Review Article

The quest for the holy grail: overcoming challenges in expanding human hematopoietic stem cells for clinical use

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Abstract: Hematopoietic stem cell (HSC) transplantation has been the golden standard for many hematological disorders. However, the number of HSCs obtained from several sources, including umbilical cord blood (UCB), often is insufficient for transplantation. For decades, maintaining or even expanding HSCs for therapeutic purposes has been a "holy grail" in stem cell biology. Different methods have been proposed to improve the efficiency of cell expansion and enhance homing potential such as co-culture with stromal cells or treatment with specific agents. Recent progress has shown that this is starting to become feasible using serum-free and well-defined media. Some of these protocols to expand HSCs along with genetic modification have been successfully applied in clinical trials and some others are studied in preclinical and clinical studies. However, the main challenges regarding *ex vivo* expansion of HSCs such as limited growth potential and tendency to differentiate in culture still need improvements. Understanding the biology of blood stem cells, their niche and signaling pathways has provided possibilities to regulate cell fate decisions and manipulate cells to optimize expansion of HSCs *in vitro*. Here, we review the plethora of HSC expansion protocols that have been proposed and indicate the current state of the art for their clinical application.

Keywords: Hematopoietic stem cells (HSCs); *ex vivo* expansion; gene therapy; Wnt; Notch

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Introduction

Hematopoietic stem cells (HSCs) produce all red and white blood cells and are responsible for maintaining hematopoiesis during life. These multipotent cells that mainly reside in bone marrow (BM), possess the capacities of self-renewal and differentiation into various mature hematopoietic cell lineages (1). This characteristic makes HSCs suitable for transplantation in patients with immunological and hematological disorders, who would benefit from a new blood cell system (2). However, the number of HSCs obtained from umbilical cord blood (UCB), as the first-line therapy, is often inadequate for transplantation. Also, only a small number of these cells have efficient homing capacity after transplantation. This indicates the importance of *ex vivo* expansion of HSCs to acquire sufficient number of cells to transplant (3,4).

There are several intrinsic and extrinsic factors that regulate HSC quiescence, self-renewal and differentiation (5). Understanding the mechanisms underlying the cell fate-decisions enables us to manipulate

cells to optimize *ex vivo* expansion of HSCs. Gene therapy, directed differentiation, and/or transplantation of HSCs require efficient protocols for long-term expansion of cells to achieve a sufficient number of cells. There are various methods to improve the efficiency of cell expansion and enhance homing potential: co-culture with stromal cells, combination of cytokines, environmental O_2 pressure, gene modification, and exposing to agents (e.g., treatment of HSCs with fucosyltransferase VI before transplantation) (6-10). The main challenges regarding *ex vivo* expansion of HSCs are limited growth potential and tendency to differentiate in culture; thus, *ex vivo* expansion of HSCs requires specific protocols to enhance proliferation and self-renewal while simultaneously controlling differentiation of HSCs (11). Development of highly efficient protocols for *ex vivo* expansion of stem cells using specific proteins and small molecules has provided better results for HSC transplantation and HSC-based gene therapy (3). These protocols have been broadly used for cell therapy in clinical trials; however, among the main challenges in functional HSC expansion are achieving sufficient number of HSCs, unfavored differentiation, loss of stemness over time, and the lack of a pure population. Further research is needed to improve the efficacy of long-term *ex vivo* expansion of stem cells. In this paper, we will review the current methods for *ex vivo* expansion of HSCs in the context of gene therapy.

Why do we need long-term expansion of HSCs *in vitro*?

Allogeneic and autologous hematopoietic stem cell transplantation (HSCT) has been used efficiently for patients with severe hematological malignancies and immunodeficiency diseases (12-14). However, there is a limitation for patients without matching donors (15). There are different sources of HSCs including BM, peripheral blood (PB), placental blood and UCB. Acquiring HSCs from BM is an invasive procedure, and mobilized peripheral blood (mPB) requires G-CSF regimens that can be toxic in some patients. In many instances, UCB transplantation is the first-line treatment (16,17). An umbilical cord contains different types of cells including HSCs, mesenchymal stromal cells, endothelial progenitors and human umbilical cord unrestricted somatic stem cells. Also, accessibility and noninvasiveness, lower incidence of graft-versus-host-disease (GvHD), lower contamination risk, and ethical issues are some of the advantages of using UCB in HSC transplantation (18). However, total isolated HSCs from one UCB unit often is less than the required amount for effective transplantation. Besides, most of the transplanted cells fail to home to their specific niche, which may diminish the efficacy of cell therapy (3).

In addition to efficient HSC transplantation, research in proteomics, metabolomics and ChIP-sequencing to identify potential drug targets also requires large numbers of expanded HSCs. Existing culture protocols usually provide short-term expansion and therefore a small number of HSCs (19,20). There have been a lot of efforts in laboratories and clinics to provide HSCs with an environment similar to the body to increase their expansion. Using recent protocols, researchers have been able to expand mouse HSCs between 236- to 899-fold over a month (21); however, a major population of these cells are non-HSCs and also there has been clone-to-clone variability in single cell-initiated cultures. Thus, identifying specific molecular characteristics that induce expansion has not been feasible yet (22). However, the identification of self-renewal regulators including HOXB4, ESAM, ANGPTL, FSTL1 and PRDM16 indicates them as potential targets to regulate self-renewal of HSCs in culture (23-28). *Ex vivo* expansion of HSCs while maintaining self-renewal and multipotency would help HSC research and would improve the outcome of HSCT with or without gene therapy, because it would be the source of enough HSCs for patients without any matching donor (29). Gene therapy using autologous HSCs with the added benefit of enhanced homing and reduced risk of GvHD has been a promising method for various hematological and immunological diseases (30,31). Of course, HSC function should be maintained during the *ex vivo* expansion of HSCs for genetic manipulation (32).

Gene therapy of human HSCs

Transplantation of gene-modified HSCs is a promising approach to treat different blood borne disorders such as primary immunodeficiencies and anemias (33-35) but interestingly also metabolic disorders affecting muscle and brain [reviewed in (36)]. Gene-modified autologous HSCs not only deliver the corrected gene to the patients but also eliminate the risk of GvHD and immune complications (36). The efficacy of viral vectors to transduce quiescent HSCs is very low. Therefore, *ex vivo* expansion and characterization of true HSCs providing sufficient numbers of functional cells for gene modification and transplantation is required. Pre-transplant conditioning as a preparative regimen can consist of chemotherapy or radiation to eliminate host cells

in order to allow donor cells to be efficiently transplanted for the long-term, however, these methods are relatively harsh and may increase the risk of malignancies (37). Even when using low-dose busulfan as the pre-conditioning agent in patients with non-malignant disorders, it has cytotoxicity effects leading to a high rate of treatment-related mortality (38,39). *Ex vivo* expansion of gene-corrected long-term HSCs may provide efficient engraftment without the need for intense pre-conditioning before transplantation. Moreover, achieving a large number of expanded gene-modified cells ensures that they are not outcompeted by the remaining host cells (40). Because only a limited number of clinical trials have been carried out in patients, it is too early to conclude anything with regards to the long-term cytogenetic abnormalities and malignancy risks following long-term expansion of HSCs in *ex vivo* conditions (41-48).

Gene modification of HSCs can be designed to be transient (high level of expression for a few weeks) or stable (permanent lower level of expression) depending on the pathophysiology of the disease. The gene delivery system can use viral or non-viral approaches. A viral-mediated gene delivery method uses certain viruses such as retroviruses, lentiviruses, and adeno-associated viruses as a vector to introduce the desired gene into host cells via transduction. On the other hand, non-viral methods for gene delivery uses one of different chemical or physical transfection techniques including calcium phosphate method, electroporation, lipofection, and nucleofection (49,50). Currently, retroviral and lentiviral vectors have been approved as a safe vector to transform the host cell genome of HSCs from patients with various inborn errors of immunity (51,52). However, accumulating studies are investigating to improve targeted integration while maintaining functional HSC characteristics (45). Moreover, using current DNA-modifying techniques including CRISPR-Cas9, transcription activator-like effector nucleases or zinc-finger nucleases could help to inactivate unfavored alleles which can be followed by homology-directed repair (HDR) or non-homologous end joining (NHEJ) to correct unfavored mutations. Following gene modification, corrected HSCs can be delivered back into patients (32,53). For these gene editing approaches, in general longer *ex vivo* culture periods are needed to allow for HDR to occur. This makes it even more important to use efficient protocols for HSC expansion that maintain stemness properties. Finally, often ignored in expansion protocols is the possibility to increase expression or accessibility of protooncogenes for viral vectors due to insertional mutagenesis. It has been demonstrated that clinically used protocols with cytokines such as TPO, SCF and FLT3L increases expression of the LMO2 protooncogene thereby increasing possibilities for genotoxicity (54,55). Clinical studies using autologous HSCT gene therapy have shown the safe and efficient multilineage engraftment of these cells in patients. However, genetic manipulation of HSCs may result in an increased risk of leukemia by overexpression of self-renewal genes or inhibition of differentiation (48,56-62). Overall, it should be considered that these methods have been recently developed, so long-term follow up of the results is warranted, certainly with regard to insertional mutagenesis and long term efficacy.

