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Feature Review

HSC Niche Biology and HSC Expansion *Ex Vivo*

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Hematopoietic stem cell (HSC) transplantation can restore a new functional hematopoietic system in recipients in cases where the system of the recipient is not functional or for example is leukemic. However, the number of available donor HSCs is often too low for successful transplantation. Expansion of HSCs and thus HSC self-renewal *ex vivo* would greatly improve transplantation therapy in the clinic. *In vivo*, HSCs expand significantly in the niche, but establishing protocols that result in HSC expansion *ex vivo* remains challenging. In this review we discuss current knowledge of niche biology, the intrinsic regulators of HSC self-renewal *in vivo*, and introduce novel niche-informed strategies of HSC expansion *ex vivo*.

Significance of *Ex Vivo* HSC Expansion

HSCs sustain blood-cell formation in a process called **hematopoiesis** (see Glossary and Box 1). This is achieved by their ability to regenerate themselves long-term, which is referred to as **self-renewal** activity, and through their ability to **differentiate** into cells of all mature blood lineages. Human HSCs are rare cells (~0.01%) primarily found in bone marrow (BM) in adults [1]. **HSC transplantation** (HSCT) can restore a new functional hematopoietic system and blood cell production in recipients [2–4]. It is used in the clinic to treat leukemia and other cancers, as well as bone-marrow failure syndromes and in gene therapy settings. The source of HSCs for a transplant is either a patient's own HSCs (**autologous transplant**) or HSCs from a **human leukocyte antigen** (HLA)-matched donor in an **allograft transplant** setting [5,6]. For HSCT, HSCs from BM or umbilical cord blood (UCB), or HSCs mobilized to blood by cytokine granulocyte-colony stimulating factor (G-CSF), can be used. **Infused donor HSCs** then home to and engraft in discrete BM **HSC niches** to reconstitute the blood system of the recipient [2]. Currently, autologous transplants have survival rates exceeding 80%, while the success rate for allogeneic transplants at 5 years fluctuates between 30% and 70%, based in part on the initial donor match [5]. The number of HSCs transplanted correlates with successful engraftment and patient survival. For successful HSCT, high numbers of **CD34$^+$ cells** (i.e., 3–4 $\times$ 10^6/kg of human body weight) are required [6,7], and thus the numbers of HSCs in a given graft may not be sufficient to allow transplantation to proceed. Protocols that result in **HSC expansion** *ex vivo* would therefore be a highly desirable tool to further increase positive outcomes in the clinic. Understanding the mechanisms of HSC self-renewal *in vivo* in depth should be a prerequisite for the development of successful protocols to expand HSCs *ex vivo* for therapeutic applications.

HSC self-renewal is regulated by a complex interplay of **cell-intrinsic** factors, such as transcription factors, cell-cycle status, and metabolic pathways, as well as extrinsically by both the local and the systemic environment. The local environment in the BM is referred to as stem cell niche [8,9]. It is believed that signals from the niche are crucial for the regulation of HSC self-renewal as well as for differentiation decisions [1,8,10,11]. In recent years numerous cellular constituents of the murine BM niche and committed hematopoietic progeny have been

Trends

HSC niches have been defined in more detail with respect to their cellular composition over the past few years. Molecular characterization of these niches has been successfully initiated.

Molecular pathways that govern the mode of division of HSCs are being discovered.

HSC intrinsic mechanisms, such as metabolic pathways regulating HSC self-renewal, can be modulated by the environment and/or niche factors.

High-throughput screening of chemical compound libraries has resulted in a few successful attempts towards HSC expansion *ex vivo*.

Attempts to engineer stem cell niches *ex vivo* in 3D matrix culture systems are promising.

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Box 1. Mammalian Hematopoiesis and HSCs

The hematopoietic system is one of the best-studied adult stem cell systems in humans and rodents:

(i) Functionally, HSCs are defined as cells that give rise to long-term multilineage engraftment that persists for at least 20 weeks after primary and secondary transplantation [171].

(ii) Multipotent progenitors can generate all major hematopoietic lineages in **transplantation assays** in lethally irradiated recipients, but fail to engraft long-term. Long-term reconstitution of hematopoiesis in a transplant setting can be achieved by a single **long-term (LT)-HSC** [3,4,32].

Recent research has been able to phenotypically define murine long-term HSCs:

(i) LT-HSCs as $Lin^-IL\text{-}7\alpha^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD34^-CD150^+CD48^-$, progenitors including short-term HSCs (ST-HSCs) as $Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-\ CD34^+CD150^+\ CD48^-$, and

(ii) multipotent progenitors (MPPs) as $Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^+c\text{-}Kit^+Flt3^{low–high}CD34^+$ [4,32,172–174].

Human long-term HSCs and MPPs have been phenotypically defined as:

(i) HSCs: $Lin^-CD34^+CD38^-CD45RA^-CD90^+Rho^{lo}CD49F^+$, and

(ii) MPPs: $CD34^+CD38^-CD45RA^-CD90^-CD49F^-$ [3,175].

nvestigated that interact either directly or indirectly with HSCs and which might contribute to the regulation of HSC self-renewal and differentiation [9,12–24]. As such, experiments usually impair genetically or pharmacologically one type of cell niche to then analyze the changes in HSC phenotype; however, much remains unknown regarding the mechanisms that regulate the complex interplay among the distinct types of stromal elements under native conditions. HSCs expand in numbers *in vivo* within their niche environment. Theoretically, the number of HSCs in the niche is determined by the frequency of **symmetric cell divisions** that lead to the generation of two stem cells or two progenitor cells, relative to the frequency of **asymmetric cell divisions** that posit a balance between HSC and daughter cell generation [25]. HSCs generally remain **quiescent** in the BM niche, while diverse stimuli that trigger loss of quiescence cause robust entry into the cell cycle, and induce proliferation often associated with stress, DNA - damage and apoptosis [26,27]. *Ex vivo* expansion will thus require approaches that result in symmetric stem cell divisions [25], and hence HSC self-renewal without further differentiation and apoptosis.

Mammalian HSCs undergo symmetric cell divisions *in vivo* during development [25] and in adulthood. For example, using mice where HSCs were labeled with a dye 'diluting' HSCs following division (label-retaining HSCs, LR-HSCs), murine HSC were found to complete four symmetric self-renewal divisions *in vivo* before re-entering a state of dormancy [28]; nevertheless, persistent inflammatory signaling can disturb HSC dormancy, resulting in **HSC exhaustion** [29]. Because adult HSCs have been shown to undergo self-renewal/expansion following chemotherapy, radiation challenge, or transplantation, thus replenishing the hematopoietic niche [4,30,31], it may be possible to achieve HSC expansion *ex vivo* once we improve our understanding of the HSC-intrinsic and niche-dependent mechanisms that are responsible for HSC expansion *in vivo*. We review below the most recent knowledge on mechanisms of HSC self-renewal, placing a particular focus on the contribution of the HSC niche.

HSC Localization within the Niche

Adult HSCs reside in specific BM locations with unique environments known as niches. A large set of data has revealed that there is vast heterogeneity of niches for HSCs within the BM (recently reviewed in [22]). Niches for HSCs comprise **endosteal** niches and vascular niches,

Glossary

Allograft transplant: stem cells from an HLA-matched donor are transplanted.
Asymmetric cell division: leads to the generation of two cells with different potential: a daughter stem cell and a daughter progenitor cell.
Ataxia telangiectasia mutated deficient ($Atm^{-/-}$) mice: ATM regulates reprogramming efficiency and genomic stability; $Atm^{-/-}$ mice exhibit pancytopenia, bone marrow (BM) failure, and hematopoietic stem cell (HSC) exhaustion.
Autologous transplant: an individual's own stem cells are collected in advance and transplanted to herself/himself after chemotherapy or radiation therapy.
Autophagy/mitophagy: process that degrades/destructs dysfunctional components of the cytoplasm (autophagy) or dysfunctional mitochondria (mitophagy) in lysosomes.
$CD34^+$ cells: human cells expressing CD34; include both stem and progenitor cell populations.
Cell-intrinsic: a property of cells that is governed/regulated by signaling/factors within, but not through, the niche environment.
Competitive transplant settings: transplantation of donor HSCs or BM cells in the presence of genetically trackable congenic competitor BM cells.
Differentiation: the generation of progenitor cells from stem cells; necessary to produce mature blood cells.
Endosteal: HSC niche in close association to a bone surface.
Extracellular matrix (ECM): includes among other elements collagen, fibronectin, dystroglycan, heparin sulfate, proteoglycans, osteopontin, and laminins.
Hemogenic endothelium: a subset of endothelial cells with the potential to differentiate into hematopoietic cells.
Hematopoiesis: the process of blood cell formation from HSCs.
HSC exhaustion: a state of turnover in which the cells are 'used up'; HSCs can undergo exhaustion due to the high demand of reconstitution in stress or serial transplant settings.
HSC expansion: the process of increasing the number of HSCs.
HSC niche: a specific BM environment that provides cellular,

further divided into arteriolar as well as **sinusoidal** components [9,18–24,32]. Deeply quiescent (dormant) HSCs are believed to localize around arterioles and closer to the endosteum in the mouse BM, while activated HSCs – which are significantly more abundant than dormant HSCs – are thought to reside in the vicinity of sinusoids [33–36]. Indeed, recent studies have revealed that most murine HSCs are present in perivascular locations in close contact with either sinusoids or arterioles [20,23,36,37]. Indeed, such new and detailed knowledge on niche architecture and on the association/proximity of HSCs to non-HSC niche cells has been based on high-resolution imaging studies in mouse BM (particularly from femur) [12,16,21,33,34,38,39]. Thus, the role of the HSC niche as a crucial contributor to the regulation of HSC cell self-renewal [1] has been confirmed in mouse models where niche cells and/or distinct soluble growth factors have been genetically modified; the cell types and factors within the niche contributing specifically to HSC self-renewal *in vivo* are detailed below.

