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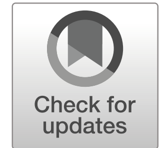

Human Hematopoietic Stem Cells: Concepts and Perspectives on the Biology and Use of Fresh Versus In Vitro–Generated Cells for Therapeutic Applications

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Abstract

Purpose of Review The in vitro production of human hematopoietic stem cells (hHSCs) has caused great interest due to its clinical impact. The main goal of the present article is to review the information existing today on the in vitro generation of hHSCs and their molecular and functional integrity as compared with fresh hHSCs.

Recent Findings By using different in vitro systems, hHSCs have been generated from fresh hHSCs (obtained from bone marrow, peripheral blood, or cord blood) and from pluripotent stem cells. Although functional and molecular gaps have been observed between fresh and in vitro–derived hHSCs, recent clinical trials indicate that hHSCs generated in vitro from cord blood are capable of long-term hematopoietic reconstitution in transplanted patients. To date, no data exist on the clinical use of hHSCs derived from human pluripotent stem cells (hPSCs).

Summary Significant achievements in hHSC expansion and manipulation, as well as in the culture and differentiation of hPSCs, have been reported. All this, together with innovative clinical trials for the treatment of hematologic disorders, will be fundamental for the in vitro generation of hHSCs and their application in clinical settings.

Keywords Cord blood · Cytokines · Embryonic stem cells · Expansion · Hematopoietic stem cells · In vitro · Pluripotent stem cells · Reprogramming

Introduction

During the last two decades, the in vitro production of human hematopoietic stem cells (hHSCs) has generated great interest due to its potential impact in the clinic. Different in vitro systems have been developed that are based on the use of recombinant hematopoietic stimulatory cytokines, with or without different types of small molecules, and in the absence or presence of stromal cells [1–3]. In most such studies, hHSCs have been generated using fresh hematopoietic cells (from bone marrow, mobilized peripheral blood, or umbilical cord blood) as the input cell population [1–3]. In the last few years, however, hHSCs have also been generated from human pluripotent stem cells (hPSCs), including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [4•]. In this context, the main goal of the present article is to review the information existing today regarding the in vitro generation of hHSCs from either hematopoietic or pluripotent cells and its current and potential impact in the treatment of a variety of human disorders.

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Hematopoiesis and Hematopoietic Stem Cells

Blood cell production—hematopoiesis—is a complex process occurring in different sites (known as hematopoietic organs) throughout development [5]. During postnatal life, mammalian hematopoiesis takes place primarily in the bone marrow, within specific microenvironments known as hematopoietic niches [6]. Evidence generated during the last 60 years indicates that all the different blood cells (erythrocytes, leukocytes, and platelets) originate from a common cell type known as HSC [7]. HSCs are immature and undifferentiated cells capable of both self-renewal and multilineage differentiation

[8•]. Evidence presented over the last few years indicates that the HSC compartment comprises not one but different cell populations [9]. Based on their proliferation and engraftment potential, there are, at least, two classes of HSCs, those with long- and short-term engraftment potential. In terms of differentiation capacities, HSC heterogeneity has also been documented, since it has been shown that some HSC clones are able to give rise to both myeloid and lymphoid progeny, whereas others are lymphoid-deficient. Under normal conditions, HSCs give rise to hematopoietic progenitor cells (HPCs), immature cells unable to self-renew, but with a large proliferative potential and multilineage, bilineage, or monolineage differentiation capacities. HPCs, in turn, can give rise to hematopoietic precursors, immature cells still present in the marrow environment that can already be identified through their morphology. Finally, maturation of precursor cells results in the production of circulating blood cells.

Although it is well known that HSCs show lymphoblastoid features, they cannot be identified through their morphology; instead, their identification is based on both immunophenotype analysis and functional assays [10]. Human HSCs express CD34, CD49f, CD90, CD117, and CD133 antigens and do not express CD38 or any lineage-restricted antigen [7]. Interestingly, within the human HSC pool, a very rare CD34 negative (CD34−) population has also been identified, whose cycling status suggests that it is located at the apex of the HSC compartment [11, 12]. In order to determine the functional integrity of HSCs, both in vivo and in vitro systems have been developed. The former consists of introducing HSCs into irradiated animals and determining the ability of such cells to repopulate the hematopoietic system of the host after several weeks post-transplant. When using hHSCs, the recipient must be an immunodeficient animal (for instance, severe combined immune-deficient [SCID], non-obese diabetic [NOD]-SCID, or NSG mice), so there will be no rejection mediated by the immune cells of the host [13]. In vitro systems, on the other hand, are based on the ability of HSCs to initiate and sustain hematopoietic cell production for several weeks in cultures containing a stromal cell layer, in the presence or in the absence of exogenous cytokines [14]. It is noteworthy, however, that this latter method does not necessarily prove that the cells sustaining hematopoiesis in vitro are actual HSCs; thus, to date, the in vivo repopulation assay is the only method validated to detect and measure HSC function.

