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HSC Niche Biology and HSC Expansion Ex Vivo

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Abstract

Hematopoietic stem cell (HSC) transplantation can restore a new functional hematopoietic system in recipients in cases where the recipient's own system is not functional, or for example, leukemic. However, the number of available donor HSCs is sometimes 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

Hematopoietic stem cells (HSCs) sustain blood-cell formation in a process called **hematopoiesis** (see Glossary, 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]. **Hematopoietic stem cell 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 in blood, mobilized to blood by the cytokine granulocyte-colony stimulating factor (G-CSF), can be used. **Infused donor HSCs** then home to and engraft in discrete BM niches to reconstitute the blood system of the recipient [2]. Currently, autologous transplants have survival rates exceeding

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80%, while the success rate for allogeneic transplants at five 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 a successful HSCT, high numbers of **CD34$^+$ cells** (i.e. 3–4 × 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. Thus, protocols that result in **expansion** of HSCs *ex vivo* would be a highly desirable tool to further increase positive outcomes in the clinic. Understanding mechanisms of HSC self-renewal *in vivo* in depth should be a pre-requisite for the development of successful protocols to expand HSCs *ex vivo* for therapeutic applications.

HSC self-renewal is regulated by a complex interplay of 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 critical 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 investigated 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]; and yet, 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 have 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 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 critical 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 (Treg) [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 Jagged1 [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 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, the 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 last couple of 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 (Key Figure, Figure 1, see also [8, 55]). However, further studies are required 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 which regulate HSC self-renewal and differentiation *in vivo* (table 1). Osteoblast-derived cytokines, including osteopontin (OPN), angiopoietin -1, -3, thrombopoietin (TPO), 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, TPO 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 TPO is mainly released by osteoblasts. Both c-Kit and c-Mpl are expressed on highly purified HSCs and genetic deletion of TPO or c-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 Jagged1) implicated in HSC regulation [20, 23, 61]. The puttaive role for angiopoietin-1 in HSC self-renewal – a cytokine well known for its role in in endothelial cell remodeling – is 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 along with impaired hematopoietic regeneration [68], while PTN can promote *in vitro* 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, could enhance *in vitro* HSC self-renewal [52].

Recent mouse studies also suggest an inhibitory role for peri-sinusoidal megakaryocytes (MKs) in HSCs 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/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 has resulted in HSC quiescence [42]; indeed, injection of TGF-β1 in MK-ablated mice restored HSC quiescence and inhibited self-renewal [42]. Also, 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 HSCs 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, 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] and 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 TPO, 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 upon 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 shown normal HSC counts, but yet 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, though they exhibited defects in differentiation [84]. Additional studies revealed that stabilization of beta-catenin in stromal cells promote 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; upon 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 cross-talk 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, as the non-canonical Wnt signaling initiated by Fz8 suppressed the Ca(2+)-NFAT- IFNγ pathway, antagonizing canonical Wnt signaling [34]; this resulted in maintenance of quiescent long-term 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 experiments [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 dependency [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 in order to elucidate the potential of modulating Wnt signaling to achieve ex-vivo human HSC expansion.

Metabolic Regulation of HSC

HSCs also exhibit a stringent regulation of their hypoxic status [91, 92] and 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 WT cells [95]. In addition, studies 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 (Hif1α); this in turn, led to an increased HSC number, while pharmacological inhibition of ROS, mitochondrial ROS, and Hif1α 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 as 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 a 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 function of both murine HSCs and progenitors upon secondary BM transplantation [102]. Others have found that 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). These data suggest that HSC self-renewal is metabolically fine-tuned, at least with respect to glycolysis. These novel findings might open up alternate 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 *Ptpmt1*$^{-/-}$ (mitochondrial phosphatase) have shown a 40-fold increase in the

number of HSCs in the BM relative to wild type animals, which has been 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].

