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Hematopoietic Stem Cells: Transcriptional Regulation, *Ex Vivo* Expansion and Clinical Application

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Abstract

Maintenance of *ex vivo* hematopoietic stem cells (HSC) pool and its differentiated progeny is regulated by complex network of transcriptional factors, cell cycle proteins, extracellular matrix, and their microenvironment through an orchestrated fashion. Strides have been made to understand the mechanisms regulating *in vivo* quiescence and proliferation of HSCs to develop strategies for *ex vivo* expansion. *Ex vivo* expansion of HSCs is important to procure sufficient number of stem cells and as easily available source for HSC transplants for patients suffering from hematological disorders and malignancies. Our lab has established a nanofiber-based *ex vivo* expansion strategy for HSCs, while preserving their stem cell characteristics. *Ex vivo* expanded cells were also found biologically functional in various disease models. However, the therapeutic potential of expanded stem cells at clinical level still needs to be verified. This review outlines transcriptional factors that regulate development of HSCs and their commitment, genes that regulate cell cycle status, studies that attempt to develop an effective and efficient protocol for *ex vivo* expansion of HSCs and application of HSC in various non-malignant and malignant disorders. Overall the goal of the current review is to deliver an understanding of factors that are critical in resolving the challenges that limit the expansion of HSCs *in vivo* and *ex vivo*.

Keywords

HSC; development; expansion; regulation; microenvironment; clinical application

INTRODUCTION

Hematopoietic stem cells (HSCs) are the multipotent stem cells, which are able to give rise to all types of blood cells including myeloid (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and dendritic cells) and lymphoid lineages (T cells, B cells, NK cells). The multipotent capability of HSCs was first described as clonogenic bone marrow (BM) cells that can reconstitute the hematopoietic system in primary and secondary recipients [1–3]. During the past several decades, hematopoietic stem cells transplantation (HSCT) has been used as standard treatment for various hematological disorders such as severe combined immunodeficiency, congenital neutropenia [4–5] and malignancies [6]. Even though HSCT has been applied in clinic for decades, regulation of HSC self-renewal and differentiation is still a major challenge. Better understanding of HSC regulation is critical to enhance the therapeutic efficiencies of

transplantation. Various transcriptional factors, which regulate the self-renewal and differentiation of HSCs were elucidated in past and these studies have increased the possibility of developing clinically applicable *ex vivo* expansion methods, which holds promise of producing sufficient number of HSCs for treating various diseases. In this review, we describe the regulation of HSCs during the development; focus on the recent improvements in regulating *ex vivo* expansion of HSCs and clinical application of HSCs in various diseases.

ORIGIN OF HEMATOPOIETIC PRECURSORS IN EARLY EMBRYO

In order to understand the regulatory mechanisms of HSCs, it is important to comprehend the origin and development of HSCs. The understanding of signaling cascades and origins of specific lineages will not only help us to accumulate knowledge on how the adult HSCs developed, but also will provide us insight on how HSCs function and being regulated in adult stage [7]. This will help us to develop methodology for efficient *ex vivo* expansion of HSCs. During embryogenesis, multiple anatomical sites are involved in hematopoiesis. These include extra-embryonic yolk sac (YS), intra-embryonic aorta-gonad-mesonephros (AGM) region, spleen, thymus and fetal liver (FL), most of which are not involved in adult hematopoiesis [8]. Of these sites, YS and AGM were identified for generating candidate progenitors for long-lasting hematopoiesis, however, other sites such as spleen, thymus fetal liver, and bone marrow do not produce *de novo* hematopoietic precursors and rather extrinsic hematopoietic cells colonize in these organs [9–11] (Fig. 1).

Yolk Sac

The yolk sac is derived from mesoderm and provides early nourishment for embryonic development. In the mouse embryo, mesoderm cells traverse the primitive streak and take an intermediate position between the primitive ectoderm and visceral endoderm germ layers, which begins at embryonic day (E) 6.5. This process is concurrent with gastrulation. Mesoderm cells together with visceral endoderm form the yolk sac at E7.5. Mesoderm cells near the visceral endoderm in yolk sac form angioblastic cord. Cells of the angioblastic cord then differentiate into erythroid cells, which are also called “blood island” [12, 13]. The importance of visceral endoderm was shown in an experiment using transcription factor GATA4^{-/-} embryoid bodies, which failed to develop an external visceral endoderm layer when cultured *ex vivo*. These Gata4^{-/-} embryoid bodies could not develop recognizable blood islands and vascular channels. These results indicated that visceral endoderm is critical in formation of yolk sac blood islands and blood vessels [14]. The close proximity of endothelial network and hematopoietic system in yolk sac made investigators to believe that blood cells and blood vessels were developed from a common precursor hemangioblast. One of the supports for this hypothesis comes from receptor tyrosine kinase, Flk-1 deficient mice, which showed that Flk-1 gene plays a critical role in endothelial development. The expression of Flk1 is restricted to endothelial cells and their embryonic precursors. Disruption of this gene resulted in absence of yolk sac “blood island” at E7.5 and severe reduction of hematopoietic progenitors [15]. Subsequent study showed that Flk-1 gene also influence migration of hematopoietic/endothelial progenitors cells [16]. It was believed that yolk sac did not contain definitive HSCs [17]. However, evidences from heterozygous mice of runt-related transcription factor 1 (Runx1) showed that yolk sac cells expressing Runx1 at E7.5 could develop into adult HSCs, which raise the possibility that yolk sac may contain definitive HSCs, which were able to reconstitute all types of hematopoietic cells in lethally irradiated adult mice [18]. However, the origin of adult HSCs is still inconclusive.

