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EDITORIAL

# *Ex vivo* expansion of hematopoietic stem and progenitor cells: Recent advances

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## Abstract

Hematopoietic stem cells (HSCs) have become the most extensively studied stem cells and HSC-based cellular therapy is promising for hematopoietic cancers and hereditary blood disorders. Successful treatment of patients with HSC cells depends on sufficient number of highly purified HSCs and progenitor cells. However, stem cells are a very rare population no matter where they come from. Thus, *ex vivo* amplification of these HSCs is essential. The heavy demands from more and more patients for HSCs also require industrial-scale expansion of HSCs with lower production cost and higher efficiency. Two main ways to reach that goal: (1) to find clinically applicable, simple and efficient methods (or reagents) to enrich HSCs; (2) to find new developmental regulators and chemical compounds in order to replace the currently used cytokine cocktails for HSCs amplification. In this Editorial review, we would like to introduce the current status of *ex vivo* expansion of HSCs, particularly focusing on enrichment and culture supplements.



**Key words:** Hematopoietic stem cell; *Ex vivo* expansion; Serum-free culture; Cell surface markers; Enrichment; Stem cell isolation

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## INTRODUCTION

The early evidence and definition of hematopoietic stem cells (HSCs) came from studies of people exposed to lethal doses of radiation in 1945 and the pioneering studies by Till and McCullock[1]. But it took until late 1980s and early 1990s for researchers to show the isolation and characterization of HSCs from human and rodents[2-4]. Since then, HSCs have become the most extensively studied stem cells (SCs), and HSC-based cellular therapy is promising to treat hematopoietic cancers, such as leukemia and lymphoma, and hereditary blood disorders such as inherited anemia.

HSCs are a small sub-set of SCs generated by the bone marrow (BM) niche which are essential for self-renewal and derivation of whole blood systems. Therefore, any deficiency of HSCs will cause serious, mostly fatal, outcomes as seen in patients of hematopoietic malignancies treated with high doses of chemotherapy and radiation. To treat patients with these complica-

tions, BM transplantation has been widely used since 1980s. Although whole BM transplantation used to be the only clinically viable option, increased numbers of *ex vivo*-expanded HSCs isolated from either the BM, or peripheral blood or cord blood cells are being used for current transplantation procedures in clinics. Currently, approximately 3000 clinical trials can be found in US with 1/3 of trials focused on the applications of HSCs for leukemia treatment (http://www.clinicaltrials.gov/). Hereafter we use the term HSCs to represent both HSCs and hematopoietic progenitor cells. We should emphasize that cells used in clinical settings are HSC-enriched but do contain progenitors. Thus, the term "HSCs" used throughout this review includes the $CD34^+$ fraction. HSCs only constitute a very minor fraction of the whole blood or BM cells, 1/10000 for BM SCs and 1/100000 for peripheral blood SCs. Thus, it is absolutely essential to develop large-scale culture methods for *ex vivo* expansion. Although HSC culture is the most advanced system among SC culture, further improvement of *ex vivo* culture for HSC expansion is important. Particularly, animal product (*e.g.*, serum)-free cell culture media will be one of the most important factors to be considered. In this short review, we would like to introduce the current status of *ex vivo* culture of HSCs and the most recently emerging reagents. Although culture devices (such as bioreactors) are another important component in *ex vivo* expansion, the main purpose of this Editorial review is to concisely summarize the new advances in enrichment of HSCs and *ex vivo* expansion of HSCs. For additional information on culture devices, we recommend reading Nielsen's excellent review[5]. We also suggest a few recent review articles that nicely and concisely summarized recent advances in the area of *ex vivo* expansion of hematopoietic stem/progenitor cells[6,7].

