistance abilities
	Tert	Quiescent SCs and radio resistant
	Ascl-2	Transcriptor factor—target of Wnt and Notch pathways
Cancer stem-like cell	CD133	Pentaspan transmembrane glycoprotein
	CD44	Hyaluronic acid receptor
	CD166	Cell adhesion molecule
	ALDH1	Enzyme
	OCT4	POU-domain transcription factor
	SOX2	Transcription factor
	c-Myc	Transcription factor
	Integrin ß1 (CD29)	Cell surface receptor—Cell adhesion molecules

1.2 Cancer Stem-like Cells

Every normal cell can accumulate mutations and become the cancer origin cell, i.e. the cancer stem-like cell (CSC) or tumor initiating cancer (TIC). In 1994, Lapidot hypothesized the existence of CSCs [17] and, in 2007 Vitiani isolated and characterized CD133 + cells as CSCs from colon cancer tumors [14]. It seems that only a small portion of cells (<2.5 % of the tumor mass) within a tumor is endowed with tumor propagation, whereas all others are not [18]. CSCs share many properties with normal adult SCs, such as expression of markers common to stem and progenitor cells, are capable to an unlimited growth in vitro and have the ability to reproduce the parental tumor in vivo. CSCs are multipotent cells capable to give rise to progenitors and differentiated cells resulting in tumor heterogeneity, and to migrate resulting in metastases. However, individual CSCs' responses to microenvironmental stimuli, epigenetic modifications and additional genetic aberrations which in turn, may lead to clonal evolution and gain or loss of CSCs' attributes [16]. Wingless/Int (Wnt) signaling pathway plays a pivotal role in the regulation of stem cell self-renewal. In normal cells, Wnt signals are transduced through Frizzled/LRP5/6 complex to stabilize ß-catenin by inhibition of its phosphorylation-dependent

degradation. In colon cancer cells, the mutations in APC or ß-catenin genes are constant but heterogeneous; CSCs have a high activity of Wnt signaling. Moreover, Wnt activity and cancer stemness can be regulated by extrinsic signals given by neighboring cells or matrix cells, such as stromal myofibroblasts [19]. Three other major pathways are altered in CSCs: TGF-ß, notch, and hedgehog signaling. The former regulates cell proliferation, differentiation, migration, apoptosis and SC maintenance and function in normal colon tissue. Its alteration induces an inhibition of its tumor suppressor effect. TGF-ß signaling alteration is found in more advanced and metastatic CRC. The second regulates cell determination during development and stem cells, and is implicated in differentiation, proliferation and apoptosis. In normal colon, a paralog of the Notch-gene, Notch-1 and its ligand Jagged-1, is abundantly expressed in the stem cell zone. In CRC, notch-1 is upregulated. Moreover, in CRC-CSCs, Notch signaling is 10–30 fold higher in comparison to commonly used colon cancer cell lines. It prevents CSCs' apoptosis through p27, a cell-kinase inhibitor, maintains CSCs renewal and represses cell lineage differentiation genes. The latter is one of the key regulators of animal embryogenesis, implicated in proliferation, migration and differentiation of cells. In CRC, hedgehog is implicated in tumor growth and CD133 + stem cells, i.e. CSCs (for a review, see Roy [19]). All these pathways are probably coordinated in CSCs.

Characterisation of CSCs' homeostasis could lead to major progress in carcinogenesis understanding. Therefore, identification and isolation of CSCs are necessary. Both are a real challenge.

Identification of CSCs is based on the markers of the normal SC, especially Lgr-5 and Bmi1, the only markers rigorously evaluated in vivo [20, 21]. Other markers have been developed for CSCs. CD133 and CD44 are two classical markers, but not specific enough. October-4 and Sox2 seem most promising. They are both a transcriptional factor implicated in cell renewal. In CRC, their high levels are correlated with poor prognosis and increased proliferation of CSCs [22, 23]. For more detailed presentation of the currently used markers for normal SC and CSCs, the reader is referred to Table 1.

Isolation of CSCs is difficult and their low number, their heterogeneity and their undifferentiated property are the major obstacles in the isolation and study of these cells, from patients' tumor as well as from in vitro cultures. In fact, developing and using different methods have been of great interest. Several methods have been developed [24] based on the expression at the cell surface of receptors or adhesion proteins known as CSCs biomarkers (for a review, see Vaiopoulos et al. [16]) (Table 1). The Flow Cytometry (Fluorescence-Activated cell sorting or FACS), or the magnetic-activated cell sorting (MACS) has been widely used [25]. These methods reside in the specific recognition of antigen expression by antibodies. But, two important disadvantages are (i) antibodies used have to own a drastic and highly specific capability of recognition and (ii) labeling could induce cell modification and differentiation, changing CSCs properties (Table 2).

Developing methods without using marker labeling is largely needed. In this way, tools based on intrinsic biophysical properties such as size or density could be of great interest. For that purpose, in the past, counterflow centrifugal elutriation

Table 2 Advantages and disadvantages of the sorting cell methods

Methods	Advantages	Disadvantages
MACS	Fast, easy to make	Cell labeling indispensable
FACS	Fast	Cell labeling indispensable, flux cytometry indispensable
CCE	Cell labeling not necessary, cell weight based method	Consuming time, specific instrumentation indispensable
SdFFF	Cell labeling not necessary, cell size and density based method	Consuming time, specific instrumentation indispensable

MACS magnetic activating cell sorting, *FACS* fluorescence activating cell sorting, *CCE* counterflow centrifugal elutriation, *SdFFF* sedimentation flux force fractionation

(CCE), which consists of cell separation by their weight, was a valuable tool for obtaining homogeneous populations [26]. But in spite of the encouraging results, no experiment was made for CSCs from CRC, to our knowledge, maybe due to sophisticated equipment needed. More recently, the ability of SdFFF (Sedimentation Field Flow Fractionation), in which cell sorting is based on their size and density, to sort CSCs from a panel of CRC cell lines was demonstrated [27].