AGM Region

The AGM region is developed from para-aortic splanchnopleura in embryonic mesoderm. Compared to the primitive hematopoiesis that appears in the “blood island” of yolk sac, the adult-type definitive hematopoiesis begins in the AGM region at E10.5 [19, 20]. At E12, the definitive HSCs can be found in close proximity to the dorsal aorta [21]. It was shown that definitive HSCs were clearly detected in E11 but the capacity of AGM region to generate hematopoietic stem cells is reduced by E12. At the same time presence of increased numbers of circulating definitive HSCs indicating HSCs developed from AGM became motile. These circulating definitive HSCs may be routed to the fetal liver *via* blood stream [22, 23]. The development of AGM and yolk sac share many common aspects with some differences. Various regulatory factors and signaling pathways affect the development of hematopoiesis in both sites, such as bone morphogenetic protein (BMP) signaling. In AGM explants, BMP4 can increase the number of HSCs, while BMP4 knock-out mice yolk sac explant showed a reduced mesoderm and erythropoiesis [24, 25]. Notch mutant mice displayed normal embryonic hematopoiesis but failed to specify adult HSCs. It was shown that transient Notch activation during embryogenesis, expanded population of HSCs in AGM region in a Runx1 depended manner. This suggests Notch signaling might selectively influence the development of HSCs in AGM, but not the yolk sac hematopoiesis [7, 26].

Fetal Liver, Thymus, Spleen and Bone Marrow

The presence of HSCs in fetal liver (FL) of mouse embryo was identified as early as E11, until the perinatal period [27]. T cells start to appear in thymus at the same time line. The fetal spleen, a minor contributor to embryonic hematopoiesis, was also found to contain HSCs from E14 until birth [9, 28]. However, HSCs were not found in the fetal bone marrow until E15, which becomes the principal site for hematopoiesis shortly after birth [29]. In mouse embryo, the fetal liver was considered as a primary hematopoietic organ, which provides all the lineages of blood and serves as the source of HSCs in thymus, spleen and bone marrow [12].

TRANSCRIPTIONAL CONTROL OF HSC IN EMBRYO

A complex network of various pathways regulates the development of HSCs, many of which are also important for regulation of adult HSCs. A better understanding of the role of transcriptional factors during development of HSCs will shed light on the later regulation of HSCs and provide new perspectives on *ex vivo* maintenance, expansion and differentiation. Several transcriptional factors were identified to be essential for development of definitive hematopoietic cells during embryogenesis, such as stem cell leukemia/T cell acute lymphocytic leukemia-1 (SCL/tal1), runt-related transcription factor1/acute myeloid leukemia1 (Runx1/AML1), LIM domain only 2 (Lmo2), friend leukemia integration1 transcription factor (FLI1), ETS related gene (ERG), purine box binding protein 1 (Pu.1), globin transcription factor-1 (GATA1) and GATA2 (Fig. 2).

SCL/tal-1 encodes a basic helix-loop-helix transcription factor, required for hematopoietic cell development, derived from YS primitive and definitive lineages. Mutation of SCL/tal-1 resulted in severe defects in blood formation and lack of any hematopoietic lineages in mice [30]. SCL was also found to play a significant role in vascular development. It was shown that SCL acts on upstream of Flk1, Tie1 and GATA1, all of which play critical role in hematopoiesis and vascularization processes [31–34].

Runx1/AML1, a core-binding factor, was shown to be required for definitive hematopoiesis and its transient expression was correlated with the emergence of HSCs [35]. Runx1 continues to be expressed in HSCs during subsequent development and in adult life [35–37]. Absence of fetal liver hematopoietic progenitors and hematopoietic clusters was observed in

Runx1 null embryos, indicating important role of Runx1 in definitive hematopoiesis [35, 38]. However, Runx1/AML1 is not essential for functioning of adult definitive HSCs, while it is critical for lymphoid and megakaryocyte functioning [39, 40].

Lmo2 is a cystein-rich two LIM domain protein that is required for erythropoiesis in the yolk sac. It was shown that *ex vivo* differentiation of erythroid development was impaired in yolk sac tissue from homozygous mutant mice. Homozygous Lmo2 null mutation led to a failure of yolk sac erythropoiesis and developed embryonic lethality around E10.5 [41]. In the later stages, Lmo2 also plays a central role in hematopoietic development. It was shown that the Lmo2 $-/-$ cells do not contribute to any of the hematopoietic lineages in adult chimeric mice and this disruption of hematopoiesis could be rescued by reintroduction of Lmo-2 expression [42].

PU.1, the hematopoietic transcription factor, belongs to the ETS family (E26 family of transcription factors) and is required for the generation of the lymphoid progenitors [43]. PU.1 mutation resulted in reduction of lymphoid-myeloid progenitors in the fetal liver, which later failed to generate B cells or macrophages *ex vivo*. These observations indicated that PU.1 is not required for specification of monocytic precursors but is important for their further functions [44]. The higher expression level of PU.1 may also play an important role in directing differentiation into macrophages instead of B cells, which suggests the effect of PU.1 is dose-dependent [43].

GATA1, a transcriptional factor, is essential for erythroid and megakaryocytic development. The role of GATA1 in lineage instruction was observed over a decade ago, where forced GATA1 expression in Myb-ETS-transformed myeloblasts induced a reprogramming of myeloblasts into cells resembling either transformed eosinophils or thromboblats [45]. When introduced GATA1 into HSCs in a level comparable to megakaryocyte/erythrocyte progenitors (MEPs), HSCs were immediately committed to megakaryocytes and erythrocytes [46]. GATA1 was also found to repress the expression and function of PU.1, mediated *via* protein-protein interaction, which in turn suppressed the myeloid gene expression [47]. Recently, an E3 ubiquitin ligase, transcription intermediate factor-1 γ (Tif-1 γ) was found to modulate the lineage specification of HSCs by favoring the expression of GATA1 over PU.1. Evidences show that erythroid population, in zebrafish model, were favored over myeloid population as Tif-1 γ maintains GATA1 expression and suppresses PU.1 expression [48]. Tif-1 γ was not required for emergence of the HSCs or primitive myeloid progenitors, however, HSCs were incapable of differentiating into erythroids when Tif-1 γ was knocked-down [48].