## SOURCES OF HSC

There are potentially three main sources of haematopoietic stem cells for transplantation - BM, peripheral blood stem cells and umbilical cord blood stem cells. Table 1 summarizes advantages and disadvantages of 3 sources. Over the past 40 years, BM transplantation and stem cells isolated from BM has been widely used to treat numerous malignant and nonmalignant diseases. In spite of increasing therapeutic applications utilizing G-CSF to mobilize HSCs into circulation and $CD34^+$ cells are collected[8], BM cell isolation is still a widely accepted HSC source worldwide[9,10]). However, isolation of HSCs from BM is not a comfortable experience for donors, and it carries the risk of infection transmission. Increasing numbers of studies have used umbilical cord blood (UCB) as an alternative source to obtain HSCs. UCB has fewer ethical issues for obtaining cells, rapid availability and reduced stringency for HLA matching[11] make them another promising source of HSC for transplantation. Probably because of lower effective infused cell dose, slower engraftment of UCB-derived HSCs than BM-derived HSCs has been reported[12,13]. In addition, some progenitor cell populations (myeloid progenitors) in UCB were reported to be more chemoresistant[14], which could be beneficial when combined with high-dose chemotherapies. The most recent study describing the isolation of single HSC capable of long-term, multi-lineage engraftment derived from UCB, which suggests UCB cell may contain functionally most primitive HSCs[15]. Although UCB is likely to be more popular as a source to obtain HSCs, further research and careful examinations will certainly be necessary to determine the pros/cons, similarities/differences, and quantity/quality of HSCs isolated from both BM and UCB.

## ENRICHMENT OF HSC

As previously mentioned, Weismann's group was the first group demonstrating isolation and purification of HSCs (from mouse BM)[2]. This first study used multiple HSC markers including Thy-$1^{low}$, $Lin^-$ and Sca-$1^+$, a widely used criteria nowadays, to purify HSCs[2]. Note that Sca-1 is not found in human HSCs. Since HSCs are a very rare population in whole BM cells (1 in 10000-15000 BM cells), enrichment of HSCs certainly is a very important step for effective *ex vivo* expansion of HSCs. CD34 is a widely recognized cell surface marker to enrich HSCs (as reviewed in[16]). Immunoselection based on cell surface CD34 expression is well accepted in clinical settings, although it should be noted that the $CD34^+$ fraction does contain many progenitor cells such as endothelial progenitor cells[17]. Since a $CD34^+$ fraction still consists of crude cell populations, a $CD34^+CD38^-$ fraction may contain a higher percentage of primitive HSCs[18]. Therefore, a $CD34^+CD38^-Lin^-Sca^+$ selection criteria may be reasonable for selecting a highly enriched, primitive fraction of HSCs. c-Kit (stem cell factor receptor/CD117)[19,20] and CD133[21,22] are also the markers for the selection, although combination of too many antibodies may not be realistic in clinical settings because of a significant loss of HSCs during purification. We would like to note that these selections would certainly help enrich for higher quality HSCs; however, the above processes are still not enough to identify a nearly pure, HSC population. Most recently, John Dick's group isolated single hematopoietic stem cells[15] whose phenotype is $Lin^-CD34^+CD38^-Thy1^+CD45RA^-Rho^{low}CD49f^+$ can regenerate the entire hematopoietic system.

It should also be noted that there is evidence showing a considerable number of HSCs can be CD34 negative[23-25]. There are two possibilities accounting for why some studies have shown apparently different trends (cite examples of CD34 negative). First of all, CD34-positive and -negative cells may be interchangeable[26]. This means that HSCs may retain CD34 in intracellular compartments upon induction of cell surface CD34 molecules. Since the majority of anti-CD34 antibodies used for flow cytometric analyses recognize the extracellular domain of CD34, CD34 expression will appear to be negative when intracellular accumulation of CD34 occurs (unless the plasma membrane of the cells are

**Table 1 Comparison of the sources for hematopoietic stem cells**

| | Advantages | Disadvantages |
|---|---|---|
| Bone marrow | 1 The longest history as a source of HSC<br>2 Well established procedure | 1 High stringency for HLA matching<br>2 Some complications associated with harvesting<br>3 Risk of GVHD |
| Umbilical cord blood | 1 Off-the-shelf availability<br>2 Reduced stringency for HLA matching<br>3 Low risk of infection/transmission<br>4 Absence of donor risk | 1 Delayed engraftment<br>2 Lower yield<br>3 Lack of additional immune cells |
| Peripheral blood | Compared to BM,<br>1 More comfortable for donors<br>2 Better yield with mobilization | 1 Extremely low level of HSC (1/100000) without "mobilization"<br>2 Risk of GVHD |

HSC: Hematopoietic stem cell; HLA: Human leukocyte antigen; BM: Bone marrow; GVHD: Graft-versus-host disease.

