Decoding Human Hematopoietic Stem Cell Self-Renewal

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Abstract

Purpose of Review Hematopoietic stem cells (HSCs) maintain blood and immune cell homeostasis by balancing quiescence, self-renewal, and differentiation. HSCs can be used in lifesaving transplantation treatments to create a healthy hematopoietic system in patients suffering from malignant or inherited blood diseases. However, lack of matching bone marrow donors, and the low quantity of HSCs in a single cord blood graft, are limitations for successful transplantation. The enormous regenerative potential of HSCs has raised the hope that HSC self-renewal could be recapitulated in culture to achieve robust expansion of HSCs for therapeutic use. Yet, when HSCs are cultured ex vivo their function becomes compromised, limiting successful expansion.

Recent Findings After decades of efforts to expand human HSCs ex vivo that resulted in minimal increase in transplantable units, recent studies have helped define culture conditions that can increase functional HSCs. These studies have provided new insights into how HSC stemness can be controlled from the nucleus by transcriptional, posttranscriptional and epigenetic regulators, or by improving the HSC microenvironment using 3D scaffolds, niche cells, or signaling molecules that mimic specific aspects of human HSC niche. Recent studies have also highlighted the importance of mitigating culture induced cellular stress and balancing mitochondrial, endoplasmic reticulum, and lysosomal functions. These discoveries have provided better markers for functional human HSCs and new insights into how HSC self-renewal and engraftment ability may be controlled ex vivo.

Summary Uncovering the mechanisms that control the human HSC self-renewal process may help improve the ex vivo expansion of HSCs for clinical purposes.

Introduction

Hematopoietic stem cells (HSCs) have the unique capacity to self-renew, which ensures lifelong blood cell production and the ability to respond to altered demands upon infection or injury. HSC self-renewal has to be carefully balanced with terminal programs such as apoptosis and differentiation to ensure sustained generation of all types of mature blood and immune cells while minimizing oncogenic transformation. Adult HSCs are mostly in a dormant state protected by their niche and self-renew only as needed. HSCs can be mobilized from their niche to circulation spontaneously or using pharmacological agents, without losing their ability to re-home and engraft in the bone marrow niche. Because of these unique properties, HSC transplantation can recreate the entire hematopoietic system in a recipient [1]. The extensive regenerative

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potential of HSCs has raised hope that HSC self-renewal could be recapitulated in culture to expand or modify HSCs for clinical use. However, when HSCs are coaxed to proliferate in culture, they are prone to differentiation, death, or losing the ability to engraft in vivo [2]. The limited success in human HSC culture expansion over the decades has raised the question whether pushing HSCs to proliferate in culture will permanently compromise their function, making ex vivo expansion of functional HSCs an unrealistic goal.

Allogeneic hematopoietic stem cell transplantation (HSCT) can be a highly effective treatment for patients with hematological malignancies and hereditary blood disorders if a suitable HSC graft can be obtained, ideally from an HLA (human leukocyte antigen)-matched donor [3]. HSCs can be harvested from placental umbilical cord blood (CB) at birth, or from bone marrow (BM) or mobilized peripheral blood (mPB) from related or unrelated donors. CB is an attractive resource for HSCT due to its accessibility, permissiveness in HLA mismatches and low incidence of chronic graft-versus-host disease, but use of single CB units has been largely limited to pediatric patients due to low numbers of HSPCs per unit [4]. For adult patients who lack an HLA matched sibling or registry donor, the standard treatment options include HSCT with BM or mPB from an HLA-haploidentical family donor or two unrelated CBs. Which HSC source is chosen depends on multiple factors, including availability of a potential donor in the family or BM registry, the disease that is being treated, patient age, and the experience of the center [5]. Recent studies have provided evidence that haploidentical marrow transplantation may be associated with lower rates of non-relapse mortality and longer overall survival compared to double CB transplantation [6]. Although some studies have suggested that adult recipients who received a double CB transplant may have a lower long-term health care burden compared with other sources of HSCs [7], treatment with two CB units has often been associated with increased incidence of graft-versus-host disease (GvHD) as well as slow hematopoietic recovery [8]. The delayed myeloid recovery with CB may be improved at least in some patients by combining CB graft(s) with haploidentical donor cells, which contribute to early neutrophil and platelet recovery before these lineages can be sustained by CB engraftment [9–11]. To also enable safe use of CB HSCT for adult patients, it has been a long-standing goal to expand CB-HSPCs (hematopoietic stem and progenitor cells) ex vivo to secure efficient long- and short-term hematopoietic recovery from a single unit with the best HLA compatibility [8, 12, 13]. In addition to CB, expansion of HSCs in culture without loss of stemness is also important with BM- and mPB-derived HSCs to maximize the effectiveness of novel HSC gene editing approaches for inherited blood and immune disorders. This would broaden the utility

of HSC based therapies for otherwise hard to treat diseases such as sickle cell anemia.