Hematopoietic Transplants

When the hematopoietic system of an individual becomes abnormal—due to hereditary or acquired genetic alterations, such as in leukemia or myelodysplasia—bone marrow cells must be replaced by healthy functional hematopoietic cells. That is to say, a hematopoietic cell transplant (HCT) is needed [15]. Hematopoietic transplantation had its origin in the discovery that mice could be protected from radiation-induced marrow aplasia by transfer of hematopoietic cells from the marrow of syngeneic mice [16]. The first HCTs in humans were performed in the mid-1950s, but it was until the 1970s that HCTs became relevant for the treatment of hematological disorders [17]. Today, allogeneic (when cells come from a different, compatible donor) [18] and autologous (when cells come from the patient itself) [19] HCTs have become one of the main therapeutic strategies for the treatment of hematologic, neoplastic, immunologic, and hereditary disorders, giving patients a real possibility of cure. Every year, over 40,000 HCTs are performed worldwide and the cumulative number of HCTs reached one million in December 2012 [15].

Three different cell sources are currently being used for hematopoietic transplantation: bone marrow (BM), mobilized peripheral blood (MPB), and umbilical cord blood (UCB) [20]. When collecting hematopoietic cells from BM, up to 1.2 l can be obtained from a single collection under general anesthesia. HLA matching criteria are usually 6/6 for a matched sibling donor, 7/8 for a matched haploidentical donor, and 8/8 for a matched unrelated donor. Risks include bleeding, infection, local pain, and a moderate risk for graft-versus-host disease [20]. Collection of peripheral blood requires prior mobilization using agents such as G-CSF or plerixafor. During apheresis collection, up to 24 l can be processed. HLA matching criteria are similar to those of BM transplants. Toxicities associated with MPB are related to mobilizing agents and mobilization procedures [20]. As compared with BM donors, MPB donors usually recover faster and show a lower incidence of adverse events [21].

For the last 30 years, UCB has been shown to be an excellent source of hematopoietic cells [22], and it has been demonstrated that it possesses several advantages over adult sources. In biological terms, UCB HSCs and HPCs are superior to their adult marrow or blood counterparts, since the relative content of hematopoietic stem and progenitor cells in a UCB unit is higher than in a BM or MPB collection, and the proliferation and expansion potentials of such UCB cells are significantly higher than the potentials observed in adult cells [23]. In practical terms, UCB collections (usually 100–120 ml) are relatively simple procedures with no risk to the mother or child. After being collected and processed, UCB cells can be stored in public or private banks [22]. UCB banking has resulted in reduced searching times for unrelated donors, as compared with adult sources, and it has developed to the point that around 800,000 units are being stored in public banks and more than 4 million units in private banks worldwide. The numbers of units that need to be kept in a bank to satisfy the public demand differ depending on the characteristics of the population; thus, the release rate among banks shows great variability. Worldwide, the release rates at UCB banks are usually low (0.5–12% of the units banked). When

properly stored, the physical and functional integrity of the banked cells can be preserved for more than 20 years [24•]. After such a time, cells can be induced to produce colonies in semisolid cultures, they can be induced to proliferate and expand in liquid suspension cultures, and they can be manipulated ex vivo to generate induced pluripotent stem cells [24•]. Because of the immaturity of some of the immune cell populations in UCB, HLA matching criteria are 4–6/6, which is less stringent than for BM or MPB. To date, over 40,000 UCB transplants (UCBTs) have been performed, in both children and adults, for the treatment of > 60 different diseases, including non-hematologic disorders. Indeed, UCB cells have been used in the treatment of specific inherited metabolic disorders and other neurologic disorders, including cerebral palsy, autism, and demyelinating brain disorders [25].