**Table 2 Cell-surface makers of undifferentiated hematopoietic stem cells**

| Mouse | Human |
|---|---|
| $CD34^{low/-}$ | $CD34^{low/-}$ |
| $Sca\text{-}1^{+}$ | $Sca\text{-}1^{+}$ |
| $CD90/Thy\text{-}1^{+/low}$ | $CD90/Thy\text{-}1^{+/low}$ |
| $c\text{-}Kit^{+}$ | $c\text{-}Kit^{+}$ |
| $CD38^{+}$ | $CD38^{-}$ |
| $CD150^{+}$ | $CD7^{-}$ |
| Side population (high hoechst-efflux activity) | $CD49f^{+}$ |
| $CD48^{-}$ | Rhodamine $123^{low}$ |
| $CD244^{-}$ | $CD133/AC133^{-}$ |
| $Lin^{-}$ | $CD45RA^{-}$ |
| | $Lin^{-}$ |

permeabilized). The exact mechanism why $CD34^{+}$ HSCs and $CD34^{-}$ HSCs have interchangeable phenotypes remains unresolved. In mouse, the most primitive HSCs can be either $CD34^{-}$ or $CD34^{low}$. Nevertheless, human HSCs can be found in $CD34^{+}$ fraction[27] (Table 2). The other potential pitfall is a technical issue related to anti-mouse CD34 monoclonal antibodies (mAbs). There are several anti-mouse CD34 mAbs available to stain cell surface CD34. At least two clones widely used among researchers, clones MEC14.7[28] and HM34[29], have different characteristics in regard to staining. HM34 is reported to be unable to stain CD34 expression on marrow cells by some unknown mechanism. In addition, clone MEC14.7 is known to show relatively low affinity against CD34; therefore, it is recommended to incubate the mAb with samples for a prolonged period of 90-120 min (in contrast, the majority of commonly used protocols in flow cytometry suggest a 30-60 min incubation). Some of the murine HSC studies might not have carefully checked these technical tips requiring careful interpretation of studies reporting $CD34^{-}$ mouse HSCs.

It should likewise be noted that positive selection leaves antibodies attached on the cell surface which may not be favorable in terms of quality control issues. Furthermore, as most recently discussed by Lodish's group[30], the large amount of antibodies used for a clinical scale ($10^{9}$ per unit for cord blood transplantation) will certainly raise the cost for purification of HSCs. Therefore, development of chemical or enzymatic activity-based substrates may be a great advantage to enrich HSCs in clinical settings. Chemical substrates could help lowering production costs, and simplifying quality control compared to mAbs. In addition, substrate-based selection may shorten the time and reduce the labors to prepare a HSC-rich fraction. Interestingly, hematopoietic progenitor cells have been shown to highly express cytosolic aldehyde dehydrogenase (ALDH)[31]. A fluorescent dye-conjugated ALDH substrate, developed by Storms and colleagues[32], enabled them to conduct a relatively simple, ALDH activity-based selection of HSCs and hematopoietic progenitor cells. Prior to this method, side populations selected based on influx of the DNA staining dye, Hoechst 33342 had been reported[33-36]. The combination of Hoechst dye efflux activity and ALDH activity was first shown by Pearce and Bonnet[37]. Their study showed that Hoechst side population (SP) only contained $CD34^{+}$ cells, thus, SP-based selection may not be suitable to enrich certain sub-populations such as $CD34^{-}CD38^{+}$ cells; which suggests that, at least in mouse, SP may not contain the most primitive HSC. Therefore, further studies need to confirm whether SP contains the primitive HSC population in humans. The authors concluded that Hoechst exclusion may not be appropriate for HSC isolation[37]. It was also reported that Hoechst 33342 is even more toxic than the other most commonly used DNA staining dye, 4',6-diamidino-2-phenyl indole dihydrochloride[38], by interrupting DNA topoisomerase I[39]. Topoisomerases play an essential role in cutting damaged DNA during DNA replication. Even temporary inhibition of a topoisomerase, might cause accumulation of unfavorable mutations during *ex vivo* expansion of HSCs; therefore, careful assessment on the long-term effects of the use of Hoechst 33342 must be assessed to pursue an application of Hoechst 33342 for HSC selection. Overall, currently ALDH-based selection may be the best option to sort/enrich HSCs.

