Review

Focus: Induced Pluripotency & Cellular Reprogramming

Transcription factor-mediated reprogramming toward hematopoietic stem cells

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Abstract

De novo generation of human hematopoietic stem cells (HSCs) from renewable cell types has been a long sought-after but elusive goal in regenerative medicine. Paralleling efforts to guide pluripotent stem cell differentiation by manipulating developmental cues, substantial progress has been made recently toward HSC generation via combinatorial transcription factor (TF)-mediated fate conversion, a paradigm established by Yamanaka’s induction of pluripotency in somatic cells by mere four TFs. This review will integrate the recently reported strategies to directly convert a variety of starting cell types toward HSCs in the context of hematopoietic transcriptional regulation and discuss how these findings could be further developed toward the ultimate generation of therapeutic human HSCs.

Keywords cell fate conversion; hematopoietic stem cells; induced reprogramming; transcription factors

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Introduction

The process by which differentiated cell types arise from more primitive stem and progenitor cells generally proceeds down a strict linear hierarchy defined by progressive functional specialization concomitant with restriction of lineage potential. From embryogenesis initiated by a single totipotent zygote to the lifelong homeostasis of organ parenchyma by tissue-specific stem cells, physiological differentiation of progenitor cells largely proceeds both unidirectionally and irreversibly, with differentiated cell types and even intermediate progenitors being remarkably fixed with respect to their cellular identity and functional potential. This paradigm, however, was challenged by the seminal works of Gurdon and others that demonstrated the sufficiency of factors present in oocyte cytoplasm to reverse differentiation of somatic nuclei and allow cloning of whole animals (Briggs & King, 1952; Gurdon, 1962; Wilmut et al., 1997). Subsequent efforts to identify the trans-acting factors capable of altering cell fate revealed the central role of transcription factors (TFs) in determining cellular identity. This was first demonstrated by the ability of a single TF MyoD to imbue myogenic identity on fibroblasts (Davis et al., 1987), which then established the foundation for the landmark discovery by Yamanaka that mere four TFs are sufficient to induce pluripotency in somatic cells (Takahashi & Yamanaka, 2006; Takahashi et al., 2007). These and other studies galvanized numerous investigators to harness the power of TFs in directly respecifying multiple cell fates: hepatocytes (Huang et al., 2011; Sekiya & Suzuki, 2011) cardiomyocytes (Ieda et al., 2010), cardiac pacemaker cells (Kapoorn et al., 2013), oligodendrocytes (Najm et al., 2013; Yang et al., 2013), various types of neurons (Vierbuchen et al., 2010; Son et al., 2011; Liu et al., 2013), neural stem cells (Han et al., 2012), pancreatic beta cells (Zhou et al., 2008), sertoli cells (Buganim et al., 2012), thymic epithelium (Bredenkamp et al., 2014), endothelial cells (Han et al., 2014), and intestinal progenitors (Morris et al., 2014).

Studies of the hematopoietic system have borne profound insights into the transcriptional regulation of cellular identity. The ability to prospectively isolate hematopoietic stem, progenitor, and effector cells with defined lineage potentials has allowed dissection of molecular mechanisms underlying blood differentiation as well as identification of characteristic TFs governing diverse hematopoietic lineages (Orkin, 1995). However, the wave of breakthroughs from cell fate conversion studies transformed the hematopoietic system from a subject of scrutiny to a destination to be reached from alternative cell types. Initial studies focused on manipulating lineage potential by means of oncogenic transformation (Beug et al., 1979; Graf et al., 1992), lineage switching of hematopoietic progenitors (Heyworth et al., 2002), and direct cell fate conversion toward non-progenitor blood cells such as macrophages (Xie et al., 2004; Laiosa et al., 2006; Feng et al., 2008); however, substantial progress has been made recently toward de novo generation of hematopoietic stem cells (HSCs) (Szabo et al., 2010; Doulatov et al., 2013; Pereira et al., 2013; Batta et al., 2014; Pulecio et al., 2014; Riddell et al., 2014; Sandler et al., 2014). HSCs reside at the apex of the hematopoietic hierarchy and serve as the lifelong reservoir for all downstream blood cells. Their remarkable regenerative capacity to durably restore the entire hematopoietic system in transplant recipients has been harnessed as the standard of care for treatment of a number of...
morbid conditions including hematological malignancies, bone marrow failure syndromes, and immunodeficiency syndromes with ~50,000 allogeneic or autologous transplants performed each year (Gratwohl et al., 2010). Many factors contribute to transplantation outcomes including relapse of primary disease, graft failure, and opportunistic infection. Moreover, in addition to the challenge of identifying histocompatible donors, allogeneic transplantation is further complicated by graft-versus-host disease (GVHD), which remains a significant cause of morbidity and mortality for a large number of patients who undergo allogeneic transplantation despite the use of prophylactic immunosuppressants (Petersdorf, 2013). Another major factor contributing to transplant success is the number of stem cells transplanted, with an increased number of CD34+ HSPCs being the strongest predictor of transplantation success as measured by rapid and durable hematopoietic recovery (Siena et al., 2000). Transplants that contain too few HSCs either fail to engraft altogether or result in delayed blood reconstitution post-transplantation that is associated increased morbidity and mortality. This is particularly clinically challenging for the 10–30% of patients from whom sufficient numbers of autologous stem cells cannot be harvested due to poor responsiveness to mobilizing agents such as GCSF (Bakanay & Demirer, 2012). Therefore, an ability to produce an inexhaustible supply of autologous HSCs from relatively dispensable and potentially expandable cell types via cell fate conversion represents an attractive solution to these challenges. This review will discuss the critical roles played by TFs in hematopoietic cell fate regulation and how this knowledge has propelled efforts to convert alternative cell types toward fully functional HSCs.