Endosteal and Vascular Niches

Both the endosteal niche and the vascular (arteriolar and sinusoidal) niches have been recognized as regulators of HSC self-renewal as well as HSC function, based in part on the cellular composition and soluble components found in both of these niches. For example, multiple heterologous mouse cell types including endosteal osteoblasts [19,33,39], sinusoidal blood vessels and leptin receptor-positive (Lepr$^+$) perivascular stromal cells [23,32], CXCL12-abundant reticular (CAR) cells [40,41], nestin$^+$ mesenchymal stem cells [18], non-myelinating Schwann cells [16], regulatory T cells (Tregs) [38], and megakaryocytes [12,42] have been shown to locate in close proximity to murine HSCs *in vivo*. The underlying assumption has been that proximity serves as a surrogate marker for the relative importance of the regulatory influence of particular types of stromal cells on HSCs [12,16,21,33,34,38,39]. However, there are also multiple examples in which cells that are, on average, relatively distant from HSCs within the BM influence HSCs, such as osteoblasts [43]. Osteoblasts have been reported to enable a 3–4-fold expansion of human **long-term culture-initiating cells** (LTC-ICs) *in vitro* [44,45], suggesting that stem cell self-renewal can be supported by osteoblast-derived factors. When parathyroid hormone (PTH/PTHrP) receptors (PPRs) were specifically introduced into murine osteoblasts, they produced a high level of the Notch ligand Jagged-1 [19]. This caused a significant increase in the number of osteoblasts, which in turn resulted in an increase in the number of HSCs *in vivo* [19]. Similarly, mice with a conditional inactivation of the gene encoding BMP receptor type IA (*Bmpr1a*) exhibited an increase in N-cadherin$^+$ osteoblasts, which also resulted in an increase in the number of HSCs *in vivo* [9]. However, the tissue-specific promoters used in these studies [9,19] could also target perivascular osteoblastic progenitors and not only osteoblasts. Moreover, genetic manipulations of mature osteoblasts have not resulted in altered HSC function [46–48]. Taken together, a controversial debate still exists regarding the role mature osteoblasts play in the putative regulation of HSCs. Accordingly, because mice devoid of N-cadherin in hematopoietic and stromal cells or osteoblastic lineages have not led to changes in HSC numbers *in vivo*, the potential role for N-cadherin signaling in the regulation of HSC expansion *in vivo* also remains controversial [49,50]. Osteoclasts in turn have been found to be dispensable for HSC maintenance, and might thus not be involved in regulating murine HSC self-renewal *in vivo* [51].

Notch ligand signaling, such as via Jagged-1 in murine endothelial cells (i.e., vascular niche), has been found to support HSC self-renewal and prevent HSC exhaustion, both *in vivo* and in serum-free co-culture assays [52,53]. BM macrophages (osteomacs) have also been reported to maintain retention of murine HSC in the BM through direct contact of perivascular cells with HSCs [14,17]. Furthermore, depletion of neutrophils in mouse BM can increase the number of CAR cells, and subsequently CXCL12 levels, reducing the size and function of the hematopoietic niche; and, neutrophil clearance by macrophages can promote hematopoietic stem and progenitor cell (HSPC) mobilization in the circulation [54]. Indeed, research over the past few

chemical, and molecular constituents and contributes to the regulation of HSC survival, self-renewal, and differentiation.

HSC transplantation (HSCT): a procedure to replenish the blood system of a recipient by providing a sufficient number of new HSCs cells from a donor.

Human leukocyte antigen (HLA): encodes the major histocompatibility complex (MHC) proteins in humans and functions as a determinant of transplant rejection.

Infused donor HSCs: intravenously injected HSCs from a donor into a recipient to reconstitute the hematopoietic system.

Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells: a distinct fraction of murine hematopoietic stem and progenitor cells in the BM; these are characterized as Lin$^-$Sca-1$^+$c-Kit$^+$ based on surface marker expression.

Long-term (LT)-HSCs: give rise to multilineage engraftment post-transplantation for a timeframe of at least 20 weeks. LT-HSCs are phenotypically characterized as Lin$^-$IL-7α^-Sca-1$^+$c-Kit$^+$Flt3$^-$CD34$^-$CD150$^+$CD48$^-$ cells.

Long-term culture-initiating cells (LTC-ICs): primitive hematopoietic cells capable of initiating and sustaining *in vitro* cultures for >5 weeks, including colony-forming cells (CFCs) or cobblestone area forming cells.

Mammalian target of rapamycin (mTOR) pathway: controls nutrient sensing, metabolism, and mitogenic signals to regulate cell quiescence, proliferation, cell survival, and longevity; important for PI3K, Akt, and insulin signal-transduction pathways.

Mitophagy: see autophagy.

NOD/SCID mice: nonobese diabetic (NOD)-severe combined immunodeficiency (SCID) mice display impaired T and B cell lymphocytes and deficient natural killer (NK) cell function. They can accept allogeneic and xenogeneic grafts, and are thus an excellent model system to study human cell transplantation and engraftment (xenotransplants).

Pancytopenia: reduction in the number of all three blood cell types: red blood cells, white blood cells, and platelets.

Pimonidazole: a nontoxic exogenous 2-nitroimidazole low molecular weight compound that

years has provided a more comprehensive characterization of the cellular composition of the murine HSC niche and the components that might influence HSC self-renewal *in vivo* with respect to both endosteal and vascular niches. Furthermore, multiple new types of niche cells have been linked to novel putative mechanisms of HSC regulation in terms of number and function within the BM (Figure 1, Key Figure; also [8,55]). However, further studies will be necessary to finely dissect the relative and specific contribution of this panoply of niche cell types in HSC self-renewal, and the functional significance in the interdependence of these signaling pathways.

Cytokine Secretion by Niche Cells

Niche cells secrete cytokines and growth factors that regulate HSC self-renewal and differentiation *in vivo* (Table 1). Osteoblast-derived cytokines, including osteopontin (OPN), angiopoietin-1, −3, thrombopoietin (THPO), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), and CXC-chemokine ligand 12 (CXCL12 or SDF-1) have been reported to regulate murine HSC self-renewal [30,41,44,56–61]. Niche-expressed SCF or Kit-ligand, THPO, and their receptors on HSCs (c-Kit and c-Mpl) are well studied with respect to their role in murine HSC expansion [23,57,62–65]. Osteoblasts and endothelial cells release SCF, while THPO is mainly released by osteoblasts. Both c-Kit and c-Mpl are expressed on highly purified HSCs and genetic deletion of *Thpo* or *Mpl* leads to a reduction of the number of murine HSCs [64,66]. Leptin receptor positive (LepR$^+$) perivascular and endothelial cells are another major component of the HSC niche and are the primary sources of CXCL12, SCF, pleiotrophin, and Notch ligands (such as Jagged-1) implicated in HSC regulation [20,23,61]. The putative role for angiopoietin-1 in HSC self-renewal – a cytokine well known for its role in endothelial cell remodeling – has been controversially discussed, and a recent report suggests that it might not directly influence HSC function [67]. Murine BM sinusoidal endothelial cells (BMECs) of the vascular niche secrete pleiotrophin (PTN) which may positively regulate HSC self-renewal [68,69]. Specifically, PTN-deficient mice harbor decreased numbers of HSCs in the BM accompanied by impaired hematopoietic regeneration [68], while PTN can promote *in vitro*

Table 1. Soluble Factors in the Bone Marrow Niche Affecting Mammalian HSCs

Soluble factor	Secreted by niche cells	Impact on HSC	Refs
SCF	Osteoblasts, endothelial cells, MSCs, nestin$^+$ MSCs	Induces HSC maintenance and self-renewal	[19,23]
THPO	Osteoblasts	Enhances HSC self-renewal and survival	[57,107,146]
G-CSF	Osteoblasts, endothelial cells, neutrophils	Induces retention and quiescence	[17,44]
CXCL-12	Osteoblasts, endothelial cells, CAR cells, MSCs, LepR$^+$ perivascular cells	Positive regulator of self-renewal, retention and function	[20,40,61]
CXCL-4	Megakaryocytes	Inhibits self-renewal and induces quiescence	[12]
Pleiotrophin	Sinusoidal endothelial cells, LepR$^+$ perivascular cells	Enhances self-renewal and BM retention	[68]
Osteopontin	Osteoblastic lineage	Negative regulation of HSC	[56]
Angiopoietin-1	Osteoblasts, osteoprogenitors, endothelial cells, nestin$^+$ MSCs	Induces self-renewal, and survival	[58–60]
TGF-β1	Nestin$^+$ Schwann cells, Megakaryocytes	Maintenance of HSC quiescence, inhibition of cell cycle activity	[16,42]
Notch ligand Jagged-1	Osteoblasts, endothelial cells, LepR$^+$ perivascular cells	Supports HSC self-renewal and prevents exhaustion	[19,52,53]

forms adducts with thiol groups in hypoxic environments and works as an effective and nontoxic hypoxia marker.