Although it is very important to explore the best set of markers to purify the most primitive fraction of HSCs for *ex vivo* expansion and transplantation, significant technical hurdles exist. The combination of many surface antigens to purify HSCs causes a considerable reduction of the number of cells, which may limit the application in clinical settings. The other potentially impor-

tant issue is that maintaining the capacity of self-renewal while preserving the primitive capabilities of these HSCs during expansion of HSCs may require paracrine signals from other less primitive progenitor cells or differentiated leukocytes in culture. As we will describe in the following section, keeping the number of primitive HSCs in HSC-enriched (but still crude) fractions is certainly important. In fact, a simple $CD34^+$ fraction gives cells with enough *ex vivo* expansion capacity[40] to be used in clinical settings. In addition, the most "primitive" HSCs are quiescent, and rapid proliferation of these cells may cause the loss of primitive HSCs[41]. Therefore, rapid expansion of the small number of HSCs may often force HSCs to exit from the resting cycle and lead to HSC exhaustion. It is worthy of note that a nearly pure HSC fraction may not be suitable for large-scale expansion. Another critical issue needs to consider is the long-term repopulation capability of *ex vivo*-expanded fractions. This capability may depend on the number of primitive HSCs after *ex vivo* culture. Therefore, from the clinical stand point, the most important goal in *ex vivo* expansion of HSC may be to develop simple and efficient protocols to obtain HSC-enriched fractions containing a sufficient number of HSCs that would result in satisfactory *in vivo* engraftment after transplantation. Faster recovery of the number of neutrophils in the body seems to be an indicator of successful engraftment of transplanted cells[42], which again justifies the possible advantage of HSC-enriched fractions over nearly pure HSCs. In summary, even great efforts have been made to search the markers for HSCs, it is important to stress that none of the surface markers is entirely specific to the long-term HSCs.

## *EX VIVO* CULTURE MEDIA FOR HSC EXPANSION

Since the limited number of HSCs is the major obstacle in clinical applications of *ex vivo*-expanded HSCs, successful *ex vivo* expansion of HSCs is one of the critical determinants emphasized throughout this review. This is particularly important for adult patients, who require increased units of HSCs in comparison to pediatric patients. The HSC field is relatively well established in comparison to the other SCs, and serum-free culture systems have already been used to expand HSCs. Development of serum-free culture is necessary to avoid the use of animal products such as bovine serum. As mentioned above, it is very important to avoid the use of animal products: (1) to prevent transmission of any possible diseases from animals, such as Creutzfeldt-Jabob disease (caused by Prion protein); (2) to achieve good quality control of expanded cells. In addition, accumulation of animal serum proteins in HSCs might increase the risk of host immune response upon transplantation of *ex vivo* cultured HSCs. The current research has focused on a cytokine cocktail-based culture to allow significant expansion of enriched HSCs.

Another major hurdle in current *ex vivo*-expanded HSCs (or HSC-enriched units) in their clinical applications is that some primitive HSCs are lost during *ex vivo* expansion of HSCs. Since the most primitive HSCs are thought to be in a resting state, the goals to achieve rapid expansion of HSCs and retain primitive HSCs stand at odds to one another. Therefore, to achieve better clinical outcomes (to reduce morbidity and mortality after transplantation of *ex vivo*-expanded cells), it is also essential to improve the number of long-term repopulating cells; *i.e.*, to maintain the number of primitive cells that retain self-renewal capacity. We will address this issue later in the section "Expansion versus *in vivo* reconstitution". Co-culture of feeder cells such as a monolayer of mesenchymal stem cells with HSCs may be a better method, since theoretically feeder cell layers should be able to provide a physiologically more relevant environment (stromal cell-HSC interaction and simultaneous feeding of sets of growth factors/cytokines that facilitate expansion of HSCs without unfavorable differentiation). However, co-culture with a feeder cell layer is not suitable for large-scale culture, and thus is difficult to translate into clinical settings. Most recently, an attempt to introduce computer simulations was made to help understand complicated paracrine mechanisms involving progenitor cells and differentiated cells in HSC culture[43]. This study may bring a paradigm shift in *ex vivo* cutulre of HSCs. In the following sub-sections, we would like to discuss emerging substrates as well as currently used soluble factors.