Cell isolation and enrichment methods

Cellular studies using novel techniques such as single-cell sequencing approaches allows to analyze cell heterogeneity and cellular evolutionary relationships of various cells, including stem cell populations with different self-renewal ability and functions and to identify specific cell populations by analyzing gene expression profiles of single hematopoietic stem and progenitor cells (HSPCs) (3,63). For instance, single-cell RNA sequencing of HSCs revealed that ADGRG1 may be considered as a potential marker for functional HSCs in *ex vivo* expanded cells under oxidative stress condition (44).

Enrichment of desired cells among the heterogenous population of cells is crucial to acquire a more pure population for *ex vivo* expansion. Due to heterogeneity in HSPC populations and a lack of potential markers to isolate functional HSCs, even the enriched cells show variable potential in expansion and transplantation results. HSC dormancy enables HSCs to retain multilineage and self-renewal capacities in a quiescent status that under stress switches into self-renewal and differentiation states that gives rise to heterogenous hematopoietic cell lineages. Label-retaining studies support this notion and help to identify and isolate HSCs. Moreover, the aged HSC compartment is highly heterogenous and consists of rare HSCs with a limited divisional history and predominant progenitors with limited self-renewal and myeloid-biased differentiation potential (3,64).

Density separation using density medium (e.g., percoll or mixtures of ficoll and hypaque) makes it easier to separate the lighter density cells including HSCs by removing erythrocytes and granulocytes (65). Further enrichment of HSCs can be carried out using antibody-mediated HSC

purification techniques (66).

Another technique suggested for isolation of HSCs is using cytotoxic drugs such as 5-fluorouracil (5-FU) which eliminates most of the rapidly dividing mature cells, and keeps HSCs and some immature progenitors which are quiescent under steady-state conditions (67). Moreover, selective cell separation using antibodies attached to specific toxins, fluorochromes, and/or solid matrices, provides high purity in cell separation (68-70).

On the other hand, negative selection allows to enrich $CD34^+$ cells and colony-forming cells (CFCs) 50- to 200-fold using antibodies against lineage markers and removing mature cells (71). Also, depleting CD71-bright erythroid progenitors and $CD38^+CD45RA^+$ myeloid progenitors yields a 5- to 10-fold greater enrichment of the long-term culture initiating cells (LTC-ICs) (72).

Using different strategies including cell surface marker expression assay using MACS and FACS, functional assays [e.g., colony formation assay, label-retaining methods, aldehyde dehydrogenases (ALDHs) activity, and side population (SP) assay], and physical properties, researchers have been able to subdivide functionally heterogeneous mixture of HSCs into homogeneous subpopulations, thereby identifying and purification of HSCs (73).

HSCs can be enriched from different sources mainly by cell surface profile using multiparameter cell-sorting systems. Though, expression of some of these markers such as CD38 of CD90 can change *in vitro*, indicating a crucial need to find novel stable markers that support the identification of HSCs subsets either *in vivo* or *in vitro* (74). Junction adhesion molecule-2 (JAM2), endothelial cell-selective adhesion molecule (ESAM), and endothelial protein C receptor (EPCR) are the examples of highly expressed genes in HSCs generating T cells (3). Purification of HSCs, primitive multipotent progenitor cells and lineage-restricted progenitors can be achieved by negative selection to remove the most mature cells, followed by positive selection using FACS and/or immunomagnetic separation techniques to isolate the desired subset. However, there is still a gap in isolating pure functional HSCs (22).

Specific surface markers used for identifying human HSPCs *in vitro* and *in vivo* are lineage markers, CD34, CD38, CD90, CD45RA, CD49f, CD133 (AC133), CDCP1, C-KIT, KDR (VEGFR-2) and VEGFR-1 (75,76). Using lineage markers such as glycophorin A, CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD56, and CD66b results in a 20- to 500-fold purification of HSPCs. Except for most of the terminally differentiating cells, other cells including the LTC-ICs and most of the repopulating cells, CFCs and lymphocyte progenitor cells express CD34 on their cell surface. Moreover, cytokine mobilization and/or cytotoxic therapy elevates the level of $CD34^+$ cells in the blood which allows to acquire adequate number of HSPCs for clinical transplantation using leukapheresis (76).

While $CD34^+CD38^-$ cells have the ability for gradual but constant multilineage hematopoietic reconstitution after transplantation having repopulation potential, $CD34^+CD38^+$ cells increase rapidly but transiently after transplantation in immunodeficient mice but lack the secondary repopulation potential (77-79). HSPCs lack or weakly express CD33, CD45RA, CD71, and HLA-DR; though, some types of lineage-committed progenitor cells highly express these markers (80).

The majority of $CD34^+$ cells—including most lineage-committed progenitor cells—express CD117 which is overexpressed in terminally differentiating erythroid cells but lacking on almost all circulating mature blood cells. Also, growth factor receptors such as the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-6 are overexpressed in HSPCs during differentiation into late precursor cells and mature blood cells.

The spleen colony-forming unit (S-CFU) assay is an *in vivo* assay based on the ability of HSPCs to form macroscopic splenic colonies following the injection of these cells into lethally irradiated mice (81). This method allows to study the proliferation and differentiation potential of HSPCs at single-cell level. The morphology and the number of the colonies give initial information about the ability of HSPCs to differentiate and proliferate. This method provides direct *in vivo* evidence of stem cell clonality and can be tested under various physiological, experimental and disease conditions (82,83).

SP assay using the certain fluorescent dyes, such as Rhodamine-123 (Rho) and Hoechst 33342 (Ho) enables to isolate primitive hematopoietic cells. Studies suggest that human HSCs can be purify by sorting $CD34^+CD38^-SP^+$ cells (22). Most of the HSCs in adult tissues are Rho-/lo and it is regulated similarly to CD34. Also, adult BM contains a rare population of Rho-/lo cells that generates a characteristic cluster of events off to the lower left side in dual wavelength FACS dot-plot profiles (84). SP in murine cells shows a significant enrichment of HSCs, while human SP demonstrates negative for CD34 and lineage antigens. Human $Lin^-/CD34^-/ALDH^+$ cells have capacity for long-

term repopulation.

Primitive hematopoietic cells express ALDH enzyme which makes them resistance to alkylating agents such as the active derivatives of cyclophosphamide [e.g., 4-hydroxyperoxycyclophosphamide (4-HC) and mafosfamide]. This provides the possibility to use fluorescent ALDH-substrates by FACS to identify and enrich HSCs (85). ALDH is overexpressed in $CD34^+CD38^-$ cells in human BM. Also, LTC-ICs can be enriched from the $ALDH^{bright}$ subset of $CD34^+$ cells two folds more than from the $CD34^+$ cells (76). Overall, studies showed that ALDH activity could identify the human HSCs better than the Hoechst exclusion technique (86).

$CD34^+$ cell selection using micro-beads and nanoparticles with/without magnetic column gives a 25- to 100-fold enrichment of HSPCs. Although positive selection methods provide efficient and pure cell separation, it may eliminate primitive cell subsets which lack the expression of selection markers such as $CD34^-$ HSCs. Moreover, most of the mature cells which are essential for function and homing of stem cells may be lost following this method (76).

Tomellini *et al.*, 2019, introduced Integrin-α3 as a functional marker for *ex vivo* expanded human long-term HSCs (LT-HSCs). They showed that measuring expression level of ITGA3 could help to separate the primitive $EPCR^+CD90^+CD133^+CD34^+CD45RA^-$ HSC population into two functionally different sub-population presenting mostly short-term ($ITGA3^-$) and both short-term and long-term ($ITGA3^+$) repopulating potential (87). ITGA3 expression is functionally essential for the long-term engraftment of UCB cells. $ITGA3^+$ cells show multilineage differentiation potential, and serial reconstitution ability in immunocompromised mice. These cells also possess a HSC-specific transcriptomic signature which has made it a potential marker for cultured LT-HSCs and to improve the accurate HSC identification in culture (87).