Quiescence: the state of being inactive or dormant in the cell division cycle.

Radioprotection ability: the capacity to confer long-term survival after lethal irradiation (e.g., mice).

Serial transplantation assay: regarded as the gold standard assay to determine HSC function *in vivo*. Serial (multiple, consecutive, up to 6) transplantations (e.g., in mice) test the ability of HSCs to undergo self-renewal *in vivo*.

SCID-repopulating cells (SRCs): human HSCs capable of long-term reconstitution in immunodeficient mice (xenotransplant approach).

Self-renewal: cell division producing two daughter stem cells.

Sinusoids: small blood vessel capillaries of irregular tubular space for blood passage within the BM. HSCs can reside near the sinusoid networks that present a sinusoidal niche.

Symmetric cell division: leads to the generation of two similar types of daughter cells: either two stem or two progenitor cells.

Transplantation assay: well-established assay to measure multilineage reconstitution and self-renewal potential of hematopoietic stem and progenitor cells in irradiated recipient mice *in vivo*.

Xenografts: a transplantation setting in which the donor of a tissue graft or organ transplant is of a species different from that of the recipient, for example human stem cell transplantation into mice. Generally, immunodeficient mice, in other words SCID, NOD/SCID, or NOD/SCID/Yc^{null} (NSG) mice, are used as recipients in human–mouse xenograft models.

Key Figure

The Mammalian Bone Marrow Hematopoietic Stem Cell (HSC) Niche

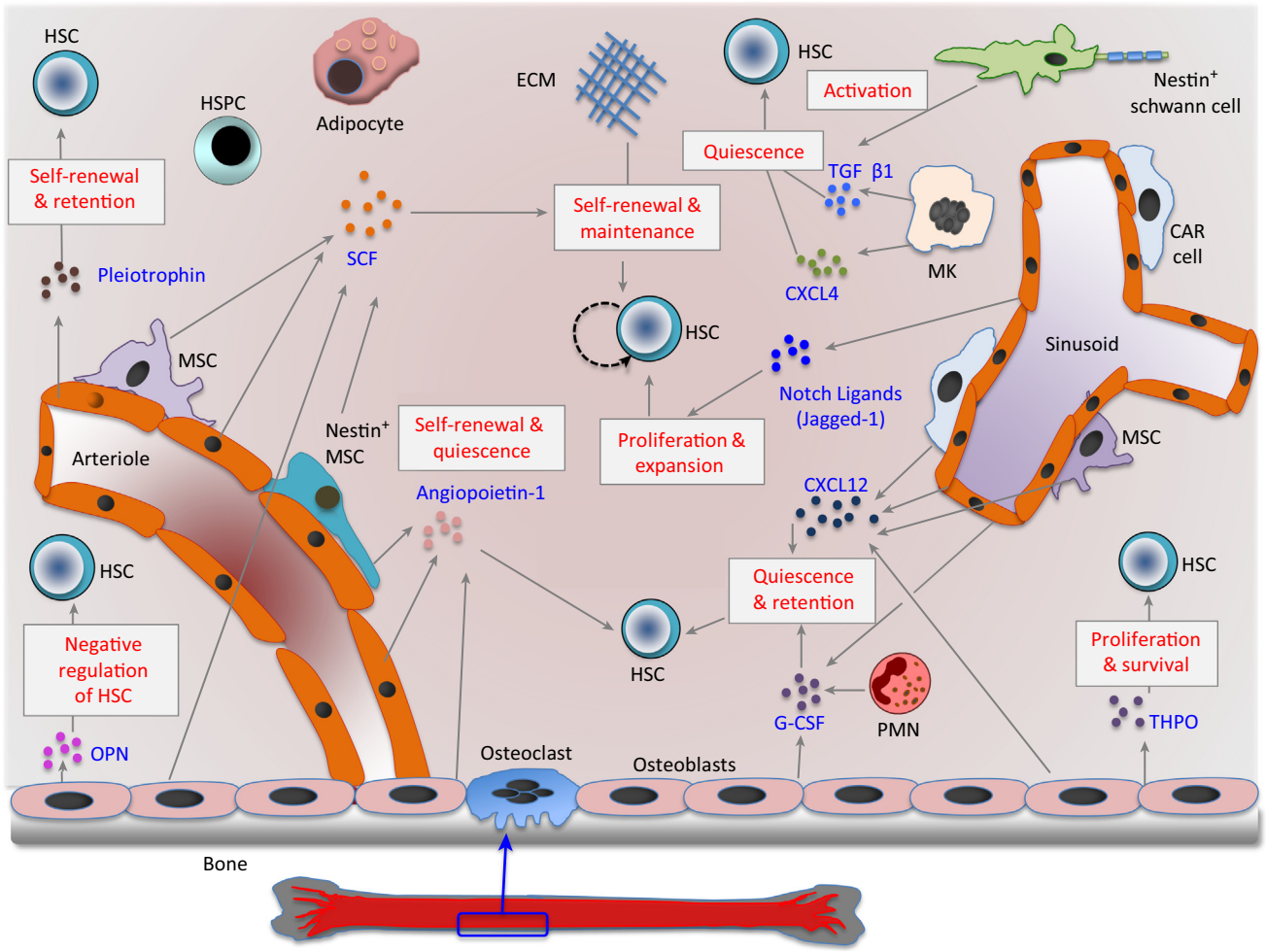

Figure 1. The diagram shows the cellular composition and cytokines/growth factors that can impact on HSC self-renewal and function in the bone marrow (BM) niche. Recent research has identified the role of diverse BM niche cells and HSC progeny including osteoblasts, nestin^{+} mesenchymal stem cells (MSCs), CXCL12-abundant reticular (CAR) cells, non-myelinating Schwann cells, BM endothelial cells and adipocytes, megakaryocytes, and neutrophils (PMN) in HSC self-renewal, differentiation, and function. Niche cells also produce/release several cytokines/growth factors, such as SCF, THPO, TGF-β1, CXCL-4, CXCL-12, G-CSF, OPN, Notch ligands, angiopoietin 1, and pleiotrophin, to regulate HSC self-renewal, maintenance, survival, retention, and function. The extracellular matrix (ECM) can also regulate HSC self-renewal and maintenance. Abbreviations: CXCL-4, CXC chemokine ligand 4; CXCL-12, CXC chemokine ligand 12; G-CSF, granulocyte-colony stimulating factor; HSPC, hematopoietic stem and progenitor cell; MK, megakaryocyte; OPN, osteopontin; PMN, polymorphonuclear cell; SCF, stem cell factor; THPO, thrombopoietin; TGF-β1, transforming growth factor β1.

expansion of long-term repopulating HSCs, both from mouse and human umbilical cord blood [69]. Moreover, PTN-induced HSC expansion could be blocked by inhibition of Notch activation through γ-secretase [69]. Another study has further implicated Notch signaling in HSC regulation by showing, in serum/cytokine-free co-culture systems, that BMECs secreting Notch ligands can enhance *in vitro* HSC self-renewal [52].

Recent mouse studies also suggest an inhibitory role for perisinusoidal megakaryocytes (MKs) in HSC expansion in the BM; for instance, depletion of MKs in the BM can cause HSC expansion due to loss of HSC quiescence [12,42]. Furthermore, MKs can secrete the chemokine CXCL4, and genetic depletion of *Cxcl4* in MKs has resulted in increased numbers of murine HSCs, while CXCL4 administration in mice can reduce HSC numbers *in vivo*, presumably via increased quiescence [12]. In addition, deletion of *Tgfb1* in MKs increased HSC activation and proliferation, and conversely, activation of TGF-β1 signaling in MKs resulted in HSC quiescence [42]; indeed, injection of TGF-β1 into MK-ablated mice restored HSC quiescence and inhibited self-renewal [42]. Furthermore, nonmyelinating nestin$^+$ Schwann cells have been found to activate TGF-β1-mediated inhibition of HSC self-renewal in mice [16]. Finally, murine BM adipocytes can also secrete soluble factors that inhibit HSC self-renewal [70], with recent reports suggesting that adipocytes can support murine HSCs *in vitro*, while not exhibiting any effects on HSCs *in vivo* [71]. Taken together, the data indicate that various cytokines and growth factors derived from the BM niche are able to regulate HSC self-renewal and differentiation (Table 1), but further functional characterization will be required.

