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Progress and obstacles towards generating hematopoietic stem cells from pluripotent stem cells

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Abstract

Purpose of review—Human pluripotent stem cells (PSCs) have the potential to provide an inexhaustible source of hematopoietic stem cells (HSCs) that could be used in disease modeling and in clinical applications such as transplantation. Although the goal of deriving definitive HSCs from PSCs has not been achieved, recent studies indicate that progress is being made. This review will provide information on the current status of deriving HSCs from PSCs, and will highlight existing challenges and obstacles.

Recent findings—Recent strides in HSC generation from PSCs has included derivation of developmental intermediates, identification of transcription factors and small molecules that support hematopoietic derivation, and the development of strategies to recapitulate niche-like conditions.

Summary—Despite considerable progress in defining the molecular events driving derivation of hematopoietic progenitor cells (HPCs) from PSCs, the generation of robust transplantable HSCs from PSCs remains elusive. We propose that this goal can be facilitated by better understanding of the regulatory pathways governing HSC identity, development of HSC supportive conditions, and examining the marrow homing properties of PSC-derived HSCs.

Keywords

hematopoietic stem cell; pluripotent stem cell; directed differentiation; pluripotency; homing; cell migration

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Introduction

HSC transplantation (HSCT) is a well-established life-saving therapy for a variety of malignant and non-malignant conditions including leukemias, lymphomas, immune system disorders, multiple myeloma, and bone marrow failure syndromes. Allogeneic and autologous HSCT is used in the treatment of ~50,000 patients/year worldwide [1], but a number of challenges still confront the clinical application of HSCT, including limited HSC numbers in donor grafts, availability of HLA-matched donors, and graft-versus host disease (GvHD). However, these challenges could be overcome if an unlimited autologous HSC source could be identified. PSCs, including human embryonic stem cells (hESCs) [2] and human induced pluripotent stem (iPS) cells [3], are able to proliferate indefinitely, and differentiate to all cell types. As such, PSCs are potentially the ideal source for deriving unlimited numbers of HSCs for transplantation. Furthermore, generating PSC-derived HSCs from patients with hematological diseases would be invaluable for gaining insights into disease etiology through *in vitro* and *in vivo* disease modeling, as well as providing a cell-based platform for therapeutic screening. However, despite enormous efforts over the past decade to generate transplantable HSCs from PSCs, robust methods have not yet been established. In this review, we discuss the recent progress in HSC generation from human PSCs and offer insight in overcoming challenges to achieving this goal.

Recapitulating hematopoietic ontogeny to generate HSCs from pluripotent stem cells

Vertebrate hematopoiesis occurs in two waves – primitive and definitive. This process is well illustrated in the mouse, where primitive hematopoiesis from the yolk sac generates nucleated primitive erythrocytes and some myeloid lineages commencing at around embryonic day 7.0–7.25. In contrast, definitive hematopoiesis is mesoderm-derived and contributes to all mature blood cell types (thrombo-erythroid, myeloid and lymphoid), beginning at embryonic day 10.5 in the aorta-gonad-mesonephros (AGM) region of the mouse embryo [4]. Definitive HSCs arise from a subset of cells called hemogenic endothelium (HE) within the AGM and subsequently migrate to sites of hematopoiesis in the fetal liver and ultimately the bone marrow. These definitive HSCs reside at the apex of the hematopoietic hierarchy and serve as the reservoir for life-long blood cell production. By definition, HSCs are capable of long-term multi-lineage differentiation, and, as such, PSC-derived early-stage hematopoietic cells that do not meet these operational criteria are referred to as hematopoietic progenitor cells (HPCs).

Using an *in vivo* teratoma model, recent proof of principal experiments have shown that human iPS cells can give rise to functional transplantable HSCs [5, 6]. In these experiments, human iPS cells were co-injected with mouse OP9 stromal cells, yielding teratomas in mice, which acted as *in vivo* ‘bioreactors’ that eventually generated transplantable HSCs (Figure 1). In one study, Suzuki et al generated teratomas from mouse or human iPS with co-injection of OP9 cells and supplementation with hematopoietic cytokines (SCF and TPO). After 8–10 weeks, donor CD45⁺ cells were detected in both the peripheral blood and bone marrow of the host mice [5]. CD45⁺CD34⁺ human cells isolated from the bone marrow of teratoma-bearing recipients were then transplanted to recipient mice, and multilineage