ERG, a member of ETS family (E26 family of transcription factors) was shown to play a significant role in hematopoietic and endothelial development [49, 50]. ERG, along with the other ETS factor such as FLI1 were shown to be direct targets of GATA2/SCL and were predicted to be involved in the regulation of RUNX1 expression *via* gene sequence analysis [51]. Furthermore, *in vivo* studies performed on ERG mutant mice confirmed that ERG is a direct positive regulator of GATA2 and RUNX1 gene expression once the hematopoiesis progressed to fetal liver and is involved in self-renewal of HSCs but was not involved in the specification of HSCs [52].

GATA2 is an another transcriptional factor that belongs to the GATA family and is important for the development of HSCs. GATA2 knockout mice were embryonic lethal around E9.5 to E10.5 due to pan-hematopoietic deficit, suggesting GATA2 as a key regulator in development, maintenance and/or function of HSCs [53]. It was suggested that GATA2 controls the proliferation rate and growth factor responses of early hematopoietic

precursors, since forced expression of GATA2 enhanced the production of hematopoietic progenitors [54]. The detailed mechanism of GATA2 regulation is still not clear.

HSC REGULATION IN ADULTS

After birth, bone marrow becomes the primary site of hematopoiesis. The maintenance, proliferation and differentiation of HSCs are mainly regulated by microenvironment in bone marrow called stem cell niche. The bone marrow stem cell niche contains two basic components: niche cells and extracellular matrix (ECM). While ECM provides the basic physical and chemical support for stem cell function, niche cells control stem cell quiescence, proliferation and differentiation [55, 56]. Many of the current *ex vivo* expansion technologies are based on study of the *in vivo* environment of HSCs. Mimicking the *in vivo* environment such as bone marrow and its microenvironment, provides better condition for survival and proliferation of HSCs [57] (Fig. 3).

Osteoblasts are believed to be one of the most important niche cells in bone marrow, which support maintenance of HSCs [58, 59]. Osteoblasts secrete various cytokines such as granulocyte-colony-stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), Interleukin (IL)-1, IL-6, IL-7, osteoprotegerin (OPG), receptor activator of nuclear factor kappa B (NF- κ B), tumor necrosis factor (TNF)- α , and vascular endothelial growth factor (VEGF), all of which can modulate the function of stem cell [60]. Osteoblasts also secrete stromal cell-derived factor (SDF)-1, which binds to CXC chemokine receptor 4 (CXCR4) of HSCs and act as a chemo-attractant [61]. SDF-1 deficient murine embryos showed a lack of bone marrow seeding [62]. SDF-1 was also shown to stimulate the growth and survival of CD34⁺ progenitor cells [63, 64].

Osteoblasts express various adhesion molecules that mediate HSC-osteoblast adhesion, such as inter-cellular adhesion molecule 1 (ICAM1), lymphocyte function associated antigen 1 (LFA1), vascular cell adhesion molecule 1 (VCAM1), very late antigen 4 (VLA4), and N-cadherin, which are critical for interaction of osteoblasts and HSCs. It was shown that quiescence and survival of HSCs was regulated by tight adhesion between ligand, angiopoietin 1 (Ang1), expressed by osteoblasts, and its receptor, Tie2 expressed on HSCs [65]. However, the detail mechanism of adhesion molecules in HSCs functioning is still not well understood.

Osteoblasts have been found to modulate HSCs *via* BMP signaling. During development, BMP signaling pathway acts as an inducer of hematopoietic system, which is important in regulating development of adult HSCs [66]. It was reported that long-term HSCs attach to N-cadherin⁺CD45[−] osteoblastic (SNO) cells through N-cadherin and β -catenin junction molecules. Increased number in SNO cells were found by conditional inactivation of BMP receptor type IA. The increment was correlated with increased number of HSCs, indicating adhesion molecules exert signals for maintenance of HSCs [66, 67]. Osteoblasts can also regulate HSCs through Notch signaling pathway. Activation of osteoblast-specific parathyroid hormone (PTH) or PTH related protein (PTHrP) receptors resulted in a higher level of Notch ligand jagged1 in osteoblasts and increased Notch1 activation in HSCs. Blocking of Notch cleavage using γ -secretase inhibitor reduced the supportive effect back to basal level, indicating that osteoblasts can regulate expansion of HSCs through Notch signaling pathway *in vivo* [56, 68].

However, the prominent role of osteoblasts in HSCs niche has been challenged recently. Endothelial cells are also suggested to be important in HSCs niche [69]. Using the cell surface receptors such as CD48, CD150 and CD244, it was shown that many HSCs were

associated with sinusoidal endothelium in the spleen and bone marrow [70]. Deletion of CXCR4, a receptor for CXC chemokine ligand (CXCL12) in mice, resulted in severe reduction in numbers of HSCs and was also shown to be required for generation of lymphocytes [71]. The cells expressing high amount of CXCL12 were found to surround sinusoidal endothelial cells and were located near the endo-osteum [72]. Whether osteoblasts and endothelial cells play an overlapping role in regulating HSCs is still unknown.

ECM has been reported to play an important role in stem cell regulation. ECM can interact with stem cells through adhesion molecules, control cell geometry, mechanical property and nanotopography. Embryos deficient in integrin molecules showed impaired migration of HSCs in the early developmental stage [73]. Adhesive segments of fibronectin were able to enhance growth and proliferation of HSCs [74]. Mechanical signal can also alter the cytoskeletal tension and regulate cell fate of HSCs causing them to proliferate, differentiate, migrate or undergo apoptosis [75].