A prior study demonstrated that HSCs, aside from residing in hypoxic niches, can also exhibit intracellular hypoxia, expressing a stabilized form of the transcription factor Hif1α within mouse BM HSCs [92]. Under normoxia, Hif1α is hydroxylated by O_2-dependent prolyl hydroxylases, followed by von Hippel-Lindau protein (VHL) and E3 ubiquitin ligase-derived Hif1α degradation [106]. However, during hypoxic conditions, Hif1α can be stabilized upon suppression of Hif1α prolyl hydroxylation [11, 106]. Stabilized Hif1α associates with Hif1β to form a transcription factor that activates the promoters of multiple glycolytic genes [11]. By contrast, Hif1α-deficient mice exhibit loss of HSC cell cycle quiescence and reduced HSC numbers upon stress, indicating that hypoxia/Hif1α-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 TPO [107], exhibiting more 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, as the pimonidazole adduct is insensitive to re-oxygenation [108], further studies will be necessary to confirm the extent to which hypoxia within HSCs can be directly correlated to a hypoxic niche. Together, a niche-regulated as well as a 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 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 present with 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 from 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], 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 critical 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 critical for the regulation of HSC quiescence, self-renewal and differentiation [117, 118]. Thus, it is possible that the pharmacological modulation of ROS concentrations as well as of signaling pathways regulated by ROS in HSCs might facilitate HSC expansion *ex vivo*, though 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 an infection, lipopolysaccharide challenge, chemotherapy, radiation or transplantation [4, 30, 31], but which 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, as higher numbers of progenitors and HSCs in transplants provide more short-term progeny 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 extra-cellular matrix 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 break-through in *ex vivo* expansion of HSCs was achieved by the Cooke laboratory [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 TPO, 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 a remarkable early neutrophil and platelet recovery and better engraftments in human recipients that received umbilical cord blood CD34$^+$ cells treated for 15 days *ex vivo* with SR-1 compared to recovery in recipients that received equal “start” numbers of CD34$^+$ cells from the same unit, but not expanded [122]. A recent study from the Sauvageau laboratory

showed that a pyrimidoindole derivative called UM171 induces human HSC self-renewal and *ex vivo* expansion in an AhR-independent manner, given that the expression of AhR targets AhRR and CYP1B1 remained un-altered 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 of 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 with either the regulation of ROS, or 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 effect 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 a 4- and 10-fold in the number of human cord blood (CB) $CD34^{+}CD38^{-}$ cells and colony forming units (CFUs), respectively, as well as a 2- to 4-fold increase in **SCID-repopulating cells (SRC)** frequency in **NOD/SCID mice** after 4 days (d) of culture with cytokines (SCF, Flt3L, G-CSF, IL-3, IL-6) [128]. However, after 9 d 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 d resulted in robust 100-fold CFU expansion, 4-fold LTC-IC, and 2-fold 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], though only resulting in a modest expansion of human HSC, 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 a 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, and 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 Reya laboratory 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 up-regulation 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 a 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, TPO) and insulin significantly expanded (~100 fold) the number of HSCs [84] after 14 d′ 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 d in the presence of rapamycin (inhibition of the mammalian target of rapamycin **(mTOR) pathway**) and CHIR99021 (activation of canonical Wnt) resulted in the maintenance in the number of human and murine HSCs, as confirmed in serial transplantation assays [135]. Also, 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 supports 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 critical 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 TPO 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 have 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* has 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, a role 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 have shown 5-fold higher day-14 spleen colony-formation efficiency as well as enhanced **radio-protection 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 Hif1α 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* has demonstrated a higher number of HSCs 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 as the HoxB4 protein is unstable in culture when provided extrinsically, as in the previous approach. TPO also positively regulates HoxB4 expression in murine and human hematopoietic cell lines [146], which might explain in part, the beneficial effect of TPO 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 up-regulated by hypoxia [147]. As such, overexpression of *Fbxw7* in murine LSK cells has been reported to cause >2 fold higher reconstitution capacity during *ex vivo* culture by maintaining HSC quiescence through a reduction in 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 HSCs generation. Adult mouse endothelial cells were fully reprogrammed to hematopoietic stem cells (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 fidning was interesting because rEC-HSCs presented a transcriptome profile and long-term self-renewal capacity similar to those found in adult HSCs [148]. Furthermore, multi-lineage 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 significant advancement in generating HSCs that do not involve altering the mode of division of existing HSCs, but rather, involve generating from “scratch” unlimited HSC numbers. 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 3-fold 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 the 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 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]. Altogether, 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 a 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 a very rapid expansion in vivo, and despite increased protein synthesis rate, 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 expressed 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 5-fold in this mouse model [154]. In general, these studies could indicate that minimizing stress to the ER might potentially contribute to successful HSC expansion *ex vivo*.