1.3 Tumor Niche, Microenvironment, and Epithelial-Mesenchymal Transition (EMT)

The niche is a dynamic milieu with stromal microenvironment surrounding the stem cells. It adapts in response to environmental cues. In non-cancerous intestinal tissues, the niche is composed of multiple types of cells such as neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts. It assures the optimal conditions for SC and regulates the stem cell proliferation and differentiation even in the absence of SC [28]. Intestinal SCs can also be affected by the components in the crypt lumen, such as bacteria or epithelial cells (for review see Vaiopoulos et al. [16]). One of the most extensively studied components is the intestinal subepithelial myofibroblasts that regulate intestinal SCs through elaboration of growth factors and cytokines. Wnt signaling is central to maintenance of the intestinal SCs [28]. Myofibroblasts produce the Wnt signaling ligands, that bind to Fizzled receptors as well as morphogenetic protein (BMP) antagonists gremlin 1 and gremlin 2 on basal epithelial SCs and modulate notch signaling [29].

Like normal intestinal SC, CSCs reside in a qualified microenvironment altered by genetic and epigenetic aberrations. CSCs can secure the microenvironment stimuli by displacing normal SCs from their niche, and interact with it to generate vascular precursors [30].

The tumorigenic niche is the supportive and connective tissue of the host tissue. It is composed of transformed myofibroblasts, recruited myeloid cells, vascular and

lymphovascular endothelial cells, infiltrating cells of immune system such as macrophages, and formed the stroma tissue. These stroma cells, especially fibroblasts, secrete numerous factors that act in an autocrine or paracrine fashion on tumor cells. These factors are various cytokines and growth factors, such as hepatocyte growth factor (HGF), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). HGF seems a major enhancer of Wnt activity [31, 32].

In epithelial-mesenchymal transition (EMT), Wnt, notch, and Hedgehog are three major pathways influencing strongly the paracrine signals. Recently, the implication of neurotrophin receptors, essentially brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinases B (TrkB) as sortilin, their transporter, initially identified in neural cells, was highlighted for CRC both in vitro and in tumors [33]. High TrkB expression is associated with more advanced disease and the worse prognosis. Moreover, some studies suggest that TrkB regulate EMT in solid cancers [34] especially in CRC [35].

Various cells compose the tumorigenic niche, and are involved in CRC progression and therapeutic sensibility. CAFs, can promote tumor growth via enhancing tumor angiogenesis, and participate to the chemoresistance, growth, progression and metastasis. These cells are able to modulate the expression of oncogenic genes in cancer cells, such as Her2, EGFR and Ras and thereby contribute to the resistance of chemotherapy [36]. CAFs can also secrete a panel of cytokines and growth factors such as CXCLA, CXCL2, interleukin-1ß (IL-1ß) and IL-6 to enhance angiogenesis and tumor progression [37]. CAFs serve as a niche promoting tumor growth and are a main actor in tumor-prone microenvironment. However, targeting them remain a challenge due to the presence of distinct CAFs populations expressing different makers such as FAP, S100, PDGFR, that cannot distinguish CAFs within tumors from fibroblasts present in non-cancerous tissues (for review see Togo [38]). However, their effects on enhancing tumor growth and angiogenesis seem less effective than MSCs effect.

MSCs are non-hematopoietic precursor cells residing in the bone marrow. They contribute to the tumor microenvironment and also influence tumor development, progression, metastatic diffusion and resistance to chemotherapy in many solid cancers such as colon [39]. The interaction of MSCs and cancer occurs early in tumor formation via numerous pathways. In colon, MSCs expressed high level of vascular endothelial growth factor (VEGF) via the HIF-1alpha pathway when they are stimulated by interferon-gamma and TNF-α, thus leading to colon cancer growth [40]. MSCs secrete CXCL12 to recruit endothelial cells after exposure to a tumor [41]. They are able to secrete IL-6 to induce non-cancer stem cells to express markers of cancer stem cells and increase the ability to form a tumor in vivo [42]. This MSC IL-6 secretion induces the secretion of endothelin-1 (ET-1) by cancer cells, which then activates the two major signaling pathways, Akt and ERK, that transduce signals at the cell surface and lead to the protein synthesis, in endothelial cells, thereby recruiting endothelial cells to promote tumor development [43]. This property has been recently demonstrated by Huang et al. [43] who induced angiogenesis by mixing non-tumorigenic MSCs and HT-29, a colorectal cancer cell lineage. Moreover, the angiogenesis, enhanced by interaction between cancer cells

and MSCs, can be blocked by IL-6 or ET-1 antibodies [43]. IL-6 and ET-1 are both important in patient tumor development: patients with colorectal cancer had significantly higher VEGF and IL-6 serum levels than healthy control. Their rates are correlated with advanced stages and metastatic disease, suggesting IL-6 is involved in tumor development [44]. The importance of IL-6 in colorectal cancer patients was highlighted in 1998, and numerous studies had proven its role [45–48]. In normal adult tissues, angiogenesis is only transiently turned-on.