### *Soluble factors (i.e., growth factors and cytokines)*

Soluble factors, such as Flt3/Flk2 ligand[44,45], stem cell factor (SCF)[46,47], interleukin-3 (IL-3)[48-50], IL-6[51], and thrombopoietin (Tpo)[52-54] are commonly used as culture supplements for HSCs. Flt3/Flk2 ligand was discovered as a factor promoting proliferation of primitive hematopoietic cells, and[55] is more effective than SCF[56], although SCF probably has the longest history as a supplement. IL-3's effect *in vitro* was first reported by Spivak *et al*[57] However, there is a report showing the suppression of the number of colony-forming cells by IL-3[58], thus, the effect of IL-3 may need careful re-evaluation. This study also showed that IL-3 reduced the reconstituting activity of HSCs in a mouse model system[58]. Other interleukins, such as IL-7 and IL-11, are also included as additional supplements in serum-free culture systems because of their potential to promote HSC proliferation. IL-7 was originally discovered as an interleukin that stimulated proliferation of B cell progenitors in mouse[59]. Although IL-7 alone did not show significant expansion of $Lin^-Sca\text{-}1^+$ HSCs, potent synergistic effect on proliferation of $Lin^-Sca\text{-}1^+$ HSCs was observed when IL-7 was combined with IL-3[60]. IL-11 was cloned as a gene product produced by BM-derived stromal cell lines in 1990[61], and the following study by Ogawa's group showed that IL-11 could also synergistically help expansion of primitive hematopoietic progenitor cells with either IL-3 or IL-4[62].

One interesting study showed that gp130 signaling

(*via* IL-6) synergistically enhanced the effect of Flt3 ligand[63] as well as SCF[64]. Interestingly, IL-6 alone was not enough to trigger this effect[48,63,64]. These findings suggest that gp130 signaling appears to be somewhat supportive in its role of potentiating the effects of SCF and Flt3 ligand as culture supplements. Commonly-used cytokine cocktails often contain IL-6. Sustained activation of the Janus kinase (JAK)-STAT signaling pathway may be an important element in *ex vivo* expansion of HSCs. It is noteworthy that thrombopoietin was reported to activate JAK-STAT signaling and thereby helping to protect $CD34^+$ cells from apoptosis in serum-free media[65]. Moreover, activation of c-Kit and gp130 was shown to synergistically induce thrombopoietin production by cord blood $CD34^+$ cells themselves[66]. It should note that there is a report describing impaired engraftment of murine BM cells cultured in the presence of IL-6[67]. Similar to IL-6, it was reported in the same year that IL-3 reduced the number of colony-forming cells[58], impaired engraftment might be mainly associated with IL-3 in the culture. It was surprising that SCF and some of the interleukins could have adverse effects upon transplantation[67]; although SCF is a well-accepted growth factor for serum-free culture of HSCs or HSC-enriched fractions. In addition to the previously mentioned cytokines, fibroblast growth factors (FGFs) were also found to be effective to help *in vitro* culture of whole blood cells or enriched hematopoietic cells[68,69]. The later study also showed that FGF receptors were not expressed on human $CD34^+$ cells[70]. Thus, the effect of FGF may be controversial.

Recent studies by Lodish have added three endothelial growth factors (angiopoietin-like 5, insulin-like growth factor-binding protein 2, and pleiotrophin) as potential soluble factors that may further help *ex vivo* expansion of HSCs[71].

Although great efforts has been made to improve the rate of HSC engraftment, *ex vivo* cytokine-based expansion protocols may have reached plateau. The use of cytokine cocktails can raise the cost for *ex vivo* expansion of HSCs, too. Therefore, there are a great need of additional factors/molecules in order to support HSC self-renewal and amplification *in vitro*.