Extra-cellular Matrix and Niche Engineering

Niches in the BM provide, besides soluble factors, specific **extracellular matrix (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 function 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 expansion *ex vivo* [158]. However, fibronectin-coated PET has shown elevated expansion in the number of human HSCs *ex vivo* in contrast to unmodified biomaterials [159, 160]. Similarly, aminated-PES substrates and cytokines (SCF, Flt3, TPO and IL-3) have been reported to support a 3- to 4-fold expansion of human $CD34^+$ HSCs derived from umbilical cord blood when compared to tissue culture polystyrene [161, 162]. Moreover, cultivation of human HSCs with BM mesenchymal stem cells (MSCs) in the absence of additional cytokines has resulted in a 5- to 7-fold increase in the number of LTC-ICs, when 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 has led to a 2 to 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 three 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 3-fold higher expansion of primitive $CD34^+$ cells under a 3D setting, in contrast to a 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 3-fold increase in human HSC self-renewal when compared to 2D cultures, which was further enhanced 7-fold in the presence of TPO and Flt-3 ligand supplementation [168]. In addition, a fibronectin-immobilized 3D PET scaffold has 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, TPO, FGF-1, angiopoietin like-5 (Angptl-5), insulin-like growth factor binding-protein 2 (IGFBP2), and heparin] following 14 d of culture, which was mirrored by a high long-term reconstitution ability (58.5%) in murine xenotransplantation models [169].

The Blau laboratory 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 TPO and IL-11, which led to higher long-term reconstitution, suggesting that Wnt3a might potentially enhance HSC self-renewal, while TPO 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 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 one 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 last 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 advancements 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 right oxygen and metabolic conditions might be 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 required 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 quite exciting to follow such advances in stem cell research.

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Glossary

Allograft transplant
Stem cells from an HLA matched donor are transplanted

Asymmetric Cell Division
leads to the generation of two cells with different potentials: 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, BM failure and HSC exhaustion

Autologous transplant
A person'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 which include both stem and progenitor cell populations

Cell-intrinsic
property of cells 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
decision to generate progenitors from stem cells necessary to produce mature blood cells

Endosteal
HSC niche in close association to a bone surface

HSC exhaustion
a state of turnover, being 'used up'; HSCs can undergo exhaustion due to a high demand of reconstitution under stress or serial transplant settings.

Extracellular Matrix (ECM)
The 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 expansion
The process of increasing the number of HSCs

HSC niche
A specific BM environment that provides cellular, chemical and molecular constituents and contributes to the regulation of HSC survival, self-renewal and differentiation

Hematopoietic stem cell 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 rejections

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

Long-term (LT)-HSC
give rise to multi-lineage engraftment post-transplantation for a time-frame of at least 20 weeks. LT-HSCs are phenotypically characterized as $Lin^{-}IL\text{-}7\alpha^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}Flt3^{-}CD34^{-}CD150^{+}CD48^{-}$ 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

LSK cells
distinct fraction of murine hematopoietic stem and progenitor cells in BM, characterized as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ based on surface marker expression

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

NOD/SCID mice
Nonobese diabetic (NOD)-severe combined immunodeficiency (SCID) mice present with impaired T and B cell lymphocyte and NK cells. 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
nontoxic exogenous 2-nitroimidazole small compound that forms adducts with thiol groups in hypoxic environments and works as an effective and nontoxic hypoxia marker

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

Radio-protection 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 (SRC)
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 tow progenitor cells

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

Xenografts
A transplantation setting where a tissue graft or organ transplant is from a donor of a different species than the recipient, e.g. human stem cell transplantation into mice. Generally, immunodeficient mice, i.e. SCID, NOD/SCID or NOD/SCID/Yc^{null} (NSG) mice are used as recipients in human-mouse xenograft models

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Trends

- Hematopoietic stem cell niches have been defined in more detail with respect to their cellular composition over the last couple of 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.