Wnt Signaling and HSC Self-Renewal

Wnt signaling is known to act in a very context-dependent manner and might also be involved in regulating murine as well as human HSC self-renewal (reviewed in [72]). Expression of constitutively active β-catenin, a component of the canonical Wnt pathway, resulted in enhanced murine HSC self-renewal [73]; accordingly, Wnt3A proteins have been shown to increase murine HSC self-renewal *ex vivo* [74]. Mice lacking Wnt3a die prenatally, and deficiency of Wnt3a has been found to impair HSC self-renewal, as evidenced by reduced reconstitution capacity of fetal liver HSCs [75]. Moreover, exogenous Wnt3a has been shown to cause reduced murine HSC proliferation relative to cells treated with THPO, but can lead to higher long-term reconstitution, suggesting an enhanced ability for self-HSC renewal [76]. Others have reported that disrupted secretion of Wnt ligands by genetic deletion of the Porcn factor – essential for Wnt secretion [77] – or deletion of β-catenin and γ-catenin does not affect adult murine hematopoiesis [78–80]; consequently, this may likely imply a context-dependent action of Wnt-signaling in hematopoiesis, but has not yet been elucidated. Specifically, HSCs from β-catenin-deficient mice have normal HSC counts but exhibit impaired long-term growth and maintenance or support of BCR–ABL-induced chronic myelogenous leukemia (CML) [81]. In other studies, constitutive β-catenin activation resulted in enforced cell-cycle entry and subsequent exhaustion of murine HSCs, with induction of multilineage differentiation *in vivo* [82,83]. In a compound genetic mouse model of *Pten* deletion and β-catenin activation in HSPCs, the number of HSCs was increased, although they exhibited defects in differentiation [84]. Additional studies revealed that stabilization of β-catenin in stromal cells promotes maintenance and self-renewal of HSCs in a contact-dependent manner, whereas direct stabilization in hematopoietic cells caused loss of HSCs [85]. Another mouse study using **serial transplantation assays** reported an increase in cell cycling, but a decline in HSC function; expression of the pan-inhibitor of canonical Wnt signaling, Dickkopf1 (Dkk1) in the niche driven by an osteoblast-specific promoter (Col1α2.3) caused inhibition of Wnt signaling in HSCs [86]. Recently Dkk-1 was also found to promote murine hematopoietic regeneration in response to irradiation, acting both directly on stem cells to regulate reactive oxygen species (ROS) levels, as well as on niche cells to regulate EGF levels via paracrine crosstalk between BM osteolineage cells and endothelial cells [87]. Genetic deficiencies of Flamingo (Fmi) or Frizzled (Fz) 8, members of non-canonical Wnt signaling cascade, have been found to reduce the frequency of murine HSCs *in vivo* [34]. In this study, Fmi regulated the distribution of Fz8 at the cell–cell interface between HSCs and N-cadherin$^+$ osteoblasts because the non-canonical Wnt signaling initiated by Fz8 suppressed the Ca^{2+}–NFAT–IFN-γ pathway, antagonizing canonical Wnt signaling [34]; this resulted in maintenance of quiescent **long-term (LT) HSCs** in the niche. Consistent with such observations, *ex vivo* cultivation of HSCs with non-canonical

Wnt5A proteins increased the HSC repopulation potential in murine **transplantation assays** [88]. The role of Wnt/β-catenin signaling with respect to murine HSC self-renewal and differentiation, while already investigated to a great extent, remains complex and controversial and is likely dependent on variables such as genetic dosage and context [72,89]. Nevertheless, the data overall suggest a distinct positive role of canonical Wnt signaling initiated by the niche to mediate HSC self-renewal [90]. However, the role of Wnt proteins and Wnt regulatory factors in the stem cell niche remain to be investigated in greater detail to elucidate the potential of modulating Wnt signaling to achieve *ex vivo* human HSC expansion.

Metabolic Regulation of HSCs

HSCs also exhibit a stringent regulation of their hypoxic status [91,92] and of their metabolic [93] and mitochondrial profiles [94] (Figure 2).

Recent research has identified regulatory pathways and probable links between HSC metabolism, mitochondrial function, energy demands, and their role in regulating HSC quiescence and self-renewal. These pathways might possibly serve as additional novel targets for HSC expansion *ex vivo*. For example, the Lkb1 tumor suppressor is a kinase that functions upstream of AMP-activated protein kinase (AMPK). Deletion of *Lkb1* in mice causes rapid HSC depletion due to loss of quiescence leading to **pancytopenia** [95–97]. Lkb1-deficient HSCs exhibit reduced mitochondrial membrane potential, alterations in lipid and nucleotide metabolism, and depletion of cellular ATP [95–97]. Furthermore, transcriptome analyses have identified decreased gene expression of the peroxisome proliferator-activating receptor (PPAR)-mediated metabolic pathway in Lkb1-deficient murine HSCs in contrast to wild-type cells [95]. In addition, studies have demonstrated a novel role for the promyelocytic leukemia (PML)-driven PPAR-δ–fatty acid oxidation (FAO) pathway in murine HSC self-renewal through regulation of cell-division symmetry, with the PML–PPAR-δ–FAO pathway being able to control the mode of HSC division [98,99]. Indeed, loss of PPAR-δ or pharmacological inhibition of mitochondrial FAO induced loss of HSC self-renewal and loss of symmetric cell division; thus, symmetric differentiation commitment was implicated as the prevailing mode of HSC maintenance [99]. The symmetric differentiation mode of HSC division was further confirmed in murine experiments where *ex vivo* daughter cells from the first HSC division were transplanted into recipient animals to assess HSC function [99].

A role for metabolic regulation of HSC self-renewal was investigated in animal models where glucose intake was altered, or where HSCs harboring a genetic deletion of an enzyme involved in glycolysis were analyzed [100–102]. Specifically, using a zebrafish embryo-to-adult transplantation model, a transient elevation in glucose levels in fish was found to accelerate the induction of functional HSCs from **hemogenic endothelium**, as identified from various murine HSC-reporter lines in contrast to control embryos following glucose exposure [Tg(runx1P1:eGFP), Tg(cmyb:eGFP), and Tg(CD41:eGFP)] [100]. Mechanistically, elevated glucose increased mitochondrial ROS which induced expression of hypoxia inducible factor-1α (HIF-1α); this in turn led to an increased HSC number, while pharmacological inhibition of ROS, mitochondrial ROS, and HIF-1α using *N*-acetylcysteine, MitoQ, or dimethyloxallyl glycine (DMOG) led to a decrease in HSC numbers [100]. Murine HSCs in the BM niche have been thought to utilize glycolysis rather than mitochondrial oxidative phosphorylation because they show low mitochondrial respiration and high glycolytic flux [94]; this suggests a unique metabolic requirement for HSCs which might enable these cells to adapt to low oxygen tension in the BM niche [94]. Murine HSCs also exhibit higher pyruvate kinase activity compared to progenitors and more differentiated BM cells through a pyruvate dehydrogenase kinase (PDK)-dependent mechanism [93]. A dependency of HSC on glycolysis has also been reported in cases where enzymes involved in aerobic glycolysis have been genetically deleted in mice. For instance, deletion of lactate dehydrogenase A (*Ldha*) was reported to block the number and

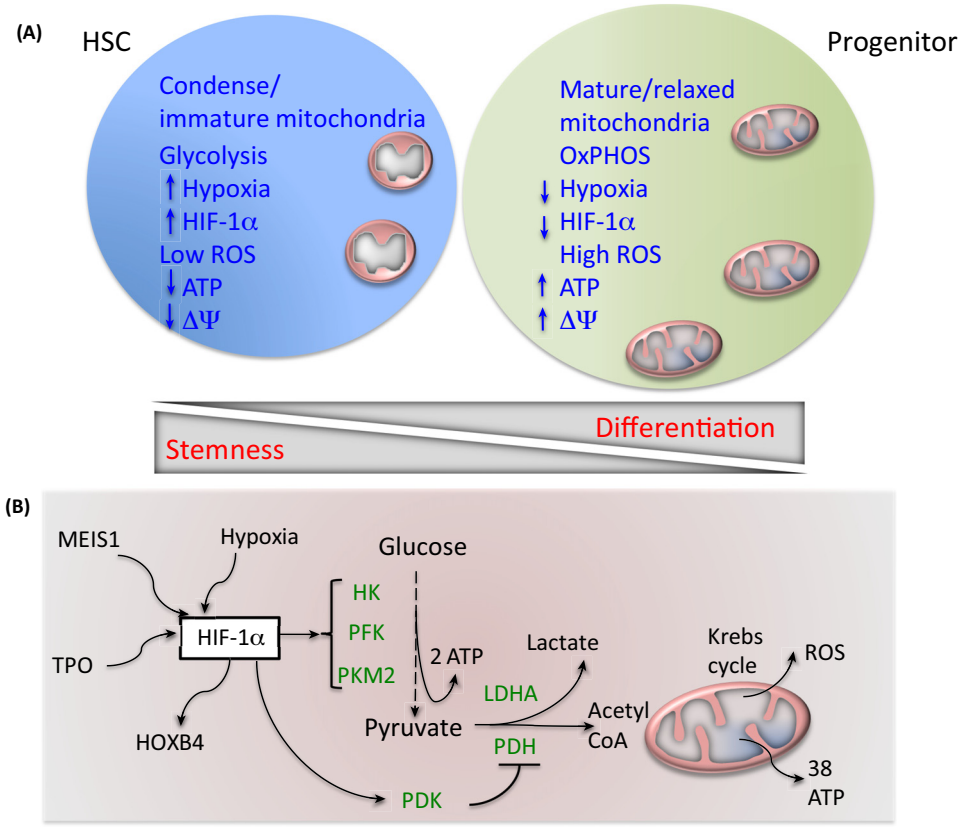

Figure 2. Metabolic Regulation of Mammalian Hematopoietic Stem Cells (HSCs). (A) HSCs exhibit condensed and immature mitochondria, low metabolic status, and high glycolytic activity, as suggested by low ATP, low ROS, and low membrane potential ($\Delta\Psi$) which maintain the stemness of the HSCs, in contrast to progenitors and more differentiated cells that exhibit high mitochondrial activity and utilize oxidative phosphorylation (OxPHOS). Furthermore, stabilized hypoxia-inducible factor 1α (HIF-1α) in HSCs can support self-renewal and stemness potential. (B) Mechanisms of metabolic regulation of HSC function. Under hypoxic conditions, cytokines THPO and MEIS1 can stabilize HIF-1α to promote glycolysis by regulating glycolytic pathway enzymes including HK, hexokinase; LDHA, lactate dehydrogenase A; PFK, phosphofructokinase 2; and PKM2, pyruvate kinase M2. HIF-1α can also controls HOXB4 and pyruvate dehydrogenase kinase (PDK) activation that prevents pyruvate oxidation by suppressing the pyruvate dehydrogenase (PDH) complex, inhibiting oxidative phosphorylation, and leading to maintenance of HSC quiescence and stemness. Abbreviations: HIF, hypoxia-inducible factor; HOXB4, homeobox B4; MEIS1, meis homeobox 1; PDK, pyruvate dehydrogenase kinase; ROS, reactive oxygen species; THPO, thrombopoietin.