Another potential source of HSCs are pluripotent stem cells (PSC), but HSC generation in culture has not been successful due to inability to recapitulate HSC development in vitro [14]. During embryogenesis, human HSCs emerge from hemogenic endothelium in the dorsal aorta and possibly adjacent major arteries between 4 and 6 developmental weeks, after which they mature and expand in the fetal liver [15]. Although HSCs from developmental tissues are not used in clinical applications, studying them may provide "the missing script" about how to generate self-renewing human HSCs from PSCs or to expand the HSC pool without losing stemness.

Despite the progress in understanding HSC biology in animal models, expansion of human HSCs in culture has remained a major challenge. This stems from the difficulty of identifying the rare human HSCs within heterogeneous cell populations for molecular and functional studies, poor understanding of the HSC microenvironment and the mechanisms that govern human HSC self-renewal in vivo, and limited ability to maintain these programs ex vivo. Here, we discuss recent discoveries of the regulatory mechanisms that control human HSC self-renewal, focusing on how "stemness" is controlled from the nucleus, and how these mechanisms may be harnessed to improve human HSPC expansion ex vivo.

Overcoming Long-standing Challenges with Studying Human HSC Self-renewal

Inconsistency Between Immunophenotype and Function with Cultured Human HSCs

Studying human HSCs has been challenging without isolation methods that allow their purification at or close to single cell level. The HSPC compartment comprises cells with long- and short-term engraftment potential and their closest downstream progeny, but distinguishing between the different cell types has been difficult due to a lack of reliable human HSC markers and assays that are sensitive enough to validate them. Using the best current flow cytometry strategies for identifying uncultured human CB HSCs (expression of CD34, CD90, and CD49f and the depletion of CD38 and C45RA), the frequency of HSCs that can repopulate immunodeficient NSG (NOD/SCID) mice was estimated at 1 in 10 cells [16]. During human fetal development, surface expression of GPI80 (encoded by *VNN2* gene) can be used to further enrich for HSCs within the CD34 + CD38-CD90 + HSPC compartment [17, 18]. Although these markers are useful for enriching uncultured HSCs, their reliability has not been confirmed for ex vivo expanded HSPCs, which

are exposed to various stressors and whose surface phenotype generally correlates poorly with HSC activity [19–22]. To this end, recent studies have identified novel human HSC surface proteins (e.g., EPCR and ITGA3), stemness associated genes (e.g., *HLF*), and markers of organelle functions (low mitochondrial membrane potential) that can be used to pinpoint HSCs in human CB expansion cultures.

Endothelial protein C receptor (EPCR/CD201), originally reported to identify mouse HSCs and regulate their retention to the bone marrow [23, 24], is a reliable surface marker for enriching for both uncultured and ex vivo expanded human fetal liver and cord blood HSCs [18, 19, 25]. However, even though the ex vivo expanded CD34 + EPCR + cells are most highly enriched for engraftable HSCs, they are functionally heterogenous [19]. Further dissection of the EPCR + population revealed that the expression of integrin-a3 (ITGA3/CD49f) fractionates the ex vivo expanded EPCR HSPCs into long- (EPCR + ITGA3 +) and short- (EPCR + ITGA3-) term repopulating HSCs [26•], providing critical information about the heterogeneity of the expanded HSPCs and their functional potential upon transplantation.

Studies in mice show that HSC enrichment based on a single fluorescent color indicating the expression of HSC genes such as *Fdg5*, *Hoxb5*, and *Ctnnal1* is feasible through the development of reporter mice [27]. A recent study with human HSCs shows that CRISPR-Cas9 targeting of a reporter transgene in the *HLF* (hepatic leukemia factor) gene, which is one of the most differentially expressed genes in long-term HSC (LT-HSC), can identify HSC-enriched and depleted populations in culture and in transplanted mice[28]. Although such knock-in reporters can be powerful tools for human HSC purification, modest homologous recombination (HR) efficiency of a repair template delivered by recombinant adeno-associated virus (rAAV6) and cell toxicity in response to viral transduction currently limit the broader use of this strategy.

These methods represent exciting progress in identifying human HSCs that have preserved functional potential in culture. Evaluating whether these markers also enrich for ex vivo expanded BM- or mPB HSCs will be important to quantify functional human HSCs after gene transfer or editing to maximize the benefit of the novel technologies to modify human HSCs for therapeutic applications.

Mitigating Culture Induced Cellular Stress

Culturing HSCs ex vivo induces a variety of stress signals as a result of proliferation, reactive oxygen species (ROS) production, and accumulation of DNA damage. Mitochondrial metabolism status is a critical feature that helps evaluate HSC activity in human HSPC cultures. Low mitochondrial activity characterized by ROS levels, mitochondrial mass and mitochondrial membrane potential can distinguish

LT-HSCs within ex vivo expanded HSPCs [29•]. Cord blood derived CD34 + CD90 + EPCR + cells show the lowest mitochondrial activity [29•] further documenting the correlation between EPCR expression and functional properties of ex vivo expanded HSCs. Many human HSC expansion protocols have a direct or indirect effect on mitochondrial metabolism (see below), highlighting the importance of controlling mitochondrial status to achieve expansion of functional HSCs.