A major disadvantage when using UCB for hematopoietic transplants in patients over 60 kg of weight is the fact that there is delayed engraftment and, in some cases, lower survival rates, as well as higher transplant-related mortality, as compared with BM or MPB transplants [26, 27]. Indeed, in adult patients, neutrophil engraftment after BM or MPB transplants can be reached in 13–18 days; in contrast, neutrophil engraftment after UCBTs is reached after > 20 days [26, 27]. A relatively low total nucleated cell content, including HSCs and HPCs, in a UCB unit has been recognized as the principal reason for delayed hematopoietic recovery and poorer engraftment. Thus, one of the major challenges with UCB is to find ways to increase the number of HSCs and HPCs to be transplanted into patients. One approach into this problem has been to develop strategies for the in vitro generation of HSCs in numbers that are clinically relevant.

In Vitro Generation of hHSCs

The relative frequency of HSCs in the bone marrow is extremely low (around 0.001% of the total number of marrow cells), and this has been a major hurdle for their identification and selection, and for carrying out biologic studies both in vivo and in vitro. On the other hand, the reduced absolute number of such cells in UCB units has resulted in major limitations when performing UCBTs. Thus, the in vitro generation of hHSCs—and their immediate cell progeny—is of great relevance in both the laboratory and clinical arenas. Today, two major biological sources exist for the in vitro generation of hHSCs: fresh (bona fide) hHSCs obtained from the marrow, peripheral blood, or UCB, and hPSCs, either from embryonic tissue or from reprogramming of somatic cells.

Generation of hHSCs from Bona Fide hHSCs

When generating hHSCs from hHSCs obtained from UCB (or an adult cell source), it is important to clearly define the cell population that will be used to initiate the cultures. Although some groups have used whole mononuclear cells, cell fractions enriched for $CD34^+$ cells (which contain both hHSCs and hHPCs) have been used in most studies. It has also been shown that primitive subpopulations of $CD34^+$ cells, e.g., $CD34^+$ Rh^{low}, $CD34^+$ $CD38^-$, $CD34^+$ $CD45RA^-$ $CD71^-$, and $CD34^+$ $CD45RA^-$ $CD71^-$ $CD90^+$ cells, possess greater expansion potentials than their more mature counterparts [28, 29] (Table 1). As clearly shown during the last five decades, the growth of HSCs is dependent on the presence of hematopoietic cytokines, particularly those acting at the early stages of hematopoiesis [30–32]. Accordingly, in vitro generation of hHSCs requires that such cytokines are part of the culture system [2]. Since early-acting cytokines favor self-renewal,

Table 1 Current approaches used for the in vitro generation of hHSCs from bona fide hHSCs

Input cell population	•Mononuclear cells •$CD133^+$ cells •$CD34^+$ cells •$CD34^+$ $CD38^-$ cells •$CD34^+$ $CD38^-$ Lin^- cells •$CD34^+$ $CD45RA^-$ $CD71^-$ cells
Recombinant cytokines	•SCF •FL •TPO •IL-6 •IL-3 •GM-CSF •G-CSF
Stromal cells	•Primary BM stromal cells •Human MSCs •Human endothelial cells •OP9 cell line •HS5 cell line •AFT024 cell line
Small molecules and Notch ligand	•DL1 •TEPA •Nicotinamide •SR-1 •UM171 •OAC1 •Valproic acid

Input cell population. In some studies, whole mononuclear cells (MNCs) have been used; however, most laboratories prefer to use $CD34^+$ cells or $CD34^+$ cell subpopulations, so that unspecific effects—due to molecules released by MNCs—are avoided. $CD133^+$ cells have also been used

Recombinant cytokines. Early-acting cytokines are usually included in the cytokine cocktail. In some studies, myeloid cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or G-CSF have also been included

Stromal cells. The presence of stromal cells has been shown to favor the generation of HSCs. Although some murine cell lines, such as OP9, have shown good results, only human stromal cells must be used for clinically oriented expansion protocols

Small molecules. Small molecules have been shown to favor self-renewal, thus, expansion of HSCs. They are always included together with recombinant cytokines

different combinations of them, including stem cell factor (SCF), FLT3 ligand (FL), and thrombopoietin (TPO), have been extensively tested (Table 1). Using this approach, significant increments in the numbers of hHPCs have been reported; however, real increments in the numbers of actual hHSCs have not been convincingly shown [2].