Transcriptional regulation of cellular identity

TFs dictate the specific gene expression pattern necessary for a cell to perform its unique functions. Mechanistically, TFs directly impact chromatin state by recruiting epigenetic modifiers to specific DNA sequence motifs present in gene regulatory regions such as promoters and enhancers (Rosenfeld et al., 2006). Depending on whether a TF recruits transcriptional coactivators or corepressors, it may either promote or suppress gene expression, respectively. Consequently, a TF may contribute to enforcing a particular cell fate by simultaneously activating genes required for maintaining the function and identity of that cell while antagonizing lineage inappropriate genes (Cantor et al., 2008; Pongubala et al., 2008; Schaffer et al., 2010; Qi et al., 2013). In addition to recruiting cofactors, TFs often bind cooperatively to DNA as components of multiprotein complexes (Huang et al., 2009; Ravasi et al., 2010; Kazemian et al., 2013). Thus, the same TF may exhibit completely different genome-wide binding patterns and regulate non-overlapping sets of target genes in different cell types (Hoffman et al., 2010; Pimkin et al., 2014). Indeed, cell fate and function is invariably the result of the combinatorial action of TF complexes that form interdependent nodes that comprise larger regulatory networks. The wiring of cell-type-specific TFs as self-reinforcing circuits allows robust, stable sustenance of specific transcriptional landscapes (Rao et al., 2002; Chew et al., 2005; Bonzanni et al., 2013). On the other hand, antagonistic relationships between TF sets governing alternate cell fates serve as barriers to cellular plasticity and thus provide a basis for their mutual exclusivity (Graf & Enver, 2009).

TFs exert differential spheres of influence over cell fate depending on their connectivity within gene regulatory networks. Some TFs function as critical hubs, and their loss may lead to the collapse of network integrity (Albert et al., 2000). The potency of TFs may also derive from their ability to act as pioneering factors that can directly trigger nucleosomal remodeling to grant chromatin access to additional TFs (Zaret & Carroll, 2011). Some of these factors can be so potent that they can single handedly convert cell fate as is the case for MyoD in establishing the myogenic transcriptome, Cebpa in activating the myeloid program (Xie et al., 2004), and Runx1 in coordinating the endothelial-to-hematopoietic transition during development (Feng et al., 2008). More often, however, multiple TFs must act in concert to access and activate cell-type-specific gene regulatory networks (Wilson et al., 2010a).

Reprogramming and cell fate conversion take advantage of the connectedness and interdependencies of TFs in orchestrating cell fate programs (Buganim et al., 2013). Ectopic expression of a subset of TFs enriched in a destination cell type can be sufficient to actuate the destination gene regulatory network in an alternate starting cell type. The destination cell gene regulatory network may then predominate over that of the starting cell type, thus altering its identity. This process depends on extensive chromatin reconfiguration including reversal of chromatin inaccessibility, installation of destination cell specific enhancers, and shutdown of regulatory elements specific to the starting cell type, the carryover of which could lead to retention of what has been termed ‘epigenetic memory’ (Hu et al., 2010; Kim et al., 2010; Apostolou & Hochdeller, 2013; Vaskova et al., 2013).

The self-reinforcing nature of gene regulatory networks implies that a small number of TFs may be sufficient to trigger their establishment and maintenance of destination cell regulatory networks. Moreover, recent evidence has demonstrated that regulatory networks governing cell identity could potentially be seeded by multiple distinct combinations of TFs. Indeed, extensive studies of TFs regulating embryonic stem cells have yielded multiple TF combinations capable of IPS cell generation that are different and even completely distinct from that originally reported by Yamanaka (Montserrat et al., 2013; Shu et al., 2013; Buganim et al., 2014; Takashima et al., 2014).

Transcriptional regulation of hematopoietic cell fates

The hematopoietic system has been studied extensively as a model tissue hierarchy for dissecting transcriptional regulation of cellular identity and cell fate transitions. As cells descend from HSCs, they are subjected to tiers of decisions that successively commit them to their final effector function. The molecular events underlying developmental cell fate decisions have been attributed in part to cross-antagonism between lineage-specific TFs, perhaps best illustrated by the activities of Gata1 and Pu.1 in promoting erythroid and myeloid differentiation programs, respectively (Arinobu et al., 2007). Physical interaction between Gata1 and Pu.1 leads to mutual extinction of transcriptional activity (Nerlov et al., 2000; Zhang et al., 2000). While they are both expressed in multipotent progenitors, offset in their relative levels, modulated by parameters such as cell cycle length (Kueh et al., 2013) and instructive cytokine signaling (Sarrazin et al., 2009), allows the higher expressed factor to
dominate cell fate decisions and hence tip the balance toward the respective lineage (Huang et al., 2007). Multiple such mutually antagonistic interactions could be assembled to form a greater landscape of attractors and transitional states that models multilineage differentiation (Krumsieck et al., 2011).

Pax5 is another TF whose role in hematopoietic lineage commitment has been studied extensively (Urbanek et al., 1994). Pax5 is essential in B-cell commitment, the lack of which arrests differentiation at the early pro-B stage (Nutt et al., 1999). Mechanistically, Pax5 locks progenitors into B-cell fate by both activating B cell promoting signaling pathways while silencing those important for the development of alternative lineages. Intriguingly, Pax5 null pro-B cells retain not only self-renewal potential but also multilineage differentiation potential spanning myeloid lineages and T cells (Mikkola et al., 2002) though Pax5-deficient progenitors do not give rise to B cells (Urbanek et al., 1994) and have very limited potential to give rise to erythrocytes or platelets (Nutt et al., 1999; Schaniel et al., 2002). The importance of Pax5 for limiting self-renewal and locking in B-cell fate is further illustrated by experiments in which deletion in CD19-positive B cells results in an aggressive and highly penetrant lymphoma in vitro (Cobaleda et al., 2007).

In addition to regulating developmental lineage commitment, TFs may impact subtype specification within hematopoietic lineages. For example, peripheral CD4 T-helper cells can develop into induced regulatory T cells (iTregs) with appropriate immunosuppressive functions upon induction of Foxp3, a TF critical for all regulatory T-cell development (Rudensky, 2011). However, inflammatory signals can extinguish Foxp3 expression in iTregs and convert them back to effector CD4 T-helper cells (Zhou et al., 2009). Furthermore, diverse subsets of macrophages are specified by the integration of Pu.1, a macrophage lineage determining TF, with transcriptional regulators downstream of tissue-specific environmental signals (Gosselin et al., 2014). These examples suggest that cellular identity can be a composite of multiple transcriptional modules each presided over by unique TFs or their combinations.