SELF-RENEWAL OF HSC

Self-renewal activities are required for sustained maintenance of HSC pool and are mediated by cross talk of signals between different intracellular and extracellular signaling molecules. Signaling pathways involving factors such as Notch, growth factor independent1 (Gfi1), B lymphoma Mo-MLV insertion region 1 homolog (Bmi1), homeobox domain B4 (HoxB4) are known to be important for maintaining the HSC progenitor pool and inhibition of differentiation *in vivo*. Thorough understanding of *in vivo* processes of HSC self-renewal in bone marrow niche would be a significant step forward in developing successful *ex vivo* expansion methods for HSCs. *In vivo*, HSCs exist in quiescent state (G0) to preserve the long-term self-renewing capacity of HSCs, thus preventing stem cell exhaustion [76]. About 75% of the long-term self-renewing HSCs (LT-HSCs) exist in G0 phase [77]. After exiting the G0 phase, HSCs enter cell cycle (divided into four phases G1-S-G2-M phase) and undergo either symmetric or asymmetric divisions giving rise to daughter HSCs and/or a committed progenitor that differentiate into blood cells [78]. Cell cycle regulator proteins (such as cyclin dependent kinase inhibitors) co-ordinate the cell cycle progression and commitment of the HSCs [79]. It was shown that G1-phase inhibitor gene (p21, p18, p27 and others) plays important role in maintaining G0 phase of HSCs (Fig. 4) [79]. In p21 knockout mice, number of HSCs in the G0 phase were decreased and concomitant increase in putative multipotent HSCs was reported, might be *via* regulation of cyclin dependent kinases (CDKs) as p21 is known to inhibit cyclin E-CDK2 activity [79]. However, recent report indicates undetectable levels of Cyclin E in HSCs [80]. The relevance of cell cycle inhibitors could be translated to *ex vivo* expansion strategies as it was reported that post-transcriptional downregulation of p21/p27 genes in cord blood derived HSCs (CD34⁺38⁻) caused HSCs to exit quiescence and undergo increased proliferation [81, 82]. Likewise, downregulation of p21 gene was observed in knockout mice experiments of a potent regulator of self-renewal of HSCs called growth factor independent1 (Gfi1) gene [83]. In Gfi1 KO mice, increased HSCs were found cycling in G2/S/M phases [79]. However, varied effects were reported in differentiated lineages of HSCs [84]. Deletion of p18, a G1-phase inhibitor, was shown to increase the self-renewal capacity of HSC [85, 86]. Similarly, downregulation of p27 was observed in HSC cell lines when exposed to proliferative cytokines such as SCF and G-CSF and were found to be expressed in cytokine non-responsive, lin⁻HSCs [87, 88]. These cytokine effects were mediated *via* STAT3 or fork-head related proteins [87]. Also, Wnt signaling pathways were linked to S phase machinery *via* Cyclin A (important for HSC survival). However, excessive cycling of HSCs lead to stem cell exhaustion as they rapidly moved into G1/S phase (important for protein/DNA synthesizes) and thus finally approach to apoptotic phase.

Also, cellular senescence seemed to be prevented by telomerase expression, an enzyme that maintains the telomeric end of the DNA [89]. Telomerase was found to be important for replicative senescence of HSCs and shortening in telomeres was observed in human HSC during aging [90]. Thus, higher activity of telomerase was correlated with enhanced survival rate of HSCs [90]. Knockout of RNA component of telomerase enzyme (mTR) in mice resulted in telomere dysfunction and reduced proliferative capacity of HSCs [91]. Human CD34⁺38⁺ cells, considered committed HSCs, showed shortening of telomere length when compared to its progenitor population of CD34⁺38⁻ HSCs [92]. Conversely, overexpression of human telomerase reverse transcriptase (hTERT) in cord blood-derived CD133⁺ and CD34⁺ cells showed no effects on telomere length activity, rather it was found to promote lineage differentiation [93]. Thus, further studies are required to exactly point out the underlying roles and mechanisms of telomerase and cell cycle regulator proteins to replicate the *in vivo* expansion of HSCs for achieving large numbers of HSCs *ex vivo*.

EX VIVO EXPANSION OF HSC

Ex vivo expansion of HSCs has been one of the most challenging techniques because of many underlying reasons. *Ex vivo* expansion of HSCs requires maintenance of sustained self-renewal with concomitant inhibition of differentiation during the course of expansion. Thus, limited understanding of physiological mechanisms that regulate the self-renewal and commitment of HSC, impedes expansion of HSCs. Sufficient number of stem cells are required for successful transplantation and engraftment. Expanded stem cell should be safe to the host. Limited availability of *ex vivo* models that exactly replicate the intricate HSC niche interactions and microenvironment. Following part of review will focus on strategies for *ex vivo* expansion of HSCs emphasizing on cytokines and biomaterials.

Cytokines in *Ex Vivo* Expansion

Numerous attempts were undertaken to define complex interplay of mitogenic stimuli mediated *via* cytokines and growth factors, which play pivotal role in maintaining and expanding the long-term repopulating HSCs (LT-HSCs) *ex vivo*. Long-term HSCs undergo numerous self-renewing divisions, thereby preserving the pool of stem cells. Various cytokines such as thrombopoietin (TPO) [94], fetal liver (FL3) [95], interleukin (IL) -3, -6, -11 [96, 97], and stem cell factor (SCF) [98] have been shown to expand HSCs *ex vivo* but were not sufficient to maintain and expand LT-HSCs. Several stromal-based cultures were also reported to expand HSCs but with limited success [99, 100]. This might be particularly due to extreme sensitivity of HSCs to their microenvironment. Role of cytokines such as IL-3 and IL-6 in expansion of HSCs is still not clear. Some investigators reported a positive effect on the *ex vivo* expansion of HSCs [96], while others reported a negative effect [101]. Thus, cocktail of cytokines was shown to be important for expansion of HSCs, along with the optimal concentration of each cytokine.

