HHS Public Access

Author manuscript

Cell Stem Cell. Author manuscript; available in PMC 2017 June 02.

Published in final edited form as:

Cell Stem Cell. 2016 June 2; 18(6): 707–720. doi:10.1016/j.stem.2016.05.016.

Engineering Hematopoietic Stem Cells: Lessons from Development

R. Grant Rowe[#,1], **Joseph Mandelbaum**[1,#], **Leonard I. Zon**[1,2,3,4,5,^], and **George Q. Daley**[1,2,5,6,7,8,*,^]

[1]Stem Cell Program and Division of Hematology/Oncology, Boston Children's Hospital, Boston MA, USA

[2]Howard Hughes Medical Institute, Boston, MA, USA

[3]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA

[4]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

[5]Harvard Stem Cell Institute, Cambridge, MA, USA

[6]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

[7]Division of Hematology, Brigham and Women's Hospital, Boston, MA, USA

[8]Manton Center for Orphan Disease Research, Boston, MA, USA

Summary

Cell engineering has brought us tantalizingly close to the goal of deriving patient-specific hematopoietic stem cells (HSCs). While directed differentiation and transcription factor-mediated conversion strategies have generated progenitor cells with multilineage potential, to date, therapy-grade engineered HSCs remain elusive due to insufficient long-term self-renewal and inadequate differentiated progeny functionality. A cross-species approach involving zebrafish and mammalian systems offers complementary methodologies to improve understanding of native HSCs. Here, we discuss the role of conserved developmental timing processes in vertebrate hematopoiesis, highlighting how identification and manipulation of stage-specific factors that specify HSC developmental state must be harnessed to engineer HSCs for therapy.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative treatment for many congenital and acquired blood disorders, and is the most widely applied

*Correspondence: george.daley@childrens.harvard.edu.
#Co-first author
^Co-senior author

cellular therapy. Although HSCT has rapidly improved over the preceding decades, impediments related to donor availability and allogenicity remain. In the absence of an optimal human leukocyte antigen (HLA)-matched donor, HSCT recipients often rely on umbilical cord blood, which typically lacks sufficient stem and progenitor cell dose for timely reconstitution of functional peripheral blood cells (Pineault and Abu-Khader, 2015). Haploidentical or mismatched HSCT expands donor options, but mandates more intense post-SCT immunosuppression (Mehta et al., 2016). Although significant progress has been made, management of allogeneic complications such as graft-versus-host disease (GVHD) remains a source of considerable morbidity for patients (Holtan et al., 2014).

Many efforts are underway to engineer ‘designer’ hematopoietic stem cells (HSCs, the functional units of HSCT) for applications in research and therapy. The ideal engineered HSC should possess long-term self-renewal capability and the ability to produce a full repertoire of differentiated progeny for effective oxygen transport, hemostasis, and innate and acquired immunity. The advent of human embryonic stem cell (ESC) research presented the theoretical opportunity to engineer HSCs for use in HSCT. Investigators developed directed differentiation strategies to differentiate mouse (Schmitt et al., 1991; Wiles and Keller, 1991) and human (Chadwick et al., 2003; Kaufman et al., 2001; Vodyanik et al., 2005) ESCs into hematopoietic lineages, despite over two decades of effort, culture protocols have produced only a limited range of primarily primitive myelo-erythroid progeny and scant evidence for definitive, adult-like multi-lineage hematopoietic stem and progenitor cells.

Reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) represented a significant step forward, providing a theoretically unlimited source of autologous patient-specific HSCs (Takahashi et al., 2007). IPSCs, combined with the emerging technology for CRISPR/Cas9-mediated gene repair of autologous cells have accelerated efforts at HSC engineering (Hendriks et al., 2016). Recently, both morphogen directed differentiation and transcription factor (TF)-mediated phenotypic conversion strategies have been applied to both human ESCs and iPSCs to derive hematopoietic cells with incremental improvement in efficiency and mature blood cell function (Doulatov et al., 2013; Elcheva et al., 2014; Kennedy et al., 2012; Sturgeon et al., 2014). However, derivation of long-term, self-renewing, adult-like HSCs of therapeutic value from pluripotent sources remains elusive.

While most prior attempts at engineering blood stem cells have sought to recapitulate embryonic hematopoietic development using morphogen signals (Kennedy et al., 2012; Sturgeon et al., 2014), more recent efforts have exploited direct cell fate conversions using TFs to overcome phenotypic and epigenetic barriers imposed by normal developmental ontogeny (Batta et al., 2014; Elcheva et al., 2014; Pereira et al., 2013; Riddell et al., 2014). However, as we discuss below, our collective understanding of normal vertebrate hematopoietic development can be further leveraged with the aim of improving strategies for engineering functional adult-like HSCs.

Recapitulating the timing of tissue development, and achieving cells and tissues that function comparably to tissues in an adult organism remains one of the dominant challenges to engineering blood cells in vitro. *In vivo*, hematopoiesis evolves sequentially in anatomic

location, stem and progenitor cell function and distribution, and progeny cell output from early embryogenesis to adulthood, synchronizing maturation of hematopoietic phenotypes with host developmental needs (Benz et al., 2012; Orkin and Zon, 2008; Rebel et al., 1996). A considerable body of knowledge of stage-specific regulators of hematopoiesis exists, including TFs, epigenetic factors, and morphogen signals (Dzierzak, 2002; Eaves, 2015; Orkin and Zon, 2008). Understanding the developmental stage of hematopoiesis and the developmental timing events mimicked in TF-mediated conversion or directed differentiation strategies in HSC engineering are essential to improve HSC functionality and therapeutic value.

Heterochrony in Hematopoietic Development

Normal development requires the temporal coordination of differentiation and morphogenesis collectively among all tissues and organ systems on a genetically determined schedule. Distinct features of developmental timing in one species relative to another is termed heterochrony (Ambros, 1989). Prototypical heterochronic genes were identified in seminal studies in *Caenorhabditis elegans* wherein mutations accelerated or retarded the morphogenesis of specific tissues relative to the remainder of the organism (Ambros and Horvitz, 1984). Mechanistically, heterochronic genes appear to control timing of developmental events by regulating the pace of stem cell differentiation and self-renewal, which manifests as the linear maturation of a tissue or organ system in time (Harandi and Ambros, 2015). In mammals, polymorphisms in highly conserved heterochronic genes impact adult height and timing of puberty (Lettre et al., 2008; Sulem et al., 2009). In a pathologic context, retarded maturation or involution of fetal tissue relative to host maturation contributes to early childhood tumors (Urbach et al., 2014).

Across evolution, the hematopoietic system reflects many aspects of heterochronic regulation. Blood lineages mature in distinct stages from early embryogenesis to adulthood in concert with organismal development, and the sequence of developmental events remains consistent across a diversity of vertebrate species, despite highly variable rates of organismal development (Figure 1, Table 1). Primitive hematopoiesis includes the earliest wave of transient embryonic erythropoiesis (Orkin and Zon, 2008). In mice, the earliest primitive erythroid cells emerge in the yolk sac blood islands at murine embryonic (E) day 7.5 to provide essential oxygenation to the growing embryo (Orkin and Zon, 2008). These macrocytic erythroid cells express embryonic globins and initially circulate as nucleated cells, but undergo enucleation beginning at approximately E12.5 (Kingsley et al., 2004). As shown in mice, there follows a second wave of yolk sac hematopoiesis that is definitive in nature, comprised of CD41+, c-kit+, CD16/32+ erythro-myeloid progenitors (EMPs) that express adult globins and possess rare B lymphoid potential, which migrate to the fetal liver (FL) to establish early myeloerythropoiesis prior to the arrival of definitive HSCs (McGrath et al., 2015), and which can produce macrophages that persist into postnatal life (Gomez Perdiguero et al., 2015).

In zebrafish hematopoietic development, which is a highly conserved process occurring at sites that are anatomically analogous to their mammalian counterparts (Jing and Zon, 2011) (Figure 1), the transient primitive wave of hematopoiesis begins at 11 hours post fertilization

(hpf) and produces erythroid and myeloid cells from mesoderm-derived hemangioblasts, analogous to the mammalian yolk sac blood islands. A wave of EMP hematopoiesis follows at the onset of definitive hematopoiesis (Bertrand et al., 2007). It has been traditionally accepted that primitive hematopoietic cells lack definitive HSC activity (Medvinsky and Dzierzak, 1996), although yolk sac cells migrate to establish definitive hematopoiesis elsewhere at later developmental time points (Samokhvalov et al., 2007).