Recent studies have also linked lysosomal biology to HSC function. Lysosomes are nutrient sensing and signaling centers that are generally most abundant in quiescent HSCs and degraded upon HSC activation. It was recently shown that suppressing lysosome degradation can dramatically enhance the potency of activated HSCs [30]. Other studies have documented that modulating endolysosomal activity in human HSCs controls their quiescence, activation, and lineage choice [31].

Endoplasmic reticulum (ER) stress is another example that links the organelle function to HSC activity. Increased protein synthesis during ex vivo HSC expansion triggers accumulation of misfolded protein in the ER resulting in activation of unfolded protein response (UPR). UPR signaling can either release the cells from stress (cryoprotect) or induce apoptosis depending on the duration and intensity of the stress signal. The pro-survival integrated stress response (ISR) sustained by high levels of activating transcription factor 4 (ATF4) is crucial for HSC survival during stress response [32]. Diminishing the adverse effects of ER stress by adding chaperones such as DNAJB8 or over expression of RNA-binding protein Dppa5 promotes protein folding and protects HSC function [33, 34]. Together, these studies highlight novel aspects of HSC biology that have relevance for developing HSC culture protocols as these basic cellular functions are challenged during culture induced stress.

Species Differences with HSC Biology Between Human and Mouse

Most mechanistic studies on HSCs have been conducted on mice or other model organisms, raising questions about direct translatability of the findings to human HSC biology. Although comparative transcriptional profiling of human and mouse hematopoietic systems has confirmed many common factors regulating hematopoiesis and lineage-commitment [35], several studies have identified major species differences in HSPC phenotype and regulation that impact the development of human HSC expansion protocols. For example, overexpression of HOXB4 results in 1000-fold expansion of HSCs in mouse, but only fourfold expansion in human [36–38]. The use of polyvinyl alcohol (PVA) as a serum albumin replacement with optimized cytokine concentrations led to 236- to 899-fold expansion of

transplantable mouse HSCs, whereas the expansion of human HSPCs in similar PVA-based culture was limited compared to uncultured cells [39, 40]. On the other hand, small molecules UM171 and SR1, which expand human CB HSPC (see the "Nuclear Mechanisms Governing Human HSC Self-renewal and Ex vivo Expansion" section), do not stimulate mouse BM HSC ex vivo expansion [41, 42].

HSC surface phenotype also differs between human and mouse. CD34 antigen is highly expressed in human HSPCs and routinely used for their purification, whereas its expression is minimal in the most quiescent mouse BM HSCs [43]. Surface antigens that are typically used to enrich for mouse HSCs are either not enriched (e.g., CD150) or lack a homologue (e.g., Sca1) in human HSPCs [44–47]. Moreover, the surface immunophenotype of both mouse and human HSCs evolves throughout ontogeny, highlighting the need to define developmental stage-specific markers and regulatory programs [48–50].

Improvement of Xenograft Models used for Assaying Human HSCs

Xenograft mouse models provide a relevant in vivo context for quantification and functional evaluation of human HSCs. For the last 10 years, primary and secondary xenotransplantation in the female NSG mouse has been the "gold standard" for assessing human HSPC activity and differentiation in vivo (Table 1 [51]). However, these studies are both labor-intensive, as large numbers of mice are needed to estimate the frequency of HSCs using limiting dilution approach, and lengthy, as the assessment of long-term repopulating cells becomes more accurate only after 20–24 weeks post-transplantation. NSG mice are not ideal models for evaluating human LT-HSC self-renewal ability, as the engraftment efficiency in secondary recipient mice is usually low. While human HSPC differentiation is partially recapitulated in NSG mice, several functional defects with human multilineage reconstitution remain. These include the absence of human erythroid cells, limited differentiation into functional myeloid and NK cells, and incomplete maturation and biased differentiation of immune cells [52]. These limitations are at least in part due to suboptimal BM and thymic microenvironments and lack of cross-reactivity of mouse cytokines between the species.

To improve the durability and quality of the human graft, modified immunodeficient mouse models have been generated, such as NSG mice that are genetically engineered to express human cytokines hSCF, hGM-CSF, hIL3 (NSG-SGM3) [53]. Nevertheless, co-transplant of human fetal thymus with fetal liver or bone is needed to support robust production of human adaptive immune cells, including T cells, B cells, and dendritic cells, which can produce significant levels of human IgM and IgG antibodies [54, 55]. Moreover,

most immunodeficient mouse models require myeloablative conditioning, which often leads to complicated hematological, gastrointestinal, and neurological side effects that may cause loss of significant numbers of recipient mice. To that end, NSG mice with a KitW41 mutation (NBSGW) have been developed to improve human multilineage engraftment, including BM glycophorin-positive erythroid cells, in the absence of irradiation [56, 57]. While this model has several advantages compared to NSG, it does not address the lack of cross-species reactivity with important regulatory proteins. Thus, improving humanized mouse models to provide a proper microenvironment that supports long-term self-renewal and differentiation of human HSCs is important to fully understand the hierarchy and function of human HSPCs, and to ensure that in vitro generated or expanded cells have the capacity for long-term engraftment if transplanted to a patient.