**Artificial hematopoietic lineage conversions toward therapeutic application**

Artificial manipulation of TFs has yielded important insights into the molecular underpinnings of lineage choice (Iwasaki et al., 2006). Overexpression together with the loss of function experiments has been used to confirm the nature of interaction between TFs and led to surprising discoveries on cellular plasticity. In addition to obtaining valuable mechanistic information, excitement in cell fate conversion research has been fueled by its enormous clinical potential. Cell fate conversion has the potential to generate patient-specific cells that are rare, inaccessible, or clinically useful from relatively dispensable autologous cells. If realized, such procedures could be applied to supplying cells for human disease modeling, therapeutic screening, and cell replacement therapy (Robinson & Daley, 2012; Cherry & Daley, 2013; Kamao et al., 2014; Nakamura et al., 2014; Stewart, 2014; Waingert et al., 2014). Thus, this strategy represents an attractive means to address both patient specificity and overcoming the rarity, and lack of means for expansion that currently limits the therapeutic use of HSCs.

A parallel strategy toward generating HSCs has been via stepwise differentiation of pluripotent stem cells such as embryonic stem cells or induced pluripotent stem cells (Sturgeon et al., 2013). However, attempts to direct differentiation of pluripotent stem cells toward HSCs by recapitulating the embryonic developmental trajectory in vitro has seen limited success. Simulating the temporal (Tober et al., 2013), spatial (Peeters et al., 2009; Wilkinson et al., 2009), mechanical (North et al., 2009), and cellular (Clements et al., 2011; Espin-Palazon et al., 2014) complexity of the embryonic milieu has proved technically challenging. Moreover, since the precise developmental intermediates en route to HSCs are only recently becoming elucidated (Rybtsov et al., 2011, 2014), the directed differentiation approach has thus far suffered from paucity of reliable developmental guideposts. For example, induction of T lymphoid potential has been used to guide directed differentiation as it correlates with definitive hematopoiesis, a temporal wave of embryonic hematopoiesis during which HSCs are specified (Kennedy et al., 2012). However, it is still unknown whether the in vitro derivatives with T lymphoid potential indeed possess the eventual capacity to produce HSCs or whether they might represent a developmental intermediate similar to embryonic T-cell progenitors that arise independently of HSCs (Yoshimoto et al., 2012). Due to such state of the field, little is known about the functional and molecular correlation between the developmental intermediates obtained in vitro and in vivo, and the knowledge is particularly lacking in the context of human HSC ontogeny (Ivanovs et al., 2014). Furthermore, the necessity to transit through multiple distinct intermediate cell types to reach HSCs means that culture conditions may need to be optimized for every intermediate and that deviation in any one step may extinguish the eventual potential to develop HSCs. Therefore, in spite of ‘forceful’ nature of TF manipulations, TF-mediated cell fate conversion may be a relatively simpler, direct, and even more tractable of a strategy for deriving HSCs as it only requires knowledge of the properties of the destination cell type.

**Generation of hematopoietic stem cells via direct cell fate conversion**

HSCs are functionally defined as cells capable of engrafting conditioned recipients and giving rise to all blood lineages (i.e., myeloid, thrombo-erythroid, and lymphoid), for an extended period (at least 4 months in mice). These functional hallmarks of HSCs, namely multilineage differentiation potential and extensive self-renewal capacity, are embodied at the clonal level such that only few HSCs are required to durably sustain the entire hematopoietic system (Holstege et al., 2014). Although fully functional human HSCs meeting the aforementioned criteria have yet to be produced in vitro, several strategies have recently been described that bring the goal of deriving fully functional HSCs from alternative cell types within reach (Table 1).

Szabo et al. (2010) and Pulecio et al. (2014) converted human fibroblasts to hematopoietic cells possessing multilineage myeloid potential aided by pluripotency-associated TFs, namely OCT4 and SOX2, respectively. The latter study also showed improved hematopoietic conversion with the addition of mir125b, a microRNA enriched in human hematopoietic progenitors. Since transient expression of pluripotency factors or OCT4 is sufficient to confer
Table 1. Summary of studies using transcription factor-mediated reprogramming to derive primitive blood progenitors.

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<td>ES cell-derived myeloid restricted progenitor</td>
<td>B-cell progenitor, myeloid progenitor, bone marrow myeloid effector</td>
<td>Umbilical vein endothelial cells, microvascular endothelial cells</td>
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<td>Transcription factors</td>
<td>OCT4</td>
<td>SOX2, mir125b</td>
<td>Gata2, Gfi1b, cFos, Etv6</td>
<td>Erg, Gata2, Runx1c, Scl, Lmo2</td>
<td>ERG, HOXA9, RORA, SOX4, MYB</td>
<td>Runx11, Hif, Lmo2, Pbx1, Zfp37, Prdm5, Mycn, Meis1</td>
<td>FOSB, GFI1, RUNX1, SPI1</td>
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<sup>a</sup>Lymphoid differentiation potential acquired with p53 deletion.
<sup>b</sup>Although modest T-cell differentiation potential was confirmed in vitro, no T cells were detected in vivo.
<sup>c</sup>Minimal in vitro T-cell differentiation possible when TFs are expressed using inducible system.
<sup>d</sup>Engrafted cells express low levels of CD45, a pan-lympho-myeloid hematopoietic marker.
<sup>e</sup>Very short-term (2 week), primarily erythroid engraftment.
<sup>f</sup>In vivo function not assayed with cells derived using inducible system.

Tri-germ layer differentiation potential on fibroblasts, fate conversion specifically to the blood lineage with OCT4 or SOX2 was likely mediated by the inductive effects of hematopoietic cytokines (Mitchell et al., 2014), which has previously been shown to be able to reprogram blood cell identity (Kondo et al., 2000). Although the resulting cells were able to engraft in vivo, the majority of them expressed low levels of the pan-hematopoietic antigen, CD45, and did not peripheralize, possibly suggesting incomplete hematopoietic conversion.