Using single clone functional transplantation results and gene expression profiling, Che *et al.*, 2022 reported EPCR as a reliable marker for functional HSCs *in vitro* to isolate HSCs from non-HSCs and determine the molecular profile of *ex vivo* expanded HSCs (22). Using a novel repopulation signature, researchers would be able to define the molecular profile of *ex vivo* expanded HSCs, thereby identifying functional HSCs in culture and providing large-scale screenings (small molecules, CRISPR, etc.) and directed differentiation. HSC gene signatures such as MolO and RepopSig provide rapid monitoring and identifying quiescent HSCs clones as well as actively cycling HSCs, in long-term expansion. A strategy proposed by Che and colleagues indicates targeted removal of megakaryocytes (MK) and erythrocyte lineages from HSC clones which predominantly are generated in long-term expanded cultures (22). Studies have shown that human HSCs are significantly enriched with homologs to mouse genes with high RepopSig scores, including Hlf and EPCR, which can identify *ex vivo* expanded human HSCs (22,74).

In addition, cell sorting using microfluidic systems allows to separate target subpopulations in a specific cell population or target cell population from a mixture of different cell types based on mechanical properties such as size and deformability (e.g., enrichment of platelets from blood) (88). Current methods for cell isolation and enrichment benefit from different characteristics of cells and choosing a method or combination of methods can be determined based on the target cells and the aim of the isolation.

The role of signaling pathways in expansion of HSCs

Several signaling pathways affect hematopoiesis, such as Wnt and Notch signaling. Understanding the molecular mechanisms of signaling pathways provides insight in improving *ex vivo* expansion of HSCs (89,90).

Wnt signaling

The Wnt signaling pathway is a prominent pathway in embryonic development, self-renewal and differentiation of adult stem cells and tissue homeostasis. Wnt signaling can be divided into two different pathways including canonical (Wnt/β-catenin) and non-canonical (planar cell polarity; PCP) pathways. The canonical Wnt/β-catenin pathway regulates proliferation, survival and cell fate (91). In fact, interaction of Wnt ligands to the membrane receptors results in inactivation of the destruction complex and therefore high levels of β-catenin that is translocated to the nucleus to regulate gene expression (92,93) by interacting with TCF factors that are turned from transcriptional repressors into activators by binding to activated β-catenin (94).

On the other hand, the non-canonical pathway acts through Ca^{2+} signals, JNK kinases, Ryk receptors and regulates cell polarity, migration, differentiation and apoptosis (95). *Ex vivo*-expanded HSCs exposed to either non-canonical Wnt5a or Wnt3a proteins represented high rate of self-renewal in mouse cells (96,97). Recent studies

showed that paracrine Wnt5a/Prox1 signaling acts as a regulator of HSC regeneration in injury and aging (98). Also, activation of canonical Wnt signaling in β-catenin-expressing HSCs showed enhanced self-renewal in a mouse model (99).

Improving β-catenin stability by using prostaglandin E2 (PGE2) triggers canonical Wnt signaling and *ex vivo* expansion of human cord blood HSCs (100,101). Moreover, inhibiting both glycogen synthase kinase 3 (GSK-3β) and the mTOR pathway activates Wnt/β-catenin pathway and results in increased numbers of murine LT-HSCs *in vivo* (102).

Stem cell fate is dose-dependently regulated by Wnt signaling. In fact, higher concentration of Wnt results in differentiation of HSC to mature blood lineage, leading to exhaustion and loss of proliferation in irradiated mice (103). In contrast, low dosage of Wnt increases long-term repopulation capacity and better maintenance of immature cells (104). Wnt signaling increases immature colony forming cells in human and mouse progenitors. Also, inhibition of Wnt signaling led to decrease in number and viability of primitive HSCs *in vivo* and *in vitro* (49). BMI1 is a polycomb group protein and an essential regulator of HSC self-renewal and differentiation. This protein regulates gene expression of Wnt pathway target genes in HSPCs (90).

In addition to differentiation, activation of Wnt/β-catenin pathway enhanced apoptosis in lymphocytes and decreased quiescence in HSCs (105). Moreover, Wnt5a and Wnt5b are crucial for hematopoiesis and BM colonization of primitive HSCs from the fetal liver, mediated by β-catenin-independent signaling. These ligands activate the proliferation of progenitors, mediate myeloid development, maintain quiescent LT-HSCs and prevent hematopoietic exhaustion (106). However, in general this non-canonical Wnt signaling does not improve self-renewal in HSC, as Wnt3a (canonical Wnt signaling) does (107). Such studies have been conducted in mice and it remains to be seen if this applies to human HSCs as well. As Wnt signaling has a crucial role in hematopoiesis and its effects are dose-dependent, tightly regulating this signaling pathway may improve HSC *ex vivo* expansion.

Notch signaling

Notch signaling acts through interaction of Notch receptors and transmembrane ligands of Delta and Jagged families, and plays a crucial role in the regulation of cell fate, development and hematopoiesis (108). The function of Notch signaling varies during life; it is crucial in early HSC development during embryogenesis in mice, less active during HSC maturation, and not essential in maintenance of adult HSC in BM (89).

Notch signaling and Notch ligands have been shown to regulate HSC fate determination (109,110). Consistent with this, expression of the Notch ligand Jagged-1 is detected in BM stromal cells and osteoblasts. Long-term reconstitution was enhanced after HSC expansion with an immobilized engineered Notch ligand, $Delta1^{Ext-IgG}$, in which the extracellular domain of Delta1 is fused to the Fc portion of human IgG1. Conversely, targeted mutation in Notch 1 or Jagged 1 resulted in embryonic lethality and impaired hematopoiesis in mice. However, Notch 2, 3, 4 knock outs did not cause impairment of hematopoiesis (3,49,111).

Notch signaling maintains HSCs in an undifferentiated state and inhibits commitment to different lineages. Moreover, Notch signaling regulates the differentiation and function of mature T lymphocytes (112). While Notch signaling increases the number of functional, self-renewing HSCs in the niche, aberrant signaling has also been associated with different hematological malignancies, most prominently T-ALL (49). The non-canonical Notch ligand Dlk has an important role in the maintenance of HSC homeostasis and quiescence by inhibiting mitochondrial metabolic activity and cell division. This protein is highly expressed in progenitor cells, HSCs and LT-HSC in mice. Also, Dlk1 is a positive regulator of Notch signaling in adult mice HSCs (113). These studies suggest that the regulation of the Notch pathway can be a potential approach for the generation of HSC from induced pluripotent stem cells or endothelial stem cells for clinical applications, but caution should be exerted for malignant transformation.

Studies indicate a crosstalk between Notch and Wnt pathways leading to cell fate decisions. For instance, in skin and mammary glands, Notch promotes lineage commitment and differentiation, while Wnt signaling controls the maintenance of stemness (3). Also, inhibition of Notch signaling disrupted the ability of Wnt signaling to maintain HSCs in undifferentiated state (114). Inhibition of Wnt signaling affects HSC fate either by Notch or Wnt target genes (115). Kim and colleagues suggested that β-catenin activated stromal cells in the niche stimulate notch signaling in the surrounding HSCs (116). These studies provide the possibility to use supplements and ligands for activation of signaling pathways and gene expression needed for self-renewal and inhibition of differentiation in the *ex vivo* expansion of HSCs (117).

Other signaling

In addition to Wnt and Notch, effective transplantation of *ex vivo*-expanded HSCs in hosts requires regulation of crucial genes including HOXB4, and cell cycling genes such as p18, p21 (118,119). Homeobox genes are crucial in the regulation of early development of hematopoiesis. *In vivo* and *in vitro* studies showed that the transcription factor HOXB4 increased the self-renewal and *in vitro* expansion of HSCs (120). Moreover, HOXB4 maintained the differentiation capacity of HSCs into lymphoid and myeloid cells (49). Activation of HOXB4 also resulted in long-term maintenance of functional HSPCs (121). In addition, the molecular interaction between the receptor tyrosine kinase (RTK)—c-KIT—and its ligand stem cell factor results in HSC survival, proliferation, differentiation, mitogenesis, adhesion, migration, and homing. Site-directed point mutations in the c-KIT gene led to its constituent activation and increased HSCs proliferation (122). Also, soluble Sonic- hedgehog moderately increases self-renewal and proliferation of human HSCs via regulation of bone morphogenic protein (BMP) and cytokines. Low concentrations of BMP-4 maintain HSCs in medium, while it induces proliferation and differentiation of human hematopoietic progenitor cells (HPCs) (123,124).