In vertebrates, including zebrafish, the first definitive HSCs with long-term engraftment capability arise via the endothelial-to-hematopoietic transition (EHT) of hemogenic endothelium (HE) in the aorta-gonad mesonephros (AGM) region as well as the placental labyrinth and vitelline and umbilical arteries, marking the onset of definitive hematopoiesis (Dzierzak and Speck, 2008; Muller et al., 1994; Orkin and Zon, 2008; Pereira et al., 2016; Robin et al., 2009)(Figure 1). In mice, following the onset of AGM hematopoiesis around E11, the yolk sac appears to acquire the capacity to produce definitive HSCs around E12, that likely contribute to the FL HSC pool (Dzierzak and Speck, 2008; Kumaravelu et al., 2002). Aortic HE is optimally located to detect the onset of blood flow, as mechanical forces from circulation provide cues that stimulate definitive HSC specification (Adamo et al., 2009). Nitric oxide may convert the biophysical forces from circulation into chemical inductive signals that trigger definitive HSC specification processes in HE (North et al., 2009). Definitive HSCs subsequently colonize the fetal liver (FL), spleen, thymus, and finally bone marrow (BM) in mammals. In zebrafish, AGM-derived HSCs colonize the caudal hematopoietic tissue (CHT; analogous to the mammalian FL) and then the thymus and kidney marrow (KM; analogous to mammalian BM), which sustain long-term hematopoiesis into adulthood. Stage-specific phenotypic and genetic variation of HSCs and progeny cells occurs across development. Mammalian hematopoietic cells have been characterized by surface phenotype and protein expression, while zebrafish cells show stage-specific reporter gene activity (Table 1).

Distinct states of developmental maturation of definitive HSCs were initially reported in 1972, when transplantation studies demonstrated more rapid self-renewal in murine FL HSCs compared to those from adult BM (Micklem et al., 1972). Subsequent studies have corroborated this finding, and found that HSCs decrease their rate of proliferation and acquire a relatively quiescent adult phenotype at the postnatal age of around three weeks in mice (Bowie et al., 2007b; Rebel et al., 1996; Szilvassy et al., 2003). HSCs also show inherent lineage priming that evolves with time during development (Nimmo et al., 2015), with BM containing a larger proportion of myeloid-restricted HSCs relative to FL (Benz et al., 2012), a phenomenon that is likely governed by changes in global transcriptional programs (McKinney-Freeman et al., 2012). The divergent phenotypes of HSCs at different developmental phases is further underlined by variations in surface markers (Morrison et al., 1995). While surface immunophenotypes of populations enriched in HSC activity at various developmental stages are becoming increasingly well enumerated in mice (Table 1), these markers are not well conserved in humans. As such, due to limited tissue availability, stage-specific markers of human HSCs are less well understood. It is generally accepted that definitive human HSC activity resides in a population of CD34+, CD38−, Thy1+, CD45RA− cells, which is reviewed in (Doulatov et al., 2012). Recently, within human fetal liver, a population enriched in HSC activity was defined using the GPI-80 surface marker, as

CD34+, CD38-lo/-, CD90+, GPI-80+ (Prashad et al., 2015). A recent report also demonstrated that HSC activity can be detected in the aorta of Carnegie stage 14-17 human embryos, with a proposed immunophenotype of CD34+ VE-cadherin+ CD45+ C-KIT+ THY-1+ Endoglin+ CD38-/lo CD45RA−, also expressing *RUNX1* (Ivanovs et al., 2014).

Stage-specific effects of the HSC niche impact HSC self-renewal, indicating that changes in HSCs that occur during hematopoietic maturation may not be entirely cell intrinsic, as is apparent in transplantation assays. Murine heterochronic transplantation models wherein HSCs isolated at various developmental time points are transplanted into hosts of differing developmental stage illustrate this concept. Murine HSCs isolated from the AGM engraft most efficiently in neonatal recipients, while FL or BM-derived HSCs engraft most efficiently in adult recipients (Arora et al., 2014). This appears to be due, in part, to stage-specific variation in HSC proliferation, as adult BM HSCs induced to cycle with interferon-α show enhanced neonatal engraftment and enrichment of FL HSC gene expression (Arora et al., 2014; Kim et al., 2016). It is possible that variation in stromal cells within different sites of hematopoiesis may underlie these effects (Charbord et al., 2002), and that the developmental stage of the hematopoietic niche impacts HSC maturation.

Zebrafish HSC heterochronic transplantation assays allow the study of cell autonomous HSC function across development. Transplantation into prethymic 48 hpf recipients evades immune rejection by circumventing a requirement for recipient conditioning (Traver et al., 2003), as $CD41^{+}$ KM HSCs or *runx1*$^{+}$ HSCs from day 3 embryos transplanted into 48 hpf recipient embryos show long-term multilineage engraftment (Ma et al., 2011; Tamplin et al., 2015). Although markers of zebrafish HSC subpopulations across development are less well defined than their mammalian counterparts, transplanting into transparent zebrafish embryos provides opportunities to visualize HSC functionality and complement mammalian studies. Zebrafish adult whole KM transplants rescue impaired multilineage hematopoiesis and lethality that occurs in day 2 hpf *gata1*$^{-/-}$ mutant embryos (Traver et al., 2003), showing that KM HSCs can rescue hematopoietic defects at the earlier CHT stage. $CD41^{+}$ HSCs isolated from zebrafish KM home to the CHT niche upon injection into 48 hour-old embryos, (Sacco et al., 2016), indicating that the developmental stage of transplant recipients regulates homing of donor KM HSCs, and more mature HSCs can engraft in hematopoietic organs that arise at earlier developmental time points.

Among lineage committed hematopoietic cells, developmental maturation was first recognized in erythroid cells, as denoted by changes in globin composition that occur with maturation. In mammals, primitive hematopoietic cells express embryonic globins but at the onset of definitive hematopoiesis, gene switching activates adult-like globin genes which continue to be expressed into adulthood. In humans, a switch in β-like globin expression occurs at birth, with silencing of the locus encoding fetal γ-globin and activation of adult β-globin, the dominant postnatal β-like chain. Primitive and definitive cells also differ in their modes of erythropoietic differentiation. As discussed above, primitive erythroid cells circulate in a nucleated state, whereas definitive erythroid cells enucleate prior to entering the circulation (Baron, 2013). Zebrafish similarly undergo embryonic to adult globin switching, but mature erythrocytes remain nucleated (Ganis et al., 2012).

Variations in patterns of lymphopoiesis occur across development. In fetal thymic repopulation models, FL HSCs are capable of producing oligoclonal innate-like CD4/CD8 double negative T-cells containing the Vγ3 T cell receptor chain, while adult BM HSCs are not (Ikuta et al., 1990). An analogous process occurs in B-cell ontogeny, with heterochronic HSC transplants demonstrating that innate-like B-1a and marginal zone B-cells arise from embryonic progenitors and conventional 'B-2' and follicular B-cells from adult HSCs (Kantor et al., 1992; Yoshimoto et al., 2011). These observations highlight fundamental divergence in hematopoietic ontogeny that exist at different developmental time points, and which may be due to inherent differences in HSC lineage capacity.

Myeloerythroid progenitor cells likewise undergo maturation from fetal to adult life. Recent dissection of myeloid progenitor lineage potential has rewritten conventional hematopoietic ontogeny by subdividing phenotypically uniform progenitor populations based on global transcriptional profiles (Paul et al., 2015). This correlates with restricted lineage fate potential ingrained at the progenitor stage, which evolves from fetal to adult life in humans (Notta et al., 2016), further emphasizing the changes that occur in stem and progenitor cell lineage capacity over time.