Instead of transitioning through a developmentally plastic state, a more direct, autonomous fate transition could be achieved by overexpressing lineage-specifying TFs enriched in destination cells. Toward this, Pereira et al. (2013) screened 18 candidate TFs enriched in quiescent mouse HSCs that could activate exogenous human CD34 promoter inserted into mouse fibroblasts. The screen identified transient expression of Gata2, Gfi1b, cFos, and Etv6 to be sufficient for generating hematopoietic cells from fibroblasts via an intermediate cell type that coexisted both endothelial and hematopoietic markers. Although the converted hematopoietic cells were similar to mouse hematopoietic stem/progenitor cells with respect to gene expression, they were devoid of in vitro clonalogenic potential unless cocultured with placental stroma, suggesting that maturation into progenitor-like blood cells required additional signals. Clonal multilineage potential or in vivo functionality was not assayed. A similar fate conversion strategy from fibroblasts was employed by Battaglia et al. (2014) who screened a curated set of 19 hematopoietic TFs for morphological change of murine fibroblasts to round hematopoietic cells. Five TFs, Erg, Gata2, Lmo2, Runx1c, and Scl, were found to robustly induce hematopoietic colonies from both embryonic and adult fibroblasts, and the reprogrammed cells were shown to possess erythroid, megakaryocytic, granulocytic, and macrophage differentiation potentials. Similar to Pereira et al., Battaglia et al. also observed that fibroblasts converted to hematopoietic cells via an endothelial intermediate. In vitro clonogenic assays confirmed the presence of cells possessing multilineage potential; upon transplantation, however, these cells only gave rise to very short-term (2 weeks) erythroid chimerism. Interestingly, p53 nullizygosity not only enhanced the efficiency of reprogramming but also increased erythroid differentiation potential in addition to permitting production of receptor rearranged B and T lineage cells.

Although iPS cells have the developmental potential to be differentiated toward potentially transplantable autologous tissues, their hematopoietic differentiation has yielded progenitors with greatly restricted self-renewal and differentiation potentials quite unlike those of true HSCs. Doulatov et al. (2013) sought to respect iPS cell-derived myeloid restricted progenitors toward HSCs using TFs enriched in both human and mouse HSCs that appeared underexpressed in the blood progenitors cells derived from pluripotent cells.
Screening nine candidate TFs and using serial plating as a readout, ectopic expressions of ERG, HOXA9, and RORA were found to instill robust in vitro clonogenic potential but not multilineage potential or engraftment capacity. However, additional ectopic expression of SOX4 and MYB enabled the acquisition of myelo-erythroid differentiation potential as well as short-term myeloid engraftment capacity in immunocompromised mice. Although modest T lineage potential was confirmed in vitro, the grafts failed to produce lymphoid lineage in vivo. Long-term engraftment was not achieved.

As opposed to respecifying embryonic-like hematopoietic cells derived from pluripotent stem cells, Riddell et al (2014) undertook reprogramming of primary adult lineage committed murine hematopoietic progenitors and effectors using gene regulatory factors exhibiting restricted expression in mouse HSCs relative to the majority of their differentiated progeny. An unbiased screen of 36 factors, which included 33 TFs and three translational regulators, was performed in the transplantation setting to take advantage of the sensitivity of the assay in reading out HSC activity at the single-cell level, and potentially co-opt signals present in the in vivo environment that might facilitate cell conversion. The screen identified six genes Hlf, Runx1, Pbx1, Lmo2, Prdm5, and Zfp37 whose transient ectopic expression was sufficient for instilling multilineage reconstituting potential on otherwise lineage committed hematopoietic cells. Inclusion of Meis1 and Mycn was found to improve reprogramming efficiency. Long-term multilineage reconstitution, serial transplantability, reconstitution of bone marrow progenitor compartments and secondary hematopoietic organs, and single-cell gene expression profiling confirmed that the reprogrammed cells possessed the functional and molecular properties of endogenous HSCs and thus were termed ‘induced HSCs’ (iHSCs).

HSCs and endothelial cells share an intimate ontological relationship as HSCs are specified from hemogenic endothelial-like intermediates during embryogenesis via endothelial-to-hematopoietic transition (EHT). Sandler et al hypothesized that EHT may be recapitulated in mature, non-hemogenic endothelial cells by ectopic expression of key TFs and provision of an inductive environment (Zovein et al, 2008; Bertrand et al, 2010; Boisset et al, 2010; Gordon-Keylock & Medvinsky, 2011; Sandler et al, 2014). Screening 25 TFs expressed at a higher level in human cord blood HSPCs relative to human umbilical vein endothelial cells (HUVECs) identified the minimal set of FOSB, GFI1, RUNX1, and SPI1 to be sufficient and necessary for robustly generating hematopoietic colonies from HUVECs and human adult dermal microvascular endothelial cells. The reprogramming was found to strictly depend on an endothelial stroma previously developed by the authors’ group for maintaining human cord blood HSPCs (Butler et al, 2012). The reprogrammed cells possessed both in vitro and in vivo multilineage differentiation potential, long-term reconstitution potential, in vivo homing/engraftment capacity, and serial transplantability, only with the caveat of defective T-cell differentiation potential, thus earning the label of multipotent progenitors (MPPs).