Although, it is known that cytokines cause HSCs to exit G0 phase and enter the cell cycle for expansion, however, very less information is available regarding the mechanism of HSC dormancy and its self-renewing divisions. Cocktail of cytokines (IL-3, TPO, SCF, EPO) were reported to play role of lipid rafts reorganization (clustering of lipid on plasma membrane, where numerous signaling and functional molecules are recruited) in maintenance quiescent (G0) phase of HSCs [102]. Quiescent HSCs (CD34⁻ cKit⁺ Sca-1⁺ Lin⁻ or CD34⁻ KSL) were found to be devoid of lipid rafts formation, however, in CD34⁺KSL cells, clusters of lipid rafts were formed and PI3K-Akt-FOXO-mediated signaling along with drastic downregulation of early G1 phase inhibitors (p57) was reported [102]. Fork-head related transcriptional factors, FOXO, were reported to be important for the self-renewing properties of HSCs [103]. Study reported that TPO also upregulates the expression of HOXB4 protein, implicated in self-renewal processes [104]. Recent study

showed that only in presence of cytokines such as TPO, SCF and FL3, overexpression of a zinc finger transcriptional factor, Sal-like protein 4 (SALL4), could self-renew peripheral blood HSCs to almost 10,000 fold in 30 days. This was associated with efficient engraftment and rapid reconstitution of HSCs *in vivo* [105]. Also, it is important to identify factors that may be inhibitory for *ex vivo* expansion of HSCs. It was shown that p38, a signal transducer, belongs to the family of mitogen activated protein kinase (MAPK kinases), may be inhibitory for *ex vivo* expansion of HSCs in normoxic culture conditions [106]. It was found that when HSCs were cultured in serum-free media supplemented with cytokines SCF, TPO and FL3, activation of p38 and p16 occurred due to the oxidative stress, which eventually caused significant reduction in numbers of murine HSCs [107]. Thus, when p38 inhibitory effects were abrogated by supplementing with specific p38 inhibitors, self-renewal of HSCs was observed *in vivo* with a 7.6-fold increment in the HSC progenitors at day30, and a significant engraftment was observed with rapid reconstitution [107]. The increased self-renewal and engraftment of HSCs after p38 inhibition was found to be due to the upregulation of HOXB4 genes and CXCR4 gene respectively. Additionally, downregulation of cell cycle inhibitors such as p21 and p16 were observed in HSCs that were cultured with p38 inhibitors [107]. However, the effects of p38 inhibitors on human derived HSCs are still unknown.

A dose dependent effect of Notch ligand (Delta 1) for the expansion of CD34 cells from the cryopreserved cord blood was reported. Though, the culture retained the ability of human hematopoietic reconstitution in murine model, however, expansion achieved was only up to 138-fold in span of 3 weeks [108, 109].

IL-6 combinations with IL-3, IL-11 and SCF-mediated downstream signaling *via* cell surface receptors (gp130), known to activate JNK/MAPK/Akt pathways [110]. Not only combinations of cytokines are important but also the concentration of each cytokine in the culture is an important determinant of HSC fate. This notion was derived from the *in vivo* observation, where FL3 serum levels were dysregulated in leukemic patients those received HSCT [111]. *Ex vivo* studies showed that negative impact of IL-3 on HSC expansion was alleviated at high concentration of FL and SCF cytokines and modulation of concentration of cytokines significantly enhanced the proliferation more than 50-fold for LT-HSCs and 500-fold for colony forming units (CFUs) [112]. Thus, all the above-mentioned attempts emphasize that further *ex vivo* studies are required for enhanced HSC expansion and *ex vivo* technology holds vast potential for ensured success in regenerative field.

Biomaterials and Substrates in *Ex Vivo* Expansion Polymers in Expansion of HSCs

Numerous polymeric biomaterial substrates have been extensively studied for *ex vivo* HSC expansion to recapitulate *in vivo* ECM structure and functionality. Synthetic polymers such as polyethylene terephthalate (PET), polyether sulfone (PES) fibers, tissue culture polystyrene (TCPS), maleic anhydride were evaluated for expansion of HSCs. These materials are advantageous because of their well-defined composition, reproducibility of surface chemistry topography, toxicity profile, degradation rates and modification of the base composition with physiological adhesion molecules.

Several biomaterials without modifications were used for the *ex vivo* expansion of HSCs, however, none of the biomaterials were shown to support expansion of HSCs in sufficient numbers. Biomaterials such as TCPS, polyethylene (PE), high-density PE (HDPE), polycarbonate (PC), PET, cellulose acetate, teflon fluorinated ethylene propylene (FEP), glass, polysulfone (PS), Teflon perfluoralkoxy (PFA), polymethylpentene (PMP), barex (polyacetonitrile-methylacrylate), polypropylene (PP), acetal (polyformaldehyde), cellulose acetate, titanium, aluminum, stainless-steel 316 and 304 were investigated for expansion of HSCs [113–114]. Some of the materials were shown to either have protein adsorption

effects or leaching of toxic chemicals, which resulted in HSCs membrane damage and subsequent cell death. Of all barex, PMP, cellulose acetate, teflon and titanium showed comparable but lesser proliferation effects compare to TCPS. This was evident from the *ex vivo* expansion studies where modified PET showed significantly higher HSC expansion [114] compare to unmodified version [113]. Clearly, the composition of base materials was critical for the expansion and cytokines are always required for survival and proliferation of HSCs [115]. Thus, modifications of base materials with ECM molecules or chemical moieties and topographical patterns were the next best possible choices for more effective HSC expansion.

Adhesion Molecules in Expansion of HSCs

Extracellular matrix molecules found in endosteal niches (such as fibronectin, collagen-I, -IV, glycosaminoglycans (GAG), heparin, heparin sulfate, and hyaluronic acid) were investigated in various studies for their potential for *ex vivo* HSC proliferation [113, 116, 117]. Expansion of cord blood-derived CD34 cells on tropocollagen-I conjugated to adhesive polymer showed enhanced adhesion and reduced mobility compared to controls. The exact reason was not known, however, expression profile for tropocollagen-I-mediated preservation of stem cell character and reduced expansion was attributed to upregulation of cell cycle inhibitors of G1-S progression and MAPK pathway [117]. Base material, maleic anhydride (MA) was coated with the above-mentioned ECM molecules to examine the proliferative effects of CD133⁺ HSCs [114]. Cell adhesion was observed on fibronectin, heparin, heparan sulfate and co-fibrils of collagen-I and hyaluronic acid, however, surprisingly, contrary to above results, tropocollagen-I was not effective in establishing cell contacts rather cell movements were observed. Strongest adhesion was observed on fibronectin and heparin coatings. Adherence was mediated *via* specific integrins ($\alpha 5\beta 1$) on fibronectin. Engagement with integrins was shown to rescue primitive HSCs from apoptosis and cause MAPK-mediated expansion and G0 to G1 phase transition. Selectin-mediated adhesion was observed on heparin molecules influencing proliferation and differentiation kinetics of HSCs. Sulfated GAG molecule-mediated adhesion maintained the survival of long-term culture initiating-cells (LTC-IC) [114]. Not only the presence of fibronectin (FN) was important but also the physical status of the FN on the substrate was thought to play a bigger role in determining fate of HSCs [116]. Studies from fibroblast cultures showed that FN could either be physically adsorbed to the substrate or chemically conjugated. In either case, two distinct patterns of focal adhesion were observed, which dictated differences in cell motility, shape, and signaling activities and are tightly related to cell function, protein expression and the status of cell cycle [118, 119].