Collectively, these studies illustrate profound changes occurring within specific hematopoietic stem and progenitor populations and differentiated lineages across development, which are physiologically timed to cooperate with the demands of other maturing tissues and the developing host organism. Therefore, for effective *in vitro* production of hematopoietic cells of therapeutic value with adult-like stem and progenitor characteristics, it is essential to understand the mechanisms by which hematopoietic heterochronicity is regulated, so that this can be leveraged into improvement of blood engineering strategies.

Control of Hematopoietic Heterochrony

Investigation into normal hematopoietic development over the last several decades has uncovered stage-specific hematopoietic regulators, including signaling pathways, transcription factors, and epigenetic factors. The power of transgenic animal models has demonstrated that these regulators are essential for timing of HSC and progenitor phenotypes at key developmental moments, but may be dispensable at others.

Several TFs exert stage-specific heterochronic effects on hematopoietic development. Expression of *Hoxb4* specifies the onset of definitive hematopoiesis, and its ectopic expression can confer definitive HSC hematopoietic characteristics on yolk sac-derived progenitors (Hills et al., 2011; Kyba et al., 2002). *Klf1* is essential for definitive, but not yolk sac, erythropoiesis (Nuez et al., 1995; Perkins et al., 1995). *Sox17* is highly expressed in FL HSCs compared to adult HSCs, and knock out in the fetal state impairs HSC self-renewal, with no apparent consequence of deletion in adults (He et al., 2011). *Runx1* is required for definitive HSC emergence in the AGM by EHT (Chen et al., 2009), but not primitive hematopoiesis (Okuda et al., 1996) or adult HSC maintenance (Ichikawa et al., 2004), with analogous stage-specific roles in zebrafish (Sood et al., 2010). HSC function requires variable *Gata2* gene dosing at different stages of development (Ling et al., 2004). *Etv6*

appears to be required for adult, but not FL, HSC maintenance in murine knock-out studies (Hock et al., 2004b).

Epigenetic regulators differentially regulate hematopoiesis across development. Polycomb repressive complex (PRC) factors appear to mechanistically regulate stage-specific hematopoietic specification. *Ezh2* function is required for effective FL HSC expansion, while loss of *Ezh2* in adult HSCs has more limited effects (Mochizuki-Kashio et al., 2011). *Ezh1* loss causes adult BM HSC failure, though fetal hematopoiesis appears to develop normally (Hidalgo et al., 2012). Deletion of the PRC1/2 component *Eed* depletes adult BM HSCs, though FL HSCs emerge normally (Xie et al., 2014). *Bmi-1* is essential for adult BM HSC self-renewal (Park et al., 2003). Consistent with developmental changes in chromatin structure, erythropoietic enhancer function varies across development (Xu et al., 2012).

Extracellular signals and cell cycle regulators influence hematopoiesis differentially throughout development. FL HSCs possess increased sensitivity to the mitogenic effects of stem cell factor (SCF) compared to adult BM HSCs (Bowie et al., 2007a), which may be due to intrinsically higher levels of pro-quiescence factors in adult HSCs (Cheng et al., 2000; Hock et al., 2004a). In zebrafish, *Scl* isoforms have stage-specific roles: whereas *scl-α* and *scl-β* are functionally redundant in primitive hematopoiesis, *scl*-β is required upstream for HE specification while *scl-α* acts post-EHT in the AGM to maintain HSCs (Zhen et al., 2013). Wnt, BMP, and activin/nodal signaling exert differential effects on primitive and definitive hematopoietic cell emergence (Kennedy et al., 2012; Sturgeon et al., 2014). *Notch1* deficiency enhances primitive erythroid cell emergence, while not affecting definitive progenitor development, but *Notch1* is required for adult long-term HSC function (Hadland et al., 2004). Similarly, zebrafish *mindbomb* mutants lacking functional Notch ligands have unaffected primitive hematopoiesis and erythromyeloid progenitor specification, but fail to produce HSCs in the AGM (Bertrand et al., 2010). Retinoic acid receptor signaling is required for HSC emergence at the AGM, but appears to be dispensable for yolk sac hematopoiesis (Chanda et al., 2013).

Various bloodless zebrafish mutants can survive for 5-15 days, which allows insight into HSC specification, function, and differentiation (reviewed in (de Jong and Zon, 2005)). *cpsf1* mutants specify HSCs normally, which then migrate to the CHT and undergo apoptosis at 72 hours (Bolli et al., 2011). Mutants with CHT-specific hematopoietic defects include *rumba* (deficient in a C2H2 zing finger factor of unknown function) and *samba* (deficient in a homolog of human augmin complex subunit 3, HAUS3), which show a decrease in HSC number during the early CHT phase and the later expansion phase respectively (Du et al., 2011). A forward genetic screen for definitive hematopoietic mutations performed by Liu and colleagues has identified heterochronic mutants such as *kri1l* with low numbers of HSPCs in the CHT at days 3 and 5, whereas AGM hematopoiesis was not impaired (Jia et al., 2015). Species-specific hematopoietic differences in models of c-myb deficiency, further highlight the advantage of the zebrafish system. Although mice lacking c-Myb die from embryonic anemia (Mucenski et al., 1991), zebrafish *c-myb* mutants exhibit unaffected primitive hematopoiesis and lack definitive hematopoiesis, but survive to 2-3 months, presumably receiving sufficient oxygenation through diffusion (Soza-Ried et

al., 2010). Thus, biological differences between species can be exploited to study conserved hematopoietic paradigms.

Recently, the highly conserved heterochronic LIN28 proteins have been shown to act as key regulators of hematopoietic timing. LIN28 proteins were initially discovered in *C. elegans* in screens for heterochronic mutants (Ambros and Horvitz, 1984). Loss of function (LOF) of *lin-28* results in precocious arrest of molting relative to the maturation of the worm (Ambros and Horvitz, 1984). This appears to be due to effects on a stem cell population termed seam cells, where *lin-28* LOF results in aberrant asymmetric cell division, which drives differentiation and maturation at the expense of symmetric divisions that sustain self-renewal (Harandi and Ambros, 2015)(Figure 2).

Biochemically, the proteins encoded by the two mammalian LIN28 paralogs (A and B) function through inhibition of processing and maturation of the *let-7* family of microRNAs and modulate translation of specific mRNAs (Shyh-Chang and Daley, 2013). Congruent with observations in worms, LIN28A/B are highly expressed in pluripotent mammalian cells (Moss and Tang, 2003), and LIN28A expression contributes to pluripotency induction in somatic cells (Yu et al., 2007). Further paralleling their control of timing in worms, polymorphisms in human *LIN28B* are associated with variations in adult height and timing of puberty (He et al., 2009; Lettre et al., 2008; Ong et al., 2009; Sulem et al., 2009; Widen et al., 2010). Within the hematopoietic system, expression of *Lin28b* varies with time. *Lin28b* is highly expressed in FL HSCs (Copley et al., 2013) and lymphoid progenitors, and its expression decreases with maturation to corresponding postnatal cell populations, coincident with accumulation of *let-7* microRNAs.

Functionally, *Lin28b* is a heterochronic regulator of HSC self-renewal. Ectopic expression of *Lin28b* in murine adult BM HSCs increases self-renewal to a FL-like state in transplantation models (Copley et al., 2013). *Lin28b* expression in adult BM HSCs represses *let-7* microRNAs, and appears to exert its effects on HSC heterochrony through the *let-7* target *Hmga2* (Copley et al., 2013). Although its developmental patterns of expression are not well defined, endogenous *Lin28a* appears to regulate hematopoietic stem and progenitor cell output and lineage selection. In transplantation models, overexpression of *Lin28a* results in diminished hematopoietic output while *Lin28a* knockdown by shRNA increases myeloid and decreases lymphoid output (Chaudhuri et al., 2012).

In committed hematopoietic lineages, the Lin28-*let-7* axis specifies fetal and adult patterns of lymphopoiesis. Ectopic *Lin28b* expression in adult BM stem and progenitor cells drives differentiation of fetal B-1a cells and innate-like T cells (Yuan et al., 2012). This is due at least in part to *let-7* repression, as ectopic *let-7* expression in FL pro-B cells is sufficient to confer adult B-2 potential (Zhou et al., 2015). These effects may be mediated through the *let-7* target Arid3a, which also regulates timing of fetal and adult B-cell phenotypes (Zhou et al., 2015).