Additionally, several reports have indicated the important role of Smad signaling pathway and ligands such as TGF-β in the regulation of HSC fate (115). TGF-β acts as a negative regulator of HSCs *in vitro*, while *in vivo*, TGF-β 1 receptor-deficient mice showed no effect on self-renewal and engraftment of HSCs (125,126). Also, Smad signaling activated by BMP has a key role in hematopoietic development in early hematopoiesis but not in adult hematopoiesis (115). Moreover, using animal models have revealed that Smad4-deficient HSCs have less proliferation capacity which indicates that Smad4 is critical regulator for self-renewal. These controversies may arise from other mechanisms involved in Smad pathway and its interaction with other signaling pathways including Wnt and/or Notch pathways (115).

Also, RepopSig analysis showed the role of sphingolipid signaling in the self-renewal of human HSCs in *ex vivo* expansion. Sphingolipid modulation inhibits *ex vivo* expansion of committed progenitors and increases self-renewal of HSCs. Moreover, modulation of sphingolipid signaling in *ex vivo* expansion culture activates proteostasis program which results in transition from quiescence to cellular activation via N-(4-hydroxyphenyl) retinamide (4HPR), and maintaining LT-HSC possessing the ability of serial repopulating in xenotransplantation (22,127).

Moreover, IL-6 and the Jak/Stat3 signaling pathway are critical mediators of HSC proliferation and function during inflammation. Hyperactivation of IL-6/Jak/Stat3 signaling pathway in chronic inflammation induces HSC expansion and reduces HSC fitness. However, inhibition of STAT3 activity was able to rescue HSC function (128). On the other hand, STAT1 is a critical regulator of interferon (IFN) signaling and is essential for HSC proliferation induced by IFN. Li and colleagues showed that in the absence of STAT1, HSC function and quiescence is disrupted, and blood cell regeneration is interrupted. These results indicated that STAT1 acts as a crucial regulator in homeostatic HSPCs, and self-renewal and maintenance of HSCs (129).

In addition, the Aryl hydrocarbon receptor (AhR) is a transcription factor responding to xenobiotic stress which also regulates the endogenous developmental functions during HSC maintenance and differentiation toward the various blood lineages via nuclear factor erythroid 2-related factor-2 (Nrf2), p53 (TRP53), retinoblastoma (RB1), and NF-κB signaling pathways (130). A study using inducible AhR knockout mice (iAHR KO) showed an increase in the number of multipotent progenitors and frequency of pregranulocyte/premonocyte committed progenitors (131).

Moreover, FMS-like tyrosine kinase 3 (FLT3) and c-KIT-knock out mouse models showed that type III RTK family including FLT3 and c-KIT regulate HSC self-renewal and early development (132,133). The $CD34^+$ cells and a subset of dendritic precursors in the BM express FLT3, and inhibition of this protein in $CD34^+$ cells inhibits cell expansion in a dose-dependent manner (134). These studies suggest the potential role of FLT3-Ligand (FLT3L) on the proliferation of primitive HPCs in expansion of HSCs *ex vivo*.

Understanding the importance of intrinsic pathways in regulation of proliferation and self-renewal of HSCs in response to internal and external signals gives a rationale to improve current approaches for *ex vivo* expansion of HSCs in order to acquire sufficient number of stem cells needed for genetic modification and therapeutic applications.

The effect of the niche on the expansion of HSCs

Several factors regulate characteristics of HSCs and determine self-renewal and differentiation. In addition to

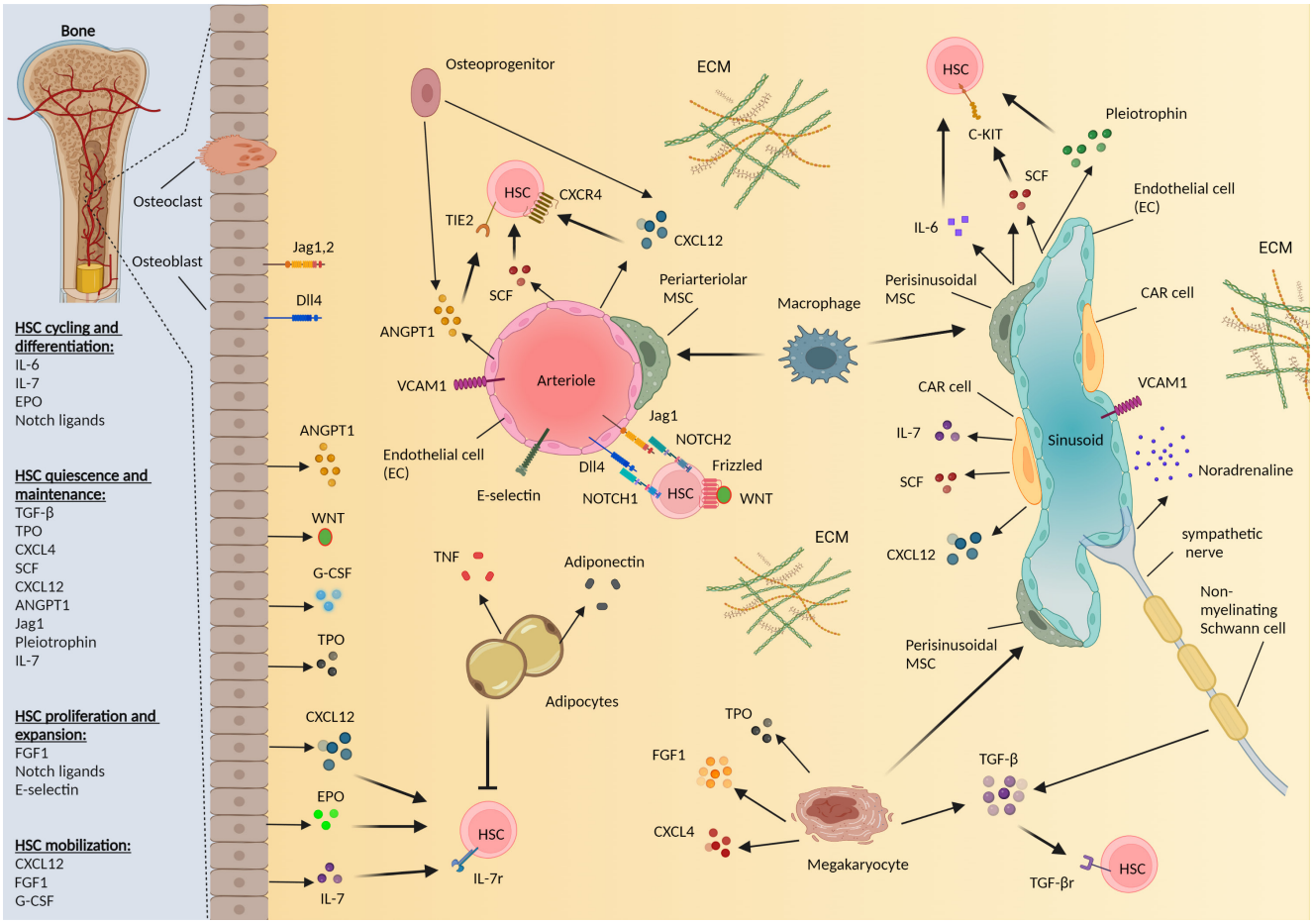

Figure 1 The hematopoietic bone marrow niche is heterogenous and includes various cell types with different interactions that influence self-renewal and expansion of HSCs (Created with BioRender.com). IL, interleukin; EPO, erythropoietin; TGF-β, transforming growth factor-β; TPO, thrombopoietin; CXCL, chemokine (C-X-C motif) ligand; ANGPT, angiopoietin; Jag, Jagged; FGF, fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; Dll, delta-like ligand; TIE1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; CXCR, C-X-C chemokine receptor; CXCL, C-X-C chemokine ligand; SCF, stem cell factor; MSC, mesenchymal stem cell; VCAM, vascular cell adhesion molecule; TNF, tumor necrosis factor; ECM, extracellular matrix; CAR cell, CXCL12-abundant reticular cells; HSC, hematopoietic stem cell.

intrinsic factors including transcription factors, cell cycle regulators and epigenetic modifiers, extrinsic factors from the niche provide a specialized microenvironment that regulates stem cell fate, self-renewal, proliferation rate, exhaustion and cell death (135). Self-renewal capacity of HSCs is diverse in different parts of the BM and also differs during aging (136,137). The niche is heterogenous and includes various cell types with different interactions that influence self-renewal and expansion of HSCs (see *Figure 1*). Therefore, identification of cell niches provides a promising way to regulate stem cell fate and hematopoiesis. However, this requires a suitable 3D model to study the various interactions between cell types that has not been fully optimized yet (138,139). In one example by Lee and colleagues, an engineered bio-mimic 3D model of the niche was designed using polymeric scaffold as synthetic extracellular matrix that enabled to study essential HSCs niche factors in a similar condition to the body (140). Also, using a 3D organoid modeling of extramedullary hematopoiesis led to investigating the potential of 3D hematopoietic organoid as a biomimetic niche to produce blood cells in laboratory. This model could support the survival and function of BM isolated from 5-FU treated mice, suggesting the efficiency of 3D model in investigating

the cell-cell and cell-extracellular matrix interactions (141).