repopulation was observed, demonstrating that HSCs had been derived. In a second study, Amabile et al reported similar results, co-injecting human iPS cells with constitutive Wnt3a expressing OP9 cells, and found that human iPS-derived teratomas can generate transplantable HSC-like cells that possessed multilineage potential, including generation of functional B- and T- cells [6]. Although not fully understood, the presumption is that signals emanating from the *in vivo* microenvironment of the developing teratoma facilitated HSC development in this setting. Nonetheless, the use of teratoma-based methodologies to derive HSCs for the clinic are, at present, not feasible owing to the very low efficiency of HSC generation, and safety issues related to zoonosis and the residual undifferentiated iPS cells [7]. Recent efforts to define cell types capable of establishing niche-like environments able to support productive and ongoing hematopoiesis may offer an alternative to using teratomas in deriving HSC from PSCs [8, 9].

Ex vivo methods to derive HSCs from PSCs typically attempt to mimic normal hematopoietic development via stepwise differentiation cultures optimized to maximize the generation of intermediate cell types (Figure 1). This has been achieved using cytokines such as BMP4, Activin A, FGF, VEGF and/or supportive stromal cells, to promote the successive generation of mesoderm, hemogenic endothelia, and HPCs [10, 11]. An early report of human PSC-derived HPCs used S17 mouse stromal cells or C166 yolk-sac endothelial cells to generate HPCs from hESCs [12]. Subsequently, additional studies have refined and improved methods for HPCs differentiation from human PSCs [13–17]. While these studies convincingly demonstrate the hematopoietic potential of PSCs *in vitro*, they all failed to produce cells that were capable of robust *in vivo* engraftment. Indeed, most differentiation protocols generate HPCs with characteristics of primitive rather than definitive hematopoiesis, and whether or not such primitive progenitors can be redirected to a definitive fate is as yet unknown (Figure 1). These data suggest that current differentiation protocols to derive definitive HSCs are insufficient to recapitulate the spatio-temporal complexity, physical stimuli, and cellular composition of the milieu critical for HSC emergence during ontogeny [18–23], and our incomplete understanding of hematopoietic ontogeny remains a major hurdle for generating engraftable HSCs from PSCs.

PSC-derived HSC generation requires appropriate culture conditions for stem cell maintenance

HSCs arise during development by transitioning through a number of distinct intermediate cell types, including subsets of lateral plate mesoderm and hemogenic endothelium that rely on activation or suppression of key developmental pathways. During *ex vivo* derivation, unique culture conditions capable of recapitulating such developmental transitions are therefore required to promote and maintain each intermediate cell type, and inappropriate conditions at any stage may inhibit their eventual potential to generate HSCs. In addition to media composition and cytokine cocktails, small molecules have been used to modulate the activity of key developmental pathways involved in distinct stages of differentiation (Figure 1). For example, the TGF β inhibitor SB431542 has been shown to inhibit primitive hematopoietic differentiation by transiently inhibiting the activin-nodal pathway to drive

definitive hematopoietic fate as defined by production of cells possessing T-cell potential *in vitro* [24, 25].

For directed differentiation protocols, besides the challenge of establishing optimal conditions allowing successful navigation through multiple developmental intermediates, it is equally critical to establish conditions that promote the successful maintenance and propagation of the target cell type [26] (Figure 1). This goal is particularly relevant for deriving transplantable HSCs from PSCs, as one of the long-standing challenges in the HSC field has been the lack of appropriate conditions to maintain HSCs *in vitro*. Indeed, despite the development of diverse culture conditions over the span of several decades, culturing HSCs invariably leads to rapid loss of self-renewal potential and irreversible commitment to differentiation. Thus, the development of conditions that support HSC maintenance *ex vivo* may be prerequisite to achieving successful PSC-derived HSC generation. Towards this end, a great deal of effort has been aimed towards identifying small molecules able to maintain/expand HSCs *ex vivo* without differentiation. The most promising recent example is the discovery of the pyrimidoindole derivative UM171 by Sauvageau and colleagues [27]. Using a fed-batch system, UM171 was demonstrated to be capable of stimulating the expansion of cord blood (CB) HSCs *ex vivo* that, when transplanted into immunocompromised mice, were capable of robust human hematopoiesis for at least 6 months post-transplant in primary and secondary recipients [27]. Additional promising compounds identified in other studies include the aryl hydrocarbon receptor antagonist StemRegenin 1 [28], the histone deacetylase inhibitor valproic acid [29], dimethyl-prostaglandin E2 [30], and the SIRT1 inhibitor nicotinamide [31]. For research to proceed towards a clinical endpoint, it will be critical to develop xeno-free and chemically defined conditions for generation of definitive HSC from PSC that will likely involve combinations of small molecules, cytokines and a defined media.