Progress in Recapitulating Human HSC Niche in Culture

HSCs receive complex signals from multiple niche cells to regulate quiescence, self-renewal, and differentiation [58, 59]. Although factors such as stem cell factor (SCF), thrombopoietin (TPO), and Fms-like tyrosine kinase 3 ligand (FLT3-L) have been adapted as standard cytokines for human culturing of HSCs. Utilizing other individual molecular constituents of the BM microenvironment, such as osteopontin (OPN), transforming growth factor-β (TGFβ), Notch ligands, fibroblast growth factor 1 (FGF1), and pleiotrophin (PTN), has also been tested for the ability to support HSCs in culture [59]. Interestingly, a recent study showed that it is possible to maintain fully functional human HSCs in a hibernating culture system containing only IL-11 without other supportive hematopoietic cytokines [60]. Nevertheless, HSCs require hematopoietic growth factors to proliferate, which is also essential for gene editing approaches that depend on HR mediated DNA-repair mechanisms.

Although recreation of the physiological BM niche in culture is not feasible, it has been possible to improve human HSC expansion by using co-culture on cells that mimic the BM niche. Examples of new developments in this area include BM-derived mesenchymal stem cells (MSCs) that have been reprogrammed to sustain properties of their native counterparts [61], and Akt-immortalized endothelial cells (E4 + ECs) [62]. Although such culture systems are limited to one type of niche cell and often have altered molecular properties compared to niche cells in vivo, they can provide support for human HSCs through secretion of niche factors, initiation of signaling, and mitigation of DNA damage and replicative stress [59].

Table 1 Strategies for controlling human HSC ex vivo expansion through the nucleus

	Name	HSPC starting population	Culture conditions	Fold expansion and assay used	Mechanism of action	Species	Transplantation model	Clinical trial outcomes	Ref
Transcriptional regulation of HSC stemness genes	Mixed-lineage leukemia translocated to chromosome 3 protein (MLLT3)	HSPC derived from CB (CD34+CD38-CD90+) or FL (CD34+CD38-CD90+GPI80+)	HSPC transduced with MLLT3 and expanded for 15 days in SFEM+50 ng/ml TPO, 100 ng/ml SCF+100 ng/ml FLT3+10 μg/ml LDL+SR1 (500 nM)+UM171 (35 nM)	12.5-fold increase in LT-HSC frequency compared with uncultured HSPCs, or 5.2 higher that control HSPCs in UM171 and SR1, assessed by LDA (24 weeks posttransplantation)	MLLT3 over-expression maintain the expression of HSC regulators in cultured HSPCs through DOT1L activity and modulating H3K79me2	Human	NSG	NA	Calvanese et al. Nature [69••]
Posttranscriptional modification	RNA-binding protein Musashi 2	CD34+CD38−cells derived from CB	HSPC transduced with MSI2 lentiviral vector and expanded for 3 or 10 days in SFEM+20 ng/ml IL6+100 ng/ml SCF+100 ng/ml FLT3-L, and 20 ng/ml TPO	17-fold increase in ST-HSC and 23-fold increase in LT-HSC frequency compared to control cells as determined by LDA (3 weeks post primary transplantation and 13 weeks post-secondary transplantation, respectively)	MSI2 over-expression expands HSPCs through post-transcriptional downregulation of the aryl-hydrocarbon receptor (AhR) signaling	Human	NSG	NA	Rentas et al. Nature [70]
Inhibition of aryl hydrocarbon receptor signaling	StemRegenin1 (SR1)	CD34+cells derived from CB/mPB	21-day culture with SR1 (1 μM) in SFEM+100 ng/mL (TPO, FLT3, SCF, and IL6)	1118-fold increase in CD34+cells relative to input cells and 17-fold increase in cells that retain the ability to engraft immunodeficient mice (16 weeks post transplantation)	SR1 inhibits HSC differentiation by antagonizing the aryl hydrocarbon receptor (AHR) and down-regulation of its target genes (AHRR and CYP1B1)	Human	NSG	Phase 1–2 clinical study shows rapid neutrophil and platelet engraftment and absence of graft failure	Boitano et al. Science [41] and Wagner et al. Cell Stem Cell [77]

Table 1 (continued)

	Name	HSPC starting population	Culture conditions	Fold expansion and assay used	Mechanism of action	Species	Transplantation model	Clinical trial outcomes	Ref
Modulation of epigenetic corepressors and histone modifiers using small molecules	Pyrimidoindole derivative: UM171/UM729	CD34+cells derived from CB/mPB	12-day culture with UM171 (35 nM) in SFEM+50 ng/ml TPO+100 ng/ml SCF+100 ng/ml FLT3L+10 μg/ml LDL	CD34+CD38-CD45RA-CD90+CD49f+cell numbers expanded 1000-fold compared to input. 13-fold increase in LT-HSC frequency assessed by LDA (20 weeks posttransplantation)	UM171 promotes retention of primitive cell phenotype and suppress mature cell output. Activates CULLIN3/KBTBD4 ubiquitin ligase that targets the LSD1-CoREST repressor complex for proteasomal degradation	Human	NSG	Phase 1–2 clinical study shows improvement in neutrophil recovery and robust T cell reconstitution with low incidence of GVHD and transplant-related mortality	Fares et al. Science [42]; Cohen et al. Lancet Haematol [78]; and Chagraoui et al. Cell Stem Cell [84••]
	Lysine-specific histone demethylase 1A (LSD1) inhibitor	CD34+cells derived from CB/BM	6-day culture in SFEM+100 ng/ml of TPO, SCF, and FLT3L+1.25 μM 2-PCPA (LSDI)	CD34+CD90+EPCR+cell numbers are higher in LSDI treated cultures compared to controls and 2.7-fold increase in LT-HSC frequency assessed by LDA (18 weeks posttransplantation)	LSDI causes similar phenotypic and molecular outcomes as UM171. Suppresses differentiation and preserves immature state	Human	NSG	NA	Subramaniam et al. Blood [83••]