However, the successful growth of metastatic cells depends on the interactions and the properties of cancer cell, and their potential target organs. This hypothesis was suggested by Paget and named the "seed and soil" [49].

1.4 CSC, Microenvironment and EMT, Implications for Physicians

During the last decade, CSCs, EMT, angiogenesis involved major therapeutic progresses for cancer and especially for CRC.

Understanding of angiogenesis pathways was a crucial step. The first antiangiogenic compound, a monoclonal antibody against VEGF, named bevacizumab, was recommended in first and second line with adjuvant chemotherapy, FOLFOX (5-Flourouracil, Leucovorin and Oxaliplatin) or FOLFIRI (5-Fluorourcil, Leucovorin and Irinotecan) in 2004. In 2009 a wide meta-analysis, including more than 3000 patients concluding that the addition of bevacizumab to chemotherapy for metastatic CRC prolongs both specific free survival and overall survival despite higher incidence in grade III/IV hypertension, arterial thromboembolic events and gastrointestinal perforations [50]. Other antiangiogenic therapies, such as aflibercept, a VEGFA,VEGFB and placenta growth factor (PIFG) decoy receptor, or ramucirumab, a VEGFR1/2/3 and Tie2 tyrosine kinase inhibitor, have been validated by clinical trials [13]. These targeted therapies opened a new era in CRC treatment.

Despite the benefits in metastatic CRC patients, antiangiogenic therapy failed to improve long-term outcome. Moreover, the AVANT study, a phase 3 randomized trial assessing the use of bevacizumab in combination with oxaliplatin-based therapy in adjuvant treatment of patients with resected stage III or high-risk stage II colon carcinoma, suggested a detrimental effect of bevacizumab that had involved more serious adverse effects without disease free survival improvement [51]. Antiangiogenic therapy could be benefit only for CCR patients with liver metastasis. Therefore, even for these patients, the administration's modalities of antiangiogenic drugs needs further evaluation. On account of the increase of the levels of plasma VEGF concentrations and EPCs after partial hepatectomy in CRC metastatic patients, Pocard claims (i) the primary colon cancer should be resected rapidly to minimize the activation of metastatic niche, (ii) surgery should be followed by systemic chemotherapy associated with anti-angiogenic drugs, (iii) any liver

metastases should be resected, (iv) immunomodulatory and anti-angiogenic treatments should be administered to minimize the risk of recurrence [52].

In fact, nor anti-angiogenic drugs nor adjuvant chemotherapy can eliminate recurrence or resistance events. It is now admitted that CSCs and EMT can induce chemoresistance. They can develop two types of mechanisms of chemoresistance, intrinsic mechanisms and indirect mechanisms. The first includes proficient DNA repair machinery, high expression of ATP-binding cassette (ABC) drug transporters, and altered cell cycle kinetics. In CRC, the overexpression of inter-leukin-4 (IL-4) amplifies the expression of antiapoptotic mediators, and blocking IL-4 increases the in vivo-efficacy of cytotoxic therapy [53]. ATP-binding cassette family is implicated in radio and chemoresistance of CRC. Blocking it improves the response to neoadjuvant radiotherapy [54]. Alterations of CSCs' cell cycle, especially the blockage in G0 phase, maintain them in quiescence. These quiescent CSCs are spared by chemotherapeutic toxicity and can reconstitute the original tumor [55–57].

The latter, includes microenvironmental influences that indirectly contributes to chemoresistance (for review see Maugeri-Sacca [11]). In fact, the interactions between the CSCs and the microenvironment are dynamic processes that result in a continuous remodeling of both compartments. These epithelio-mesenchymal interactions take place in the EMT. EMT has a main role in chemoresistance as in the metastases development. In addition to the EMT, hypoxia, derived from various tumor factors, such as chaotic and dysfunctional vasculature, poor oxygen and nutrients supply, leads to a suboptimal concentration of chemotherapeutic agents within the tumor [11]. Cotargeting intrinsic and indirect mechanisms with antiangiogenic agents or inhibitors of EMT/hypoxia-associated effectors could lead to depletion of CSC pool and contribute to increase the chemotherapeutic response.

The type of predominant cells could have a main importance for prognosis and therapy in CRC. Traditionally, CRC classification of AJCC (American Joint Committee on Cancer) is the base of prognostic and care. Surgery is curative for stages 1–3, and adjuvant chemotherapy is ordered for high-risk stage 2 and stage 3 CRC, anti-angiogenic drugs are recommended for metastatic patients. Unfortunately, it is still difficult to predict disease progression or treatment response. Numerous studies have been conducted in CRC to determine a signature capable to identify patient populations with high risk of recurrence who need adjuvant therapy, from those who can be spared from chemotherapy. Currently, in order to select patients that will respond to targeted treatment, a mutation in the KRAS or BRAF gene is analyzed, a mutation signing a resistance of therapy. Despite this marker, a large proportion of patients with wild-type KRAS are chemoresistant. Different molecular subtypes have been determined in CCR [58, 59]. However, Sadanandam propose a new CRC classification system based on the subtype cellular phenotype and the therapeutic response. Six CRC subtypes were defined, based on the combined analysis of gene expression and differential response to cetuximab. The CRC subtypes were associated with distinctive anatomical regions of the colon crypts and with the differentiation states and Wnt signaling activity. The characteristics of each subtype were analyzed on cultured

cell lines and on patients' tissues. The stem-like subtype had the poorer prognosis and the need for adjuvant chemotherapy (FOLFIRI) even in case of metastases, whereas, the most differentiated CRC subtypes, named transit-amplified and goblet-like subtypes, had a good prognosis, and do not need adjuvant therapy [59].