Tapping transcriptional networks as a means of generating HSCs

The developmental process by which differentiated cell types arise from more primitive progenitor cells is guided in part by the successive establishment of cell type-specific transcriptional networks. Generally, lineage specification is unidirectional and irreversible with differentiated cell types, and even intermediate progenitors, being remarkably fixed with respect to their cellular identity and developmental potential. That said, seminal studies by Gurdon and others demonstrated that the process of differentiation is reversible, in experiments that showed that the nuclei of differentiated cell types could be reprogrammed to totipotency when exposed to the primitive cellular milieu of enucleated *Xenopus* oocytes [32, 33]. This process, known as somatic cell nuclear transfer (SCNT), was subsequently shown to be capable of reprogramming nuclei from differentiated mammalian cells back to a pluripotent state [34]. That ectopic expression of defined transcription factors was sufficient to convert cell fate was first shown in 1989 with the demonstration that enforced expression of a single transcription factor, *MyoD*, could convert fibroblasts to the myogenic lineage [35]. Enormous progress in this field has been made since then, culminating with the discovery by Yamanaka and colleagues that ectopic expression of four transcription factors, *c-Myc*, *Oct4*, *Klf4*, *Sox2*, could reprogram diverse cell types from mice and humans into iPS cells [3, 36, 37]. This dramatic demonstration of reprogramming has sparked an intense

interest in identifying factors able to reprogram diverse cell types to HSCs. Using a murine system, Riddell et al identified factors capable of reprogramming multiple differentiated blood cell types to cells possessing the functional and molecular properties of HSCs [38]. The combination of *Runx1t1*, *Hlf*, *Lmo2*, *Prdm5*, *Pbx1*, and *Zfp37* were found to be sufficient for reprogramming, while *Mycn* and *Meis1* increased reprogramming efficacy [38]. In a recent study, the combination of *Erg*, *Gata2*, *Lmo2*, *Runx1c*, and *Scl* were found to be sufficient to reprogram mouse fibroblasts to cells possessing short-term reconstitution ability *in vivo* [39]. Another recent study reported that *Gata2*, *Gfi1b*, *cFos*, and *Etv6*, could generate hemogenic endothelial-like precursor cells with the concomitant appearance of hematopoietic cells from mouse fibroblasts [40]. Similar studies have also been attempted in human cells. Ectopic expression of *OCT4* alone was reported to induce human dermal fibroblasts to express HSC markers and generated granulocytic, monocytic, megakaryocytic and erythroid lineages, but produced only minimal short-term repopulation when transplanted *in vivo* [41]. More recently, it was shown that the combination of *FOSB*, *GFII*, *RUNX1* and *SPII*, followed by co-culture with E4EC endothelial cells, could reprogram somatic human cells into functional multipotent hematopoietic progenitor cells possessing colony-forming-cell potential *in vitro* and a robust engraftment in primary and secondary transplantation [42]. Collectively, these studies provide evidence that transcription factor-mediated reprogramming to generate HSCs is possible, and give credence to the idea that HSC generation from human PSCs by transcription factor manipulation may also be attainable (Figure 1). In support of this notion, ectopic expression of *HoxB4* in murine ES cells was capable of generating progenitors capable of long-term multilineage hematopoiesis in lethally irradiated primary and secondary recipients [43]. However, transduction of *HOXB4* in human ESCs was not sufficient to generate transplantable HSCs [16, 44], suggesting that mouse and human PSCs may have unique differentiation processes and presumably require different transcription factors. Indeed, progress has also been made in identifying transcription factors able to promote hematopoietic differentiation of human PSCs. Overexpression of *SOX17* increases hemogenic endothelium differentiation without altering hematopoietic differentiation [45], while *HOXA9* specifically regulates the hemogenic endothelial to hematopoietic transition [46]. Transduction of *GATA2* together with either *ETV2* or *TALI* induced human PSCs to differentiate into hemogenic endothelium that could generate blood cells with myeloid or thrombo-erythroid lineage potential, respectively [47], whereas ectopic expression of a single transcription factor *RUNX1a* enhances emergence of HPCs from human PSCs [48]. Hematopoietic progenitor cells expressing *RUNX1a* show increased expansion ability *in vitro*, while retaining multilineage differentiation potential *in vitro* and *in vivo* for 9 weeks [48]. In a combinatorial approach, the ectopic expression of *HOXA9*, *ERG*, *RORA*, *SOX4* and *MYB* was sufficient to re-specify PSC-derived myeloid precursors to progenitors capable of short-term myeloid and erythroid engraftment [49]. However, though iPS cell reprogramming from diverse cell types can invariably be achieved by enforced expression of a core set of four reprogramming factors [3], a unified combination of transcription factors have yet to be identified that enable HSC derivation from multiple cell types [50]. Whether or not a core set of transcription factors exist that can impart HSC identity onto multiple other cell types is as yet unclear. Nonetheless, it is hoped that continued research in this area might eventually identify a set of factors that enable efficient and robust generation of HSCs from PSCs (Figure 1).