### Exposure to developmental regulators

It has been suggested recently that some developmentally conserved pathways or transcriptional factors are important in the regulation of the adult stem cell compartment, such as wingless-type (Wnt), Notch, Hox transcription factors and Sonic hedge hog Shh/BMP signaling[72]. Among them, Notch signaling is the most extensively studied. Notch ligand may be the most promising recombinant protein product to assist in *ex vivo* expansion of HSCs. In 1994, $CD34^+$ cells were shown to express high levels of the human homolog of *Drosophila* Notch[73], and this initial observation led to hypothesize the role of Notch signaling in maintenance of undifferentiated status of HSCs. Immobilized Notch ligand (Delta-1) was shown to dramatically increase (up to approximately 100-fold) the number of $CD34^+$ cells[74]. More importantly, the expanded cells enhanced repopulating ability of hematopoietic cells in NOD/SCID mice[74]. The effectiveness of Notch ligand was further confirmed by a follow-up study by the same group[75]. The outcome of their clinical trial is very encouraging, because preliminary data of a phase I clinical trial showed engraftment of the $CD34^+$ cord blood cells has significant advantage over the controls including shorter periods for neutrophil recovery after transplantation[75]. One potential side-effect of Delta-1 is that it has potential to cause density-dependent apoptosis of cells[76], and it is necessary to carefully control ligand density to minimize the loss of cells or unfavorable phenotypic changes during expansion. Interestingly, the most recent study by Rafii's group highlighted the critical role of Notch signaling in the BM microenvironment, where Notch activation by BM endothelium is essential for self-renewal of long-term HSCs[77]. This study clearly indicates that Notch signaling is a naturally-occurring, critical signal for maintenance of HSCs.

Besides Notch 1 ligand, TAT (HIV virus-derived cell permeable peptide)-tagged HOXB4 protein was shown to be effective to expand HSCs *in vitro*[78]. The advantage of TAT-HOXB4 protein over Notch ligands is the lack of a requirement to immobilize it onto the culture apparatus. Overexpression of HOXB4 had been shown to be one of the most potent stimulators of HSC expansion[79,80]; however, recently it was reported that there was high incidence of leukemia in large animals two years after receiving HOXB4-carrying retrovirus-introduced *HSC* gene therapy[81]. Therefore, the use of the plasma membrane-permeable recombinant HOXB4 protein may be a reasonable alternative for *ex vivo* expansion of HSCs. Although Krosl *et al*[78] study showed the effectiveness of recombinant HOXB4, the study did not show long-term repopulating activity of expanded cells. Therefore, further studies examining any potential safety issues caused by HOXB4 would be necessary. Overall, Notch ligands seem to be the most promising peptide-type supplements for HSC expansion at current moment.

### Stromal support

In the BM niche, HSCs interact with stromal cells, and direct interaction of HSCs with stromal cells or extracellular matrices in the BM may help maintain small populations of primitive HSCs. Thus, it was quite reasonable to hypothesize that major extracellular matrices in the BM niche facilitated sustained hematopoiesis. In fact, a major extracellular matrix, fibronectin, was found to be perhaps the most important extracellular matrix protein to facilitate proliferation/self-renewal as well as adhesion of HSCs and progenitor cells in the BM niche[82,83]. The initial studies showed that stromal support increased not only gene transduction efficiency but also successfully preserved the ability of human $CD34^+$ cells to sustain long-term hematopoiesis in immune deficient mice[84]. This study also showed that a C-terminal fragment of

fibronectin could help successful long-term engraftment of human HSCs and progenitor cells to bnx/hu mice. This C-terminal fragment contains both CS-1 and RGD(S) domains. The CS-1 domain is known to interact with VLA-4 (integrin α4β1)[85], and interestingly, Verfaillie *et al*[82] reported that primitive progenitors bound to fibronectin CS-1 *via* VLA-4, but expression of VLA-4 was lost upon differentiation of the cells. Immobilized fibronectin peptides were also shown to help *ex vivo* expansion of human cord blood HSCs ($CD34^+$ fraction)[86]. In this study, the authors used several fibronectin peptides including those carrying mutations on binding domains. Although all peptides they used showed significant increase in colony forming units as well as expansion of cells, only the peptide containing the intact CS-1 domain could give successful long-term engraftment of transplanted cells and survival of NOD/SCID mice[86]. Thus, short C-terminal fragments of fibronectin containing the CS-1 domain may be a good coating material for *ex vivo* expansion of HSCs or progenitor cells.