Box-1

Mammalian Hematopoiesis and HSCs

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

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

- (LT-HSCs) as Lin^{-}IL-7α^{-}Sca-1^{+}c-$Kit^{+}Flt3^{-}CD34^{-}CD150^{+}CD48^{-}$, progenitors including short-term HSCs (ST-HSCs) as Lin^{-}IL-7Rα^{-}Sca-1^{+}c-Kit^{+}Flt3 $CD34^{+}CD150^{+}$ $CD48^{-}$,
- and multipotent progenitors (MPPs) as Lin^{-}IL-7Rα^{-}Sca-1^{+}c-$Kit^{+}Flt3^{low\text{-}high}CD34^{+}$ [4, 32, 172–174].

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

- HSCs: $Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}Rho^{lo}CD49F^{+}$ and
- MPPs: $CD34^{+}CD38^{-}CD45RA^{-}CD90^{-}CD49F^{-}$, respectively [3, 175].

BOX 2

Extracellular Matrix Modeling and HSC Niche Reconstitution

The extracellular matrix (ECM) consists of, among others, collagen, fibronectin, dystroglycan, heparin sulfate, proteoglycans, osteopontin and laminin [56, 158, 159, 169, 176] and can bind adhesion molecules such as integrins, on HSCs. Several polymeric biomaterial substrates such as polyethylene terephthalate (PET), tissue culture polystyrene (TCPS) and polyether sulfone (PES) fibers exhibit the advantage of a 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; blockage 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 also include microfluidic trap devices for capturing individual HSCs to perform post-culture single-cell analysis [177].

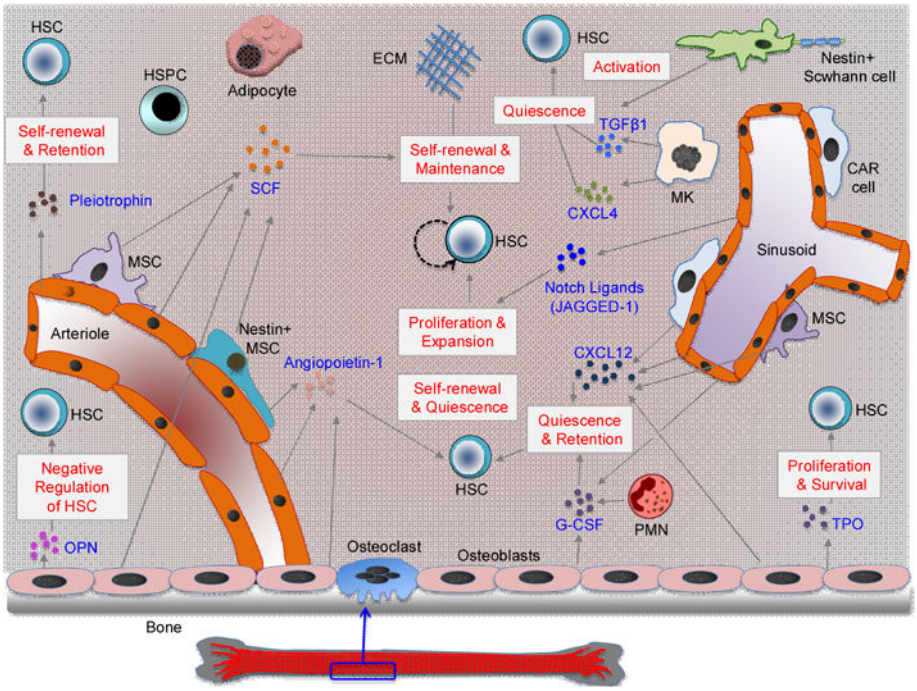
Box 3

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 be potentially achievable [120–122].
- Similarly, co-cultures of cord blood $CD34^+$ cells with mesenchymal stromal cells has led to expansion of $CD34^+$ cells by a median factor of 30.1, improving time to neutrophil engraftment at 15 days, as compared to 24 days in recipients who received un-manipulated 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 Box

- What is the relative contribution of cellular (stem cell intrinsic) and niche factors (stem cell extrinisic) towards 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 extra-cellular matrix environments to modulate HSC self-renewal ex vivo?