BM cells in the niche include MSCs, osteoclast, adipocytes, megakaryocytes, and especially osteoblasts and vascular endothelial cells that secrete cytokines such as IL-3, IL-6, granulocyte-colony stimulating factor (G-CSF), macrophage stimulating factor (M-CSF), stromal derived factor (SDF), leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), osteoprotegrin (OPG), and receptor activator of NF-κB ligand (RANK-L) (49,136,138,142-145).

These factors regulate HSCs in different ways, for instance, OPG has an inhibitory effect on proliferation of HSCs, whereas G-CSF plays a significant role for maintenance and differentiation of HSCs towards the hematopoietic lineages (49,146,147). Also, SDF-1 interacts with its receptor called CXC chemokine receptor 4 (CXCR4) expressed on HSCs and regulates HSC maintenance and homing within the BM (148). These cells regulate different signaling pathways including Wnt and Notch, leading to activation or inhibition of HSC expansion. As an example, osteoblasts regulate HSC expansion by activation of bone morphogenetic protein receptor (BMPR1A) signaling (149). Also, activation of the p21 signaling pathway leads to quiescence of HSCs, while its inactivation leads to increased stem cell cycling that results in stem cell exhaustion in irradiated p21 deficient mice (49).

The vascular niches of HSCs including vascular endothelial cells (VECs) and perivascular mesenchymal cells release regulatory factors such as CXC motif chemokine ligand 12 (CXCL12), stem cell factor (SCF), VCAM1, Semaphorin, and Pleiotrophin that regulate HSCs proliferation, maintenance, and retention in adult BM. Hemogenic specification of VECs is promoted by BMP4 via BMP binding endothelial regulator (BMPER) which is also regulated by other niche factors including SCF, FGF, Noggin and sonic hedgehog. These extrinsic factors also modulate intrinsic factors such as SCL, RUNX1, miR-223, LMO2, and GATA1-6, thereby regulating the fate of hemogenic VECs (150).

In addition, BM macrophages collaborate with other cells in the HSC niche by initiating the expression of CXCL12 on osteoblasts and MSCs, thereby supporting the maintenance of HSCs in BM (151). Moreover, the role of stem cell receptor tyrosine kinase (Tie-2) and its ligand angiopoietin (Ang1) in tight adhesion of HSCs to their niche and expansion of the LT-HSCs was reported (152). Expression of Ang-1 on mature osteoblasts is higher than that on HSCs. Also, most of the side-population HSCs that express Tie-2 receptors adhered to the Ang-1 expressing cells, indicating the importance of osteoblasts in adhesion of HSCs in their niche. Consistent with this, a higher number of osteoblasts resulted in more LT-HSCs and interaction through Tie-2/Ang1, which is crucial for maintenance of quiescent HSCs (152). A recent study showed that overexpression of Ang1 disrupts megakaryopoiesis through activation of Ang1/Tie2 signaling, and impairs erythropoiesis via HSC senescence, inflammation, and disruption of the SDF-1/CXCR4 axis. These results showed that to maintain suitable HSC function, megakaryopoiesis and erythropoiesis, optimal activity of the Ang1/Tie2 pathway and/or an appropriate level of Ang1 (e.g., a local supplementation of rhCOMP-Ang1, not overexpression of Ang1 gene) are required (153).

Inflammatory regulators also affect hematopoiesis and HSCs development. Interferon regulatory factor 1 (IRF1) is the crucial HSC regulator which modulates various inflammatory signaling pathways and its loss results in impaired self-renewal of HSC and an activated stress-induced cell cycle. Moreover, IRF1-deficient HSCs showed reduced megakaryocytic/erythroid priming and antigen presentation (154).

Interaction of HSCs and niche cells through different types of integrins including very late antigens [VLA-4 (α4β1-integrin) and VLA-5 (α5β1-integrin)], and LFA-1 (leukocyte function-associated antigen-1, αLβ2 integrin, CD11a/CD18) are crucial for survival and engraftment of the HSCs (155). Additionally, extracellular ionic calcium in endo-osteal sites interacts with calcium-sensing receptor (CaR) on HSCs. A knockout study revealed that that $CaR^{-/-}$ HSCs lack the ability to adhere to collagen I secreted by osteoblasts in BM (152). Additionally, oxygen partial pressure has a crucial role the in maintenance of the primitive HSCs. Transplantation of HSCs in irradiated mice showed that HSC culture in low oxygen is necessary for maintenance of multi-lineage engraftment potential. Under low-oxygen conditions, survival signaling pathways are mainly activated by phosphorylation of AKT downstream to the VLA-4 and VCAM-1 interactions (156,157). These findings show the importance of the niche and the interaction of HSCs with various cells with regards to their function and viability, which can be applied to provide a suitable microenvironment for enhancing and maintaining homing potential (e.g., Fucosylation of HSCs before expansion, CXCR4-SDF-1 axis partnership and homing gene involvement) in the long-term expansion of HSCs (158).

Novel and traditional *ex vivo* approaches

Successful engraftment requires sufficient numbers of HSCs transplanted into the host. Reports have shown that at least 2×10^8 nucleated bone marrow cells/kg human body weight is generally needed for efficient transplantation, of UCB, this corresponds to an often used minimal number of 1.0×10^6 $CD34^+$ cells/kg (159). As HSCs isolated from UCB is in most cases not enough for a successful transplantation, it is important to expand these cells *in vitro*. However, it has been a great challenge to achieve efficient protocols for long-term maintenance and enhanced number of functional HSCs in culture. Balancing various biological and chemical agents in culture medium can affect the ratio of processes leading to various cell fates including quiescence, self-renewal, apoptosis, differentiation and migration in order to expand HSCs for a longer time. Studying the molecular mechanisms involved in self-renewal and differentiation of HSCs enables to provide the optimal environment for long-term expansion. Using new techniques such as single-cell RNA-seq (scRNA-seq) to analyze the naïve and stimulated HSCs gives a deeper insight in the various phenomena such as stemness (160). Moreover, development of microfluidic systems enables to create similar patterns to that of the niche to investigate the influence of different biophysical factors and niche cell paracrine signaling on cell fate decision. These approaches provide optimization of culture medium for expansion of HSCs (161). Different protocols have been developed for long-term expansion of human HSCs, using various biomolecules and co-culture methods to promote self-renewal and homing, and inhibit differentiation and apoptosis.

Hematopoietic cytokine treatment is considered a traditional method for *ex vivo* expansion of HSCs in which different cytokines and growth factors including SCF, FLT3L, thrombopoietin (TPO), IL-3 or IL-6 are used to support proliferation and survival of HSPCs (43,162,163). Also, co-culturing HSCs with stromal cells or osteoblastic feeder layers as a 2D hematopoietic niche with/without cytokines provides a microenvironment for the expansion of HSCs (164-172). Still, it is difficult to determine the optimal amount of cytokines needed in culture. Also, it can be complicated to separate feeder cells from HSCs at the end of the harvesting (172).

Using small molecules in culture medium is a cost-effective, rapid and safe method for expansion of HSPCs. However, they have to be carefully tested because they may cause unexpected or irreversible side effects after engraftment. Various compounds used in *ex vivo* expansion of HSCs maintain the self-renewal program including SR1, UM171, P18IN003, P18IN011, CHIR99021 + Rapamycin, BIO, NR-101, 5azaD/TSA, GAR, and TEPA by acting through either the activation and/or maintenance of cell proliferation pathways or the inhibition of differentiation and cell death pathways (132,173). These compounds also regulate signaling pathways including Wnt, Notch, Shh/BMP, TGF-β/smad4, pleiotrophin, FGF, IGF and Angiopoietin-Like Proteins (ANGPTLs) pathways (173).

Moreover, various microRNAs (miR-125a/b) and proteins such as transcription factors (e.g., HOX family, GATA2, GFI1, AML1, JUNB, NF-κ), chromatin-associated factors (e.g., BMI1, EZH2, DMNT3A, CBX7), cell cycle regulators (INK4/p18, WAF1/p21, KIP1/p27, KIP2/p57, PTEN, MYC, FBXW7) modulate self-renewal and expansion of HSCs (173). Treatment with different agents acting through these proteins and signaling pathways have increased HSCs self-renewal rate in *ex vivo* cultures, however, there has been usually unfavored differentiation and exhaustion during culture which has been a real limitation in clinical trials (174). As symmetrical division of HSCs results in expansion of these cells without further differentiation, many efforts have been made to create *in vitro* environment promoting symmetrical HSC division. Li and colleagues suggested that the small molecule inhibitor of p18, 005A, upregulated Notch signaling genes including HEY1, HES1, HES5, NOTCH1, and FOXO3, thereby increasing the frequency of symmetric cell division and delaying cell division, leading to retaining the stemness and increasing LT-HSC self-renewal in *ex vivo* expanded HSCs (175).