Finally, the heterogeneity of CRC imposes to change our therapeutic schemas. Personalized therapeutic approaches could improve the survival, not only by an increase of specific survival, but also by a decrease in the adverse effects induced by unnecessary chemotherapy.

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Insight into Cancer Stem Cell Niche; Lessons from Cancer Stem Cell Models Generated In Vitro

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Abbreviations

CSC	Cancer stem cell
miPSC	Mouse induced pluripotent stem cell
LLC	Lewis lung carcinoma
CM	Conditioned medium
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
DAPT	N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
GFP	Green fluorescent protein
LIF	Leukemia inhibitory factor

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1 Introduction

Cells that possess self-renewal and differentiation capacities in various tissues are recognized as somatic/adult stem cells. These stem cells are contributing to the organogenesis in development and to the maintenance of tissues by supplying differentiated and functional progeny cells for tissue homeostasis. It is well documented that fates of stem cells are tightly regulated by their environments, "niche", composed of extacellular matrix (ECM), soluble factors, and various kinds of cells including stem cell itself and differentiated cells [1–5]. Similar to normal tissues, tumor could be also described as a tissue consisting of undifferentiated and differentiated cells in a cellular hierarchy. The cell on the apex of this hierarchy is called "cancer stem cell (CSC)" [6–10]. Along with the capacity of self-renewal, the differentiation capacity of CSCs is contributing to giving rise to heterogeneous population of cancer cell in a tumor, and to maintenance of tumor mass. Further, the growing number of evidences that indicate the contribution of CSCs to cancer pathology, such as resistance to the conventional chemo-/radio-therapies, relapse, and metastasis, are making a new avenue for the understanding cancer and for the establishment of complete cure of cancer by targeting CSCs [11–14]. Cells with defined CSC properties have been isolated from various kinds of tumor and cell lines, and vigorous investigation are on going to understand and overcome cancer from the view of CSCs [11, 15–20]. However, large part of CSCs themselves is still veiled and is under controversies: where, when and how are they generated in human body? What are critical stimuli for the expression of their character? And how can we eliminate them from human body?

As in the case of normal stem cells, the fate of CSCs is considered to be controlled by the niche of CSCs [4, 21–23]. Recently, we have reported that CSCs and their progenies mutually depend to form a CSC niche, which controls the self-renewal and differentiation of CSC, using a CSC model derived from mouse induced pluripotent stem (miPS) cells [24, 25]. In addition, our miPS derived CSCs and other model CSCs from other laboratories might shed light on the niche where cells acquire the properties of CSCs. In this chapter, we would like to focus on cancer stem-like cells generated in vitro and the recent progress to understand the CSC niche, including vascular endothelial cells differentiated from CSCs. And also we would like to prospect for the niche to generate CSC from recently literatures.

2 CSC Niche, Contribution of Vascular Endothelial Cell to CSC Properties

CSCs, like normal tissue stem cells, possess the self-renewal capacity and the differentiation capacity to sustain their own tissue, a tumor. The fate of CSCs, whether they maintain dormant state, self-renew or start to give rise to differentiated cancer cells by symmetric/asymmetric division [26–28], is thought to be regulated

by the signals from their environment, CSC niche. The components of CSC niche should play roles in the CSC regulation and contribute to the physiology and the pathology of cancer. Thus, the comprehensive understanding of the mutual relation between CSCs and components of CSC niche, including stroma cells, and penetrated immune cells, is required for the drawing up therapeutic strategy [11–13].

One of the notable target components in CSC niche to understand the cancer development is vascular endothelial cell in tumor. It has been shown that the CSCs could be found close to the blood vessels in tumor, which are proposed the perivascular CSC niche [29–33]. The cells positive for Nestin, which is a marker of normal neural stem and progenitor cells, and also is a marker of brain CSCs, were detected to be associated with tumor vasculature in various brain tumors, such as medulloblastoma, ependymoma, oligodendrogiloma and glioblastoma [29]. $CD133^+$ CSCs could be found in association with endothelial tubes in vitro. Like neural stem cells, the properties of brain tumor stem cells also seemed to be maintained by vascular niche. Furthermore, the sphere forming activity, which indicates the self-renewal property of CSCs in vitro [34], of these brain tumor stem cells was enhanced in the co-culture system with primary human endothelial cells (PHECs) [29]. Similar results were reported when C6 rat glioma cells were treated with conditioned media prepared from human umbilical vein endothelial cells (HUVECs) [30]. In both studies, an anti-angiogenic agent successfully reduced the cell population that possesses tumor sphere formation [29, 30]. In addition, the stem-like cells of medulloblastomas presented in vascular niche could be resistant to radiation and undergo p53-dependent cell cycle arrest through PI3 K/Akt pathway [31], implying the contribution of vascular niche to the cancer pathology related to CSC character. Vascular niche could be supply not only for the promotion self-renewal of CSCs, but also for the regulation of tumor dormancy in the case of breast tumor metastasis [32]. Interestingly, dormant state of metastasized cancer cells, presumably CSCs, was canceled by TGF-β1. This cytokine is secreted from endothelial tip cells of sprouting vessels and to allow cancer cells to grow [32]. Thus, beyond the classical roles of tumor blood vessel such that the vessels are supplying nutrients and oxygen to maintain and to develop a tumor, we should re-consider the tumor angiogenesis as an activity of CSC niche, which could regulate various aspects of cancer pathology.