Despite sharing the same destination identity, the studies summarized above show striking diversity with respect to experimental design and the TF combinations identified (Fig 1). However, in totality, the reported TFs are highly enriched for both developmental genes involved in embryonic specification of hematopoiesis and those implicated in leukemogenesis. Classical developmental hematopoiesis genes such as Scl, Gata2, Gfi1, Runx1, and Sp1 (Pu.1) (Wilson et al, 2010b) appear to be important in fate conversions that involve lineage switching, such as from fibroblasts or endothelial cells, whereas they do not appear to be important in reprogramming or respecification within the blood lineage. This could reflect that even post-embryogenesis, the same small set of TFs that specified hematopoiesis earlier in life can pioneer the establishment of hematopoietic program in non-hematopoietic adult cell types, though such activity may be redundant in conversions within hematopoietic lineage. The requirement for TFs such as HOXA9, MYB, Lmo2, Pbx1, Mycn, and Meis1 in reprogramming of committed blood cells is consistent with their classical roles in tumor development (Thorsteinsson et al, 2001; Kawagoe et al, 2007; Jin et al, 2010; McCormack et al, 2010). In particular, components of Hox effectors, namely HOXA9, Pbx1, and Meis1, are found in TF sets reported by both Doulavot et al and Riddell et al. This particular set of TFs has been shown to function as a heterotrimeric complex (Shen et al, 1997) and is frequently represented in regenerative processes (Mercader et al, 2005; Capellini et al, 2006; Chen et al, 2013; Roensch et al, 2013) and across multiple types of cancers (Morgan et al, 2007; Shears et al, 2008; Plowright et al, 2009; Sun et al, 2013), suggesting that it may regulate generic properties of stem/progenitors such as self-renewal, anti-apoptosis, and differentiation arrest. In spite of the apparently central role played by the Hox complex in regulating stemness, it is intriguing that ectopic stimulation of this pathway was not required to generate progenitor cells as highly functional as the MPPs directly from endothelial cells (Sandler et al, 2014). However, this could be explained by the fact that endothelial cells exhibit intrinsic HoxA cluster activity, which is integral to vascular development and function (Rössig et al, 2005; Bandyopadhyay et al, 2012).

Species-specific differences may also contribute to the identification of distinct HSPC-inducing TF combinations. It is possible, though not rigorously studied, that divergent gene regulatory networks may govern mouse and human HSCs. For example, although constitutive ectopic expression of Hoxb4 allows the generation of engraftable mouse HSPCs from mouse embryonic stem cells (Wang et al, 2005b; Matsumoto et al, 2009), the same robust effect was not seen using human cells (Wang et al, 2005a). In the erythroid lineage, noticeable transcriptional divergence was found between mouse and human cells isolated from comparative stages of differentiation (Pishesha et al, 2014). Furthermore, overexpression of a dominant-negative isoform of IKAROS (IKZF1) was recently found to impart diameentric effects on mouse and human HSPCs (Beer et al, 2014). Whereas IKZF1 overexpression in mouse HSPCs suppressed B but enhanced T lineage outputs, the same manipulation in human cord blood HSPCs significantly increased B lineage cell production without affecting T lineage. Therefore, although the majority of TFs discovered in the studies using murine cells (Pereira et al, 2013; Riddell et al, 2014) are highly homologous between mouse and human, it is uncertain whether the same set of factors capable of inducing mouse HSPCs would also be sufficient for inducing human HSCs.

Another major variable among the studies is the system used for ectopic expression of TFs. An important criterion for complete cell fate conversion is transgene-independent sustenance of destination cell gene regulatory networks. Although transduction with viruses encoding TFs under constitutive promoters may become passively silenced over time, the use of an inducible transgene
system (i.e., doxycycline inducible) can give better temporal control over TF expression as well as ensure that the ectopic genes are turned off upon completion of cell fate conversion. Importantly, continued ectopic expression of TFs involved in ‘respecification/reprogramming’ may also interfere with the function of the destination cells. For example, continued ectopic expression of SPI1 in endothelial cell-derived multipotent progenitors (MPPs) was shown to block T-cell differentiation potential (Sandler et al., 2014). In the same study, as only MPPs obtained using constitutive expression vectors were assayed in vivo, it remains to be shown definitively whether the MPPs can stably maintain their identity independent of residual transgene expression.

Since environmental responsiveness and developmental plasticity represent cardinal properties of HSCs, it is conceivable that environmental cues may strongly influence cell fate conversion toward HSCs. Reasoning that reprogramming in the context of the native HSC niche (Morrison & Scadden, 2014) may facilitate the acquisition of HSC identity, Riddell et al. (2014) conducted reprogramming experiments in vivo. Similarly, Sandler et al. took advantage of an endothelial stromal coculture system developed for ex vivo maintenance of human HSCs to provide an inductive environment for de novo HSC generation (Butler et al., 2012; Sandler et al., 2014). Intriguingly, both of these studies gave rise to serially transplantable cells possessing lympho-myeloid multilineage potential, a feat unattained by any of the other cell fate conversion strategies, which were all conducted in the absence of HSC supportive milieu. Although Riddell et al. did not show the absolute requirement of the in vivo environment for HSC induction, an HSC supportive environment was shown to be a necessity in the conversion of endothelial cells to MPPs by Sandler et al. Insights from other cell fate conversion systems also point to the importance of the environment. In particular, STAT3, a transcription factor directly activated by growth factor signaling, has been shown to play an central role in embryonic stem cells as well as in induction of pluripotency (Niwa et al., 1998; Raz et al., 1999; Yang et al., 2010; van Oosten et al., 2012), thus emphasizing that cell extrinsic cues can be as important as the intrinsic ones.

**Figure 1. Categorization of transcription factors employed toward de novo HSC generation.**

TFs employed by Szabo et al. (Sz), Pulecio et al. (Pu), Pereira et al. (Pe), Batta et al. (Ba), Doulatov et al. (Do), Riddell et al. (Ri), and Sandler et al. (Sa) are classified into categories based on previously described activities. ‘Heptad’ factors refer to classical hematopoietic TFs known for their physical interactions (Wilson et al., 2010a). Individual loss of function of TFs highlighted in pink has been shown to impair HSC activity.

**Toward understanding the transcriptional regulation of HSC identity**

In spite of enormous progress, the era of HSC induction has only dawned. With little consensus on the optimal combination of TFs and/or environmental cues for inducing HSCs, it may be necessary to perform comparative studies or even ‘mix and match’ findings from hitherto studies for even better results. With respect to reevaluating TF combinations for human HSC induction, it may be worth noting the heterogeneity of primary human HSCs. Although immunophenotypically defined single human HSC has been successfully isolated (Notta et al., 2011), a number of studies show that the human HSC compartment can still be fractionated into subpopulations with distinct functional potentials (Anjos-Afonso et al., 2013; Chitteti et al., 2014) similar to the HSC subfractionation that has been demonstrated in the mouse (Dykstra et al., 2007; Beerman et al., 2010; Morita et al., 2010; Babovic & Eaves, 2014). Therefore,
TFs enriched in each of these subpopulations may differentially impact HSC induction.