Epigenetic regulation such as DNA methylation and post-translational histone modifications may increase self-renewal and inhibit unfavored differentiation. Therefore, various small molecule inhibitors of histone deacetylase (HDAC) and DNA methyltransferase have been developed to increase the efficiency of *ex vivo* expansion. Some of the compounds such as valproic acid (VPA) and diethylaminobenzaldehyde (DEAB) mainly act through the inhibition of lineage commitment differentiation (173). In addition, inhibition of HSC apoptosis using agents such as zVADfmk/zLLYfmk and 5-HT has a crucial role in increasing *ex vivo* expansion of HSCs (173). Moreover, increasing HSC homing has been considered as a potential method to improve engraftment using compounds such as nicotinamide (NAM) by inhibiting Sirtuin1 (SIRT1) class III nicotinamide adenine dinucleotide (NAD)-dependent HDAC and PGE2 via interaction with Wnt signaling (173).

Development of serum-free media makes it easier to translate HSC expansion protocols to GMP conditions needed for clinical application in humans (43). As serum used for expansion of stem cells may contain unknown factors, contaminants, and lot-to-lot variability, it results in clinical restrictions/issues. Also, different serum sources may have varying effects in terms of HSC differentiation (176). Thus, it is essential to optimize human HSC expansion through regulation of different signaling pathways in serum-free culture medium. Therefore, several cytokine- and albumin-free culture methods have been proposed for long-term expansion of HSCs. In a recent study, a cytokine- and albumin-free system was optimized using 740Y-P (1 μM), butyzamide (0.1 μM) and UM171 (70 nM) to expand HSCs by activating PI3K and TPO signaling while repressing megakaryocyte differentiation, respectively. In the presence of the synthetic polymer polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG), the compounds revealed significantly enhanced expansion of functional HSPCs for at least one month. This functional study supported serial engraftment in xenotransplantations and provided a new platform to expand and study HSCs (177).

3D-culture mimics the actual microenvironment of HSCs in BM and may reveal different physical factors such as temperature and pressure on cell fate decision. Therefore, a microfluidic system provides a biomimetic condition to culture and analyze cells in a controlled manner. This technique also offers optimal conditions for efficient symmetrical division and protects against contamination. Engineered HSPCs niches including Matrigel, alginate and GelMA (methacrylamide-functionalized) provide support to expand HSCs *in vitro* with or without adding cytokines (178-180).

Another technique to expand HSCs in the laboratory called automatic fed-batch media dilution approach that controls inhibitory feedback signals during culture. Using this technique, researchers expanded SCID repopulating cells 11-fold while maintaining the self-renewing, multilineage repopulating ability (179). Also, the fed-batch negative feedback regulation system, artificial 3D niches with ECM proteins and functionalized hydrogels, and also novel culture medium such as F12 PVA-based cultures have been promising to expand HSCs for clinical application (22,178). Moreover, Xu and colleagues used low-intensity pulsed ultrasound (LIPUS) strategy to regulate HSPCs fate decision and found increased proliferation of HSPCs and colony formation after ultrasound pulses. Also, it could promote cell differentiation of HSPCs to early erythroid progenitors without affecting the proportion of $CD34^+$ and $CD14^+$ cells (180).

Recently, Igarashi and colleagues demonstrated that optimizing O_2 concentration (physioxia) can improve the selectivity of PVA-based mouse HSC expansion cultures. They also showed that HSC-selective cultures reduce GvHD-causing T cells, while it is highly selective for HSCs over more mature hematopoietic cell types. This can be translated to human implications to develop selective HSC expansion systems for allogeneic HSCT (181).

Several potential compounds have been approved to be tested for clinical studies including SR1 (AhR antagonist), tetraethylenepentamine (TEPA, copper chelator), and Nicord (SIRT1 inhibitor) (173,182). A cohort-controlled study showed that transplantation of StemEx (or so called, Carlecortemcel-L, *ex vivo* expanded allogeneic UCB cells using cytokines and TEPA led to 225% expansion of the $CD34^+$ HSCs (183). Also, a single-arm, phase 1–2 safety and feasibility study showed that HSC expansion treated by UM171 as a feasible and safe method, increased HSC cell renewal and reconstitution ability without compromising engraftment (184). Moreover, a clinical trial in patients with hematological malignancies showed 89-fold expansion of $CD133^+$ HSCs in presence of IL-6, TPO, SCF, FLT3L and TEPA with a well-tolerated transplantation compared to control group (43,185-187). Also, a recent multicenter long-term follow-up of allogeneic hematopoietic cell transplantation with Omidubicel (a UCB-derived cellular therapy product expanded using NAM) showed long-term trilineage hematopoiesis, immune competence, and graft durability in extended follow-up (188). Most of the protocols are being investigated in preclinical and clinical studies (44).

Regarding the necessity for long-term expansion of HSCs, many *ex vivo* culture protocols have been developed. However, most of the current methods are not cost-effective or have insufficient efficiency. Besides, using the current protocols usually modify cell-fate decision temporarily in which cell fate changes over time (189). Moreover, the majority of these protocols support only short-term expansion of HSCs, which highlights the importance of developing protocols that support *ex vivo* expansion of HSCs in long-term cultures (190). *Tables 1,2* summarize *ex vivo* human HSC expansion protocols that have been reported during the last ten years; additionally, *Table 1* consists of studies that reported *in vivo* HSC functionality by demonstrating serial engraftment in xenotransplantation models.

Table 1 Summary of protocols for *ex vivo* expansion of human HSCs from the last 10 years (clinical trials and *in vivo* studies)

Expansion method	Findings	Source of human HSC	Mechanism	Reference
Co-culture with hFLSECs-E4orf1	Expansion of HSCs	UCB HSC	Growth factors and Notch signaling	(168)
Co-culture with M2-MΦs	(I) Induction of self-renewal; (II) expansion of HPCs and LT-HSCs; (III) multilineage engraftment	UCB HSC	(I) Induction of autophagy; (II) production of regulatory factors; (III) expression of adhesion molecules	(169)
Co-culture with M-OST cell	(I) Induction of homing; (II) expansion of HSCs	UCB HSC	(I) Wnt and β-catenin signaling pathways; (II) Notch-dependent pathway; (III) expression of *CXCR4*; (IV) reduced SDF-1/CXCR4 signaling	(164)
Co-culture with fetal liver $CD34^{lo}CD133^{lo}$ cells	(I) Expansion of HSCs; (II) induction of self-renewal	Fetal liver and UCB HSC	Production of SCF, IGF2, CXCL12, and ANGPTLs	(171)
Co-culture with hkirre-AFT024 cells	(I) Expansion of HSCs; (II) induction of self-renewal	UCB HSC	Upregulation of the expressions of *Wnt-5A, BMP4, DLK1, SDF-1, FL, SCF, N-cadherin*, and *VEGF,* and downregulation of *TGF-β*	(165)
3D scaffold of fucosylated HSCs	(I) Promotion of homing; (II) expansion of HSCs	UCB HSC	Increased expression of homing genes, e.g., integrins and other adhesion molecules	(191)
3D zwitterionic hydrogel culture	(I) Expansion of $CD34^+$ cells and LT-HSCs; (II) increased self-renewal	UCB and BM-derived HSPCs	Inhibition of excessive ROS production via suppression of O_2-related metabolism	(178)
Treatment with UM171	Expansion of HSCs	UCB HSC	(I) Enhancing the human LT-HSC self-renewal machinery; (II) suppression of transcripts associated with erythroid and megakaryocytic differentiation; (III) increasing the expression of *PROCR/CD201/EPCR*; (IV) NF-κB activation and protein C receptor-dependent ROS detoxification; (V) decreasing the apoptosis; (VI) regulation of the H3K4me2 and H3K27ac marks; (VII) proteasomal degradation of LSD1-CoREST repressor complex; (VIII) activation of the CRL3 complex	(74,184,192-195)
Treatment with UM171	(I) Retained the multi-lineage differentiation capacity of LT-HSCs and long-term reconstitution; (II) expansion of HSCs; (III) suppression of erythroid and megakaryocytic differentiation	mobilized PB $CD34^+CD45R$ cells	(I) Upregulation of the surface molecules genes such as *TMEM183A* and *PROCR/CD201/EPCR*; (II) suppression of expression of inflammatory mediated chemokine; (III) upregulation of HSC-, mast cell-specific genes and non-canonical Wnt signaling related genes	(192,196)
Treatment with UM171 and SR1	(I) Expansion of progenitor cells; (II) suppression of the differentiation	UCB HSC	Downregulation of AhR target genes, e.g., *CYP1B1, CYP1A1*, and *AhRR*	(192,197)
Treatment with 740Y-P + butyzamide + UM171, supported by PCL-PVAc-PEG	(I) Expansion of functional HSPCs; (II) clonal expansion of HSCs	UCB-derived $CD34^+$ cells	(I) Activation of the PI3K-AKT signaling by 740Y-P; (II) activation of the TPO signaling by butyzamide; (III) suppression of transcripts associated with erythroid and megakaryocytic differentiation; (IV) activation of mitochondria	(177)
Treatment with NAM or EX-527	(I) Improvement in homing and engraftment; (II) inhibition of differentiation; (III) expansion of HPCs	UCB HSC	(I) Inhibition of the SIRT family of the NAD-dependent class III HDAC; (II) inhibition of SIRT1 and NAD-dependent ribosyl transferases; (III) reduction of *p21* expression	(198,199)
Treatment with LSD1 inhibitor	(I) Expansion of HSPCs; (II) increased the long-term repopulating HSCs	UCB HSC	Targeting and depletion of the LSD1-containing CoREST complex	(200,201)
Treatment with resveratrol	(I) Expansion of functional $CD34^+$ cells; (II) preserved more primitive HSCs	UCB HSC	(I) Increased the scavenging of ROS and decreased intracellular ROS level; (II) decreased the percentages of apoptotic cells	(202)