The conventional idea of tumor angiogenesis is that the blood vessels in tumor, composed of both endothelial cells and pericytes, penetrate from vessels surrounding a tumor. Sproution from pre-existing vessels is stimulated by various growth factors secreted from caner cells [21, 35, 36]. Now, it should be noted that the vascular endothelial cell could be driven from CSCs in the tumor as one of the differentiation lineage of CSCs, parts of tumor vessels are formed by progenies of CSCs themselves in some tumors. The studies reported in 2010–2011 clearly indicated that a population of endothelial cells in glioblastoma harbored the identical somatic mutations to that in the cancer cells [37–39]. Moreover, the $CD133^+$ glioblastoma stem cells could differentiate into the vascular endothelial cells in vitro. Interestingly, the differentiation of $CD133^+$ glioblastoma stem cells into $CD133^+/CD144^+$ (VE-cadherin) endothelial progenitors was induced by

co-culturing with tumor cells. This observation suggested that either the direct interaction between cancer stem cell and the differentiated cancer cell or the soluble factor(s) secreted from the cancer cells promoted the differentiation. Furthermore, the generation of endothelial progenitors did not depend on VEGF, but depended on Notch (we will discuss Notch later), although the terminal differentiation into mature endothelial cells required VEGF signaling pathway [38]. In addition, another group has also shown that the origin of tumor endothelial cells could be tumor initiating CSCs [39]. Glioblastoma stem cells are also reported to differentiate into vascular pericytes [40].

Given the differentiation capacity of CSCs into the vascular endothelial cells in a tumor, it raised a possibility that these differentiated progenies could take part in CSC niche for regulating CSC's fate; in other words, CSC generates their own niche by giving rise to the vascular endothelial cells. We have addressed the question by using a mouse iPS-derived cancer stem model cell, miPS-LLCcm (described below) [24]. miPS-LLCcm also revealed the differentiation capacity into the vascular endothelial cells [25]. Interestingly, the endothelial differentiation of miPS-LLCcm was abrogated by repeating selection of undifferentiated cells followed by spontaneous differentiation. This result implicated that some differentiation lineage of CSCs was affected by the secreted factor(s) from the differentiated cells from CSCs, especially differentiation into vascular endothelial cells from CSC were induced by endothelial cells themselves [25].

The self-renewal of CSCs is usually described to be under the control of Notch, WNT and Hedgehog signaling pathways. These signals are known to maintain stemness of CSCs and promote CSC's self-renewal in various cancers [12, 13, 41]. In this case, the ligand recipient cells are certainly CSCs, whereas the donor of the ligands for these signaling pathways may be any cell in the CSC niche. In the case of Notch signaling, it has been reported that vascular endothelial cells play a role for the activation of Notch signaling in CSCs as a supplier of ligands. Expression of Notch ligands in endothelial cells and some tumor cells has been shown, and these cells were localized around the Notch receptor positive CSCs in primary glioblastoma [42]. In this study, the authors found the expression of Dll1 in tumor cells, whereas Dll4 in $CD31^+$ endothelial cells. In addition, Jag1 and Jag2 were shown to be expressed in both of tumor cells and endothelial cells, and Nestin positive cells in glioblastoma expressed Notch receptors. Intriguingly, the authors also found neurosphere of glioblastoma cells (presumably reflects self-renewing CSCs) expressed Notch ligands (discuss later). In the study with human colorectal CSCs, endothelial cells isolated from liver could promote CSC phenotype via supplying soluble form of Jag1 [43]. Also, nitric oxide (NO) produced by endothelial cells is involved in activation of Jag1-Notch signaling pathway through enhancement of inhibitor of differentiation 4 (ID4), and in turn, suppression of miR-129 targeted to Jag1 [44, 45]. Thus, the stem cell-like characters of glioma CSC were promoted.

In addition to Notch signaling, various signaling and signal mediators from endothelial cells are involved in expression/promotion of CSC phenotypes. Activation of Hedgehog signaling pathway by endothelial cells in tumor environment have also been shown to promote stem-like phenotype of glioma cells [46].

The perivascular expression of osteopontin, which is one of the ligands for CD44, can promote the stem cell-like properties and radiation resistance through enhancement of HIF-2 [47]. All the above reported observations suggest the vascular endothelial cells are components of CSC niche to promote self-renewal and other properties of CSCs.