The major disadvantage of these proteins/peptides, except TAT-HOXB4 protein, is their requirement of immobilization onto the surface of cell culture apparatuses. This will increase culture volume as well as cost of industry-scale production of cells; thus, development of suspension culture systems that allow both self-renewal of HSCs and a reasonable level of expansion would be desirable.

### *Small molecules*

It would be desirable to use chemical compounds as supplements for serum-free culture systems, which may significantly help reducproduction costs of HSCs at the industrial scale and at the same time, facilitate efficient *ex vivo* expansion of HSCs.

Most recently, one study has shown that a chemical compound could also be used for *ex vivo* expansion of HSCs[87]. Chemical compounds would be superior to biological compounds because of potentially lower production costs and easier quality control.

These promising chemical compounds include the retinoic acid receptor agonist all-trans retinoic acid, copper chelator tetraethylenepentamine (TEPA), histone deacetylase inhibitors, acryl hydrocarbon receptor antagonist [referred to as StemRegenin1 (SR1)] and, PGE2[72]. Among them, TEPA are the most promising chemical to be used for expansion of human HSCs at current moment. TEPA-supplemented, *ex vivo* cultures of $CD34^+$ cord blood cells significantly increased the number of HSCs and enhanced NOD/SCID repopulating capacity[88]. A phase I/II clinical trial of TEPA-cultured cord blood cells showed the safety of this approach[89]. The efficiency of TEPA-cultured HSPC is currently under investigation in an ongoing phase Ⅱ/Ⅲ study.

Aryl hydrocarbon receptor antagonists (SR1) were identified as potential drug candidates for promoting *ex vivo* expansion of HSCs by microscopy-based high-throughput screening[87]. The antagonist was subsequently tested with a feedback culture system[43]. Aryl hydrocarbon receptor signaling emerged as an important element in HSC functions[87]. Currently, these compounds require the supplementation of cytokines, and thus further studies will be necessary to apply this strategy for large scale expansion of HSCs without animal serum. Nevertheless, this discovery may eventually pave a way for establishing industrial-scale production of HSCs with chemically defined culture media.

## EXPANSION *VS IN VIVO* RECONSTITUTION

Although finding the optimum conditions for clinical-scale *ex vivo* expansion culture is an absolutely important determinant to use HSCs in clinical settings, another important factor in determining the clinical outcomes of HSC transplantation is engraftment and reconstitution of the hematopoietic system in a recipient. Successful engraftment requires repopulation of transplanted cells to irradiated BM, of which success may mainly rely on the ability of transplanted cells to migrate toward BM. (SDF-1/CXCR4 axis). Therefore, it is also essential to test repopulation and engraftment of expanded cells by immunologically incompetent small animal models such as severe combined immunodeficient (SCID) mice. On the other hand, reconstitution of the hematopoietic system requires *de novo* production of hematopoietic lineages from donor-derived HSCs. This function requires self-renewable, primitive HSCs in the BM after transplantation to prevent exhaustion of self-renewable HSCs in BM niche. The $CD34^+$ fraction, which is commonly used in the current clinical settings as a source of HSC-enriched cells, should be T cell-free to avoid graft-versus-host-disease. Successful engraftment is likewise likely to be associated positively with the number of neutrophils present in the body[42].

Before closing this section, we also would like to comment on the quiescence of stem cells under normal conditions. HSCs retain labeled thymidine, indicating replicative quiescence in their niche[41]. We speculate that this may be because HSCs need to maintain longevity and to minimize mutations concomitant with replication errors. Therefore, a line of studies may be necessary to confirm that rapid *ex vivo* expansion of HSCs does not generate and accumulate genetic mutations that can lead to the formation of cancerous cells. In addition, we should always keep in our mind that rapid *ex vivo* expansion of HSCs may often cause the loss of long-term engraftment capacity of HSCs or exhaustion of the most primitive HSC pool. It was demonstrated for more than a decade ago that HSCs proliferate slow *in vivo*[90,91]. Therefore, it is postulated that HSC quiescence is critical in maintaining the stem cell compartment. Weissman's group estimated that approximately 8% of long-term self-renewing HSCs enter the cell cycle per day[91]. However, contradictory results appeared later showing traditionally used DNA labeling dye, BrdU (5-bromo-2-deoxyuridine), may not be specific enough to label slow-dividing cells[92]. More recently, different labeling

strategies have been proposed as alternative methods to trace slowly dividing HSC fractions[93,94]. Proliferation of cells requires the entry to $G_1$ cycle, thus, it is quite natural to hypothesize that perturbation of cyclin dependent kinase inhibitors may initiate dramatic cell proliferation followed by the exhaustion of HSC pools. Scadden's group showed for the first time, $p21^{cip/waf1}$ deletion eventually causes exhaustion of HSCs in a mouse model system[95].