Table 1 *(continued)*

Table 1 *(continued)*

Expansion method	Findings	Source of human HSC	Mechanism	Reference
Treatment with 005A	(I) Retained the stemness; (II) increased LT-HSC self-renewal	UCB HSCs	Delayed cell division and activated both the Notch signaling pathway and expression of transcription factor *HOXB4*	(175)
Treatment with GDNF/GFRα1	(I) Expansion of HSC; (II) long-term engraftment; (III) improved progenitor and LT-HSC function	UCB HSC	(I) Activation of RET; (II) reduced the apoptosis; (III) sustained cellular growth, resistance to stress, and improved cell survival; (IV) activation of PKB, ERK1/2, NF-κB, and p53 pathways	(203,204)
Treatment with Notch ligands	(I) Induction of self-renewal; (II) expansion of HSC; (III) promoted HSC engraftment	BM $CD34^+$, Lin-, $CD38^-$ cells	(I) Notch signaling pathway; (II) influence on stem cell niches	(205)
Serum-free culture with certain cytokines and immobilized anti-LILRB2	Expansion of HSC	UCB HSC	Prevented the internalization of LILRB2 and prolonged the receptor activation	(206)
Treatment with VPA	(I) Induction of proliferation and self-renewal of HSC; (II) increased SCID repopulating cells; (III) increased stemness; (IV) established the multilineage hematopoiesis	UCB HSC	(I) Inhibition of HDACI; (II) increased the expression of pluripotent genes, including *SOX2, OCT4, NANOG*, and *Z1C3*; (III) cell cycle progression of HSC accompanied by a downregulation of *p21cip-1/waf-1*; (IV) activation of Wnt signaling pathway and inhibition of GSK-3β; (V) upregulation of *HOXB4*, a target gene of Wnt signaling; (VI) increased ALDH activity; (VII) enhanced expression of *CD90, c-Kit (CD117)*, *integrin α6 (CD49f)*, and *CXCR4 (CD184)*; (VIII) increased the expression of *KDR (VEGF2), AC133, c-kitR, GATA1*, and *HOXB4*	(207,208)
Treatment with chromatin modifying agents 5azaD and TSA	(I) Expansion of HSC; (II) induction of self-renewal; (III) increased the $CD34^+CD38^-$ cell population; (IV) decreased the cell division rate and cell cycling; (V) maintained hematopoietic potential	UCB HSC	(I) Inhibition of DNA methyl transferase; (II) upregulation of the self-renewal related genes, such as *HOXB4*, *BMI-1*, *GATA2*, and cell cycle regulating genes, such as *p21* and *p27*; (III) downregulation of the proliferation related gene *C-MYC*	(207)
Treatment with Azole-based small molecule C7	(I) Expansion of HSC; (II) increased engraftment	UCB HSC	Inhibition of p38-MAPK along with casein kinase-1	(209)
Treatment with GAGs	Increased engraftment	UCB HSC	Modulation of growth factor effects in the hematopoietic niche	(210)
Treatment with TEPA (a Cu chelator)	(I) Expansion of HSC; (II) inhibition of differentiation; (III) increased long-term self-renewal; (IV) increased initial neutrophil engraftment and reconstitution capacity; (V) increased the number of CFUs	UCB HSC	(I) Copper elimination; (II) reduction in the activity of cytochrome oxidase; (III) reduced the oxidative stress; (IV) deactivation of S-adenosylhomocysteine hydrolase and protein arginine methyltransferase 1	(211)
Treatment with StemRegenin1 (SR1) (an AhR antagonist)	(I) Induction of homing; (II) expansion of $CD34^+$ cells; (III) rapid recovery of neutrophils and platelets *in vivo*; (IV) increased multipotent progenitors and erythroid/megakaryocytic	Mobilized PB $CD34^+$ cells	(I) Induction of *CXCR4*; (II) inhibition of AhR	(212)

Table 1 *(continued)*

Table 1 *(continued)*

Expansion method	Findings	Source of human HSC	Mechanism	Reference
Treatment with CH223191 (an AhR antagonist)	(I) Enhanced the expansion of phenotypic functional HSCs; (II) preserved the stemness; (III) triggered MK lineage-skewed differentiation; (IV) expansion of the $CD41^+$ MK-lineage populations	UCB HSPCs	(I) Activation of MK/platelet signaling pathways in HSPCs; (II) increased gene expression related to platelet activation and formation, coagulation cascade and complement; (III) blockade of AhR, resulting in HSPCs expansion	(213)
Treatment with BC (a phosphatase inhibitor of STS1/STS2)	(I) Increased the multipotency and hematopoietic function of HSPCs; (II) enhanced the colony-forming capacity; (III) expansion of primitive HSCs	UCB $CD34^+$ cells	(I) Suppression of TCR signaling; (II) downregulation of FLT3 and c-KIT signaling, resulting in decrease in self-renewal and differentiation of HSPCs	(214)
Treatment with GSK-3 inhibitor	(I) Accumulation of late dividing cells enriched with primitive progenitor cells; (II) retained the ability for sustained proliferation of $CD34^+$ cells; (III) promoted the engraftment	UCB HSC	(I) Downregulation of cyclin D1 and upregulation of β-catenin, c-myc, CDKi p57; (II) modulation of the genes related to stem cell function including *ADAMDEC1, ANGPT2, RARRES2*, and *HMOX*; (III) delayed cell cycle progression by increasing cell cycle time; (IV) increased the expression of genes regulating Notch and Tie2 signaling	(215)
Treatment with CHIR99021 (GSK-3 inhibitor) + SB431542 (TGF-β inhibitor) + bpV (PTEN inhibitor)	(I) Increased the number of $CD34^+CD38^-$ cells; (II) increased the clonogenic potential; (III) increased the engraftment	UCB HSCs	Upregulation of *CXCR4*, *HOXB4*, *GATA2*, and *CD34* genes	(216)
Treatment with 10074-G5 (c-Myc inhibitor) + TUDCA + L-NIL (i-NOS inhibitor)	(I) Increased $CD34^+$ and $CD133^+$ HSC; (II) increased efficiency of CRISPR/CAS9 mediated gene editing	UCB HSCs	(I) Downregulation of CDKi gene expression; (II) upregulation of major HDR modulator Rad51 expression; (III) modulation of HSC glycolytic gene expression	(217)
Treatment with BET inhibitor CPI203	(I) Enhanced the expansion of phenotypic HSCs and megakaryocytes; (II) supported megakaryocyte maturation; (III) enhanced platelet production; (IV) retained the cell viability and self-renewal; (V) improved engraftment and multilineage reconstitution	$CD133^+$ human UCB cells	(I) Increased differentiation from committed progenitors; (II) increased β-catenin/Wnt signaling	(218)