Table 1 (continued)

Name	HSPC starting population	Culture conditions	Fold expansion and assay used	Mechanism of action	Species	Transplantation model	Clinical trial outcomes	Ref
Valproic acid (VPA)	CD34+cells derived from CB/mPB/BM	16 h cytokine priming (SFEM+100 ng/ml TPO, 150 ng/ml SCF, 100 ng/ml FLT3, and 50 ng/ml IL3) followed by addition of VPA (1 mM) for 7 days	1507- and 3000-fold increase in the numbers of CD34+CD45RA-CD90+CD49f+cells of VPA-treated BM and mPB, respectively. Higher percentage of chimerism of VPA-treated cells compared to control cultures (16 weeks posttransplantation)	Enhances histone acetylation, induces aldehyde dehydrogenase activity and reduces mitochondrial activity: decreases mitochondrial mass, membrane potential, oxygen consumption and production of ROS	Human	NSG	Ongoing phase I clinical trial (NCT03885947). Initial data shows persistent multilineage donor cell reconstitution with rapid T cell and platelet engraftment	Papa et al. Front Cell Dev Biol [29●] and Zimran et al. Stem Cells Transl Med [89●●]
Bromodomain and extra-terminal (BET) motif inhibitor CPI203	CD133+cells derived from CB	5-day culture in SFEM+20 ng/ml TPO+100 ng/ml SCF+100 ng/ml FLT3, and CPI203	threefold increase in LT-HSC frequency assessed by LDA (20 weeks post transplantation)	Inhibits bromodomain-containing proteins (BCPs)	Human	NSG	NA	Hua et al. Blood [94●●]

HSCs hematopoietic stem cells, *HSPCs* hematopoietic stem and progenitor cells, *CB* cord blood, *BM* bone marrow, *mPB* mobilized peripheral blood, *ST-HSC* short-term HSC, *LT-HSC* long-term HSC, *SFEM* serum-free expansion medium, *FLT3L* FMS-like tyrosine kinase 3 ligand, *TPO* thrombopoietin, *SCF* stem cell factor, *IL6* interleukin 6, *LDA* limit dilution assay

In addition to stromal components and endothelial cells that are now well-established in HSC niche, the nervous system also modulates hematopoietic activity in the BM microenvironment by regulating HSC mobilization, cytokine production, and HSC self-renewal gene expression (63). Recent studies show that human HSC expansion may benefit from activating regulating pathways that are shared between neural cells and HSCs. RET, a neuronal tyrosine kinase receptor that receives signals from the glial-derived neurotrophic factor (GDNF) and its coreceptor (GFRa1), is expressed in mouse and human HSPCs and their environment [64, 65••]. In fact, HSC frequency is four-fold higher within the RET[high] subset compared to RET^{low} cells. RET activation in mouse HSPCs induces Bcl2 and Bcl2l1 anti-apoptotic genes resulting in improved HSPC survival, proliferation, and engraftment [64]. These observations are also valid in human HSPCs, where kinome profiling identified RET as one of the most differentially active kinases in the HSPC compartment. RET activation by GDNF/GFRa1 induced proliferation programs and sustained a phosphorylation cascade of NF-kB/p53/BCL2 and IL-2 signaling that activated anti-apoptotic, anti-oxidative stress and anti-inflammatory programs in cultured HSPCs. Treating human CB HSPCs with RET ligands (GDNF/GFRa1) enhanced the engraftment potential of cultured HSCs, and combining with small molecules SR1/UM171 further improved these outcomes [65••]. Although additional studies are needed to define the optimal RET activation dose, culture time, and the effect on long-term repopulating cells [66], activation of RET by GDNF/GFRa1 may be a useful addition to complement various HSC culture protocols.

In order to provide more supportive surface for culturing HSCs, various studies have examined the use of extracellular matrix components and polymeric biomaterials to mimic the physiological niche [39, 58, 67]. Such materials may be used as scaffolds to help recreate 3D niche for culturing HSCs. Most cell culture studies are performed on hydrophobic surfaces, which differ greatly from the in vivo BM niche, which is dominated by hydrophilic or zwitterionic cell membrane lipids. To mimic such niche conditions, a novel 3-D culture system formed from super-hydrophilic matrix consisting of degradable zwitterionic hydrogel (3D-ZTG) was developed. These conditions protected cultured human HSPCs and promoted the expansion of transplantable CB LT-HSCs up to 73-fold [68••]. Importantly, similar effects were also observed when culturing adult BM HSPCs. The 3D-ZTG culture system protected the cultured HSCs by reducing differentiation and metabolic activity and limiting excessive production of ROS. Both the zwitterionic surface and the 3D structure were important to achieve HSPC expansion.