3 CSC Niche Created by CSC Itself

It would be noted that, 'normal' endothelial cells were used for the evaluation of their function as CSC niche in many investigations, especially in vitro analysis [29, 30, 42, 43, 45]. As we and others have shown the differentiation capacity of CSC into vascular endothelial cells [25, 37–39], the tumor derived endothelial cells should play some roles on in the CSC niche. We tested whether the self-renewal capacity of CSCs in miPS-LLCcm that is an CSC model cell established in our lab [24], were affected by the differentiated progenies of CSCs. Our recent results show the vascular endothelial progenies of CSCs as suppliers of the ligands for Notch signaling pathways to propagate CSC population [25]. In brief, the self-renewal of CSCs, which assessed by the sphere formation capacity, were enhanced in the presence of the conditioned medium from the bulk culture of miPS-LLCcm containing $CD144^{+}$/$VEGFR2^{+}$ vascular endothelial progenitors (CM-ad) and this promotion of self-renewal was partially depended on Notch signaling, inferred from suppression by DAPT, an famous inhibitor of γ-secretase. Involvement of Notch activation in the promotion of sphere formation was not observed when miPS-LLCcm were cultured in suspension with the conditioned medium of only CSC population (CM-sp), or when spheres were formed in the conditioned medium prepared from bulk culture which appeared not contain endothelial cells but contains undefined differentiated cells from CSCs. This implicated that the endothelial cells differentiated from CSCs, like 'normal' endothelial cells do [29, 30, 42, 43, 45], actually function as CSC niche by supplying Notch ligands [25].

As for Notch activation, the unconventional activation of this signal might be involved in CSCs self-renewal. In our study, stem cell population of miPS-LLCcm also express Dll1, Dll4, Jag1 and Jag4. And soluble forms of some Notch ligands were detected in the conditioned media, both of CM-ad and CM-sp. However, the Notch-dependent promotion of self-renewal was observed only in the presence of CM-ad [25]. This implicated that the additional factor other than typical Notch ligands or the mechanism(s) could take part in this signaling. When the ligand and receptor of Notch are expressed in the same cell, downstream of this signal cascade won't be activated, known as cis-inhibition [48–50]. Perhaps, the vascular endothelial cells in CSC niche may secrete factor(s) to release or control of cis-inhibition of Notch signaling for the initiating/promoting self-renewal of CSC, such as Fibulin-3 [51, 52].

Not surprisingly, many investigations reveal the mutual dependence between CSCs and their vascular niche. For example, in contrast to more differentiated cell population, tumor derived stem cell-like glioma cells have been shown to secret

significant amount of VEGF, which is a well-known factor of angiogenesis, stimulated the tumor vascularization [53]. In our recent research, as mentioned above, it was suggested that the differentiation capacity of CSCs of miPS-LLCcm into endothelial cells might be regulated by the factors secreted from progeny cells of CSCs [25]. Thus, CSCs give rise to differentiated cells including endothelial cells, and the differentiated progenies support not only self-renewal but also differentiation lineages of CSCs. This feedback circuit(s) between CSC and non-stem cancer cells is supposed to maintain stem cell population and heterogeneity in CSC self-created niche. It might be speculated that CSCs can change their differentiation lineage if they put a different niche or particular lineages of cells are eliminated from CSC niche by administration of particular anticancer drugs [54]. Thus, further understanding interactions between CSCs and their niche components should be needed for revealing mechanisms of drug resistance and developing the novel clinical strategy against CSCs and CSC niche for the complete cur of cancer.

4 The Niche Responsible for CSC Generation

Along with vigorous efforts to understand the nature of CSC and CSC niche, many investigators has tried to address events at the very beginning of cancer, the generation of CSCs or the origin of CSCs. Classically, cancer is though to be a disease of cells with gene alterations, accumulation of mutations or abnormal chromosome formations. Especially mutations on oncogenes or tumor suppressor genes are believed to cause of cancer. In addition, epigenetic abnormality is also considered to contribute to transformation of cells to malignant cancer cells [55–58]. Very recently, insufficient in vivo reprogramming using iPS technology cause embryonic carcinomas in mouse [58]. These tumors did not have oncogenic mutations, clearly indicating that the contribution of epigenetic abnormality for carcinogenesis.

The gene mutations could be introduced by exposure to various kinds of mutagens, and some of them are actually carcinogens. However, epigenetic change should depend on the environments of cells, when we think about normal differentiation and development. In normal differentiation or development, stem cells/progenitors are tightly regulated their behavior by surrounding environments, niche [1–5]. Through this regulation, epigenetic activation of gene expression and/or epigenetic silencing of genes for proper differentiation or maintain the state of cells should be achieved as well as transient regulation of lineage specific gene expression by lineage specific transcription factors [59]. Take consideration this, it could be speculated that the cancer-related epigenetic changes are results of niche abnormality that cannot stimulate cells in properly. And when stem cells/progenitors are exposed to an abnormal niche, they might be transformed or could acquire CSC properties.

Through the works with multipotent skeletal muscle-derived stem cells, it has been proposed that stem cell/progenitors could be transformed when they were exposed to an environment in which cells were stimulated by improper signals for

intrinsic lineage-commitment [60]. For example, in vitro osteogenic-primed skeletal muscle-derived stem cells were tumorigenic when they were implanted into myogenic, but not osteogenic environment in vivo. In following study from the same group, they could show the differentiation capacity of muscle-derived stem cells into neuronal lineage, and the regeneration of wounded nerve by implantation of this cell [61]. However, without commitment to differentiate into neurogenic lineage before implantation in vitro, the cells formed malignant tumors in mice. Furthermore, the cells isolated form primary tumor, although the authors found chromosome instability in these cells, could generate secondary tumor in new recipient mice. The lineage-committed cells were not tumorigenic when they were implanted into proper site. Thus, the parental cells should not have genetic abnormality. Although these studies did not mention about generation of CSCs, the secondary tumor formation are likely due to the presence of cells with CSCs properties [60, 61]. Collectively, the conflict between signals required for committed cell to further differentiation and signals provided from niche could be one of the crucial factors for malignant transformation of cell, further for generation of CSCs.