function of both murine HSCs and progenitors upon secondary BM transplantation [102]. Others have found, by blocking glutaminolysis with 6-diazo-5-oxo-L-norleucine (DON) *in vitro*, or *in vivo* in mice, that erythroid specification of human and murine HSCs requires glutamine-dependent *de novo* nucleotide biosynthesis [101]. Furthermore, supplementation with nucleosides rescued erythropoiesis [101]. This suggests a broader regulatory input of metabolic pathways in terms of glycolysis and glutaminolysis on HSC self-renewal and differentiation [101]. In addition, starvation-induced metabolic stress in murine HSCs appears to be reduced by active **autophagy**, resulting in improved HSC maintenance [103], but the role of autophagy in regulating HSC self-renewal requires further investigation. Finally, stem cell divisions can result in an asymmetric allocation of mitochondria to one daughter cell versus another; daughter cells receiving aged mitochondria will differentiate, while daughter cells receiving low amounts of mitochondria maintain stemness in human mammary stem-like cells [104]

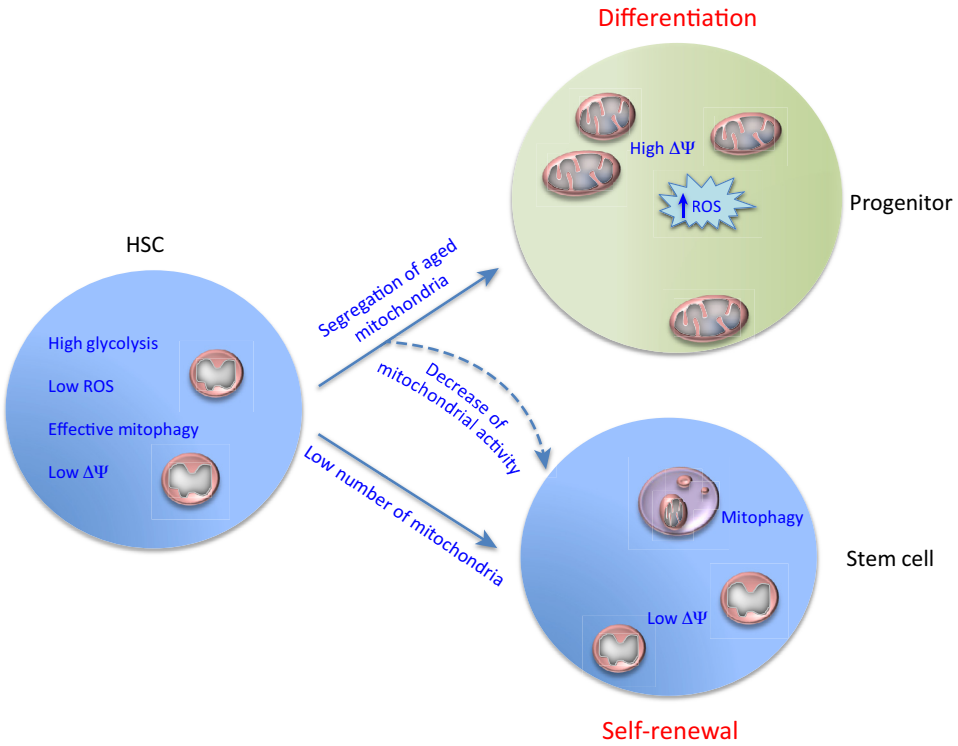

Figure 3. Metabolic Regulation of Mammalian Hematopoietic Stem Cell (HSC) Self-Renewal. HSCs maintain low metabolic status and high glycolytic activity with low levels of reactive oxygen species (ROS) and low membrane potential ($\Delta\Psi$) that help to maintain stemness during steady-state or stress conditions. HSCs divide to produce stem and progenitor cells. Recent research has suggested that daughter cells fated to perform as progenitors through differentiation exhibit high mitochondrial activity with increased mitochondrial numbers and high membrane potential, and utilize oxidative phosphorylation to produce more ROS, while daughter cells that receive low mitochondrial activity are fated to perform as stem cells through self-renewal decisions. Furthermore, pharmacological modulation of mitochondrial activity using uncoupling agents or mitophagy can lead to increased stem cell self-renewal decisions.

(Figure 3). These data suggest that HSC self-renewal is metabolically fine-tuned, at least with respect to glycolysis. These novel findings might open up alternative avenues to explore the potential of enhancing *ex vivo* human HSC self-renewal.

Mitochondria, Hypoxia, and ROS

Mitochondria are indispensable for energy generation. Mammalian HSCs exhibit low mitochondrial content and mitochondrial potential, with reduced rates of oxygen consumption and low ATP content but higher lactate production [93,94] (Figure 2); this suggests that HSC utilize glycolysis rather than oxidative phosphorylation. Moreover, mice devoid of mitochondrial phosphatase (*Ptpmt1*$^{-/-}$) have shown a 40-fold increase in the number of HSCs in the BM relative to wild-type animals, and this was attributed to defective HSC differentiation [105]. Consequently, it is possible that mitochondrial bioenergetics may be directly involved in the mode of HSC division. These studies further imply a distinct mitochondrial activity profile in HSCs relative to more differentiated cells – a process which may be necessary to meet the energy demands of HSCs upon activation, and to favor self-renewal over differentiation [93,94,105].

An earlier study demonstrated that HSCs, in addition to residing in hypoxic niches, can also exhibit intracellular hypoxia, and express a stabilized form of the transcription factor HIF-1α within mouse BM HSCs [92]. Under normoxia, HIF-1α is hydroxylated by O_2-dependent prolyl hydroxylases, followed by von Hippel–Lindau protein (VHL) and E3 ubiquitin ligase-derived HIF-1α degradation [106]. However, during hypoxic conditions, HIF-1α can be stabilized upon suppression of HIF-1α prolyl hydroxylation [11,106]. Stabilized HIF-1α associates with HIF-1β to form a transcription factor that activates the promoters of multiple glycolytic genes [11]. By contrast, HIF-1α-deficient mice exhibit loss of HSC cell-cycle quiescence and reduced HSC numbers upon stress, indicating that hypoxia/HIF-1α-dependent regulation of HSC quiescence and self-renewal may occur [92]. A positive role of HIF-1α in HSC self-renewal has been further supported *in vitro* in human hematopoietic cell lines or murine primitive cells treated with either SCF [63] or THPO [107], and these exhibit higher stabilized levels of HIF-1α than cells without treatment. Another study also reported that HSPCs in murine femur BM could maintain a hypoxic profile cell-intrinsically regardless of their localization in the vicinity of vascular structures or of their cell-cycle status, as evidenced from imaging cytometry revealing HIF-1α expression and reduced **pimonidazole** levels (a surrogate marker for hypoxia) [39]. However, because the pimonidazole adduct is insensitive to reoxygenation [108], further studies will be necessary to confirm the extent to which hypoxia within HSCs can be directly correlated to a hypoxic niche. Together, both a niche-regulated and cell-intrinsic hypoxic status have been implicated in HSC maintenance *in vivo* and might be exploited during *ex vivo* expansion protocols.

From another angle, mitochondrial aerobic metabolism is the main source of ROS generation in HSCs [109]. Ablation of the Polycomb repressor protein (Bmi1) in mice has led to defects in stem cell self-renewal and has been mechanistically linked to impaired mitochondrial function, reduced ATP generation, and increased intracellular ROS levels [110]. Indeed, ROS at low levels can also function in signaling [111]. Quiescent HSCs exhibit a low level of ROS that contributes to higher self-renewal potential and long-term stemness, while a higher level of ROS within HSCs or in the niche can result in loss of HSC from differentiation, proliferation, or apoptosis [112–114]. Consequently, mice that lack components of the ROS regulatory system frequently display a loss of HSC self-renewal [113–115]. For example, **ataxia telangiectasia mutated deficient (*Atm*$^{-/-}$) mice** have shown progressive BM failure (HSC), as evidenced by failed BM reconstitution and a decline in hematocrit levels in old mice relative to young, which has been attributed to elevated ROS levels in HSCs [115], and which in addition lead to p38 activation [116]. Moroever, Forkhead transcription factors (FoxO) that act downstream of the Pten/PI3K/Akt pathway and are activated in response to ROS, have been found to be crucial for HSC self-renewal in mice, specifically for the *in vivo* maintenance of the HSC pool that depends on self-renewal [113,114]. Indeed, *Foxo1/3/4* triple-knockout (KO) mice exhibited a reduced HSC pool size, and HSCs exhibited defective long-term repopulation as well as increased cell cycling and apoptosis associated with high ROS [114]. Similarly, the BM of *Foxo3a* KO mice exhibited decreased HSC repopulation potential, as well as defective maintenance of quiescence associated with elevated ROS, leading to p38 activation [113]. In this study the antioxidant *N*-acetyl-L-cysteine decreased p38 activation while inhibition of p38 restored the colony-forming capacity of *Foxo3a* KO **Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells**, at least *in vitro* [113]. These data support the notion that appropriate levels of ROS and antioxidant enzyme activity may be crucial for the regulation of HSC quiescence, self-renewal, and differentiation [117,118]. Thus, it is possible that pharmacological modulation of ROS concentrations as well as of signaling pathways regulated by ROS in HSCs might facilitate HSC expansion *ex vivo*, although this remains to be tested.