These studies provide hope that it may be possible to identify the critical crosstalk mechanisms between human HSC and their niche that enable HSC amplification without a major decline in function.

Nuclear Mechanisms Governing Human HSC Self-renewal and Ex vivo Expansion

Despite the challenges in human HSC research, there has been exciting recent progress in identifying diverse regulatory mechanisms that control human HSC stemness and can be harnessed to improve human HSC expansion (Table 1 and Fig. 1). These include transcriptional regulators and chromatin modifiers that control HSC fate from the nucleus and small molecules that can modulate these HSC regulatory mechanisms. Although their targets or mechanisms of action are still not fully understood, recent studies have begun to uncover important mechanisms by which they can support human HSPC expansion. Together, these discoveries are beginning to decode the process of human HSC self-renewal.
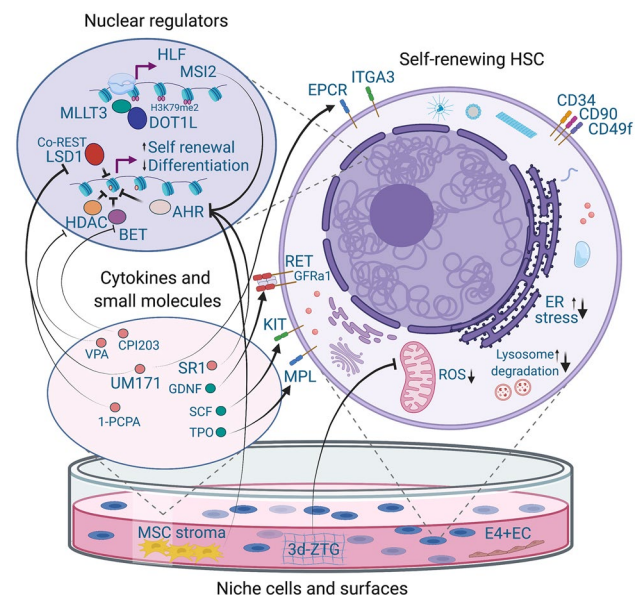
Fig. 1 Regulatory pathways and interventions to expand human HSCs in culture. Cultured human HSCs expressing the HSC surface antigens and receptors and evidencing cellular processes involved in maintaining stemness. Inset to the nucleus (top left) shows gene regulatory mechanisms involved in maintaining HSC self-renewal genes and suppression of differentiation. An inset to the culture milieu (bottom left) shows interventions aimed to promote human HSC expansion, including small molecules (pink), growth factors, and signaling molecules (cerulean). Culture plate (bottom) shows co-culture systems and biomaterials mimicking the HSC niche. Created with BioRender.com

Maintaining MLLT3 (AF9) Levels in Cultured Human HSCs

The expression of MLLT3 (AF9), a transcriptional regulator highly enriched in human HSCs, is gradually downregulated in culture, leading to loss of HSC activity. Restoring physiological MLLT3 levels in cultured human HSCs using lentiviral vectors mitigates this decline and allows over tenfold expansion of transplantable CB-HSCs with no evidence of cellular reprogramming or oncogenic transformation [69••]. This was important to show, as the leukemic fusion protein involving MLLT3, MLL-AF9, is known for its ability to transform progenitors to leukemia stem cells (LSC), leading to aggressive leukemia. Gene expression profiling and ChIP-seq data of human HSCs cultured with MLLT3 shows MLLT3 binding to and upregulation of genes encoding powerful HSC regulatory factors, including MECOM, HLF, and Musashi-2 (MSI2). Interestingly. MSI2 overexpression alone can sustain HSC stemness in cultured CB HSCs through posttranscriptional downregulation of the aryl hydrocarbon receptor pathway [70] (see also below). MLLT3 safeguards the HSC transcriptional program by cooperating with DOT1L to maintain H3K79me2, a gene body-associated active mark, in HSC regulatory genes, thereby supporting symmetric self-renewal in culture [69••]. The binding pattern of overexpressed MLLT3 in cultured human HSCs was strikingly similar to that of native MLLT3 in freshly isolated human FL HSPCs, highlighting the ability of MLLT3 YEATS domain to guide overexpressed MLLT3 correctly to active transcription starting site (TSS) in HSC regulatory genes. The full-length MLLT3 does not lock HSCs into self-renewing mode, as these cells differentiated readily in vivo in NSG mice and in vitro when placed into differentiation conditions. However, the DOT1L dependent mechanism does not explain how all MLLT3 target genes are regulated, as some gene groups are downregulated by MLLT3 and show minimal H3K79me2 in MLLT3 binding sites. This suggests that MLLT3 binds distinct groups of target genes in HSPCs as part of different activator and repressor complexes. Although lentiviral expression of MLLT3 is a safe approach to achieve ex vivo expansion of human HSCs in an experimental model, it is not directly translatable to the clinic. Therefore, a more detailed understanding of the molecular mechanisms that control MLLT3 expression and its mode of action in human HSCs may help develop novel tools to enhance human HSC expansion for clinical purposes.