This proposal exactly has been considered for the iPS cell-based regenerative therapy. iPS-derived tissue progenitors are expected to be used for the regeneration of damaged/wounded tissue [62–67]. For the purpose of this, methods for the induction of various lineages of progenitors are reported day by day. Once cells are induced the differentiation into particular lineage, investigators are trying to isolate their desired cells according to cell surface markers. This is a crucial point before clinical use of iPS-derived cells since it has been alerted the contaminated undifferentiated or differentiation defective cells formed neoplasia [58, 68]. Given the fact that the conflict niche lead the transformation of multipoint stem cells [60, 61], remaining stem cell/progenitors of unwilling lineages which are induced from iPS cells simultaneously could form malignant tumors, and the complete undifferentiated pluripotent stem cell which is supposed to be formed teratomas. According to widely accepted definition [7, 8], the self-renewable, multipotent, and tumorigenic cells could be called CSCs. In this context, the improper environment for a stem cell/progenitors should be a CSC generating niche. In the situation of implantation of the iPS cell-based regenerative therapy, the implanted place should be a normal niche for desired stem cells/progenitors, but the same place will be a CSC generating niche for contaminated stem cells/progenitors. In addition, the alien stem cells/progenitors are possible origin of CSCs in this case.

5 Generation of CSCs In Vitro by Modified Culture System as CSC Generating Niche

Not only for basic researches but also for clinical studies, it would be quite valuable to have model cell lines of CSC. Number of reports demonstrated establishment of cancer stem-like cells in vitro. Most of them are using gene manipulation, such as

oncogenic gene or miRNA transduction or reprogramming of cancer cells. Cells with cancer stem cell properties established by Scaffidi and Misteli were transformed human primary skin fibroblasts by retroviral introduction of the genes for telomerase, oncogenic H-RasV12, simian virus 40 large T, and small T antigens [69]. According to the stage-specific embryonic antigen (SSEA-1) expression as a marker for isolation, cells showing multipotency, self-renewal and tumor initiation properties were obtained. In other case, reprograming of cancer cells which undoubtedly carry oncogenic mutation in their chromosomes by forcing expression of Yamanaka factors [70–74].

In contrast to those efforts, we have generated several cells with CSC's properties from mouse iPS cells, miPS-CSCs, without any artificial gene manipulations [24, 75]. The generations of these cells are likely dependent on the culture conditions of iPS cells, in other words, dependent on niche. These miPS-CSCs were generated by culturing mouse iPS cells in the presence of conditioned medium prepared from various kinds of cancer cell lines [24] or microvesicles/exosomes secreted from cancer cells [75], as candidates of CSC inducing components in niche. It is likely that the spontaneously 'differentiating cells' finally acquired CSC phenotypes in our studies, since we set the experimental condition without LIF, which is needed for maintaining undifferentiated state of mouse ES/iPS cells. Essentially, LIF was removed 1 or 3 days prior to the addition of conditioned medium [24, 75]. Considering this spontaneous differentiation of iPS cells, a possible explanation for causing conversion into CSCs is the conflict of differentiation signal described above [60, 61]. Cancer cells could secrete a various kinds of molecules, such as cytokines, chemokine, cancer-related metabolites, exosomes and so on, and affect surrounding cells and promote tumor growth. Thus, these secreted factors are used/proposed for biomarkers for diagnosis [76–83]. In our investigations, in the presence of conditioned medium of cancer cells, differentiating iPS cells could be stimulated and activated simultaneously various kinds of intrinsic signaling pathways, but these signals could not be orchestrated as seen in normal niche for normal differentiation process. In such condition, in other word 'niche', cells might acquire CSCs phenotype eventually.

In addition, tumor-derived microvesicles/exosomes are considerable as a factor in CSC generating niche. Growing evidences reveal that the microvesicles/exosomes mediated cell-cell communication are involved not only in normal development and homeostasis, but also in various diseases [77]. Microvesicles/exosomes carry cellular contents such as proteins, mRNAs, miRNAs from secreted cells to recipient cells [84]. Thus, upon receive microvesicles/exosomes, recipient cells could change their characters or behaviors. In the aspect of cancer, the pivotal roles of microvesicles/exosomes secreted from cancer cells and communication between surrounding stroma cells are reported [85–88]. For examples, EGFRvIII, an oncogenic receptor, transferred from aggressive glioma tumor cells by microvesicles let non-aggressive population transform morphologically and bring them capacity of anchorage-independent growth, through the activation of MAPK and Akt signaling pathways [85]. Highly metastatic melanomas also secrete exosomes to educate bone marrow cells for creation of pre-metastatic niche, result in enhancement of the

metastatic behavior of primary tumors [86]. It is well described the contribution of cancer associate fibroblast to tumorigenicity, cancer associate fibroblasts should be also controlled by tumor-derived microvesicles as shown in the case of human prostate cancer cell line [87]. Further, exosomes from breast cancer cell lines brought tumor associate myofibroblastic characters to adipose tissue-derived mesenchymal stem cells [88]. In addition, profiles of the miRNA in tumor-derived microvesicles/exosomes are proposed as diagnostic markers, implicating that cancer cells express specific microRNAs [77–79]. The functions of miRNAs in various pathologic feature of cancer are well described [89, 90].