As mentioned above, the expansion of progenitor cells can be achieved by culturing such cells in the presence of different combinations of recombinant hematopoietic cytokines. In contrast, expansion of actual hHSCs has proved to be more difficult because, besides their need for recombinant cytokines, they seem to deeply depend on the presence of stromal cells and/or molecules that promote the activation of particular self-renewal signaling pathways. In keeping with the fact that the in vivo development of HSCs takes place in close association with microenvironment cells [33••], ex vivo systems have been established in which stromal cells are used as feeder layers, to allow the expansion of primitive hematopoietic cells. Different types of stromal cells have been assessed, including primary bone marrow stroma, endothelial cells, stromal cell lines, and mesenchymal stromal cells (MSCs), the latter from different tissues (Table 1) [34–36]. These studies have demonstrated that stromal cells, particularly MSCs, are capable of promoting the ex vivo expansion of primitive cells in a process that may involve both cell-to-cell contact and cytokine secretion. When MSC-based cultures are supplemented with recombinant cytokines, the increments observed in HPCs and $CD34^+$ cells are even higher, particularly in the presence of early-acting cytokines [37]. Interestingly, evidence indicates that when cultures are supplemented with early-, intermediate-, and late-acting cytokines, the presence of MSCs is not necessary for the production of committed HPCs or mature cells [35]. However, MSCs are still required for the ex vivo generation of hHSCs. Besides MSCs, other types of stromal cells, including endothelial cells, as well as the OP9 and AFT024 cell lines, have been used in experimental protocols aimed at the ex vivo expansion of primitive hematopoietic cells [38, 39]. These studies have confirmed the importance of stromal cells for generation of hHSCs in culture.

In the last few years, different small molecules have been tested in combination with recombinant cytokines and have demonstrated significant positive effects on the in vitro generation of immature UCB cells (including HSCs and different types of HPCs). Such molecules include the copper chelator tetraethylenepentamine (TEPA) [40]; the ligand for Notch known as Delta-like ligand-1 (DL1) [39, 41•]; nicotinamide, a form of vitamin B_3 [42]; the purine derivative known as StemRegenin-1 (SR1) that acts via engagement of the aryl hydrocarbon receptor [43]; and UM171, a pyrimidoindole derivative [44]. Other compounds include OAC1, a small compound that activates the pluripotent transcription factor Oct4 [45]; a PPAR-γ antagonist that enhances glycolysis [46]; and HDAC inhibitors, such as valproic acid [47] (Table 1).

Some of these laboratory approaches have already been included in clinical trials. The first clinical trials with in vitro–generated cells were performed over 15 years ago. In such studies, UCB cells were generated in vitro using different recombinant cytokines (SCF, FL, TPO, and erythropoietin [EPO]) and then were infused into patients. No positive effects were observed in terms of myeloid, erythroid, or platelet engraftment; however, those studies demonstrated that the procedure was feasible and safe [48, 49]. Trials in which cells were generated with TEPA, MSCs, DL1, nicotinamide, or SR1 were further reported [41, 50–53]. Among them, those in which cells were generated in the presence of MSCs, DL1, nicotinamide, or SR1 have shown encouraging results, since in all of them a significant expansion of $CD34^+$ cells ($CD133^+$ cells for the study using nicotinamide), and significant reductions in the times to engraftment, compared with historical controls, were observed.

Generation of Hematopoietic Cells from PSCs

PSCs are immature, undifferentiated cells capable of extensive self-renewal and able to give rise to cells of the three germ layers (ectoderm, mesoderm, and endoderm) [4•, 54]. In vitro, they have the potential to form any fully differentiated cell of the body; however, under specific culture conditions, they can be induced to unlimited proliferation without differentiation [55]. Two types of PSCs have been characterized so far: those obtained from the inner cell mass of the blastocyst, also known as embryonic stem cells (ESCs) [56•], and those generated experimentally by reprogramming somatic cells via introduction of specific genes (e.g., Oct 4, Sox 2, Nanog, Klf4, and c-Myc), known as induced-PSCs (iPSCs) [57••, 58••].

Generation of hHPCs and mature blood cells from hPSCs has been approached using three different methods: through the formation of embryoid bodies (EBs); through feeder cell co-culture; and through an extracellular matrix–coated dish that supports PSCs differentiation [59]. EBs are three-dimensional cell aggregates formed in methylcellulose or suspension cultures that mimic the spatial organization of the embryo; that is to say, the three germ layers are formed within the EB. Under the appropriate culture conditions, the formation of EBs resembles the sequential development of the hematopoietic system. Indeed, it has been shown that when EBs were cultured in the presence of endothelial cell conditioned medium, together with basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), SCF, IL-6, EPO, IL-11, IL-3, and insulin-like growth factor (IGF), hemangioblasts (primitive bipotent progenitors capable of giving rise to both hematopoietic and endothelial cells) were developed [60]. Such hemangioblasts were identified by their ability to produce blast colonies which represent a transient population that develops in the presence of bone morphogenic