Posttranscriptional Regulation by RNA-Binding Protein MSI2

RNA-binding protein Musashi 2 (MSI2) plays a critical role in HSC homeostasis by controlling the expression of key HSC regulators posttranscriptionally [70]. MSI2 expression is highest in undifferentiated hematopoietic cells and gradually decreases upon lineage commitment. Mice deficient for Msi2 show significant reduction in the number of early hematopoietic precursors (ST-HSC and LMPPs) and knockdown of Msi2 in mouse HSCs results in a loss of quiescence and significant reduction in HSC engraftment potential [71, 72]. Msi2 overexpression in mouse HSCs compromises their function by driving proliferation and asymmetric cell division, resulting in a reduced HSC pool [73]. Other studies show that moderate overexpression of Msi2 improves mouse HSC function as evidenced by their increased reconstitution ability [71]. A similar effect is observed with human CB HSCs, as lentiviral overexpression of MSI2 increases both short and long-term repopulating CB-HSCs. MSI2 overexpression promotes HSPC expansion through downregulation of aryl hydrocarbon receptor signaling. MSI2 directly binds to the 3′UTRs of AHR pathway components (CYP1B1 and HSP90) and mediates the posttranscriptional repression of these genes [70]. Two transcription factors, PLAG1 and USF2, bind to the MSI2 promoter, which results in significant increase in MSI2 while its downstream target (CYP1B1) is reduced, thereby supporting human HSPC maintenance and expansion [74].

Inhibition of Aryl Hydrocarbon Receptor Signaling

The aryl hydrocarbon receptor (AHR) signaling pathway was first identified as a negative regulator of human HSPC from an unbiased screen of heterocyclic compounds that can promote the expansion of CB CD34 + cells. Suppression of AHR signaling using a small molecule antagonist StemReginin (SR1) or through posttranscriptional downregulation of its pathway components (AHRR and CYP1B1) promotes ex vivo expansion of transplantable human HSPC [41, 70]. Suppression of the AHR pathway was also identified as one of the mechanisms by which OP9M2 stroma co-culture supports the expansion of multipotent human fetal liver and CB HSPCs, although HSPCs expanded using this system did not demonstrate increased engraftment to NSG mice [22]. Suppressing AHR signaling also enhances HSPC production from human embryonic stem cells and induced pluripotent stem cells, and improves lymphoid specification and differentiation to functional natural killer cells [75, 76]. Phase I/II clinical trials showed that patients who were transplanted with SR1 expanded CB HSPCs in addition to unmanipulated HSPCs demonstrated faster neutrophil engraftment compared to patients treated with two unmanipulated CB units [77]. However, the SR1 treated unit did not demonstrate significant improvement in hematopoietic recovery when compared with the simultaneously infused unmanipulated unit in a double CB transplant setting. Thus, further studies are required to fully evaluate the impact of

SR1 on the speed of immune recovery and ability to sustain long-term hematopoietic engraftment.

Regulating Epigenetic Corepressors and Histone Modifiers Using Small Molecules

Pyrimidoindole Derivatives (UM171) and Destabilization of CoREST Complex UM171 was discovered from a small molecule screen as a human HSC agonist preserving CB HSCs in an undifferentiated state independent of AHR suppression [41, 42]. Short-term [7-day] expansion of CB HSCs with UM171 is under clinical investigation and has been shown to be a safe and feasible approach to treat patients with hematological malignancies who lack a suitable HLA-matched BM donor [78]. Transcriptome analysis of CD34 + cells following UM171 treatment showed reduced levels of transcripts associated with lineage differentiation and induction of the expression of genes encoding for HSC membrane proteins. One of the most differentially expressed genes was EPCR, which was subsequently shown to be a reliable marker for ex vivo expanded human HSPCs [19]. UM171 also expands distinct myeloid and lymphoid progenitors, enhances derivation of hematopoietic progenitors from human PSCs, and increases lentiviral gene transfer of human HSPCs [79–81]. One of the mechanisms by which UM171 stimulates HSPC expansion is by readjusting NF-kB proinflammatory and anti-inflammatory signals through EPCR, which diminishes toxic accumulation of ROS [82]. In-depth investigation of UM171-dependent HSC supportive mechanisms revealed that UM171 selectively destabilizes LSD1 and RCOR1, a core member of LSD1-containing chromatin remodeling complexes (CoREST) [83••], by activating CULLIN3/ KBTBD4 ubiquitin ligase [84••]. UM171-induced activation of this complex leads to the re-establishment of H3K4me2 and H3K27ac epigenetic marks, which are otherwise rapidly decreased in human HSCs during ex vivo culture [84••].