In addition to above functions, we have recently shown the effect of tumor-derived microvesicles/exosomes on differentiating iPS cells, result in the generation of cells with CSC properties [75]. iPS cells were treated with microvesicles/exosomes isolated from culture medium of mouse LLC cells. We found that the colonies with Nanog positive, which monitored Nanog promoter controlled-GFP expression [63], were re-emerged from differentiated population. Together with pathological features of tumor formed in immunodeficient mice, we concluded the resultant cells were CSC of liposarcoma. Although we have not yet revealed the molecular mechanism(s) involved in this CSC generation, we could speculate that miRNAs transferred by microvesicles/exosomes from LLC cells contribute to the malignant transformation of differentiating iPS. miRNAs are shown to play pivotal roles in tumorigenesis through the tuning of amount of target mRNAs, and/or induction of epigenetic modifications of genes [89, 90]. It should be noted that miRNAs are involved in the regulation of epithelial-mesenchymal transition (EMT), which is frequently described as an event related to cancer metastasis, cellular reprogramming and acquisition CSCs properties [91, 92].

Similar to, but might be essentially different from our studies, it has been shown that mouse ES cells could be transformed into CSC-like cells under the abnormal differentiation conditions [93]. In the report, differentiation of stem cells induced by culturing without LIF in newborn bovine serum caused cancerous transformation with immortality. The generation of CSC-like cells from ES cells in newborn bovine serum condition apparently depend on DNA damage-related cellular senescence of differentiated cells. It was likely that cells which could overcome the senescent-induced cell cycle arrest acquired immortality [93]. The resultant cells exhibit genomic instability with dysfunction of the Arf/p53 pathway, which preferentially due to mutations in p53 gene. Dysfunction of p53 pathway is widely accepted as a risk of cancer. However, the genomic instability and dysfunction of Arf/p53 pathway was also observed in immortalized, but non-tumorigenic MEFs [94]. This implies that alteration(s) other than genomic instability caused by Arf/p53 disruption in cells was indispensable for acquisition of tumorigenicity. In an aspect of CSC properties, stem cells should be immortal. Thus, immortalization might be result of (re-)acquisition of stemness in differentiated cells in this case. Although differentiating ES cells in newborn bovine serum conditions apparently responded to DNA damage, why differentiation of ES cells in different serum induced DNA damages, and how cells re-acquired stemness by DNA damages were

not elucidated. Revealing molecular mechanisms are necessary, but the environment where cells are stimulated to escape senescence-induced cell cycle arrest could be a CSC generating niche [95, 96].

6 Insight into 'Native' In Vivo CSC Generation Niche

It must quite difficult to identify the actual cell in vivo which can be transform into CSCs, so there is a big argument about the origin of CSCs, mainly whether they are generate from somatic/adult stem cells or from differentiated cells by reprogramming [97–101]. Either possibility, we must consider the change of environment surrounding cells. So, what is the 'native' CSC niche? When could it be emerged in human life?

We know that chronic inflammations are one of the situations that bring the risk of cancer to us. Recently, intriguing researches approaching to the initiation of cancer were performed, indicating that the generation of cancer stem cells was triggered by inflammation in molecular and cellular levels [102–104]. Iliopoulos et al. have been shown that the malignant transformation of non-malignant MCF10A cell was achieved by transient activation of an oncoprotein, Src [102]. This transient activation established the feedback loop consistent with NF-κB, Lin28, miRNA Let-7, and Interleukin-6 (IL-6), result in up-regulation of IL-6 production. Inflammatoric IL-6 appeared to be necessary for transformation of MCF10A and for maintenance of the CSC phenotype of transformed cells. It is likely that the epigenetic changes contribute to establishment of this CSC generating/maintenance molecular circuit, since the Src activation was required as short as 5 min and transformation could be achieved within 36 h in this case [102]. It was unlikely that such short periods cause accumulation of mutations on DNA. In addition to this, the report from He et al. indicates the presence of liver cancer progenitors (termed HcPC) in experimentally chronic damaged and cirrhosis mouse liver [103]. These cells also produced IL-6 and malignant progression was depended on IL-6. Of interest, hepatocarcinomas were developed only when HcPC was transplanted into damaged liver, not into healthy liver or other organs. Although it was implied that factors other than IL-6 related to chronic inflammation were involved in development of hepatocarcinomas, the premalignant/dysplastic lesions in chronic/cirrhosis liver should be niche for generating CSCs. And undoubtedly, IL-6 is one of the key players for CSC generation in the niche [104].

7 Closing Remarks

It is still under the argument what type of cell is the origin of CSC. Given the fact that tumors are tissues with highly heterogenetic cell population and that the heterogeneity is also observed among patients even they are diagnosed the same

cancer, it is not surprising that the actual cell of origin of CSCs might be different in each case of tumor, in each case of patient. The single cell lineage tracing technique and lineage specific activation of oncogenes/disruption of tumor suppressors in model animal will be helpful to identify a cell of origin of CSCs in some cases [99, 101]. However, we should also capture the change(s) of niche that induce the acquisition of CSC property and the malignant transformation of cells. In addition, the change(s) of niche related to various cancer pathologies must be revealed. Collectively, cancer should be considered a disease of cellular environment abnormality, issue of niche.

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