protein-4 (BMP-4). It is noteworthy that two distinct types of hemangioblasts were identified, those giving rise to primitive erythroid cells, macrophages, and endothelial cells, and those giving rise to erythroid cells and endothelial cells only. Early progenitor cells can also be generated from EBs. When ESC-derived EBs are cultured in the presence of hematopoietic cytokines—i.e., FL, SCF, IL-3, IL-6, and granulocyte-colony-stimulating factor (G-CSF)—and BMP-4, hematopoietic progenitors from multiple lineages are generated [61]. Similarly, when iPSC-derived EBs are cultured in the presence of SCF, FL, IL-3, IL-6, G-CSF, and BMP-4, $CD34^+$ $CD45^+$ cells, as well as colony-forming cells, are produced [62].

Regarding the second method, ESCs and iPSCs can be induced to proliferate and differentiate without the formation of EBs by culturing them on feeder cell layers, such as OP9, AGM-S3, or fetal liver stromal cells. This method is extensively used when working with cell lines derived from PSCs. In this co-culture system, stromal cells give growth support to the PSCs by means of direct cell-to-cell interactions and by secreting stimulatory cytokines [59]. This experimental system has been extensively used for generating cells of the hematopoietic lineage. Human pluripotent cell lines (either from ESCs or iPSCs) co-cultured on OP9 cells—without the need for exogenous cytokines—generated $CD34^+$ $CD43^+$ hematopoietic progenitors capable of giving rise to mixed, erythroid, and myeloid colonies in semisolid cultures [63]. It is noteworthy that in this in vitro system, endothelial cells were also generated. A co-culture system based on AGM-S3 feeder cells and the presence of recombinant cytokines favoring erythropoiesis, such as SCF, IL-3, IL-6, TPO, and EPO, has been successfully used for the in vitro generation of human erythroblasts [64]. Similarly, a system using fetal liver–derived stromal cells as a feeder layer was used for the generation of erythroid progenitors that were subsequently induced to differentiate into fully mature enucleated erythrocytes when cultured in suspension cultures supplemented with recombinant erythropoietic cytokines [65].

The third method for the generation of hematopoietic cells from PSCs consists of culturing PSCs on extracellular matrix proteins in the presence of recombinant cytokines. In this system, there is no need for EB formation or co-culture with feeder cells. In a study by Salvagiotto and colleagues, ESCs or iPSCs were cultured on collagen or fibronectin in TeSR1 culture medium for 24 h. Then, the initial culture medium was replaced with a differentiation medium containing BMP-4, VEGF, and bFGF, which favors early hematopoietic and endothelial differentiation. After 6 days of culture, the culture medium was changed to a medium containing TPO, FL, IL-6, IL-3, and SCF. Using this experimental approach, the authors were able to generate $CD34^+$ $CD43^+$ hematopoietic progenitors that developed into erythroid cells, megakaryocytes, macrophages, neutrophils, and dendritic cells [66].

Generation of hHSCs from hPSCs

The reports described above clearly show that it is feasible to produce hematopoietic progenitor and mature cells in culture from hPSCs. The question is, is it possible to generate functional hHSCs from hPSCs? Considering the limited number of donors for HCTs and the reduced frequency of HSCs in the marrow and blood, the idea of producing hHSCs from hPSCs has gained significant clinical relevance. In the study by Doulatov et al. [67], committed hematopoietic progenitors ($CD34^+$ $CD45^+$ cells) were generated by culturing hPSC-derived EBs in the presence of hematopoietic cytokines and BMP-4. Such hHPCs were reprogrammed by introducing specific genes, including HoxA9, Erg, Rora, Sox4, and Myb. HOXA9, ERG, and RORA reactivated the expression of HSC transcription factors, such as RUNX1, HLF, FOS, JUN, MAFF, and GFI1B, thus giving rise to self-renewing $CD34^+$ $CD38^-$ $CD90^+$ $CD49f^+$ multipotent hHSCs. SOX4 and MYB, on the other hand, were required for in vivo engraftment. However, these hHSCs showed short- but not long-term engraftment potential in vivo when introduced into immunodeficient mice [67].