Key Figure, Figure 1. Mammalian Bone Marrow HSC Niche

The diagram shows the cellular composition and cytokines/growth factors that can impact 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, CAR cells, non-myelinating Schwann cells, BM endothelial cells and adipocytes, macrophages, megakaryocytes and neutrophils (PMN) in HSC self-renewal, differentiation and function. Niche cells also produce/release several cytokines/growth factors, such as SCF, TPO, 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 extra-cellular matrix (ECM) can also regulate HSC self-renewal and maintenance. SCF, stem cell factor; TPO, thrombopoietin; TGF-β1, transforming growth factor beta 1;CXCL-4, CXC chemokine ligand 4; CXCL-12, CXC chemokine ligand 12; G-CSF, Granulocyte-colony stimulating factor; OPN, osteopontin.

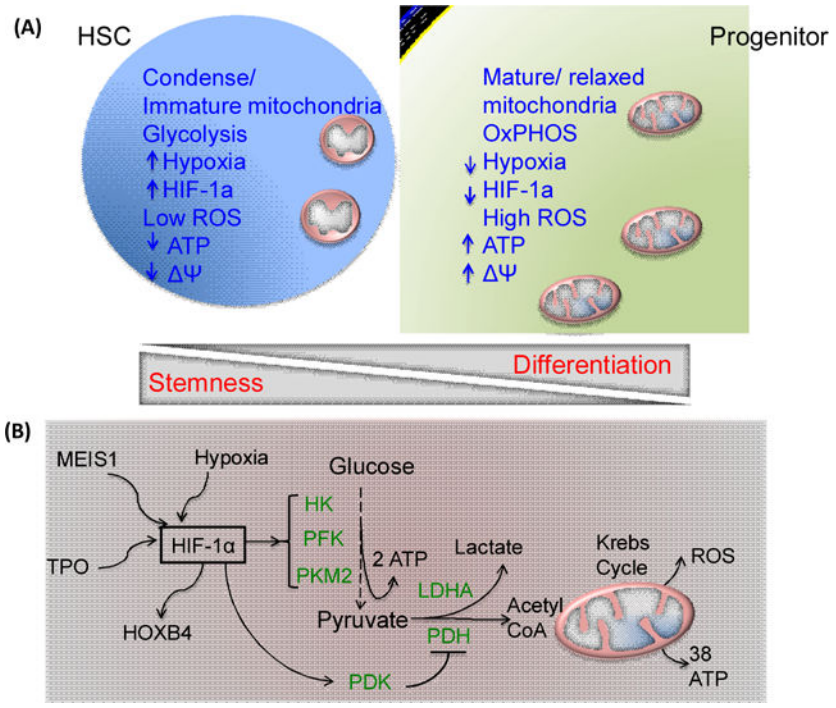


Figure 2. Metabolic Regulation of Mammalian HSCs

(A) Hematopoietic stem cells (HSCs) exhibit condense and immature mitochondria, low metabolic status and high glycolytic activity, as suggested by low ATP, low ROS and low membrane potential (ΔΨ) which uphold 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 (HIF1α) in HSCs can support self-renewal and stemness potential. **(B)** Mechanisms of metabolic regulation of HSC function. Under hypoxic conditions, cytokine TPO and MEIS1 can stabilize HIF1α to promote glycolysis by regulating glycolytic pathway enzymes including HK, hexokinase; LDHA, lactate dehydrogenase A; PFK, phosphofructokinase 2; PKM2, pyruvate kinase M2. HIF1α 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. ROS, Reactive Oxygen Species: MEIS1, Meis Homeobox 1; TPO, thrombopoietin; HIF, hypoxia-inducible factor; HOXB4, Homeobox B4; PDK, pyruvate dehydrogenase kinase;