Furthermore, fibronectin (FN) domains; connecting segment-1 (CS-1) and RGD moieties were shown to bind to the HSC progenitors *via* specific receptors domains [120–122]. These receptors domains were linked on to the PET base material *ex vivo*. CD34⁺ cells in presence of cytokines and serum-free media showed highest expansion on CS-1 conjugated fibers (590-fold for total cell number and 76-fold for LT-HSCs) [123]. Increased engraftment in mice was observed with the cells that expanded on the CS-1 immobilized on PET fibers [123]. Morphologically, the cells cultured on PET were shown to grow in clusters compare to those growing on immobilized CS-1 and RGD fragments. Thus, it was suggested that in the absence of suitable substrates, HSCs would still engage their receptors to form suitable adhesive interactions, which may cause rapid cell differentiation leading to production of cells that form colony-like aggregates [123].

Substrate Topography and Dimensionality in Expansion of HSCs

Studies have demonstrated that surface chemistry and topography also affect the rate of HSC proliferation and expansion [116, 124–126]. Human UCB-derived CD34⁺ cells were *ex vivo*

expanded on chemically modified PES (poly ether sulfone) substrate. The PES was conjugated with either carboxylic, hydroxyl or amine group as these were shown to have different patterns of focal adhesion [124]. Of all the modifications, aminated PES substrates showed the highest expansion of CD34+ cells compared to other chemically modified PES or unmodified PES. Number of possible factors might have resulted in such a high proliferation potential are as follows. Positively charged amine groups could selectively adsorb protein components from medium and functional conformation of adsorbed fibronectin was different on various modified substrate, thus, leading to presentation of immobilized proteins similar to those in the bone marrow [124]. Possibility of direct electrostatic interaction between negatively charged sialylated CD34 receptor with positively charged immobilized amine groups was thought to enhance the self-renewal and inhibit the differentiation pathways. CD34+ cell proliferation has been reported to be highest in aminated PES nanofiber (almost 250- fold) compared to carboxylated or hydroxylated PES fibres [124–126].

Dimensionality was another influencing factor in expansion of HSCs. It was argued that 3D culture systems (e.g., nonwoven porous carriers, large cellular porous disks, polyvinyl formal and porous microspheres) provided greater surface area for cell proliferation and may cause superior reconstitution of HSCs compared to the 2D cultures [127–129]. 3D culture techniques using polyurethane foam (PUF) were described [128]. Proliferation of HSCs was significantly higher in 3D pores and sustained for longer time periods compared to 2D cultures. The PUF matrices that had larger pore size and lesser contact angle demonstrated more production of cells. However, lesser colony forming units (CFUs) were observed in PUF matrices that had larger pores than those with smaller pores. This could be due to the combined differences of surface characteristics, porosity and dimensionality for each matrix influencing the cell culture microenvironment [128]. A more profound difference was reported for the CD34+ cell cultured on 3D PET conjugated with FN compared to 2D (100-fold expansion vs. 20-fold), however, engraftment efficiencies were similar [116]. Overall 3D seems to give better adhesion and expansion than the 2D matrices, which indicates that 3D better mimics the *in vivo* bone marrow microenvironment. Although, above studies point to definitive and overlapping role of substrate topography, dimensionality, substrate's chemical composition, role of adhesion molecules combined with the role of signaling molecules (Wnt, Notch and HoxB4) and cytokines (SCF, FLT3, IL3) in determining fate of HSCs but underlying mechanisms are still largely unknown.

CLINICAL APPLICATION OF HSC

Enormous amount of efforts are underway to develop effective stem cell therapy towards various hematological disorders that may result from genetic abnormalities, metabolic dysfunction, immune disorders, degenerative conditions or malignant transformation of normal cells [130–132].

Success of HSC transplantation in clinic is limited due to number of reasons. Limited availability of HLA-matched donor to perform successful transplant with out any immune rejections from the host [133]. Even though matched HLA donor is found, success of transplant is impaired by reconstitution and engraftment abilities due to lack of sufficient numbers of available HSCs from one donor source. Thus, there's an urgent need to develop an alternative source of stem cells, mainly *ex vivo* expansion of HSCs to meet the clinical criterion of HSC transplant-related success. Allogeneic and autologous transplantations of HSCs were shown to be effective for diseases such as leukemia, sickle cell anemia, fanconi anemia, autoimmune disorders and other hematopoietic disorders [134–136]. Generally, allogeneic transplantations from a matched healthy donor have been proven successful for leukemic patients due to possibility of absence of any malignant stem cell in donor cells;

however sometime cancerous patients receive autologous HSCT after complete elimination of cancerous cells [137, 138]. In autologous transplantation, the patient's bone marrow cells could be collected by mobilizing bone marrow resident HSCs into the peripheral circulation with the granulocyte colony stimulating factor (G-CSF) injection [139] or pretreatment with synthetic compounds (AMD 3100) [140, 141]. Mobilization of resident HSCs is facilitated due to the blockage of binding of chemokine cell surface receptor (CXCR4) with its ligand stromal-derived factor (SDF-1), which is produced by bone marrow stromal cells. This results in the disruption of SDF-1 and CXCR4 binding causing an increased mobilization of resident HSCs to peripheral circulation in G0 or G1 phase [142, 143].