HSC Expansion *Ex vivo*: Status and Perspective

HSCs undergo massive expansion in numbers *in vivo* during the process of hematopoietic reconstitution after stress, such as from infection, lipopolysaccharide challenge, chemotherapy, radiation, or transplantation [4,30,31], but this still cannot be recapitulated by *ex vivo* expansion approaches. Successful protocols for the expansion of both HSCs as well as hematopoietic progenitor cells *ex vivo* are warranted in the clinic because higher numbers of progenitors and HSCs in transplants provide more short-term progeny that are required for better cell survival, and at the same time generate more robust long-term reconstitution [119]. HSC *ex vivo* expansion efforts are primarily based on protocols to expand both murine LSK cells (containing stem and progenitor cells) and human $CD34^+$ cells (also containing both stem and progenitor cell populations). High-throughput screening of chemical compound libraries (see below) has resulted in a few successful attempts towards human HSC expansion *ex vivo* [120,121] (Table 2). In addition, protocols for bioengineering HSC niches using **extracellular matrix** (ECM) components and 3D cultures have been established for human and mouse HSC expansion. In this section we discuss these recent findings and other niche-informed approaches for HSC expansion *ex vivo* that aim to conserve the functional and molecular characteristics of HSCs.

High-Throughput Screening of Compounds for Expansion

A major breakthrough in *ex vivo* expansion of HSCs was achieved by the laboratory of Cooke [120]. They utilized high-throughput technology for an unbiased screen to search for factors that could expand human HSCs *ex vivo*, using a library of 100 000 small molecules and serum-free expansion medium containing THPO, SCF, Flt3L and IL-6 [120]. A purine derivative, StemRegenin 1 (SR1), was found to promote a 50-fold *ex vivo* expansion of human cord blood-derived $CD34^+$ cells and a 17-fold increase in the number of human HSCs engrafting long-term in immunodeficient mice [120]. SR1 antagonizes the aryl hydrocarbon receptor (AHR) [120]. Recently, a clinical study using SR-1 demonstrated remarkable early neutrophil and platelet recovery and better engraftment in human recipients who received umbilical cord blood $CD34^+$ cells treated for 15 days *ex vivo* with SR-1 compared to recovery in recipients who received equal 'start' numbers of $CD34^+$ cells from the same unit but that were not expanded [122]. A recent study from the laboratory of Sauvageau showed that a pyrimidoindole derivative, UM171, induces human HSC self-renewal and *ex vivo* expansion in an AHR-independent manner, given that the expression of the AHR targets AhRR and CYP1B1 remained unaltered upon UM171 treatment [121]. A library of 5280 low molecular weight compounds and 300 analogs were screened to identify UM171. UM171 resulted in a better expansion of more primitive human $CD34^+CD45RA^-$ cells from mobilized peripheral blood (mPB) than SR1 [121]. Consequently, UM171 and SR1 may represent promising chemical compounds for *ex vivo* expansion of human HSCs. Of note, they do not act on murine HSCs [120,121]. However, the mechanisms underlying the SR-1 or UM171-mediated HSC self-renewal and differentiation block are not known. We speculate that, given their putative role in determining the mode of HSC division, these might interfere either with the regulation of ROS or with the mitochondrial or metabolic function that allows HSC self-renewal and expansion.

Reliance on Cytokines and Growth Factors

Cytokines were among the first drugs tested for HSC *ex vivo* expansion (Table 2). As mentioned, numerous cytokines have been shown to influence murine HSC numbers, at least *in vivo* [1,58,64–66,69,123–130]. Several cytokines singly, or in combination, have been investigated for their effects on murine and human HSC cultures and expansion *ex vivo;* however, only a maximum of 2–4-fold expansion of murine and human HSCs with long-term repopulation potential has been achieved (see also [126]). One study reported a modest increase of fourfold and 10-fold in the number of human cord blood (CB) $CD34^+CD38^-$ cells

Table 2. Successful Protocols for HSC Expansion *Ex Vivo*[a]

Factors	Cells tested	Species	Supplement	Culture period	Assay	Fold expansion	Refs
Cytokine-derived expansion							
Cytokines	CB $CD34^+38^-$	H	SCF, Flt3L, G-CSF, IL-3, IL-6	4 days	CFU and CRU (HSC frequency)	15-fold CFU; fourfold chimera	[128]
Cytokines	CB $CD34^+38^-$	H	Flt-3, SCF, IL-3, IL-6, and G-CSF	5–8 days	CFU, LTC-IC, CRU (HSC frequency)	100-fold CFU; fourfold LTC-IC; twofold CRU	[131]
Angiopoietin	SP $CD45^+$ $Sca\text{-}1^+$	M	SCF, THPO, FGF-1, IGF-2	10 days	CRU (HSC frequency)	24–30-fold	[130]
Pleiotrophin	$CD34^-$ LSK, CB $CD34^+$ 38^-	M and H	SCF, Flt3L, THPO	7 days	CRU frequency/LT engraftment	Fourfold CRU; 10-fold chimera	[69]
Virus-mediated overexpression, TFs, etc.							
HOXB4	CB $CD34^+$	H	Co-culture on MS-5 mouse stromal cells	4–5 weeks	LTC-IC assay, reconstitution analysis	20-fold LTC-ICs; 2.5-fold long-term repopulation	[145]
Fbxw7	LSK	M	SCF, THPO	10 days	Competitive reconstitution analysis	>Twofold long-term repopulation	[147]
Dppa5	$CD34^-48^-$ LSK	M	SCF, THPO	14 days	Competitive reconstitution analysis	6–10-fold	[154]
Hypoxia, ROS, and metabolic modulations							
PDH inhibitor (1-aminoethylphosphinic acid, 1-AA)	$CD34^-Flts^-$ LSK	M	SF-O3 medium 1.0% BSA, serum-free, SCF, THPO	2–4 weeks	CFU/competitive reconstitution analysis	Twofold CFUs; fivefold LT repopulation	[93]
Mitochondrial phosphatase Ptpmt1 inhibitor, alexidine dihydrochloride (AD)	LSK and *in vivo* treatment $CD34^+$ 38^-	M H	SCF, THPO, Flk-3	7 days	CFU and LT chimera 34^+38^- number/CFU	Twofold CFUs; 3–5-fold LT repopulation Twofold number; twofold CFUs	[150]
GSK-3β inhibitor, CHIR99021	LSK and CB $CD34^+$ LSK Flk^-	M and H M	With rapamycin in cytokine-free X-VIVO medium SCF, THPO, and insulin	7 days 14 days	HSC frequency/LT engraftment Competitive reconstitution analysis	10–20-fold HSC number; 2–5-fold LT repopulation 100-fold number; 2–10-fold LT repopulation	[135] [84]
High-throughput chemical screens							
SR-1	$CD34^+$ MPB UBC	H	SCF, Flt3L, THPO, IL-6	7–21 days	Number/CFU/ competitive reconstitution analysis	65-fold CFUs; 17-fold enhanced chimera	[120]
UM171	$CD34^+$ MPB UBC	H	SCF, Flt3L, THPO	7–21 days	Number/CFU/ competitive reconstitution analysis	>100-fold LT-HSC; 35-fold enhanced chimera	[121]

[a]Abbreviations: CB, cord blood; CFU, colony forming unit; CRU, competitive repopulating unit; CXCL-4, CXC chemokine ligand 4; CXCL-12, CXC chemokine ligand 12; Dppa5, developmental pluripotency associated 5; Fbxw7, F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase; FGF-1, fibroblast growth factor 1; Flt3L, FMS-like tyrosine kinase 3 ligand; G-CSF, granulocyte-colony stimulating factor; IGF-2, insulin-like growth factor 2; IL-3, interleukin 3; OPN, osteopontin; LT, long-term; LTC-IC, long-term culture initiating cell; MPB, mobilized peripheral blood; MS-5, murine MS-5 stromal cell line; SCF, stem cell factor; SP, side population; SR-1, StemRegenin1; TGF-β1, transforming growth factor β1; THPO, thrombopoietin; TFs, transcription factors; UCB, umbilical cord blood.

and colony forming units (CFUs), respectively, as well as a 2–4-fold increase in **SCID-repopulating cells** (SRCs) in **NOD/SCID mice** after 4 days of culture with cytokines (SCF, Flt3L, G-CSF, IL-3, IL-6) [128]. However, after 9 days of culture, despite further increase in the total number of $CD34^+$ cells, the reconstitution ability was lost [128]. In another study, human CB

$CD34^+CD38^-$ cells in serum-free medium containing Flt-3, SCF, IL-3, IL-6, and G-CSF for 5–8 days resulted in robust 100-fold CFU expansion, fourfold LTC-IC, and twofold increase in the competitive repopulating unit (CRU) [131].