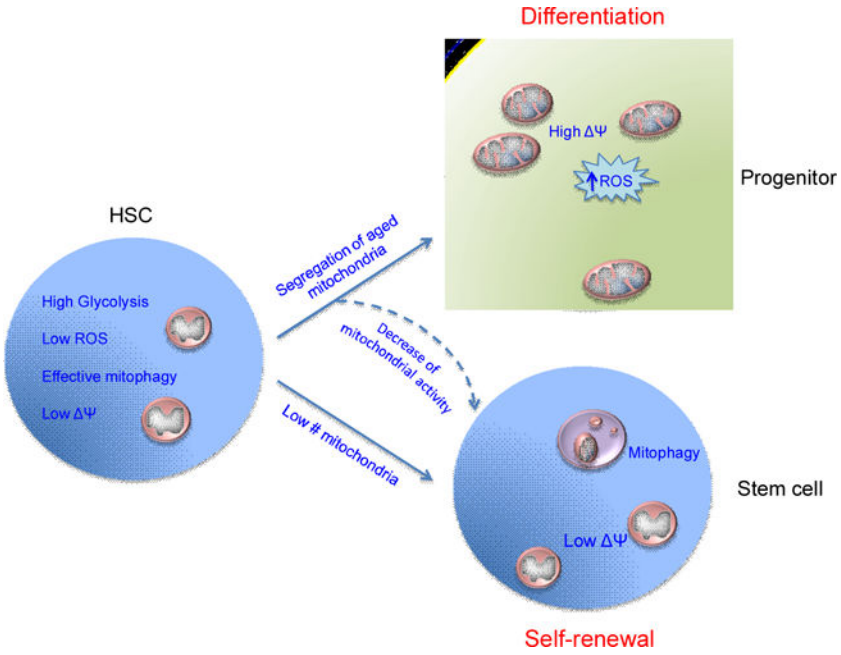


Figure 3. Metabolic Regulation of Mammalian HSC Self-Renewal

HSCs maintain low metabolic status and high glycolytic activity with low ROS and low membrane potential (ΔΨ) that helps 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, high membrane potential and utilize oxidative phosphorylation to produce more ROS, while daughter cells that receive low mitochondrial activity fate 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.

Table 1

Soluble Factors in the Bone Marrow Niche Affecting Mammalian HSCs

Soluble Factor	Secreted by Niche cells	Impact on HSC	References
SCF	Osteoblasts, Endothelial cells, MSCs and Nestin+ MSCs	Induces HSC maintenance and self-renewal	[19, 23]
TPO	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]

Table 2

Successful Protocols of HSC Expansion *Ex-Vivo*

Factors	Cells tested	Species	Supplement	Culture period	Assay	Fold expansion	References
Cytokine derived expansion							
Cytokines	CB CD34+38−	H	SCF, Flt3L, G-CSF, IL-3, IL-6	4 days	CFU & CRU (HSC frequency)	15 fold CFU/4 fold 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/4-fold LTC-IC, 2-fold CRU	[131]
Angiopoietin	SP CD45+ Sca-1+	M	SCF, TPO, FGF-1, IGF-2	10 days	CRU (HSC frequency)	24–30-fold	[130]
Pleiotrophin	CD34− LSK, CB CD34+ 38−	M & H	SCF, Flt3L, TPO	7 days	CRU frequency/LT engraftment	4 fold 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, TPO	10 days	Competitive reconstitution analysis	>2 fold Long term repopulation	[147]
Dppa5	CD34–48− LSK	M	SCF, TPO	14 days	Competitive reconstitution analysis	6–10 fold	[154]
Hypoxia, ROS and metabolic modulations							
PDH inhibitor [1-aminoethylphos phinic acid (1-AA)	CD34-Flts-LSK	M	SF-O3 medium 1.0% BSA, Serum free, SCF, TPO	2–4 Weeks	CFU/Competitive reconstitution analysis	2 fold CFUs/5 fold LT repopulation	[93]
Mitochondrial phosphatase Ptpmt1 inhibitor, alexidine dihydrochloride (AD)	LSK & In vivo treatment CD34+ 38−	M H	SCF, TPO, Flk-3	7 days	CFU/and LT Chimera 34+38− number/CFU	2 fold CFUs/3–5 fold LT repopulation 2 fold number/2 fold CFUs	[150]
GSK-3β inhibitor, CHIR99021	LSK & CB CD34+ LSK Flk−	M & H M	With Rapamycin in cytokine free X-VIVO medium SCF, TPO & 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, TPO, IL-6	7–21 days	Number/CFU/Competitive reconstitution analysis	65 fold CFUs/17 fold enhanced chimera	[120]

Factors	Cells tested	Species	Supplement	Culture period	Assay	Fold expansion	References
UM171	CD34+ MPB UBC	H	SCF, Flt3L, TPO	7–21 days	Number/CFU/Competitive reconstitution analysis	>100 fold LT-HSC/35 fold enhanced chimera	[121]

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