Lin28/*let-7* also heterochronically modulates the myeloerythroid compartment. Compared to human umbilical cord erythroid cells, adult peripheral blood red blood cells contain higher levels of *let-7* microRNAs (Noh et al., 2009). Ectopic expression of *LIN28B* in adult

erythroblasts reduces *let-7* levels and increases levels of fetal hemoglobin, possibly via control of *BCL11A* levels (Lee et al., 2013). LIN28B appears to also function in mast cell differentiation (Wang et al., 2015). Consistent with its role in regulation of cell potency, *let-7* activation drives granulocytic differentiation of leukemic myeloblasts (Pelosi et al., 2013). Thus, within the hematopoietic system, the Lin28/*let-7* axis likely plays orthogonal roles: in regulating heterochronic hematopoietic maturation across time within phenotypically and equipotent stem and progenitor populations, and in regulation of stem cell maintenance and differentiation, paralleling its role in worms (Figure 2).

Blood engineering: bypassing the normal constraints of hematopoietic heterochrony

Strategies for blood cell derivation via directed differentiation utilize systems designed to recapitulate normal developmental events in culture. In contrast, TF-mediated conversion strategies use unbiased screening to traverse barriers imposed by normal differentiation and maturation. Strategies that combine these methodologies may prove to be an optimal approach. In all of these systems, enhanced understanding of the heterochronic mechanisms of blood development could yield rational strategies to unlock efficient definitive adult-like hematopoiesis for therapeutic purposes. Below we examine current methods of blood cell engineering through the lens of normal hematopoietic heterochrony. Specific examples representative of each general approach are highlighted in Figure 3.

Reiteration of the early embryonic germ layer formation and tissue patterning by morphogen and environmental cues in culture systems elicits hematopoietic progenitors with limited engraftment and differentiation capability (Murry and Keller, 2008). For such approaches to provide a foundation to generate long-term HSCs, definitive hematopoietic cells must emerge and be distinguished from primitive cells. Seminal studies have demonstrated that, in the presence of hematopoietic cytokines or stromal cells, mouse and human embryonic stem (ES) cells can be induced to develop a mesoderm-derived yolk sac-like primitive hematopoietic program typically within one week of culture (reviewed in (Murry and Keller, 2008)). In these models, definitive-like hematopoietic cells develop later, possibly via an EHT-like process or maturation of primitive cells (Chadwick et al., 2003; Murry and Keller, 2008; Vodyanik et al., 2005).

Lymphoid differentiation capacity has been proposed to be a key criterion defining the onset of definitive hematopoiesis directed differentiation models that employ pluripotent stem cells (Murry and Keller, 2008). As a prototypical directed differentiation strategy, Keller and colleagues developed a system wherein human ESC or iPSC embryoid bodies (EBs) were cultured initially in the presence of BMP-4 (days 0-4) to induce mesoderm formation, with the addition of activin to drive primitive hematopoiesis (days 2-4), and subsequently with pro-hematopoietic cytokines to promote definitive hematopoietic cell emergence, modeling the morphogen expression patterns that occur during early mammalian embryogenesis (Kennedy et al., 2012). Consistent with the onset of adult-like hematopoiesis, *SOX17* expression could be detected at day 9 of culture. T-lymphoid potential could be detected as early as day 6 (but not at day 3 when only yolk sac-like primitive hematopoietic cells are present) and appeared to emerge independently of activin/nodal signaling, arguing against direct maturation of primitive cells contributing to this effect and in favor of recapitulation of

normal definitive-like cell emergence, possibly via EHT (Kennedy et al., 2012). More recently, this group demonstrated that definitive-like cells developed in this system via a HE-like intermediate (Sturgeon et al., 2014). Interestingly, definitive hematopoietic progenitors derived using this strategy possess the potential to express adult-like T-cell markers and polyclonal TCRαβ rearrangements, and can acquire definitive globin expression (Kennedy et al., 2012). These strategies, rationally constructed based upon understanding of normal embryogenesis, are proof-of-principle that application of normal hematopoietic heterochrony can be leveraged in blood engineering, but as yet have not yielded progenitor cells capable of engrafting and producing a full complement of functional progeny *in vivo*.

As compared to this EB system, engraftment of ESCs or iPSCs as teratomas represents a complementary model of early embryogenesis. Teratomas engrafted in immunocompromised murine hosts contain active hematopoiesis within a niche bearing similarities to mature BM after 8 weeks, with some evidence for myeloid and lymphoid lineages as well as transplantable HSPC-like cells with multilineage potential (Amabile et al., 2013). B- and T-cells arise from these teratomas with apparent adult-like immunoglobulin and cytokine production (Amabile et al., 2013). Further investigation of earlier time points in the model is required to determine the intermediate phases, such as primitive hematopoiesis, EHT, and FL-like hematopoiesis, that lead to the formation of an apparently functional pluripotent cell-derived BM environment.

Respecification of cell phenotypes by TF-mediated reprogramming is central to many blood derivation strategies. This powerful technique has been applied to committed hematopoietic cells, pluripotent cells, as well as terminally differentiated somatic cells to reverse lineage commitment, drive normal maturation-like events, bypass or reverse normal hematopoietic maturation schedules, and cross lineage compartments normally insulated from one another by normal patterns of differentiation. Several of these approaches appear to recapitulate EHT as an intermediate stage of conversion. In one of the earliest studies utilizing this approach, expression of *Hoxb4* – a factor known to function in definitive HSC renewal – in murine primitive yolk sac or EB-derived progenitors conferred definitive hematopoietic features and limited engraftment capability (Kyba et al., 2002).

More recently, reprogramming of fully differentiated somatic cells has been demonstrated. Moore and colleagues used a screening strategy whereby mouse embryonic fibroblasts (MEFs) bearing a CD34 reporter mechanism were transduced with a library of 18 hematopoietic TFs, identifying the heterochronic genes *Gata2* and *Etv6*, as well as *Gfi1b*, and *Fos*, as sufficient to activate this reporter (Pereira et al., 2013). Long-term culture (30-40 days) of MEFs ectopically expressing these factors generated cells with myeloid colony forming capacity and definitive hematopoietic markers via an HE-like intermediate stage, which did not appear to be preceded by primitive-like hematopoiesis (Pereira et al., 2013). Similarly, Lacaud and colleagues transduced MEFs and adult fibroblasts with six hematopoietic TFs (including *Gata2* and *Runx1c*) to generate cells with hematopoietic colony forming potential by day 21 post-transduction, a process accelerated by loss of p53 and which also occurred via an HE-like intermediate (Batta et al., 2014). These cells expressed a mixture of primitive and definitive globins, underwent rearrangement of TCRβ and immunoglobulin loci, and showed short-term engraftment *in vivo* (Batta et al., 2014).

Although it is possible that these respecification strategies recapitulate only aspects of yolk sac-like hematopoiesis, via primitive-like hemangioblasts/HE, the use of stage-specific definitive TFs (*Gata2, Etv6* and *Runx1*), which are dispensable for primitive hematopoiesis, supports the specification of definitive programs.

It could be inferred that TF-mediated reprogramming of committed hematopoietic lineages to HSC-like cells may enhance efficiency of the conversion process, given the relatively smaller distance in developmental ontogeny of hematopoietic cells from HSCs compared to fibroblasts. Another somatic cell reprogramming approach showed that ectopic expression of six TFs is sufficient to reprogram murine adult pre-/pro-B cells and myeloid progenitors to serially transplantable, multilineage reconstituting 'induced HSCs', within 48 hours, although the stage of definitive hematopoiesis achieved by this conversion strategy has not been clearly elucidated (Riddell et al., 2014). This process appears to occur entirely within the confines of definitive hematopoiesis, uniquely without an HE-like intermediate (Riddell et al., 2014).

Reprogramming approaches have also been applied to pluripotent cells. An innovative strategy incorporated aspects of morphogen-directed differentiation from human pluripotent cells to hematopoietic progenitors and TF-mediated reprogramming. Human myeloid progenitors isolated from 14 day-old ESC and iPSC EBs cultured in the presence of hematopoietic cytokines were transduced with five TFs to confer short-term myeloid and erythroid engraftment in humanized mice (Doulatov et al., 2013). *In vitro* lymphoid potential as well as adult globin expression were consistent with definitive phenotypes (Doulatov et al., 2013). Slukvin and colleagues used a TF screen to specify endothelial fate in human ESCs and iPSCs, demonstrating emergence of endothelial cells after 5-7 days, which possessed the capacity for hematopoietic colony formation (Elcheva et al., 2014).