Homing

An often overlooked yet essential component of HSC function is their ability to “home” upon intravenous transplantation, i.e., to migrate to bone marrow and to lodge within relevant hematopoietic growth microenvironments. This function is critical for a successful clinical transplantation, but also for assaying HSC potential in transplantation assays. Homing is a multistep process that initially involves tethering and rolling interactions of HSCs onto the bone marrow microvasculature, firm adherence to the endothelial wall, transendothelial migration, and lodgment in the appropriate niche within the bone marrow. Each step of the homing process is governed by discrete molecular events, and insufficiency at any of these steps could result in failure to engraft.

The initial tethering and rolling of HSCs along marrow microvasculature is critical to slow cells from the velocity of the prevailing blood flow, thereby enabling HSCs to make direct contact with the endothelial surface and be exposed to chemokines in the local environment. This tethering and rolling is mediated primarily by interactions between the selectins and their ligands. Selectins, as their name suggests, bind to specific carbohydrate moieties such as the tetrasaccharide sialylated Lewis X (sLe^x), which is created on protein or lipid acceptors via specific Golgi glycosyltransferases. Notably, marrow sinusoidal microvessels specialized to recruit HSCs constitutively express E-selectin, and expression of E-selectin ligands on circulating HSCs dictates the tropism of cells to the marrow. In human HSCs, the principal E-selectin ligand is a glycovariant of CD44 known as HCELL [51]. HCELL is found on human HSCs, but is not present on mouse HSCs, and is the most potent E-selectin ligand expressed on any mammalian cell [52].

E-selectin-dependent tethering and rolling enable cellular proximity to chemokines which are present within target endothelial beds. For homing to marrow, HSCs recognize the chemokine CXCL12 (SDF-1), which is co-localized at marrow microvessels that express E-selectin [53]. CXCL12 binds to its receptor CXCR4, which is constitutively expressed on HSCs. Through a G-protein coupled cascade, engagement of CXCR4 results in activation of integrins such as VLA-4 ($\alpha_4\beta_1$) and LFA-1 ($\alpha_L\beta_2$) [54]. VLA-4 and LFA-1 activation enables high-affinity interactions to their ligands (VCAM-1 and ICAM-1, respectively), and results in firm adherence of the cell to the endothelial surface [54].

Once firmly adhered, cells are then able to sense and respond to chemokine gradients, and transmigrate through the endothelial wall into the perivascular space of the bone marrow. The best-studied chemokine in this regard is CXCL12 (SDF-1), which, via interaction with its cellular receptor CXCR4, is critical for transendothelial migration of murine and human HSCs [55–57]. Besides SDF-1, additional chemokines have also been identified as important for transmigration of human HSCs, including CCL2 (MCP-1), CCL5 (RANTES), and CXCL10 (IP-10) [58].