LSD1 Inhibition Lysine-specific demethylase 1 (LSD1/ KDM1a) is critical for repressing HSC genes during hematopoietic differentiation. Loss of LSD1 in mice resulted in increased H3K4me1 and H3K4me2 methylation and de-repression of genes encoding for HSC regulators such as Gfi1b, Hoxa9, and Meis1 [85, 86]. LSD1 inhibition using small molecule (1-PCPA) showed similar phenotypic (expansion of CD34 + EPCR +) and molecular responses as UM171, including increased H3K4 methylation and expression of HSC-related genes while progenitor-specific genes were suppressed. Compared to LSD1 inhibitors that directly inhibit LSD1 enzymatic function, UM171 abrogates LSD1 by targeting the LSD1-containing CoREST complex [83, 84••]. Notably, permanent Lsd1 loss in adult mice impaired HSC differentiation and negatively impacted the progenitor pool, particularly erythroid

and megakaryocytic progenitors which were converted to myeloid fate [85–87]. Thus, clinical translation of LSD1 inhibition will require a more thorough analysis on hematopoietic system to assure safety.

Histone Deacetylase Inhibition HDAC inhibition is another example of how targeting epigenetic regulators may help promote human HSC expansion. Valproic acid (VPA), an HDAC class I inhibitor, triggers a rapid acquisition of HSC surface phenotype by promoting the reprogramming of CD34+CD90− cells into CD34+CD90+ HSC-like cells. Moreover, VPA promotes the maintenance of mitochondrial and transcriptomic profiles reminiscent of uncultured HSCs [29, 88, 89••]. Specifically, VPA regulates the expression of HSC genes that mark their surface phenotype (CD90, EPCR), quiescent state (CDK6, CDKN1A), and self-renewal activity (TIE2, ALDH1, PBX1, HES1, MEIS1, GATA2) [88]. Ex vivo culture of HSCs causes proliferative and differentiation stress, which leads to a metabolic switch from glycolysis (utilized by quiescent HSCs in the BM niche) to mitochondrial oxidative phosphorylation (OXPHOS) and increased generation of ROS [90, 91]. Addition of VPA to HSC cultures improves the metabolic, mitochondrial and low ROS status of primitive HSCs [88]. Whether these mitochondrial alterations are a direct effect of VPA treatment, or a consequence of downstream events, needs further investigation.

Inhibition of BET Bromodomain and extra-terminal (BET) family proteins, which regulate gene transcription and cell cycle by binding to acetylated histones, are also powerful modulators of HSC function [92, 93]. BET inhibition in human HSCs through the small molecule CPI203 displaced bromodomain protein (BRD4) from chromatin, resulting in HSC expansion and enhancement of megakaryocyte differentiation potential [94••]. CPI203-expanded HSPCs maintained HSC-associated genes such as PROM1, EMCN, and HLF, and suppressed differentiation-related genes. Interestingly, the most differentially expressed genes in CPI203 conditions were genes associated with megakaryocyte differentiation (e.g., CXCR4, PF4, and C6orf25). Although further studies are needed to fully dissect the CPI203-mediated mechanism governing HSC expansion and megakaryocytic differentiation, there is evidence that Wnt/β-catenin signaling could be involved. Previous studies demonstrated that resistance to BET inhibitors in both human and mouse leukemia cells was in part caused by increased Wnt/β-catenin and TGF-β signaling, and that negative regulation of these pathways restores sensitivity to BET inhibitors [95–97]. This approach holds potential for clinical applications, as it may increase the numbers of HSCs and enhance platelet production, although additional studies evaluating its effects on other blood lineages will be important to understand the full effects of BET inhibition.

Conclusions

Efforts to optimize culture conditions for expansion of various sources of human HSCs for clinical purposes are still actively ongoing, in an effort to provide adequate HSC numbers from a single CB unit for successful CB transplantation in adults and maximize the success of gene manipulation on mPB or BM HSC. After decades of efforts to expand human HSCs with little impact on clinical practice, recent progress and ongoing clinical trials provide hope that human HSC expansion for the clinic may become a reality. Despite promising results with various approaches, most of the culture strategies have so far only led to modest expansion of transplantable units compared to much greater expansion of immunophenotypic HSPCs, suggesting that the functional activity of the majority of the ex vivo expanded human HSPCs is still compromised [58]. Moreover, very few of these studies have shown a robust expansion of human adult BM- or mPB-derived HSCs, which have very different proliferative properties than CB or fetal liver HSCs, and may require different culture conditions. Finally, as enhanced self-renewal is also a property of leukemia stem cells, and every cell division poses a risk of mutation, it will be important to monitor the effects of expansion protocols to ensure that the cultured HSCs are not prone to malignant transformation. The rapid development of new techniques such single cell transcriptome, epigenome, and metabolome profiling may help understand the molecular defects in cultured HSPCs and optimize protocols and markers for human HSC expansion. Such methods can be exploited to resolve the molecular heterogeneity of cultured HSPCs and identify changes in regulatory mechanisms and metabolic pathways that may still compromise HSC function in specific culture conditions. With increased understanding of human HSC self-renewal mechanisms and how they become disrupted in culture, it may ultimately become possible to combine various ex vivo expansion approaches to tailor protocols that preserve the identity and functional potential of cultured human HSCs from different sources.

Declarations

Conflict of Interest Dr. Fares has a patent WO 2013110198 A1 with royalties paid. Dr. Calvanese has a patent WO2017216775A3 pending. Dr. Mikkola has a patent WO2017216775A3 pending.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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