HSCs, hematopoietic stem cells; hFLSECs-E4orf1, human fetal liver sinusoidal endothelial cells, expressing the adenoviral E4orf1 gene; M2-MΦs, (M2)-polarized macrophages; M-OST, mesenchymal stromal cell (MSC)-derived osteoblasts; PCL-PVAc-PEG, polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol; NAM, nicotinamide; LSD1, lysine specific demethylase; VPA, valproic acid; 5azaD, 5-aza-2'-deoxycytidine; TSA, trichostatin A; GAGs, glycosaminoglycans; TEPA, tetraethylenepentamine, known as StemEx; BC, Baicalein; GSK-3, glycogen synthase kinase 3; TGF-β, transforming growth factor-β; PTEN, phosphatase and tensin homolog; TUDCA, tauroursodeoxycholic acid; BET, Bromodomain and extraterminal domain; LT-HSCs, long-term hematopoietic stem cells; HPCs, hematopoietic progenitor cells; HSPCs, hematopoietic stem and progenitor cells; SCID, severe combined immunodeficiency; MK, megakaryocytes; UCB, umbilical cord blood; BM, bone marrow; PB, peripheral blood; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; CRL3 complex, Cullin-RING ubiquitin ligase 3 complex; SIRT, Sirtuin; NAD, nicotinamide adenine dinucleotide; HDAC, histone deacetylase; RET, receptor tyrosine kinase; PKB, protein kinase B; ERK1/2, extracellular signal-regulated kinase 1/2; NF-κB, nuclear factor kappa B; TCR, T cell receptor; CDKi, cyclin-dependent kinase inhibitor.

Concluding remarks

HSCT has been the golden standard for many uncurable hematological disorders. However, HSCs acquired from UCB, as the first-line HSCT therapy, often provide limited cell numbers and thus are not adequate for an efficient engraftment. *Ex vivo* expansion of human HSCs while maintaining stem cell properties facilitates effective engraftment by increasing the amount of functional HSCs. During *ex vivo* expansion, proliferation of self-renewing cells needs to be stimulated and differentiation inhibited. This can be achieved using the correct combination of

Table 2 Summary of protocols for *ex vivo* expansion of human HSCs from the last 10 years (*in vitro* studies)

Expansion method	Findings	Source of human HSC	Mechanism	Reference
Co-culture with MSCs	(I) Induction of self-renewal; (II) expansion of HSCs	UCB HSC	(I) Cell-to-cell interaction; (II) cytokine secretion; (III) Wnt and β-catenin signaling pathways; (IV) higher expression of *CXCR4, EpHB4, FOXO1, Myc* and *HPRT1*, and a lower expression of *HOXC8, SDF-1, SOX17* and *SOX9* genes	(219,220)
3D scaffold of fucosylated HSCs	(I) Increased homing genes; (II) expansion of HSCs	UCB HSC	Increased expression of homing genes, e.g., integrins and other adhesion molecules	(221)
3D alginate-gelatin hydrogel scaffold	(I) Expansion of HSCs; (II) increased the proliferation; (III) maintained the stemness	UCB HSC	(I) Facilitating the exchange of the nutrients and oxygen; (II) increasing the expression of *VLA-4* and *VLA-5*	(222)
3D scaffold of gallic acid grafted-chitosan (2c) + osteoblasts	(I) Expansion of HSCs; (II) increased the colony forming potential; (III) maintained self-renewal	UCB HSC	(I) Protection against apoptosis and promoted HSCs division; (II) biomimetic HSCs niche	(223)
3D scaffold of hydroxyapatite coated zirconium oxide	(I) Expansion of HSCs; (II) maintained the stemness; (III) retained multilineage differentiation potential	UCB HSC	Biomimetic HSCs niche	(224)
PCL scaffold coated with fibronectin	(I) Expansion of HSCs; (II) increased proliferation; (III) increased homing genes; (IV) maintained self-renewal	UCB HSC	Biomimetic HSCs niche	(225)
3D culture based on alginate hydrogel	(I) Expansion of HSCs; (II) maintained the cellular phenotype	BM $CD34^+$ HSCs	(I) Reduced electron capture and cellular oxygen stress; (II) increased cell survival by upregulating TNF, and NF-κB signaling pathways	(172)
Co-culture system based on WJ-MSC sphere under hypoxia condition	(I) High proliferation; (II) maintained self-renewal; (III) maintained stemness; (IV) enhanced homing potential	UCB HSC	Increased expression of *EPCR, NS, Nfix, CXCR4* and *VLA-4*	(6)
Treatment with UM171	(I) Retained the multi-lineage differentiation capacity of LT-HSCs and long-term reconstitution; (II) expansion of HSCs; (III) suppression of erythroid and megakaryocytic differentiation	Mobilized PB $CD34^+$ cells	(I) Upregulation of the surface molecules genes such as *TMEM183A* and *PROCR*/*CD201*/*EPCR*; (II) suppression of expression of inflammatory mediated chemokine; (III) upregulation of HSC-, mast cell-specific genes and non-canonical Wnt signaling related genes	(56)
Treatment with UM171	(I) Expansion of HPCs; (II) increased NK progenitor production	hPSCs-derived HPCs	(I) Increased the number of cycling $CD34^+CD45^+$ cells; (II) decreasing the apoptosis	(226)
Treatment with Resveratrol	(I) Expansion of functional $CD34^+$ cells; (II) preserved more primitive HSCs	UCB HSC	(I) Increased the scavenging of ROS and decreased intracellular ROS level; (II) decreased the percentages of apoptotic cells	(227)

Table 2 *(continued)*

Table 2 *(continued)*

Expansion method	Findings	Source of human HSC	Mechanism	Reference
Treatment with TEPA (a Cu chelator)	(I) Expansion of HSC; (II) inhibition of differentiation; (III) increased long-term self-renewal; (IV) increased initial neutrophil engraftment and reconstitution capacity; (V) increased the number of CFUs	UCB HSC	(I) Copper elimination; (II) reduction in the activity of cytochrome oxidase; (III) reduced the oxidative stress; (IV) deactivation of S-adenosylhomocysteine hydrolase and protein arginine methyltransferase 1	(228)
Treatment with small molecule CAPE	(I) Expansion of HSC; (II) increased total CFU	UCB HSC	Upregulation of *SCF* and *HIF1-α* genes expression	(229)
Treatment with GSK-3 inhibitor	(I) Accumulation of late dividing cells enriched with primitive progenitor cells; (II) retained the ability for sustained proliferation of $CD34^+$ cells	UCB HSC	(I) Downregulation of cyclin D1 and upregulation of β-catenin, c-myc, CDKi p57; (II) modulation of the genes related to stem cell function including *ADAMDEC1, ANGPT2, RARRES2*, and *HMOX*; (III) delayed cell cycle progression by increasing cell cycle time; (IV) increased the expression of genes regulating Notch and Tie2 signaling	(230)

HSCs, hematopoietic stem cells; MSCs, mesenchymal stromal cells; PCL, polycaprolactone; WJ, Wharton jelly; TEPA, tetraethylenepentamine; CAPE, caffeic acid phenethyl ester; GSK-3, glycogen synthase kinase 3; LT-HSCs, long-term hematopoietic stem cells; HPCs, hematopoietic progenitor cells; NK, natural killer; CFUs, colony-forming units; UCB, umbilical cord blood; BM, bone marrow; PB, peripheral blood; hPSCs, human pluripotent stem cells; VLA, very late antigen; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; SCF, stem cell factor; HIF1-α, hypoxia inducible factor 1 subunit alpha; CDKi, cyclin-dependent kinase inhibitor.

cytokines, growth factors and environmental factors such as cell-cell interactions and oxygen concentration, but has been a great challenge for decades. For clinical applications, the preferred expansion would be achieved without the use of animal-derived products or biological factors including cytokines with lot-to-lot variation. Therefore, completely chemically-defined protocols to activate and inhibit the appropriate signaling pathways may be the key to successful HSC expansion in the future (177). Current methods for *ex vivo* expansion of human HSCs aim to provide conditions similar to the HSC niche in the BM, mainly by using different cytokines, growth factors, signaling molecules, transcription factors, copper chelators, biosynthetic scaffolds and stromal cells. Ultimately, technical and clinical considerations determine the choice of the method or combination of the methods used for expansion and engraftment of HSCs.

Some of the above-mentioned protocols to expand HSCs along with genetic modification have been successfully applied in clinical trials and some others are being studied in preclinical and clinical studies. However, more investigation and validation to develop current HSC expansion protocols would greatly benefit clinical HSC transplantations, while characterization and isolation of true HSCs would enable downscaling of costly gene therapy procedures.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://sci.amegroups.com/article/view/10.21037/sci-2023-016/coif). All authors report grants from EU H2020 and ZonMW. Besides, FJTS is a SAB member of GOSH, thymus program, institute Imagine, Gt program and a coordinator and scientific leader of the RECOMB and CURE4LIFE consortia. The authors

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