Once the cells have transmigrated through the marrow endothelium, the final step involves transit through the marrow parenchyma and lodgment in an appropriate BM niche. This process is still poorly understood, in part due to challenges in measuring localization of transmigrated cells over time. Hyaluronic acid (HA), an extracellular matrix component, as

well as CD44, the primary HA receptor, have been shown to be important for lodging within bone marrow niches [59–61]. Other factors reported to be important in murine HSC lodgment include transmembrane-bound stem cell factor [62] and osteopontin, which can bind to CD44, as well as $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins, and can act as a chemoattractant [63].

In order to be capable of engraftment *in vivo*, it is critical that the molecular effectors of homing are operable on transplanted HSCs. Though little studied in the context of directed differentiation, the lack of robust engraftment of PSC-derived HPCs suggests that homing defects may be a contributing factor (Figure 1). Therefore, when attempting to generate functional HSCs from PSCs, it may be prudent assess the expression and function of molecules involved in each of the steps outlined above. To date, experimental approaches to overcome HSC homing deficiencies have focused on creation of selectin ligands via *ex vivo* glycan engineering using recombinant glycosyltransferases [64, 65], modulation of integrins [66], and enhancing the SDF-CXCR4 axis [67, 68]. Similar efforts have been made to engineer homing in mesenchymal stem cells (MSCs) [69, 70], which represent a useful model system in this regard since MSCs have great potential as a cellular therapeutic, yet like PSC-derived HSCs, do not natively home efficiently to marrow. Optimally, technical manipulations and culture conditions for differentiating HSCs from PSCs will evolve that will maintain and/or enhance expression and function of molecules that mediate marrow homing. Alternatively, techniques to improve intra-marrow delivery of HSC may facilitate engraftment of PSC-derived HSC, without requiring systemic homing capabilities [71].

Conclusion and Future Directions

Allogeneic and autologous HSC transplantation is used widely in the treatment of hematologic and genetic conditions [1]. IPS-derived HSCs could be an ideal source for resolving current limitations related to HSC transplantation. In addition, an ability to generate bona fide HSCs from iPS lines derived from patients with genetic diseases such as sickle cell anemia [72], Fanconi anemia [73], chronic myelogenous leukemia [74], etc., could be invaluable for disease modeling. Although generating human HSCs from PSCs still remains elusive, efforts directed at identification of transcription factors regulating HSC development, small molecules to augment HSC expansion, cell culture conditions to mimic niche-like microenvironments [75], HSC-specific regulatory signaling pathways [76], and HSC homing molecule expression/activities are overcoming the current hurdles in generating functional HSCs. Collectively, these efforts should provide the needed cumulative understandings about *in vitro* conditions of HSC development from PSC that will make the promise of this approach into a clinical reality.

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Abbreviations

HSC	hematopoietic stem cells
PSCs	pluripotent stem cells
HPCs	hematopoietic progenitor cells
HCELL	hematopoietic cell E-/L-selectin ligand

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Key points

- Human PSC is an invaluable source for generating functional HSCs that could overcome current limitations associated with HSC transplantation, as well as being invaluable for disease modeling. Though enormous efforts have been directed toward deriving functional HSCs from PSCs, the ultimate goal has not been achieved.
- Current efforts to derive HSCs from PSCs have largely focused on recapitulating normal developmental progression in an *ex vivo* setting.
- The development of culture conditions that support HSC *ex vivo* maintenance and propagation will be necessary to enable *ex vivo* HSC derivation strategies.
- A better understanding of the transcriptional networks governing HSC identity may enable transcription factor-mediated approaches to deriving HSCs from PSC, and indeed lessons can be learned from a number of studies focused on deriving HSCs via reprogramming strategies from other cell types.
- As homing and lodgment of HSCs is requisite to enabling their clinical transplantation potential, optimizing the homing properties of PSC-derived hematopoietic progenitors will be required to realize the clinical potential of PSC-derived HSCs.

Figure 1. Overview of promise, challenges and future strategies for generating hematopoietic stem cells (HSCs) from pluripotent stem cells (PSCs)

(A) Method of *in vivo* teratoma formation to derive transplantable HSCs from PSCs (limitations noted). (B) Small molecule and transcription factors can be used at multiple stages of differentiation to promote developmental progression towards definitive HSCs. (C) Going forward, conditions that support PSC-derived HSC maintenance and propagation need to be developed. (D) Though not well studied, it is possible that PSC-derived HSCs may need to be engineered for effective homing and lodgment *in vivo*.