Since phosphatase and tensin homolog (PTEN)/Akt pathway plays a critical role in cell proliferation, survival and growth, it was predicted that mis-regulation of this pathway could cause problems on hematopoiesis. In fact, PTEN knockout mice study showed decreased numbers of HSCs[96]. PTEN knockout mice also developed leukemia, suggesting there is a critical factors suppressing cell cycle progression. The phenotype of Foxo3a knockout mice is quite interesting. Foxo3a-KO significantly decreased the number of colony forming cells in BM. Age-dependent decrease of HSC pool was also observed. However, Foxo3a-KO little effects on hematopoietic progenitor cells except a decreased number of erythrocytes[97]. More interestingly, distribution of Foxo3a in a cell further implies Foxo3a's specific role in maintaining primitive HSCs; Foxo3a accumulate in inside of a nucleus in a $CD34^-c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cell, however, Foxo3a no longer accumulates in a nucleus and translocates to a nuclear envelop in a $CD34^+c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cell, which is less primitive than $CD34^-c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cells. Thus, it appears that Foxo3a may hold a key role in the maintenance of HSCs in BM.

Although c-Kit may be dispensable for extensive proliferation of HSCs, mice carrying c-Kit mutation (W41/W41) were reported to show approximately 2-fold reduction of long term-HSCs[98]. This study was further reinforced with the result that multipotent progenitor cells were less affected by this mutation, suggesting that c-Kit function may also be very important for maintenance of primitive HSCs for sustained hematopoiesis.

Thus, we should keep in our mind that our attempt to improve *ex vivo* expansion of HSCs by manipulating some signaling pathways could be a "double-edged sword".

## CONCLUSION

HSC-based cellular therapy is promising for hematopoietic cancers and hereditary blood disorders. Adaptive transfer of sufficient number of enriched HSCs is the key for successful transplantation. Besides BM, peripheral blood and umbilical cord blood also are the sources of HSCs. Traditionally, BM is the main source of HSCs, but peripheral blood and UCB are increasingly used as sources due to non-invasive harvesting procedures. Soluble growth factors, signaling molecules, small molecules and extracellular matrix support are commonly-used methods to amplify isolated HSCs, but chemical compounds such as TEPA is promising because of its lower cost and easier quality control.

A rapidly increasing number of studies and clinical trials have demonstrated very promising applications of HSCs in clinical settings. Currently, applications of HSCs are often limited to children mostly due to restrictions in the quantity of HSCs that can be obtained. Increased demand of HSC therapies, including those for adult patients, is expected. Therefore, it is necessary to establish large-scale production of HSCs to supply enough units of HSCs in the near future. In addition to cytokine cocktails, most recent studies have added several other options such as recombinant proteins and chemical compounds for *ex vivo* expansion of HSCs. Chemical-based culture would be advantageous, however, it will take some time until it is ready for clinical application.

Using multiple cell surface markers, including CD34, CD38, Thy-1, CD45, c-kit, CD133, CD49f and Lineage cocktail, it is possible to isolate single HSCs with the full capacity to regenerate the entire hematopoietic system. However, it is worthy to note that purification of ultimately primitive HSCs may not be necessary for clinical-scale production of cells. Instead, "HSC-enriched" fractions would be useful because of the possible positive feedback by the other less primitive cells in the culture. Thus, the effective *ex vivo* expansion of HSCs or hematopoietic progenitor cells may require inclusion of precise feedback regulation (*i.e.*, autocrine and paracrine), since the hematopoietic system is a highly complicated system requiring maintenance of a highly ordered hierarchy. Further investigation should develop more promising methods which will substantially enhance generation of HSCs with high efficiency for clinical application.

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