For therapeutic purposes, *ex vivo* expanded cord blood, bone marrow cells, and peripherally mobilized cells were employed in various clinical trials [144, 145]. The technique of *ex vivo* expansion is currently being explored to evade the problems of low cell dose, purge the contaminating tumor cells during expansion of bone marrow from tumor patients, reduced engraftment time and long-term reconstitution [146–148]. Genetic modification of HSCs was also performed for diseases that require corrective genetic dosage for synthesizing fully functional and active protein [149, 150]. However, the results of various clinical trials that employed *ex vivo* expansion strategies varied due to cell source chosen for expansion, expansion methods, choice and concentration of cytokines for expansion, phase of the cell cycle during expansion and transplantation, initiating cell density and type (as to whether they were multipotent HSCs or not) and or culture conditions [151–155].

Non-Malignant Diseases

Non-malignant diseases such as severe combined immune deficiencies (SCID) involve immune disorders of T and B cells making the patients highly immunocompromised. Infectious agents, reaction to certain drugs, development of anti-idiotypic reactions, severe immune reactions to self-antigens and T cells (CD4⁺) appear to be involved in most of autoimmune disorders [156]. Small clinical trials were reported by European Group for Blood and Marrow Transplantation (EBMT) and the European League Against Rheumatism (EULAR) involved several autoimmune disorders such as scleroderma, systemic lupus erythematosus (SLE), rheumatoid arthritis, refractory thrombocytopenia and pure red cell aplasia, multiple sclerosis, Sjogern syndrome, inflammatory bowel disease to treat with autologous graft of HSCs with event-free survival and enhanced engraftment rates [157–159]. For other non-malignant diseases such as sickle cell anemia (SCA) or beta-thalassemia (β -thalassemia), replacing defective HSCs by transplantation combined with gene therapy for inherited blood disorders were reported to be best curative option available [160, 161]. Another treatment option is to transduce HSCs with the corrective gene. In diseases such as SCA, in which 6th codon of β -globin gene is transversed to a non-polar valine from glutamic acid, clinical trials with HSC transduction with corrected copy of β -globin gene using viral vector has been reported [162, 163]. However, insertional mutagenesis was observed to give rise to leukemic cell clones [164–166]. Thus, newer techniques have been proposed, which involves gene replacement rather than gene addition. Gene replacement technique is based on the knock-in experiments where sickle globin gene is replaced with normal copy of the globin gene, however, these experiments were carried out using induced pluripotent cell (iPS)-derived HSCs from skin fibroblasts and murine embryonic stem cells and were not tested in humans [167]. The disadvantage of this technique is that differentiation of iPS cells into HSCs could never be 100% and there is possibility of teratoma formation. Gene replacement techniques will be advantageous if only new therapeutic HSCs confer complete genetic match to the patients existing HSCs except defected sickle β -globin gene [168]. In another, hematological disorder β -thalassemia, clinical trials with *ex vivo* transduction of autologous CD34 cells using globin gene (4 million cells/kg) of β -thalassemia patient has shown successful hematological reconstitution,

thus avoiding the need of repeated blood transfusions [169, 170]. However, this *ex vivo* transduction of autologous CD34 cells with β -globin genes caused upregulation of potential oncogene (HMGA2) expression resulting in clonal expansion of genetically modified cells was observed [171]. Thus cure of non-malignant diseases *via* HSCs transplantation still needs further experimentation to avoid any graft-versus host disease and bio-safe genetic modification of HSCs.

Malignant Diseases

Malignant diseases in cellular compartment of HSCs occurs due to the dysregulation of genetic factors or abnormal proliferation. HSC transplantation seems to be advantageous in patients with cancerous and/or infectious diseases, especially those who have undergone myeloablative therapies (exposure to ionizing radiations) [132]. Other, treatment strategies involve use of HSCs transplants coupled with antibodies that bind and block expression of adhesion molecule (such as CD44) [172]. Furthermore, autologous stem cell transplantation is the treatment of choice for patients suffering from Hodgkin diseases (HD), whereas for non-Hodgkin lymphoma (NHL) (malignancies of white blood cells; B and T cells), both allogeneic and autologous HSC transplants are treatment of choice, however, autologous transplants are less preferred due to higher rate of disease relapse [173–176]. In some cases, high-dose therapy and allogeneic transplant is associated with acute and chronic GVHD and risk of infections [177, 178]. For leukemia such as chronic lymphocytic leukemia (CLL), in which malignant B lymphocytes become resistant to chemotherapy, allogeneic stem cell transplantation was shown to be a reliable treatment [179, 180]. Stem cell transplantation is also used as an adjuvant with other chemotherapeutic agents for treating cancer patients suffering from acute myeloid leukemia and high-grade lymphoma with the hope of replacing the endogenous healthy stem cells that were destroyed due to high dose of therapeutic regimens [181, 182]. Solid tumors such as those affecting pediatric patients (Wilm's tumor, retinoblastoma, Ewing sarcoma of bone or germ cell tumors and ovarian cancers) were treated with the adjuvant hematopoietic stem cell therapy with high rate of survival and lower immunological graft rejection [183–185].

Further, clinical trials with *ex vivo* expanded HSCs for malignant diseases gave encouraging results. Hematopoietic reconstitution with *ex vivo* expanded mobilized peripheral blood occurred 3–4 days earlier compared to the standard HSCT [153]. Furthermore, *ex vivo* expanded stem cells mobilized *via* agents such G-CSF or SCF transplanted into breast cancer patients, replaced neutrophils and platelets in a significantly shorter time period compared to standard control [186]. However, some trials showed no changes in the engraftment times when compared with the unexpanded peripheral blood control [154]. This could be due to loss of adhesion molecules required for engraftment and homing during expansion, or cell cycle status of the expanded cells at time of transplantation [187]. Thus, there's an urgent need of successful *ex vivo* expansion protocols for HSCs from above mentioned sources to meet the clinical criterion of HSC transplant-related success.