Generation of hHSCs with the capacity to reconstitute the hematopoietic system in primary and secondary immunodeficient mice was achieved in Daley's lab by inducing differentiation of hPSCs into hemogenic endothelium (HE). To this end, hESCs were cultured in the presence of FGF, VEGF, BMP-4, IGF-1, SB431542 (an inhibitor of activin/nodal signaling), and CHIR99021 (a GSK inhibitor). Once HE cells were generated, 26 HSC-specific transcription factors were screened for their capacity to promote multilineage hematopoietic engraftment in mouse hosts. Seven transcription factors were selected (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, and SPI1) and induced to convert HE into HSCs. The resulting HSCs showed B and T lymphoid, as well as myeloid engrafting potential in primary and secondary recipients [68••]. Interestingly, induction of these 7 transcription factors prior to generation of HE was not sufficient to permit multilineage engraftment, indicating the importance of developmental context and HSC ontogeny recreation in vitro.

An alternative approach has been used by Rafii's laboratory. These authors reprogrammed human endothelial cells into engraftable hHSCs without transition through a pluripotent state [69••]. Human umbilical vein endothelial cells or adult dermal microvascular endothelial cells were transduced with four transcription factors (FOSB, GFI1, RUNX1, and SPI1) and grown on serum-free vascular cell monolayers resulting in the generation of hematopoietic colonies that contained cells with immunophenotypic and functional features of multipotent progenitor cells. These cells were able to form hematopoietic colonies of different lineages and displayed durable, multilineage engraftment capacity in primary and secondary immunodeficient mice. The above studies highlight

the importance of an endothelial state, either as an intermediate state from hPSCs or as a direct reprogramming step, for the generation of functional long-term hHSCs.

Fresh (Bona Fide) or In Vitro–Generated HSCs?

Although significant advances in the development of ex vivo systems for the generation of hHSCs have been reported in the last two decades, it is still unclear to what extent those hHSCs generated in vitro retain the functional and genomic integrity of their freshly isolated (bona fide) counterparts. In other words, how similar are those hHSCs (and hHPCs) generated in vitro to their equivalent cell populations obtained directly from human sources (BM, MPB, or UCB)? The fact that ex vivo–expanded stem and progenitor cells are capable of engrafting and restoring hematopoiesis in immunodeficient mice seems to argue that these cells are similar, in biological terms, to their initially obtained counterparts. However, this hypothesis has not been conclusively demonstrated. Indeed, it is well known that when primitive hematopoietic cells are cultured in vitro, they experience a variety of phenotypic and functional changes induced by the culture conditions, including a tendency of HSCs to lose their stemness when they enter the cell cycle and an external pressure on HPCs to mature [70–72]. Thus, even though they may retain their original immunophenotype after ex vivo expansion, their genomic integrity and functional capacities may be altered [73]. This notion, in fact, seems to be the reason why UCB-derived expanded cells transplanted into patients have always been infused together with an unmanipulated UCB unit [74].

In a recent study, Dircio-Maldonado et al. [75•] addressed this issue by comparing the functional integrity in vitro and the gene expression profiles of hHSCs ($CD34^{+}$ $CD38^{-}$ $CD45RA^{-}$ $CD71^{-}$ Lin^{-} cells), myeloid progenitor cells (MPCs; $CD34^{+}$ $CD38^{+}$ $CD45RA^{+}$ $CD71^{-}$ Lin^{-} cells), and erythroid progenitor cells (EPCs; $CD34^{+}$ $CD38^{+}$ $CD45RA^{-}$ $CD71^{+}$ Lin^{-} cells), obtained directly from fresh UCB units and those generated in vitro under particular culture conditions. The results of such a study indicated that in spite of being immunophenotypically similar, fresh and in vitro–generated cells showed significant differences, both in functional and genetic terms. As compared with their fresh counterparts, hHSCs generated in culture showed a deficient content of long-term culture-initiating cells and a marked differentiation bias towards the myeloid lineage. In addition, in vitro–generated hHSCs and hHPCs showed a limited expansion potential. Such functional alterations correlated with differences in their gene expression profiles. It is noteworthy, however, that in this study, the authors cultured hHSCs in the presence of recombinant cytokines and OP9 stromal cells, but in the absence of small molecules, such as UM171, SR1, OAC1, nicotinamide, or resveratrol, which have been shown to induce self-renewal and favor expansion of hHSCs. Thus, it would be important to determine whether in the presence of any of such small molecules, in vitro–generated hHSCs retain their functional integrity in vitro and their gene expression profiles.