Recent conversion strategies have leveraged understanding of EHT. The Rafii group used non-hemogenic umbilical cord or adult endothelial cells ectopically expressing TFs (including *Runx1* and the adult HSC quiescence factor *Gfi-1* (Hock et al., 2004a)) cultured in an *in vitro* vascular niche; after 4 weeks, hematopoietic colony forming activity could be detected, as well as multilineage engraftment of primary and secondary humanized mice recipients, although the developmental stage of the generated hematopoietic cells is unclear (Sandler et al., 2014). The Moore group cleverly extended their findings discussed above (Pereira et al., 2013) by using surface markers to establish an immunophenotype (Prom1^{+}, Sca1^{+}, CD34^{+}, CD45^{-}) of hemogenic precursors in their reprogrammed fibroblasts. They used this immunophenotype to identify an endogenous endothelial population in mouse placentas and AGM with hemogenic activity, capable of differentiating to engraftable hematopoietic cells following stromal coculture (Pereira et al., 2016) raising the possibility of using placental endothelial cells as a source of engineered hematopoietic cells. Together, these studies illustrate the applicability of TF-mediated conversion in blood engineering, and reliance on recapitulation of normal developmental events to establish definitive adult-like hematopoiesis.

Strategies for Discovery

Decades of study in HSC developmental biology have laid the groundwork for the rapid progress made in the past five years in HSC engineering. Although we collectively have the capability to derive hematopoietic cells from a variety of cell sources, limitations remain in the therapeutic value of derived ‘HSCs’. Future investigation should focus on developing methodologies to encourage the derived definitive hematopoietic cells to discard embryonic/fetal characteristics and acquire adult like HSC self-renewal, lymphopoietic output, and erythroid phenotypes for effective cell therapy. Only then can schemes be further optimized to enhance cell yield for translation into clinical trials.

Extensive investigation has dissected the heterochronic events occurring in morphogen directed differentiation schemes that generate hematopoietic cells from pluripotent cells (Kennedy et al., 2012; Sturgeon et al., 2014). These studies illustrate the power of this methodology in enhancing understanding of HSC development. Screening approaches may be applied to directed differentiation models to identify further factors that improve efficiency of HSC generation in these systems, as well as for optimization of their developmental state. Coupled with use of stage-specific hematopoietic markers, this may be an impactful strategy to derive adult-like HSCs. Small molecules may offer more economical alternatives to the use of cytokines in directed differentiation, and zebrafish systems could offer a tractable model to screen compounds in a high throughput manner (MacRae and Peterson, 2015). As such, we propose that directed differentiation in zebrafish systems may provide further insights into early developmental hematopoiesis to inform cell engineering. Co-culture approaches with supporting cells that can enhance hematopoietic induction such as mouse OP9 stromal cells, zebrafish kidney stromal cells, and zebrafish endotome cells, can be further incorporated in screening platforms (Nguyen et al., 2014; Stachura et al., 2009; Vodyanik et al., 2005). Improved characterization of stage-specific markers of zebrafish hematopoiesis (Table 1) is essential to understand the developmental state of the cells produced, and novel prohematopoietic factors can be validated in mammalian culture systems.

TF-mediated direct conversion has taken a prominent role in blood engineering strategies that use somatic cells and pluripotent cells. It has been shown that directed differentiation and TF-mediated conversion could be combined to generate human hematopoietic progenitors (Doulatov et al., 2013). Directed differentiation approaches recapitulate the developmental processes that bridge pluripotency and definitive hematopoiesis (Murry and Keller, 2008), but the developmental stage of the derived hematopoietic cells is unclear, and are unlikely to bear full adult hematopoietic potential, including effective engraftment. Moreover, the hematopoietic cells produced from ‘pure’ direct conversion strategies are limited in engraftment and self-renewal, possibly due to inadequate adult specification or the lack of ordered maturation imparted by sequential passage through normal developmental stages of definitive hematopoiesis (Orkin and Zon, 2008).

Whereas the objective of engineering approaches is to produce self-renewing HSCs that beget adult-like differentiated cell progeny for cell therapy, the power of TF-mediated phenotypic conversion may be the ability to bypass heterochronic barriers to reach adult-like HSC phenotypes. As such, further TF respecification approaches should focus on

identification of factors specific to adult definitive HSCs relative to FL or AGM HSCs for incorporation into engineering models. As both directed differentiation and conversion approaches effectively traverse developmental EHT to mature beyond primitive hematopoiesis and reach definitive hematopoietic cell emergence, efforts must focus on accelerating the developmental maturation of these nascent HSCs to acquire adult-like quiescence, globin expression, and adaptive immune capacity for use in cell therapy.

For improvement on the approaches discussed thus far, further understanding of stage-specific markers of HSCs and HSC-specific reporters are required (Table 1). Although gene expression can be used to classify HSC developmental stage (McKinney-Freeman et al., 2012), determining an adult-specific immunophenotype for mouse and human HSCs would be an important tool to query outcomes of engineering strategies. While HSC reporter systems have been developed in zebrafish (Stachura and Traver, 2011), identification of mammalian HSC-specific genes for development of HSC-specific reporters should prove invaluable in blood engineering, as has recently been reported in the cases of murine *Fgd5* and *Hoxb5* (Chen et al., 2016; Gazit et al., 2014).

Due to its role in specification of developmental stage of definitive hematopoiesis, the LIN28/*let-7* axis can be harnessed to interrogate and possibly define the developmental stage identity of induced HSCs and potentially regulate their differentiated cell output. Combined with assessment of differentiated cell output, querying the status of LIN28 expression, *let-7* microRNA abundance, and *let-7* target gene activity in engineered HSCs could provide a readout of developmental state. Given its key roles in specification of HSC self-renewal, lymphoid output, and globin switching (Copley et al., 2013; Lee et al., 2013; Yuan et al., 2012; Zhou et al., 2015), fine-tuning *let-7* levels in engineered hematopoietic cells may be a key to unlocking effective adult-like hematopoiesis for cell therapy.

Novel transplantation models could provide additional tools for discovery. Heterochronic transplantation can be used to assess the self-renewal and progeny output of engineered cells by comparing derived HSC engraftment to published benchmarks of the *in vivo* behavior of normal HSCs across development when engrafted into recipients of different ages (Arora et al., 2014). Other model organisms can be used to mechanistically dissect aspects of HSC function. Human $CD34^+$ or $CD138^+$ cell xenotransplants into 48 hpf zebrafish embryos enable the *in vivo* visualization of cell homing to hematopoietic organs (Sacco et al., 2016; Staal et al., 2016), taking advantage of the high level of conservation of hematopoiesis across species (Figure 1), and may provide novel models of heterochronic HSC transplantation.

Heterochronic mutants of *C. elegans* identified by screening have proven invaluable to the identification of highly conserved regulators of developmental timing, such as LIN28, which has also proven to be a hematopoietic regulator (Ambros and Horvitz, 1984). Other model organisms such as two species of frogs, *Liophryne schlaginhaufeni* and *Sphenophryne cornuta*, appear to be natural heterochronic ‘mutants’ which bypass the tadpole stage, developing from larvae to adults while inside the egg (Bickford, 2002). Study of hematopoiesis in these organisms may provide novel paradigms of hematopoietic

development and provide strategies to accelerate definitive hematopoietic maturation to adulthood for engineering purposes.

As several chromatin regulators appear to play stage-specific roles in hematopoiesis, and as chromatin architecture changes with hematopoietic development (Mochizuki-Kashio et al., 2011; Xie et al., 2014; Xu et al., 2012), it could by hypothesized that the changes in chromatin structure that evolve with hematopoietic maturation may impose epigenetic barriers to reprogramming between developmental stages. As such, manipulating the epigenetic landscape of directly differentiated HSCs may be a mandatory approach in blood engineering. Approaches involving reprogramming committed hematopoietic cells into HSCs seek to minimize the 'epigenetic distance' from HSCs, relative to pluripotent cells (Doulatov et al., 2013; Riddell et al., 2014), highlighting the importance of this concept.