CONCLUSION AND FUTURE DIRECTIONS

Given the importance of HSC in regenerative field, not much is known about interplay of intrinsic and extrinsic regulators. HSCs in embryonic phase, prenatal and postnatal phase have been extensively studied. Their origin, transcriptional regulation in hematopoiesis and microenvironment regulation is still debatable. However, these studies provide us with important information, which can be used to avoid disease development and progression in prenatal and postnatal life of humans and animals. Although, much progress has been made in the last decade, still the optimal development of HSC expansion technology methods *via* soluble growth factors, biomaterial fabrication remains an elusive goal. None of the studies so far were able to formulate a protocol that would work universally with the given HSC

population for use in pre-clinical and clinical studies. Our lab demonstrated ~250-fold expansion of CD34⁺ cells isolated from human umbilical cord blood using aminated PES nanofiber in stromal-free, serum-free media supplemented with cytokines in relatively short period of time (10 days). The nanofiber expanded stem cells preserved their stem cell characteristics and were functionally superior compared to their freshly isolated counterparts as evident from our animal models. These nanofiber-expanded HSCs were able to migrate, engraft and restore angiogenesis without any tumorigenic development in ischemic tissues in murine model of hind limb ischemia and rat model of myocardial ischemia. However, nonetheless challenges remain as to what are the optimal conditions in generating clinically relevant HSCs. It is not clear yet that whether expansion strategy should differ according to the source of HSCs, population of HSCs, cell cycle status of HSCs, age of donor as it influence the therapeutic outcomes. Defining all these parameters is extremely hard due to the extreme sensitivity of HSC to their surroundings and the failure to maintain the undifferentiated state of HSCs. Still number of questions remains unanswered, as to what is the exact regulatory reparative mechanism of HSC regeneration. Given that HSCs home to the damaged tissue, how long do they survive in the damaged area as they were shown to be highly sensitive to their surroundings? Do they generate teratomas *in vivo*, if no, is it tissue specific or dependent on the cell cycle status of transplanted HSCs. Cell cycle status and self-renewal gives us enough answers as to why some patients are more resistant to myeloablative therapy but at the same time present with problem of undesired proliferation or differentiation. Pertinent to the cell cycle and self-renewal issues, whether repair mechanisms of the transplanted HSCs are permanent, if not, then how often does transplantation is required? Since, most of the studies are still in preclinical stage, it's hard to answer the therapeutic potential of HSCs in humans due to their large genetic, geographical, age, gender and lifestyle variations. Thus more focused research is required for understanding the HSC regulatory pathways and their therapeutic implications.

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Fig. 1. Schematics of murine embryonic development, and localization of hematopoietic stem cells during the course of development. E = embryonic day, AMG = aorta-gonad-mesonephros.

Fig. 2.

Transcriptional regulation of hematopoiesis. Crosstalks of various transcriptional factors regulate the self-renewal HSCs or commit to either lymphoid or myeloid lineages depending on the expression levels. HSC=hematopoietic stem cells; CMP= common myeloid progenitor; CLP=common lymphoid progenitor; MEP=megakaryocyte erythroid progenitor; GMP= granulocyte macrophage progenitor; LMO2=LIM domain only 2; SCL; tal-1 =T cell acute lymphocytic leukemia-1; NK =natural killer; Rb2=retinoblastoma 2; HOXB4=homeobox protein 4.

Fig. 3.

Schematics of complex signaling within the niche of HSCs. The niche of adult HSC, within bone marrow consists of various types of cells (osteoblastic, endothelial and bone marrow stromal) and extracellular matrix proteins. Fate of HSC is determined by the interaction between the extracellular molecules and the cell surface receptor expressed on HSCs. HSCs receive signals *via* Wnt signaling pathways, BMP pathways, chemokine ligand receptors, Notch signaling, Parathyroid hormone signaling, cytokine mitogenic stimulation, and also signaling *via* adhesion molecules. PTH-PTHrP=Parathyroid hormone-parathyroid hormone receptor; CXCR4 = chemokine receptor, CXCL12 = chemokine ligand 12; TPO = thrombopoietin; Mpl = myeloproliferative leukemia; SCF = stem cell factor; Ang-2 = angiopoietin-2; ECM = extracellular matrix.

Fig. 4.

Factors regulate cell cycle of HSC. Cell cycle consists of four phases growth 1 (G1), synthesis (S), growth 2 (G2), and mitosis (M). Activities and duration in each phase is regulated by interplay of several factors. HSC exist in G0 phase in bone marrow milieu and exit quiescent phase upon concerted actions of cytokines and cell cycle protein complexes (Cyclins and CDKs). Several positive regulators (p16, p15, p18, p21, p27, PBX1, C/EBP α 1, PTEN, Gfi1, Rb, E2F1, TGF- β 1) of HSCs have been identified, which mainly serve as checkpoint regulators and inhibitors for early transition to G1 phase. Progression of G1 phase is mediated by increased level of expression of Cyclin D2, D3; CDK4/6 complexes. Once HSC is in G1 phase, move to enter S-phase, synthesize replication machinery and progress through G2 and M phase to undergo self-renewal divisions giving rise to daughter HSCs. This is modulated by over expression of certain genes such HOXB4, GATA-2, Notch1, 4; STAT3, 5; hTERT, Bmi-1, MAP kinase, Wnt, BMP2, Fork-head proteins. The cell cycle modulators such as Cyclin A and CDK1 were upregulated during the cell cycle progression. HOXB4 = homeobox B4; STAT3, 5 = signal transducer and activator of transcription 3, 5; hTERT = human telomerase reverse transcriptase, PBX1 = pre-B-cell leukemia homeobox1; C/EBP α 1 = CCAAT/enhancer binding protein, PTEN = phosphatase and tensin homolog; Rb =retinoblastoma, E2F1 = E2 transcription factor, TGF- β 1 = transforming growth factor, β 1; Gfi1=growth factor independent 1; MAPK = mitogen activated protein kinase.