Interestingly, Horwitz and colleagues [76••] just reported a study in which 36 patients with hematological malignancies (AML, ALL, CML, MDS, non-Hodgkin's lymphoma, and Hodgkin's disease) were transplanted, each one receiving a single UCB unit previously expanded ex vivo in the presence of nicotinamide, together with FL, SCF, TPO, and IL-6. The authors observed a 33-fold increase in the number of $CD34^{+}$ cells after ex vivo expansion. Patients receiving the expanded UCB unit showed a median time to neutrophil and platelet recovery of 11.5 and 34 days, respectively (10 and 12 days, respectively, earlier than in the control group—i.e., patients receiving a single unexpanded UCB unit). Such numbers are relevant considering that, in general, the median time to neutrophil recovery is 18 days after myeloablative HLA-identical allogeneic bone marrow transplantation and 15 days after HLA-identical mobilized peripheral blood transplantation [77]. Overall and disease-free survivals at 2 years were 51% and 43%, respectively, which were statistically similar to those in the control group. This study is the first to show the feasibility of transplanting hematologic patients with a single, ex vivo–expanded UCB unit as a stand-alone graft and suggests that those hHSCs generated in vitro in the presence of early-acting cytokines and nicotinamide retain the self-renewal and multipotential capacities that are intrinsic to HSCs. Further studies will be required to see if other small molecules have the same effect as nicotinamide on the biology of human HSCs.

Are hHSCs generated in vitro from hPSCs genetically and functionally similar to those obtained from marrow or blood? As discussed in the previous section, in vivo studies in animal models suggest that they are, indeed, as long as they were generated via the HE as an intermediate differentiation stage [68]. It is noteworthy, however, that there is a functional and molecular gap between hPSC-derived hHSCs and those obtained from UCB. In functional terms, there are differences in the robustness of engraftment and the recapitulation of terminally differentiated cells. In molecular terms, the global transcriptomic analysis showed that although there was a significant correlation in gene expression profiles, distinct gene clustering was observed when comparing the 500 most variable genes. Thus, such profiles were not identical and this may explain, at least in part, the functional differences observed.

Functionally mature erythroid cells have been generated from hPSCs [65]. This finding could argue in favor of the fact that hHSCs generated from hPSCs are similar to bona fide hHSCs. However, such erythrocytes were functionally assessed using an in vitro system and it is not known if they could perform normally in vivo and if they have a normal life span.

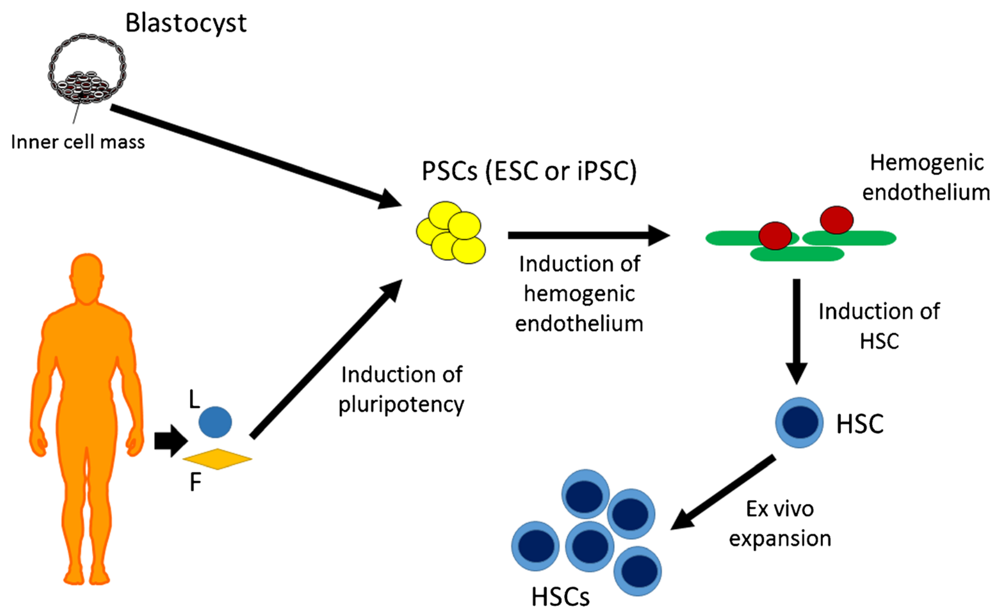

Fig. 1 Human HSCs generated from hPSCs. PSCs can be obtained directly from the inner cell mass of the blastocyst (ESCs) or by reprogramming of fibroblasts (F) or lymphocytes (L) obtained from a donor by expression of transcription factors such as OCT4, SOX2, NANOG, KLF4, or c-MYC (iPSCs). By culturing these cells in the presence of FGF, VEGF, BMP-4, IGF-1, SB431542, and CHIR99021, PSCs can be induced to develop into hemogenic endothelium (HE). Then, HE can give rise to HSCs by transduction of specific transcription factors, such as ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, and SPI1. Once generated, HSCs can be expanded by different approaches, including hematopoietic recombinant cytokines, stromal cells, and/or small molecules (see Table 1)