Detailed elucidation of stage-specific chromatin modifications is likely required to accurately model these variables to specify adult-like hematopoiesis. Quiescence is a hallmark of adult LT-HSCs, which preserves replicative potential and prevents exhaustion until activated by stress, and appears to be linked to the epigenetic state of HSCs. The polycomb group factor Bmi-1 appears to provide a link between chromatin modification and quiescence, as it appears to exert stage-specific effects of HSC function (Park et al., 2003). Moreover, *Ezh1* appears to be dispensable for fetal hematopoietic development, but its ablation causes BM failure in adult mice due to excessive HSC senescence (Hidalgo et al., 2012). Further study of the mechanistic links between chromatin regulation and adult HSC cycling is warranted. DNA methylation appears to play a key role in HSC progeny cell output during postnatal aging (reviewed in (Beerman and Rossi, 2015)), but the methylation changes that occur in HSCs during development from embryogenesis to adulthood are uncharacterized. Further understanding of the epigenetic changes that occur across development, and the mechanisms of hematopoietic stage specification by epigenetic regulators is required to faithfully reproduce the epigenetic state of functional adult HSCs in engineering strategies.

Conclusions

Remarkable progress has been made toward the goal of engineering customized HSCs for research applications through use of directed differentiation and reprogramming approaches, but ultimate success in deriving *bona fide* HSCs for clinical applications has remained elusive. For effective cellular therapy, patient-specific HSCs with adult-like patterns of cell renewal and progeny output must be generated in sufficient quantity for rapid, effective establishment of transfusion independence, innate immunity, and the acquisition of a diverse repertoire of adaptive immune cells. Many current HSC engineering approaches are grounded in recapitulation of the normal developmental events that lead to definitive HSC differentiation, and the potential synergy of the methodologies described above suggest that therapeutic HSC engineering may be an attainable goal. Self-renewing, multilineage-capable HSC-like cells have been generated from pluripotent and somatic sources, but the potential therapeutic value of these cells remains unknown, as the developmental state of the engineered cells are for the most part incompletely characterized.

To produce personalized HSCs of therapeutic value, our understanding of naturally arising human adult HSCs must improve. We must comprehensively characterize the epigenetic and transcriptional profiles of normal adult HSCs relative to their prenatal counterparts in order to fully recapitulate the factors required to specify their specialized state. Genetic and phenotypic comparisons of HSCs arising normally across development, and enhanced understanding of normal hematopoietic developmental maturation, are the first steps toward this goal. Engineered HSCs specified to the adult state must be rigorously evaluated with stage-specific markers of HSCs and progeny cells to prognosticate their potential value in cellular therapy.

The challenge of generating human HSCs *ex vivo* for therapeutic use requires further understanding of normal HSC biology using multi-disciplinary strategies and cross-species approaches. Use of zebrafish development in high-throughput chemical genetic screening to define factors that promote HSC specification and expansion, complemented with translation of results to mammalian systems, should identify factors that are conserved across species. As the studies discussed above have begun to demonstrate, a multi-step approach combining relevant chemical or morphogen factors and TFs involved in normal adult HSC specification, possibly with manipulation of epigenetic factors, is likely required to successfully differentiate engineered adult HSCs. By this approach, we speculate that derivation of adult-like HSCs of therapeutic value is an achievable goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Adamo L, Naveiras O, Wenzel PL, McKinney-Freeman S, Mack PJ, Gracia-Sancho J, Suchy-Dicey A, Yoshimoto M, Lensch MW, Yoder MC, et al. Biomechanical forces promote embryonic haematopoiesis. Nature. 2009; 459:1131–1135. [PubMed: 19440194]

Amabile G, Welner RS, Nombela-Arrieta C, D'Alise AM, Di Ruscio A, Ebralidze AK, Kraytsberg Y, Ye M, Kocher O, Neuberg DS, et al. In vivo generation of transplantable human hematopoietic cells from induced pluripotent stem cells. Blood. 2013; 121:1255–1264. [PubMed: 23212524]

Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell. 1989; 57:49–57. [PubMed: 2702689]

Ambros V, Horvitz HR. Heterochronic mutants of the nematode Caenorhabditis elegans. Science. 1984; 226:409–416. [PubMed: 6494891]

Arora N, Wenzel PL, McKinney-Freeman SL, Ross SJ, Kim PG, Chou SS, Yoshimoto M, Yoder MC, Daley GQ. Effect of developmental stage of HSC and recipient on transplant outcomes. Dev Cell. 2014; 29:621–628. [PubMed: 24914562]

Baron MH. Concise Review: early embryonic erythropoiesis: not so primitive after all. Stem Cells. 2013; 31:849–856. [PubMed: 23361843]

Batta K, Florkowska M, Kouskoff V, Lacaud G. Direct reprogramming of murine fibroblasts to hematopoietic progenitor cells. Cell Rep. 2014; 9:1871–1884. [PubMed: 25466247]

Beerman I, Rossi DJ. Epigenetic Control of Stem Cell Potential during Homeostasis, Aging, and Disease. Cell Stem Cell. 2015; 16:613–625. [PubMed: 26046761]

Benz C, Copley MR, Kent DG, Wohrer S, Cortes A, Aghaeepour N, Ma E, Mader H, Rowe K, Day C, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell Stem Cell. 2012; 10:273–283. [PubMed: 22385655]

Bertrand JY, Cisson JL, Stachura DL, Traver D. Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. Blood. 2010; 115:2777–2783. [PubMed: 20107232]

Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. Development. 2007; 134:4147–4156. [PubMed: 17959717]

Bickford D. Animal behavior: male parenting of New Guinea froglets. Nature. 2002; 418:601–602. [PubMed: 12167847]

Bolli N, Payne EM, Rhodes J, Gjini E, Johnston AB, Guo F, Lee JS, Stewart RA, Kanki JP, Chen AT, et al. cpsf1 is required for definitive HSC survival in zebrafish. Blood. 2011; 117:3996–4007. [PubMed: 21330472]

Bowie MB, Kent DG, Copley MR, Eaves CJ. Steel factor responsiveness regulates the high self-renewal phenotype of fetal hematopoietic stem cells. Blood. 2007a; 109:5043–5048. [PubMed: 17327414]

Bowie MB, Kent DG, Dykstra B, McKnight KD, McCaffrey L, Hoodless PA, Eaves CJ. Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. Proc Natl Acad Sci U S A. 2007b; 104:5878–5882. [PubMed: 17379664]

Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A, Bhatia M. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood. 2003; 102:906–915. [PubMed: 12702499]

Chanda B, Ditadi A, Iscove NN, Keller G. Retinoic acid signaling is essential for embryonic hematopoietic stem cell development. Cell. 2013; 155:215–227. [PubMed: 24074870]

Charbord P, Oostendorp R, Pang W, Herault O, Noel F, Tsuji T, Dzierzak E, Peault B. Comparative study of stromal cell lines derived from embryonic, fetal, and postnatal mouse blood-forming tissues. Exp Hematol. 2002; 30:1202–1210. [PubMed: 12384152]

Chaudhuri AA, So AY, Mehta A, Minisandram A, Sinha N, Jonsson VD, Rao DS, O'Connell RM, Baltimore D. Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A. Proc Natl Acad Sci U S A. 2012; 109:4233–4238. [PubMed: 22366319]

Chen JY, Miyanishi M, Wang SK, Yamazaki S, Sinha R, Kao KS, Seita J, Sahoo D, Nakauchi H, Weissman IL. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. Nature. 2016; 530:223–227. [PubMed: 26863982]

Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature. 2009; 457:887–891. [PubMed: 19129762]

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science. 2000; 287:1804–1808. [PubMed: 10710306]

Copley MR, Babovic S, Benz C, Knapp DJ, Beer PA, Kent DG, Wohrer S, Treloar DQ, Day C, Rowe K, et al. The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. Nat Cell Biol. 2013; 15:916–925. [PubMed: 23811688]

de Jong JL, Zon LI. Use of the zebrafish system to study primitive and definitive hematopoiesis. Annual review of genetics. 2005; 39:481–501.

Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. Cell Stem Cell. 2012; 10:120–136. [PubMed: 22305562]

Doulatov S, Vo LT, Chou SS, Kim PG, Arora N, Li H, Hadland BK, Bernstein ID, Collins JJ, Zon LI, et al. Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. Cell Stem Cell. 2013; 13:459–470. [PubMed: 24094326]

Du L, Xu J, Li X, Ma N, Liu Y, Peng J, Osato M, Zhang W, Wen Z. Rumba and Haus3 are essential factors for the maintenance of hematopoietic stem/progenitor cells during zebrafish hematopoiesis. Development. 2011; 138:619–629. [PubMed: 21228005]

Dzierzak E. Hematopoietic stem cells and their precursors: developmental diversity and lineage relationships. Immunol Rev. 2002; 187:126–138. [PubMed: 12366688]

Dzierzak E, Speck NA. Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol. 2008; 9:129–136. [PubMed: 18204427]

Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. Blood. 2015; 125:2605–2613. [PubMed: 25762175]

Elcheva I, Brok-Volchanskaya V, Kumar A, Liu P, Lee JH, Tong L, Vodyanik M, Swanson S, Stewart R, Kyba M, et al. Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. Nat Commun. 2014; 5:4372. [PubMed: 25019369]

Ganis JJ, Hsia N, Trompouki E, de Jong JL, DiBiase A, Lambert JS, Jia Z, Sabo PJ, Weaver M, Sandstrom R, et al. Zebrafish globin switching occurs in two developmental stages and is controlled by the LCR. Dev Biol. 2012; 366:185–194. [PubMed: 22537494]

Gazit R, Mandal PK, Ebina W, Ben-Zvi A, Nombela-Arrieta C, Silberstein LE, Rossi DJ. Fgd5 identifies hematopoietic stem cells in the murine bone marrow. J Exp Med. 2014; 211:1315–1331. [PubMed: 24958848]

Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. Nature. 2015; 518:547–551. [PubMed: 25470051]

Hadland BK, Huppert SS, Kanungo J, Xue Y, Jiang R, Gridley T, Conlon RA, Cheng AM, Kopan R, Longmore GD. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. Blood. 2004; 104:3097–3105. [PubMed: 15251982]

Harandi OF, Ambros VR. Control of stem cell self-renewal and differentiation by the heterochronic genes and the cellular asymmetry machinery in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2015; 112:E287–296. [PubMed: 25561544]

He C, Kraft P, Chen C, Buring JE, Pare G, Hankinson SE, Chanock SJ, Ridker PM, Hunter DJ, Chasman DI. Genome-wide association studies identify loci associated with age at menarche and age at natural menopause. Nat Genet. 2009; 41:724–728. [PubMed: 19448621]

He S, Kim I, Lim MS, Morrison SJ. Sox17 expression confers self-renewal potential and fetal stem cell characteristics upon adult hematopoietic progenitors. Genes Dev. 2011; 25:1613–1627. [PubMed: 21828271]

Hendriks WT, Warren CR, Cowan CA. Genome Editing in Human Pluripotent Stem Cells: Approaches, Pitfalls, and Solutions. Cell Stem Cell. 2016; 18:53–65. [PubMed: 26748756]

Hidalgo I, Herrera-Merchan A, Ligos JM, Carramolino L, Nunez J, Martinez F, Dominguez O, Torres M, Gonzalez S. Ezh1 is required for hematopoietic stem cell maintenance and prevents senescence-like cell cycle arrest. Cell Stem Cell. 2012; 11:649–662. [PubMed: 23122289]

Hills D, Gribi R, Ure J, Buza-Vidas N, Luc S, Jacobsen SE, Medvinsky A. Hoxb4-YFP reporter mouse model: a novel tool for tracking HSC development and studying the role of Hoxb4 in hematopoiesis. Blood. 2011; 117:3521–3528. [PubMed: 21278354]

Hock H, Hamblen MJ, Rooke HM, Schindler JW, Saleque S, Fujiwara Y, Orkin SH. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature. 2004a; 431:1002–1007. [PubMed: 15457180]

Hock H, Meade E, Medeiros S, Schindler JW, Valk PJ, Fujiwara Y, Orkin SH. Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. Genes Dev. 2004b; 18:2336–2341. [PubMed: 15371326]

Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. Blood. 2014; 124:363–373. [PubMed: 24914140]

Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, Yamagata T, Mitani K, Chiba S, Ogawa S, Kurokawa M, et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med. 2004; 10:299–304. [PubMed: 14966519]

Ikuta K, Kina T, MacNeil I, Uchida N, Peault B, Chien YH, Weissman IL. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. Cell. 1990; 62:863–874. [PubMed: 1975515]

Ivanovs A, Rybtsov S, Anderson RA, Turner ML, Medvinsky A. Identification of the niche and phenotype of the first human hematopoietic stem cells. Stem Cell Reports. 2014; 2:449–456. [PubMed: 24749070]

Jia XE, Ma K, Xu T, Gao L, Wu S, Fu C, Zhang W, Wang Z, Liu K, Dong M, et al. Mutation of kri1l causes definitive hematopoiesis failure via PERK-dependent excessive autophagy induction. Cell research. 2015; 25:946–962. [PubMed: 26138676]

Jing L, Zon LI. Zebrafish as a model for normal and malignant hematopoiesis. Disease models & mechanisms. 2011; 4:433–438. [PubMed: 21708900]

Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. Proc Natl Acad Sci U S A. 1992; 89:3320–3324. [PubMed: 1565622]

Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 2001; 98:10716–10721. [PubMed: 11535826]

Kennedy M, Awong G, Sturgeon CM, Ditadi A, LaMotte-Mohs R, Zuniga-Pflucker JC, Keller G. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. Cell Rep. 2012; 2:1722–1735. [PubMed: 23219550]

Kim PG, Canver MC, Rhee C, Ross SJ, Harriss JV, Tu HC, Orkin SH, Tucker HO, Daley GQ. Interferon-alpha signaling promotes embryonic HSC maturation. Blood. 2016

Kingsley PD, Malik J, Fantauzzo KA, Palis J. Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. Blood. 2004; 104:19–25. [PubMed: 15031208]

Kumaravelu P, Hook L, Morrison AM, Ure J, Zhao S, Zuyev S, Ansell J, Medvinsky A. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development. 2002; 129:4891–4899. [PubMed: 12397098]

Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell. 2002; 109:29–37. [PubMed: 11955444]

Lee YT, de Vasconcellos JF, Yuan J, Byrnes C, Noh SJ, Meier ER, Kim KS, Rabel A, Kaushal M, Muljo SA, et al. LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo. Blood. 2013; 122:1034–1041. [PubMed: 23798711]

Lettre G, Jackson AU, Gieger C, Schumacher FR, Berndt SI, Sanna S, Eyheramendy S, Voight BF, Butler JL, Guiducci C, et al. Identification of ten loci associated with height highlights new biological pathways in human growth. Nat Genet. 2008; 40:584–591. [PubMed: 18391950]

Ling KW, Ottersbach K, van Hamburg JP, Oziemlak A, Tsai FY, Orkin SH, Ploemacher R, Hendriks RW, Dzierzak E. GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. J Exp Med. 2004; 200:871–882. [PubMed: 15466621]

Ma D, Zhang J, Lin HF, Italiano J, Handin RI. The identification and characterization of zebrafish hematopoietic stem cells. Blood. 2011; 118:289–297. [PubMed: 21586750]

MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nature reviews Drug discovery. 2015; 14:721–731. [PubMed: 26361349]

McGrath KE, Frame JM, Fegan KH, Bowen JR, Conway SJ, Catherman SC, Kingsley PD, Koniski AD, Palis J. Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. Cell Rep. 2015; 11:1892–1904. [PubMed: 26095363]

McKinney-Freeman S, Cahan P, Li H, Lacadie SA, Huang HT, Curran M, Loewer S, Naveiras O, Kathrein KL, Konantz M, et al. The transcriptional landscape of hematopoietic stem cell ontogeny. Cell Stem Cell. 2012; 11:701–714. [PubMed: 23122293]

Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell. 1996; 86:897–906. [PubMed: 8808625]