Overall, these cytokines and their combinations can maintain HSCs and progenitors, and most likely protect them against apoptosis during *ex vivo* proliferation, as evidenced from flow cytometric assays [66,132], although only resulting in a modest expansion of human HSCs, albeit a multilog expansion of progenitor cells [126,132]. Thus, additional factors are clearly necessary for successful expansion of human HSCs *ex vivo*. Newly identified factors in stroma-conditioned medium, such as nerve growth factor and collagen 1, have resulted in better expansion of murine HSCs compared to the 'standard' cocktail listed above [133]. This suggests that the stroma still harbors additional yet-to-be-determined factors that alone, or in combination, might result in significant *ex vivo* expansion of HSCs, representing an exciting novel area of research to improve HSCs expansion *ex vivo*.

Several laboratories have also tested the activation of Wnt signaling for stem cell expansion. The laboratory of Reya reported an 8–80-fold expansion of functional murine HSCs upon short-term culture in serum-free medium supplemented with low concentrations of cytokines (SLF, Flt-3L, IL-6), transducing HSCs with constitutively active β-catenin through upregulation of the self-renewal gene homeobox B4 (*Hoxb4*) [73]. Moreover, short-term pretreatment of cells with a GSK-3β inhibitor (6-bromoindirubin 3′-oxime or BIO) that activates β-catenin was demonstrated to enhance the engraftment of *ex vivo*-expanded human cord blood $CD34^+$ HSCs in murine **xenograft** models [134]. Another study reported that pharmacological, and thus reversible, activation of both Wnt/β-catenin and PI3K/Akt signaling in HSCs using another type of GSK-3β inhibitor (CHIR99021) in combination with cytokines (SCF, THPO) and insulin significantly expanded (~100-fold) the number of HSCs [84] after 14 days of cultivation of murine HSCs in serum-free medium, suggesting that cooperation between these pathways might be beneficial for HSC self-renewal and expansion [84]. Cultivation of human $CD34^+$ cells as well as murine LSK cells in cytokine-free medium for 7 days in the presence of rapamycin [inhibiting the **mammalian target of rapamycin (mTOR) pathway**] and CHIR99021 (activating the canonical Wnt pathway) resulted in maintenance of the number of human and murine HSCs, as confirmed in serial transplantation assays [135]. In addition, when provided in excess, prostaglandin E2 (PGE_2) – which modifies the Wnt signaling cascade at the level of β-catenin degradation [136] – has been shown to result in an increase in HSC numbers in zebrafish and mouse embryos [137]. Treating HSCs with PGE_2 increased the number of human CFUs *in vitro*, and enhanced the engraftment of unfractionated and human cord blood $CD34^+$ HSPCs upon xenotransplantation [138], which may support a role for Wnt signaling in HSC expansion. Another study used an automatic fed-batch media dilution approach to control inhibitory feedback signals during culture of human cord blood HSPCs; this led to an 11-fold expansion of SCID repopulating cells with self-renewing, multilineage repopulating ability [139], implying a crucial role for inhibitory feedback loops in mitigating HSCs expansion *ex vivo*.

Regulation of ROS, Antioxidants, and Hypoxia

Cytokines such as GM-CSF, IL-3, SCF, and THPO have been shown to increase murine and human HSC proliferation through a rapid increase in the level of ROS in quiescent cells [118]. Specifically, elevation of ROS induced HSC-specific phosphorylation of p38 MAPK upon culture in serum-free media supplemented with cytokines including SCF, IL-3, and EPO, while antioxidant treatment or inhibition of p38 MAPK *ex vivo* rescued ROS-induced defects in HSC repopulating capacity, preventing exhaustion of murine HSCs in serial transplantation experiments [116]. These data suggest that p38 MAPK or ROS inhibition in *ex vivo* cultures might be able to contribute to HSC expansion. In addition, roles of hypoxia and fine-tuned regulation of HIF-1α stabilization in HSC maintenance have been established by both biochemical and

genetic approaches [91,92,140,141]. For instance, mouse BM cells cultured under hypoxia showed a fivefold higher day 14 spleen colony-forming efficiency as well as enhanced **radioprotection ability** than under normoxic conditions [140], suggesting better maintenance and expansion of HSCs. Upon cultivation under hypoxic conditions, murine HSCs have also been shown to accumulate at the G_0 stage of the cell division cycle, which results in an increase in HSCs with long-term engraftment potential relative to non-hypoxic conditions [141]. Furthermore, hypoxia induces HIF-1α-dependent expression of the cell-cycle regulators p21, p27, and p57 in murine HSCs [141]. Isolating and manipulating murine BM and human cord blood under strict hypoxic conditions *in vitro* demonstrated that a higher number of HSCs can be recovered from the BM under these conditions [142], while a brief exposure to ambient oxygen was found to decrease the number of HSCs upon BM harvest, through an extraphysiologic oxygen shock/stress (EPHOSS) mechanism [142]. Together, it is therefore possible that maintaining a strict hypoxic environment might be beneficial for *ex vivo* expansion of HSCs, but robust validation of this hypothesis is still warranted.

Retrovirus-Mediated Introduction of Stem Cell Regulators and Reprogramming

Multiple approaches have been reported for *ex vivo* HSC expansion based on retrovirus-mediated expression of HSC maintenance or expansion genes. For example, overexpression of *Hoxb4* expands murine HSCs approximately 40–1000-fold *in vitro* and *in vivo*, respectively [126,143,144], without stem cell transformation. Human cord blood $CD34^+$ HSCs have been expanded approximately 2.5-fold using a HOXB4 fusion protein expressed by the stromal cell line MS-5 [145]. A challenge remains because the HOXB4 protein is unstable in culture when provided extrinsically, as in the previous approach. THPO also positively regulates HOXB4 expression in murine and human hematopoietic cell lines [146], and this might explain in part the beneficial effect of THPO on HSC maintenance *ex vivo*, although this remains speculative. The expression of the ubiquitin-ligase, F-box, and WD-40 domain protein 7 (Fbxw7) that mediates degradation of cell-cycle activators in HSCs is upregulated by hypoxia [147]. As such, overexpression of *Fbxw7* in murine LSK cells has been reported to cause >twofold higher reconstitution capacity during *ex vivo* culture by maintaining HSC quiescence through a reduction in the expression of c-Myc, Notch1, and mTOR [147]. While the transduction of HSCs with the above-mentioned regulators expand HSCs to small but distinct levels, there is a risk of insertion-mediated oncogene activation (as with all transgenic approaches), which will most likely preclude the application of these current approaches in the clinic.

Recently, two studies have described the generation of functional HSCs via reprogramming from adult endothelium and human pluripotent stem cells [148,149], introducing novel exciting technologies into the field of HSC generation. Adult mouse endothelial cells were fully reprogrammed to HSCs (rEC-HSCs) through transient ectopic expression of four transcription factors (*Fosb*, *Gfi1*, *Runx1*, and *Spi1*; FGRS) and vascular-niche-derived angiocrine factors from a feeder layer [148]. This finding was interesting because rEC-HSCs present a transcriptome profile and long-term self-renewal capacity similar to those found in adult HSCs [148]. Furthermore, multilineage reconstitution was achieved in both primary and secondary bone-marrow transplantation settings [148]. In another study, human pluripotent stem cells were first directed towards the hemogenic endothelium using chemical signals, and seven transcription factors (*ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1*, *and SPI1)* pushed the hemogenic endothelium towards a blood stem cell state that provided *in vivo* multilineage reconstitution in a xenograft mouse model [149]. These findings provide a significant advance in the generation of HSCs that does not involve altering the mode of division of existing HSCs, but instead involves generating unlimited HSC numbers 'from scratch'. Nevertheless, the translational potential of these approaches and newly generated cells currently remains limited due to the use of reprogramming factors bearing oncogenic potential.

Targeting Metabolic Pathways

HSC metabolism has also been targeted to investigate HSC expansion, but with mixed success. When glycolysis was favored in murine HSCs using a pyruvate dehydrogenase (PDH) inhibitor (1-aminoethylphosphinic acid, 1-AA), the cycling and colony growth of HSCs was suppressed during *ex vivo* culture, while HSC frequency and reconstitution ability was maintained even after 4 weeks of culture [93]. Alexidine dihydrochloride (AD) inhibits mitochondrial phosphatase Ptpmt1 [150] and can shift mitochondrial aerobic metabolism to glycolysis through AMPK [150]. Thus, treatment of murine LSK cells with AD under normoxic conditions increased their transplantation efficiency about threefold in **competitive transplant settings** relative to untreated control cells [150]. Consequently, it will be interesting to test if there is a synergistic effect of hypoxia and treatment with AD with respect to HSC expansion. Recent reports demonstrate that chemical uncoupling of the electron transport chain, which decreases mitochondrial activity, resulted in increased murine HSC self-renewal under *ex vivo* culture conditions, generally causing rapid differentiation [151]. In general, active **mitophagy** appears to be a mechanism necessary for directing HSCs towards self-renewal and away from differentiation (at least in mice) [152] (Figure 3). Indeed, murine HSCs have been demonstrated to exhibit high mitophagy function via the PPAR–FAO pathway, preferentially undergoing symmetric divisions to self-renew. Accordingly, GW501516, a PPAR–FAO agonist, can enhance LTC-IC frequency via mitophagy activation in human HSCs [152]. Together, these studies suggest that targeting the 'metabolic switch' to enhance HSC glycolysis during an *ex vivo* culture might potentially enhance *ex vivo* self-renewal and perhaps even HSC expansion, a hypothesis awaiting robust validation.