Understanding the function of individual TFs in HSCs constitutes an important step toward elucidating the mechanism of HSC induction. Consistent with the ability to access the HSC gene regulatory program, many of the TFs identified by the studies highlighted in this review have been previously implicated in HSC and progenitor biology. Individual loss of function of Hoxa9 (Lawrence et al., 2005), Gata2 (Lim et al., 2012), Pbx1 (Ficara et al., 2008), Meis1 (Unnisa et al., 2012), Gfi1 (Hock et al., 2004; Zeng et al., 2004), Etv6 (Wang et al., 1998), SPI1 (Pu.1) (Staber et al., 2013), Myb (Lieu & Reddy, 2009), or Erg (Ng et al., 2011) in HSCs has each been shown to compromise their homeostatic maintenance and/or competitive repopulation potential. Although direct loss of function of Lmo2 in HSCs has not been conducted, its critical role in hematopoiesis is underscored by the inability of Lmo2 null ES cells to contribute to postnatal hematopoiesis in chimeric blastocysts (Yamada et al., 1998). Similarly, Runx1 null ES cells cannot contribute to adult hematopoiesis (Okuda et al., 1996) due to impairment of EHT (Chen et al., 2009). Intriguingly, however, conditional loss of Runx1 in the adult hematopoietic system only impairs megakaryocytic and lymphoid differentiation with minimal impact on HSC activity (Ichikawa et al., 2004; Cai et al., 2011). Although the consequence of loss of function of Sox4, Rora, Runx1L1, Zfp37, Prdm5, or Hlf in HSCs has yet to be reported, overexpression and leukemia studies have implicated some of these factors in HSC function. For example, Sox4 has been implicated in self-renewal of leukemic cells (Zhang et al., 2013) and ectopic expression of Hlf, a leukemia-associated TF (Hunger et al., 1992), has been shown to enhance repopulation potential of human HSCs (Shojai et al., 2005) and allows maintenance of in vitro multilineage differentiation potential and serial plating capacity of murine HSCs and progenitors (Gazit et al., 2013). Also of note, many of these understudied factors, namely Runx1L1 (Lindberg et al., 2003), Zfp37 (Dreyer et al., 1998), and Prdm5 (Duan et al., 2007), identified by Riddell et al. (Riddell et al., 2014) function as transcriptional repressors, suggesting that active repression of differentiation-associated genes may be an important component of reprogramming differentiated blood cells back to HSCs. Finally, loss of function of some TFs may not result in HSC defects due to functional redundancies with other TFs. For example, Mycn deletion in HSCs is compensated by the presence of c-Myc (Laurenti et al., 2008). However, combined deletion of Mycn and c-Myc demonstrated the requirement of the Myc genes in the exit from stem cell state as well as preventing apoptosis of HSCs. Similarly, Scl nullizygosity has no effect on HSCs due to its functional redundancy with Lyl1, but their combined ablation leads to loss of HSCs via apoptosis (Souroulias et al., 2009).

Detailing the interactions between the TFs used in HSC induction may provide deeper insights into the gene regulatory network governing HSC identity, which could lead to discoveries of more efficient or alternative TF combinations for inducing HSCs. Many of the reported TFs have already been shown to interact physically and/or at the transcriptional level. As mentioned previously, Hoxa9, Meis1, and Pbx1 directly interact to coregulate transcription (Shen et al., 1997), although they may also function independently as their individual deletions result in differential HSC phenotypes (Lawrence et al., 2005; Ficara et al., 2008; Unnisa et al., 2012). The Hox complex cannot only autoregulate Hox gene expression (Trivedi et al., 2008; Horman et al., 2009) but also enhance the expression of a number of other reprogramming TFs such as Erg, Myb, Sox4, Lmo2, Etv6, Mycn, and Hlf in blood cells (Palmqvist et al., 2007; Nagel et al., 2011; Huang et al., 2012) although there also appears to be redundancies among these targets such as the cross-regulation between Lmo2 and Erg (Oram et al., 2010) and induction of Lmo2 by Hlf (de Boer et al., 2011). However, the diverse and wide-ranging targets of the Hox complex combined with the fact that it regulates distinct processes in non-hematopoietic tissues (i.e., body segmentation, blastema formation, and non-hematopoietic cancers, mentioned previously) suggests that the complex may require interaction with lineage-restricted TFs to exert tissue-specific functions. In support of this, Hoxa9/Meis1 has been shown to specifically bind and activate myeloid enhancers in cooperation with hematopoietic TFs such as SPI1 (Pu.1), and Runx1 (Huang et al., 2012). This model implies that HSC specification by the Hox complex requires either a priori patterning of relevant enhancers or coexpression of hematopoietic-specific TFs that can pioneer enhancer establishment. Following this logic, under certain circumstances, overexpression of the Hox complex may impede cell fate conversion by promoting maintenance of enhancers specific to the starting cell type, akin to the antagonism of EHT by Hoxa3 during embryogenesis (Iacovino et al., 2011).