Conclusions and Perspectives

HCTs constitute a common therapeutic strategy for the treatment of a variety of disorders, including hematologic, neoplastic, metabolic, and immunologic diseases. However, finding a perfect match between donor and recipient and obtaining a large number of HSCs remain as two major barriers in hematopoietic transplantation. Thus, in vitro generation of hHSCs has become a priority in the hematologic field. By using different experimental in vitro systems, it has been possible to generate hHSCs in the laboratory. Such systems usually depend on the presence of hematopoietic stimulatory cytokines that act at early stages of hematopoiesis. Microenvironment stromal cells have also been shown to favor HSC development in vitro. More recently, several small molecules have been shown to further expand HSCs in culture; thus, they are being included in clinically oriented expansion protocols. Human HSCs have been generated from two biological sources: bona fide hHSCs (obtained from BM, MPB, or UCB) and hPSCs (ESCs or iPSCs).

A major concern for the clinical application of hHSCs produced in vitro is the fact that when primitive hematopoietic cells are cultured in vitro, they experience a variety of phenotypic and functional changes induced by the culture conditions. Thus, even though they may retain their original HSC immunophenotype after ex vivo expansion, their genomic and functional capacities may be altered [70–73, 75•]. On the other hand, the genomic instability of hPSCs, observed in culture by several groups [78], points to the possibility of producing hHSCs that may not be genetically and functionally equivalent to bona fide hHSCs. These are issues that must be taken into consideration when planning on using such in vitro–generated cells in clinical settings. Nevertheless, it is noteworthy that hHSCs generated from UCB-derived hHSCs have already been used as stand-alone cells for hematopoietic transplants with very encouraging results [76••]. This suggests that such cells are molecularly and functionally similar to hHSCs obtained from conventional sources. In contrast, at the moment, no data exist on the clinical use of hHSCs derived from hPSCs. Based on the results obtained so far and the relevance of this field, it is expected that those studies will be carried out in the near future (Fig. 1).

It is important to point out that in the last few years, novel in vitro culture systems have been developed for the generation of hHSCs. Indeed, researchers in Toronto have developed an automated closed-system process in which a controlled fed-batch media dilution approach is used [79]. In this system, there is continuous removal from the culture of Lin^+ cells, so that accumulation of negative regulators is prevented, whereas primitive lineage-negative cells are reselected and cultured throughout several days. The authors have reported significant increments in the numbers of total nucleated cells (179-fold), colony-forming cells (64-fold), $CD34^+$ cells (80-fold), and long-term culture-initiating cells (29-fold). Importantly, SCID-repopulating cells were also significantly expanded (11-fold), and they were capable of multilineage engraftment when transplanted into secondary animals. These results clearly suggest that such a bioprocess approach may have clinical relevance in the near future.

Three-dimensional (3D) culture systems have been recently developed in which generation of HSCs and HPCs can be achieved under conditions that mimic the bone marrow microenvironment. Such 3D cultures are based not only on the use of different stromal cell types, such as mesenchymal, endothelial, and osteoblastic cells, but also on the presence of extracellular matrix molecules and molecular scaffolds [80–83]. 3D cultures have also been used as models to study leukemic growth and chemoresistance [84]. Hematopoietic cell culture in 3D cultures can result in significant increments in the numbers of myeloid and erythroid progenitors, as well as repopulating cells [80]. When assessing the growth of hematopoietic cells in 3D and 2D cultures, the former seems to be superior as compared with the latter [85]. In vitro approaches such as the ones described above need to be assessed to determine whether they are capable of producing functional and genetically stable hHSCs that can be taken to clinical settings.

Taking advantage of the technical achievements in hHSC identification, selection, culture, expansion, and molecular manipulation; exploring new avenues for the culture and differentiation of hPSCs; and conducting innovative clinical approaches for the treatment of hematologic disorders will be fundamental for the in vitro generation of functionally, genetically, and molecularly normal hHSCs, and for their clinical application in the near future.

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Compliance with Ethical Standards

Conflict of Interest The author declares that he has no conflict of interest.

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

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