Targeting ER Stress Pathways

HSCs can encounter diverse types of stress such as elevated ROS and DNA damage, but also endoplasmic reticulum (ER)-dependent stress stemming from the unfolded protein response (UPR). Indeed, recent work demonstrates that an overall appropriate response to ER stress from unfolded proteins can support HSC maintenance, self-renewal, and expansion [153–155]. For example, human HSCs, but not progenitors, are highly predisposed to undergo apoptosis through PERK-mediated UPR to ER stress, while overexpression of the co-chaperone ERDJ4 (that increases ER protein folding) has been found to enhance human HSC engraftment in a mouse xenograft model [153]. Moreover, in contrast to BM HSCs, fetal liver (FL) HSCs undergo very rapid expansion *in vivo*, and despite an increased rate of protein synthesis, they do not exhibit ER stress [155]. Instead, taurocholic acid, the major maternal and fetal liver bile acid (BA) form, has been shown to serve as a chemical chaperone that can inhibit protein aggregation and support HSC growth in mice [155]. Such recently identified chaperones might thus comprise a novel class of compounds to be tested in *ex vivo* expansion approaches for adult HSCs. Developmental pluripotency-associated 5 (Dppa5), an RNA-binding protein, is highly enriched in HSCs [154]. Murine HSCs that ectopically express Dppa5 have been reported to robustly increase their reconstitution potential in transplantation experiments, reducing (ER) stress and apoptosis during *ex vivo* culture for 14 days [154]. Correspondingly, tauroursodeoxycholic acid (TUDCA), a chemical chaperone that reduces ER stress, was shown to enhance HSC engraftment approximately fivefold in this mouse model [154]. In general, these studies could indicate that minimizing ER stress might potentially contribute to successful HSC expansion *ex vivo.*

The ECM and Niche Engineering

Niches in the BM provide, in addition to soluble factors, specific ECM components and structural 3D architectures [8,156,157]. Several polymeric biomaterial substrates that mimic the structure of the ECM have been explored with respect to their ability to enhance HSC expansion. Diverse ECM substrates including polyethylene terephthalate (PET), tissue culture polystyrene (TCPS), and polyether sulfone (PES) (Box 2) have failed to enhance HSCs

Box 2. ECM Modeling and HSC Niche Reconstitution

The ECM consists of, among others, collagen, fibronectin, dystroglycan, heparin sulfate, proteoglycans, osteopontin, and laminin [56,158,159,169,176], and can bind to adhesion molecules such as integrins on HSCs. Polymeric biomaterial substrates such as polyethylene terephthalate (PET), tissue culture polystyrene (TCPS), and polyether sulfone (PES) fibers have the advantage of defined composition, surface chemistry, and toxicity profile. Recent evidence further suggests that substrate elasticity can influence self-renewal versus differentiation outcomes of murine and human HSC divisions *ex vivo* [156,164,165]. For example, the tropoelastin substrate can enhance HSC self-renewal through mechanotransduction machinery; blockade of mechanotransduction using myosin II inhibition abrogated tropoelastin-induced expansion effects [165]. Currently the field focuses on developing 3D biomaterials of low density, with open-cell foam structure scaffolds and distinct levels of elasticity, using stromal cells to support HSC expansion and which could be adopted as analogs of the trabecular bone [170,176]. Novel approaches in the area of 3D ECM research for stem cell expansion include microfluidic trap devices for capturing individual HSCs to perform post-culture single-cell analysis [177].

expansion *ex vivo* [158]. However, fibronectin-coated PET has shown elevated expansion in the number of human HSCs *ex vivo* versus unmodified biomaterials [159,160]. Similarly, aminated-PES substrates and cytokines (SCF, Flt3, THPO, and IL-3) have been reported to support a 3–4-fold expansion of human $CD34^+$ HSCs derived from umbilical cord blood compared to tissue culture polystyrene [161,162]. Moreover, cultivation of human HSCs with BM mesenchymal stem cells (MSCs) in the absence of additional cytokines resulted in a 5–7-fold increase in the number of LTC-ICs compared to HSCs cultivated in the absence of MSCs [163]. MSCs might thus provide niche components, including soluble cytokines, that support HSC expansion *ex vivo.*

Various studies have demonstrated that elasticity, dimensionality, and topography of the matrix positively influences HSC proliferation and expansion [156,164,165]. Specifically, cultivation of mouse or human primitive hematopoietic cells on a tropoelastin substrate led to a 2–3-fold expansion of HSCs compared to cultivation on bare tissue culture plates due to changes in substrate elasticity [165]. 3D collagen-coated porous reticulated polyvinyl formal (PVF) resin scaffolds with low oxygen have also led to murine HSC expansion over 3 weeks in the presence of BM stromal cells without exogenous cytokines [166]. Furthermore, 3D PVF resin scaffolds that produced an oxygen gradient, as opposed to a constant hypoxic environment, mimicked key features of marrow physiology [91], leading to threefold higher expansion of primitive $CD34^+$ cells in a 3D setting, in contrast to 2D culture systems [167]. Accordingly, 3D culture systems (e.g., nonwoven porous carriers, macroporous collagen carriers, and porous microspheres such as PET and collagen) have resulted in a threefold increase in human HSC self-renewal compared to 2D cultures, and this was further enhanced sevenfold by THPO and Flt-3 ligand supplementation [168]. In addition, a fibronectin-immobilized 3D PET scaffold led to a remarkable 100-fold expansion of human HSCs [160]. A high immuno-phenotypic expansion (10^{14} vs 10^6 input cells) of cord blood $CD34^+$ HSCs was also observed with fibrin scaffolds in the presence of human umbilical cord (UC)-MSCs and cytokine supplementation [SCF, THPO, FGF-1, angiopoietin like-5 (Angptl-5), insulin-like growth factor binding-protein 2 (IGFBP2), and heparin] following 14 days of culture, which was mirrored by a high long-term reconstitution ability (58.5%) in murine xenotransplantation models [169].

The laboratory of Blau utilized a hydrogel microwell array for rapid analysis of murine HSC proliferation kinetics which correlated well with subsequent serial long-term blood reconstitution in mice *in vivo* [76]. In such assays Wnt3a resulted in slow HSC proliferation compared to several other tested proteins such as THPO and IL-11, and this led to higher long-term reconstitution, suggesting that Wnt3a might potentially enhance HSC self-renewal, while THPO and IL-11 induced robust proliferation as well as differentiation [76]. Others have described a bone-marrow-on-a-chip platform to replicate murine BM niche-like analogs for HSC *in vitro* cultures [170]. To generate an artificial niche, they combined demineralized bone powder and

Box 3. Clinician's Corner

A recent clinical trial of *ex vivo* expanded umbilical cord blood $CD34^+$ cells used SR-1 and demonstrated better engraftment and improved early recovery of leukocytes, suggesting that *ex vivo* expansion of HSCs might potentially be achievable [120–122].

Similarly, co-cultures of cord blood $CD34^+$ cells with mesenchymal stromal cells led to expansion of $CD34^+$ cells by a median factor of 30.1, improving the time required for neutrophil engraftment to 15 days, compared to 24 days in recipients who received unmanipulated cord blood $CD34^+$ cells [178].

Growing evidence indicates that targeting metabolism/mitophagy and cellular stress for HSC expansion might potentially lead to successful HSC expansion approaches for transplantation therapies in the future [150,152].

Outstanding Questions

What are the relative contributions of cellular (stem cell intrinsic) and niche factors (stem cell extrinsic) to the regulation of HSC self-renewal?

What is the role of a hypoxic environment on HSC expansion *in vivo*, and what is the best model to use *ex vivo*?

Can ROS/stress accumulation usually associated with HSC proliferation *ex vivo* be efficiently targeted/suppressed?

Can we harvest novel knowledge on HSC metabolism for HSC expansion strategies?

Do novel compounds such as SR1 and UM171 that result in HSC expansion also regulate HSC metabolism? If yes, how?

Which molecular mechanisms are triggered by engineered 3D ECM environments to modulate HSC self-renewal *ex vivo*?

BMP2/4 into a collagen scaffold, which was subcutaneously transplanted into mice to form into a bone-encased marrow compartment containing hematopoietic cells. This engineered bone marrow (eBM), when used in a microfluidic device *ex vivo*, retained the number of HSCs after 1 week of culture [170]. This system may represent a promising novel platform for screening diverse drugs for *ex vivo* HSC expansion in an *in vivo*-like artificial niche setting. Collectively, the data suggest that novel ECM and niche engineering approaches in the presence of stromal cells might support HSC self-renewal and expansion *ex vivo* (Box 2).

Concluding Remarks

Over the past decade several novel studies have suggested that HSC expansion *ex vivo* might actually be feasible. This is based on a better understanding of HSC-intrinsic as well as niche-specific factors regulating HSC self-renewal *in vivo*, and which have led to novel putative strategies to expand HSCs *ex vivo*. Significant advances in HSC expansion have also been made recently in high-throughput screening approaches of low molecular weight compound libraries. Ultimately, a combination of 3D scaffolds mimicking the niche 3D architecture, mixed with cytokines/chemokines and stromal cells under the appropriate oxygen and metabolic conditions, might provide a solid option for achieving robust expansion of adult HSCs *ex vivo*. However, many mechanisms of HSC regulation in the niche remain poorly understood (see Outstanding Questions and Box 3), and extensive and robust validation of these platforms will be necessary as a first essential step for clinical translation. Nevertheless, various modalities, strategies, and methodologies will undoubtedly emerge for HSC expansion in the near future, and it will be exciting to follow such advances in stem cell research.

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