Extensive interactions have also been reported between TFs classically associated with hematopoiesis such as Scl, Runx1, Gata2, Gfi1, and SPI1 (Pu.1) that appear in the reprogramming TF cocktails. For example, a heptad of TFs regulating HSPCs, SCL, LYL1, LMO2, GATA2, RUNX1, ERG, and FLI1 have been shown to directly bind each other and cooperatively regulate hematopoietic genes (Wilson et al., 2010a) as well as autoregulate their own expression (Grass et al., 2003; Pimanda et al., 2007; Diffner et al., 2013). While the majority of these TFs is implicated in both hematopoietic and endothelial development (De Val & Black, 2009), Runx1 has received much attention for its specific and critical role in embryonic HSC specification (North et al., 2002). During mid-gestation, Runx1 specifies HSCs by acting as a pioneering TF that initiates expression of hematopoietic genes such as SPI1 (Pu.1) in an endothelial-like cell (Huang et al., 2008; Chen et al., 2009; Lichtinger et al., 2012; Tanaka et al., 2012). The activity of Runx1 is modulated not only by its obligate binding partner Cbfβ and other members of the heptad TFs but also by the API complex, whose motifs are highly enriched at genomic Runx1 binding peaks (Pencovich et al., 2011; Lie et al., 2014). API complex has been shown to physically interact with Runx1 (Hess et al., 2001; D’Alonzo et al., 2002) and is itself a heterodimer of JUN and FOS family proteins, the latter of which were found to be necessary to induce hematopoiesis from non-hematopoietic cells in the studies by Pereira et al. (2013) and Sandler et al. (2014). The same studies also identified Gfi1/Gfi1b and SPI1 (Pu.1), direct targets of Runx1 that promote the loss of endothelial identity associated with EHT (Lancrin et al., 2012; Pereira et al., 2013; Sandler et al., 2014; Wilkinson et al., 2014). Interestingly, Gfi1 has been shown to directly repress Hoxa9, Pbx1, and Meis1 (Horman et al., 2009), which could support the aforementioned notion that suppression of Hox complex may be important to facilitate lineage switching toward blood. Ultimately, the kinetics and expression levels of TFs may need to be regulated for optimal HSC induction.
TFs mediate cell fate conversion by rewriting epigenetic information, which has been shown to be facilitated by directly modulating nuclear enzymes responsible for chromatin modifications. A number of small molecules that increase chromatin accessibility have been shown to enhance the efficiency of iPSC generation (Federation et al., 2014). One study even demonstrated that a G9a histone methyltransferase inhibitor was able to replace Oct4 in inducing pluripotency from mouse fetal neural progenitors (Shi et al., 2008). Although the mechanisms governing induction of pluripotency or HSCs may differ, barriers to accessing the HSC program may be similarly lowered by the use of such small molecules. Epigenetics has also been shown to underlie functional heterogeneity within the HSC compartment, especially with respect to aging-induced increase in self-renewal and myeloid lineage bias (Beerman et al., 2013; Sun et al., 2014a). Although reprogramming to pluripotency has been demonstrated to be sufficient in reversing epigenetic components of HSC aging (Wahlestedt et al., 2013), other studies have shown functional and molecular heterogeneity of iPSCs that originate from retention of epigenetic information associated with the starting cell type (Hu et al., 2010; Kim et al., 2010; Vaskova et al., 2013). Therefore, an intriguing question would be to determine whether the starting cell age is reset upon direct cell fate conversion to HSCs and whether HSCs induced from committed blood cell lineages exhibit differentiation bias toward the respective lineages. This latter point was not however evident in iHSCs generated in the Riddell et al. (2014) study as iHSCs derived from either B-cell progenitors or myeloid progenitor or effector cells appeared comparable in their capacity to generate HSCs and myeloid effector cells in vivo.

Complementary to overexpressing HSC-specific TFs, direct extinction of gene regulatory networks governing starting cell types may further augment cell fate transition. Since deletion of Pax5 has already been shown to be sufficient for liberation from B lineage fate (Nutt et al., 1999), such manipulation may enhance HSC induction from B-cell progenitors. The use of RUNX1 and GFI1 by Sandler et al. in inducing hematopoiesis from endothelial cells indeed follows the logic of their developmental role in extinguishing the endothelial program (Iacovino et al., 2011; Lancrin et al., 2012).

**Toward human HSCs**

Given the rapid progress in the field, derivation of the first human HSCs via cell fate conversion appears increasingly attainable. The diverse strategies presented thus far provide lessons that may help refine the approach to generating human HSCs as well as gain deeper insights into the mechanism of HSC induction.

Though already mentioned earlier, the importance of using inducible expression vectors should be reemphasized. Inducibility is critical to ensure transient ectopic expression of TFs in order to demonstrate that resulting HSCs possess self-sustaining and stable identity. Another advantage of this system is the re-inducibility of the TFs, which can be exploited for secondary reprogramming experiments (Wernig et al., 2008; Riddell et al., 2014).

To accelerate the study of HSC induction, it would be critical to perform experiments in defined media. Although the endothelial stromal system used by Sandler et al. appears to provide a powerful inductive milieu, it remains to be seen whether it is generally applicable. Nonetheless, it is also possible that a special environment may not be necessary for certain starting cell types and/or TF combinations. In either case, with an ability to perform the entirety of cell fate conversion in vitro, it should be possible to gain finer control over experimental parameters as well as to obtain kinetic information on the induction of HSC identity.

The crux of HSC induction is the functional evaluation of test cells. Rigorous assays should be employed to confirm the in vivo potential of induced HSCs to self-renew and generate multilineage progeny at the clonal level. Long-term reconstitution and serial transplantation are commonly employed to assess the in vivo self-renewal potential of HSCs. These assays also indirectly provide indications on the ability of test cells to properly home to and engraft in the niches. In vivo functionality of HSCs is contingent upon their successful engraftment. To arrive at bone marrow niches, HSCs must home to the correct vasculature, extravasate, and then migrate to gain contact with the niche components (Lapidot et al., 2005). The surface molecules and signal transduction components necessary for orchestrating this process are thus integral to HSC function. Although it is possible that this facet of HSC identity is within the domains governed by HSC-specific TFs, it could represent a generic functional module shared with other bone marrow resident cell types. In the latter scenario, orthogonally acting TFs or specific environmental signals may be required in addition to ectopic expression of HSC-enriched TFs to induce engraftable HSCs. For instance, it has been shown that expression of Cxcr4, an essential chemokine receptor for HSC migration, is regulated by Hif1a, suggesting that Cxcr4 induction may depend on hypoxia rather than HSC-specific TFs (Speth et al., 2014).

The prerequisite for homing and engraftment complicates functional assessment of human HSCs in the setting of xenotransplantation experiments. To circumvent graft rejection, immunodeficient mice that lack B, T, and NK cells are used as hosts for human HSCs (Shultz et al., 2012). Even then, the engraftment potential of human HSC in murine hosts is inferior to that of mouse HSCS, likely due to cross-species differences between cytokines and signaling/homing receptors or even due to the incompatibility of human CD47, a ‘don’t eat me’ signal, with host phagocytes (Jaiswal et al., 2009; Kwong et al., 2014). To obtain robust chimerism, human HSCs may need to be injected directly into the bone marrow cavity via intrafemoral injection, thus bypassing the complex orchestration of cellular maneuvers leading to engraftment. The caveat, however, is that lowering the hurdle to engraftment or differences in transplantation procedures may result in incongruous definitions for human HSCs. Although intrafemoral injection of induced HSCs could be justified if one were to claim the necessity of the in vivo environment in inducing proper homing/engraftment capacity, this needs to be confirmed with secondary transplantation via intravenous route of injection. The difference in the rigor of in vivo assays used to define HSCs as well as cross-species mismatches in signaling may also underlie molecular differences between mouse and human HSCs, and it is unknown whether experimentally derived cells tested in xenograft models will ultimately function effectively in human patients. An important step, therefore, is to develop better models for native engraftment of human HSCs, such as better humanized mice or reconstituted human bone marrow, so that their functional definition could be refined (Drake et al., 2012; Scotti et al., 2013; Cosgun et al., 2014; Torisawa et al., 2014).
The question of in vivo clonal multilineage differentiation potential, though challenging, can potentially be addressed using two molecular approaches. First, if HSCs can be induced from B or T lineage cells that have undergone receptor rearrangement, it would be possible to use the unique sequences of the recombined loci as a bar code to track the clonal progenitor cell origin of effector cell progeny as was done by Riddell et al (2014). Receptor rearrangement also provides direct evidence for the cell of origin of the reprogrammed cells, an important consideration given that even a single contaminating HSC inadvertently introduced during transplantation experiments has the potential to confound interpretations. The second approach is viral integration analysis that takes advantage of viral insertion sites that can serve as cellular barcodes. Finally, single-cell transplantation experiments can be used to assure clonal multilineage potential in vivo—though this is a very high bar to surmount, especially if cell fate conversion efficacy is low.

**Toward clinical translation**

Despite the progress, a number of challenges must be met before de novo generated human HSCs can reach clinical translation (Fig 2). Despite the diversity of TF combinations hitherto used for hematopoietic induction, a commonality among most of them is their proto-oncogenicity. Although no tumors have thus far been reported by hematopoietic cell fate conversion studies, the potential dangers of these potent TFs combined with the risks of insertional mutagenesis by lentiviral vectors cannot be tolerated for use in generating clinical-grade human HSCs. Therefore, the current lentiviral methods for HSC induction may be most applicable for such uses as disease modeling.

Given the risks associated with lentiviral transduction, non-integrating approaches will need to be considered toward clinical translation. A number of such methods developed for generating integration-free iPSCs including protein transduction, non-integrating viruses, and mRNA based transient protein expression systems could potentially be applied to HSC induction (Fusaki et al, 2009; Warren et al, 2010; Zhang et al, 2012; Mandal & Rossi, 2013; Yoshioka et al, 2013; Elcheva et al, 2014). However, as the non-integrating approaches are highly variable with respect to the duration of ectopic factor expression, the kinetics of respecification/reprogramming will need to be investigated in parallel to optimize the generation of integration-free HSCs. Furthermore, the level of TF induction may also need to be controlled since the stoichiometry of TFs may impact reprogramming efficiency as well as the functionality of reprogrammed cells as has been reported in other systems (Carey et al, 2011).

In addition to guaranteeing safety, it will be necessary to increase the efficiency of HSC induction to obtain sufficient numbers for transplantation. Although the efficiency of generating iPSCs has increased dramatically, their utility is augmented by their limitless self-renewal potential and well-defined culture conditions that support it. Since it is unclear whether HSCs could ever be expanded to the same extent as iPSCs while avoiding functional decline as seen with aging (Rossi et al, 2005; Beerman et al, 2013; Beerman & Rossi, 2014; Sun et al, 2014a), an ideal solution would entail near deterministic induction of HSCs from readily available somatic cells such as peripheral blood or fibroblasts. Until such efficiency can be reached, robust, defined means for ex vivo HSC expansion should be pursued.

Finally, the assays used to confirm HSC identity should be reevaluated. Currently, the only reliable, accepted method for assessing HSC function is competitive transplantation, a resource and time draining procedure incompatible with routine quality control of patient-derived HSCs. Therefore, a bioinformatic metric, like those developed for iPSC quality control (Nestor & Noggle, 2013), predictive of HSC functionality will not only benefit clinical translation but also accelerate the pace of HSC induction research.

**Figure 2. Road map to clinical translation of human induced HSCs.**

Upon establishment of a method for generating human HSCs from patient-derived cells, a number of critical steps must be taken en route to clinical translation. Although the initial, suboptimal method may be sufficient for initiating patient-specific disease modeling research, substantial improvements in the efficiency of HSC generation must be made to obtain sufficient numbers of HSCs toward therapeutic screening and for use in reconstituting adult patients. Prior to preclinical testing, a non-integrating approach to generating HSCs must be established in order to eliminate the risks associated with insertional mutagenesis and accidental re-induction of reprogramming TFs, many of which are potentially oncogenic. During the preclinical phase, quality control and safety testing should be performed at molecular and functional levels. Functional testing should involve the best methods for modeling human HSC engraftment such as humanized immunodeficient animal recipients.
Conclusion

“What I cannot create, I do not understand.”
Richard Feynman

HSCs have captivated generations of researchers not only because of their clinical importance but also for their intriguing yet elusive biological properties. Arguably, a major contributor to this allure has been the challenges associated with the seemingly simple task of obtaining more HSCs. Even while the biology of HSCs continues to be elucidated in staggering resolution with the advent of increasingly sensitive and high throughput methods (Lu et al., 2011; Gazit et al., 2013; Cabezas-Wallscheid et al., 2014; Lara-Astiaso et al., 2014; Sun et al., 2014a,b; Wu et al., 2014) as well as more accurate description of the HSC niche (Morrison & Scadden, 2014), robust ex vivo expansion or de novo generation of human HSCs still remains elusive. However, major strides made by the recent cohort of fate conversion studies have introduced new hope and perspectives to a field historically reignited by attempts to mimic the natural processes that regulate and specify HSCs. Despite having to induce nonphysiologic cell fate transitions, fate conversion toward HSC has begun to yield relevant insights into HSC biology that may synergize with preexisting paradigms to better understand the ontogeny, maintenance, dysregulation, and therapeutic potential of HSCs.

Conflict of interest

The authors declare that they have no conflict of interest.

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