Mehta RS, Saliba RM, Chen J, Rondon G, Hammerstrom AE, Alousi A, Qazilbash M, Bashir Q, Ahmed S, Popat U, et al. Post-transplantation cyclophosphamide versus conventional graft-versus-host disease prophylaxis in mismatched unrelated donor haematopoietic cell transplantation. Br J Haematol. 2016

Micklem HS, Ford CE, Evans EP, Ogden DA, Papworth DS. Competitive in vivo proliferation of foetal and adult haematopoietic cells in lethally irradiated mice. J Cell Physiol. 1972; 79:293–298. [PubMed: 4554679]

Mochizuki-Kashio M, Mishima Y, Miyagi S, Negishi M, Saraya A, Konuma T, Shinga J, Koseki H, Iwama A. Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. Blood. 2011; 118:6553–6561. [PubMed: 22042701]

Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci U S A. 1995; 92:10302–10306. [PubMed: 7479772]

Moss EG, Tang L. Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. Dev Biol. 2003; 258:432–442. [PubMed: 12798299]

Mucenski ML, McLain K, Kier AB, Swerdlow SH, Schreiner CM, Miller TA, Pietryga DW, Scott WJ Jr. Potter SS. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell. 1991; 65:677–689. [PubMed: 1709592]

Muller AM, Medvinsky A, Strouboulis J, Grosveld F, Dzierzak E. Development of hematopoietic stem cell activity in the mouse embryo. Immunity. 1994; 1:291–301. [PubMed: 7889417]

Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell. 2008; 132:661–680. [PubMed: 18295582]

Nguyen PD, Hollway GE, Sonntag C, Miles LB, Hall TE, Berger S, Fernandez KJ, Gurevich DB, Cole NJ, Alaei S, et al. Haematopoietic stem cell induction by somite-derived endothelial cells controlled by meox1. Nature. 2014; 512:314–318. [PubMed: 25119043]

Nimmo RA, May GE, Enver T. Primed and ready: understanding lineage commitment through single cell analysis. Trends in cell biology. 2015; 25:459–467. [PubMed: 26004869]

Noh SJ, Miller SH, Lee YT, Goh SH, Marincola FM, Stroncek DF, Reed C, Wang E, Miller JL. Let-7 microRNAs are developmentally regulated in circulating human erythroid cells. J Transl Med. 2009; 7:98. [PubMed: 19939273]

North TE, Goessling W, Peeters M, Li P, Ceol C, Lord AM, Weber GJ, Harris J, Cutting CC, Huang P, et al. Hematopoietic stem cell development is dependent on blood flow. Cell. 2009; 137:736–748. [PubMed: 19450519]

Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, Kaufmann KB, McLeod J, Laurenti E, Dunant CF, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science. 2016; 351:aab2116. [PubMed: 26541609]

Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature. 1995; 375:316–318. [PubMed: 7753194]

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996; 84:321–330. [PubMed: 8565077]

Ong KK, Elks CE, Li S, Zhao JH, Luan J, Andersen LB, Bingham SA, Brage S, Smith GD, Ekelund U, et al. Genetic variation in LIN28B is associated with the timing of puberty. Nat Genet. 2009; 41:729–733. [PubMed: 19448623]

Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008; 132:631–644. [PubMed: 18295580]

Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature. 2003; 423:302–305. [PubMed: 12714971]

Paul F, Arkin Y, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H, Winter D, Lara-Astiaso D, Gury M, Weiner A, et al. Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. Cell. 2015; 163:1663–1677. [PubMed: 26627738]

Pelosi A, Careccia S, Lulli V, Romania P, Marziali G, Testa U, Lavorgna S, Lo-Coco F, Petti MC, Calabretta B, et al. miRNA let-7c promotes granulocytic differentiation in acute myeloid leukemia. Oncogene. 2013; 32:3648–3654. [PubMed: 22964640]

Pereira CF, Chang B, Gomes A, Bernitz J, Papatsenko D, Niu X, Swiers G, Azzoni E, de Bruijn MF, Schaniel C, et al. Hematopoietic Reprogramming In Vitro Informs In Vivo Identification of

Hemogenic Precursors to Definitive Hematopoietic Stem Cells. Dev Cell. 2016; 36:525–539. [PubMed: 26954547]

Pereira CF, Chang B, Qiu J, Niu X, Papatsenko D, Hendry CE, Clark NR, Nomura-Kitabayashi A, Kovacic JC, Ma'ayan A, et al. Induction of a hemogenic program in mouse fibroblasts. Cell Stem Cell. 2013; 13:205–218. [PubMed: 23770078]

Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 1995; 375:318–322. [PubMed: 7753195]

Pineault N, Abu-Khader A. Advances in umbilical cord blood stem cell expansion and clinical translation. Exp Hematol. 2015; 43:498–513. [PubMed: 25970610]

Prashad SL, Calvanese V, Yao CY, Kaiser J, Wang Y, Sasidharan R, Crooks G, Magnusson M, Mikkola HK. GPI-80 defines self-renewal ability in hematopoietic stem cells during human development. Cell Stem Cell. 2015; 16:80–87. [PubMed: 25465114]

Rebel VI, Miller CL, Eaves CJ, Lansdorp PM. The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. Blood. 1996; 87:3500–3507. [PubMed: 8605370]

Riddell J, Gazit R, Garrison BS, Guo G, Saadatpour A, Mandal PK, Ebina W, Volchkov P, Yuan GC, Orkin SH, et al. Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. Cell. 2014; 157:549–564. [PubMed: 24766805]

Robin C, Bollerot K, Mendes S, Haak E, Crisan M, Cerisoli F, Lauw I, Kaimakis P, Jorna R, Vermeulen M, et al. Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. Cell Stem Cell. 2009; 5:385–395. [PubMed: 19796619]

Sacco A, Roccaro AM, Ma D, Shi J, Mishima Y, Moschetta M, Chiarini M, Munshi N, Handin RI, Ghobrial IM. Cancer Cell Dissemination and Homing to the Bone Marrow in a Zebrafish Model. Cancer research. 2016; 76:463–471. [PubMed: 26744527]

Samokhvalov IM, Samokhvalova NI, Nishikawa S. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. Nature. 2007; 446:1056–1061. [PubMed: 17377529]

Sandler VM, Lis R, Liu Y, Kedem A, James D, Elemento O, Butler JM, Scandura JM, Rafii S. Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. Nature. 2014; 511:312–318. [PubMed: 25030167]

Schmitt RM, Bruyns E, Snodgrass HR. Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. Genes Dev. 1991; 5:728–740. [PubMed: 1709130]

Shyh-Chang N, Daley GQ. Lin28: primal regulator of growth and metabolism in stem cells. Cell Stem Cell. 2013; 12:395–406. [PubMed: 23561442]

Sood R, English MA, Belele CL, Jin H, Bishop K, Haskins R, McKinney MC, Chahal J, Weinstein BM, Wen Z, et al. Development of multilineage adult hematopoiesis in the zebrafish with a runx1 truncation mutation. Blood. 2010; 115:2806–2809. [PubMed: 20154212]

Soza-Ried C, Hess I, Netuschil N, Schorpp M, Boehm T. Essential role of c-myb in definitive hematopoiesis is evolutionarily conserved. Proc Natl Acad Sci U S A. 2010; 107:17304–17308. [PubMed: 20823231]

Staal FJ, Spaink HP, Fibbe WE. Visualizing Human Hematopoietic Stem Cell Trafficking In Vivo Using a Zebrafish Xenograft Model. Stem Cells Dev. 2016; 25:360–365. [PubMed: 26650921]

Stachura DL, Reyes JR, Bartunek P, Paw BH, Zon LI, Traver D. Zebrafish kidney stromal cell lines support multilineage hematopoiesis. Blood. 2009; 114:279–289. [PubMed: 19433857]

Stachura DL, Traver D. Cellular dissection of zebrafish hematopoiesis. Methods in cell biology. 2011; 101:75–110. [PubMed: 21550440]

Sturgeon CM, Ditadi A, Awong G, Kennedy M, Keller G. Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. Nat Biotechnol. 2014; 32:554–561. [PubMed: 24837661]

Sulem P, Gudbjartsson DF, Rafnar T, Holm H, Olafsdottir EJ, Olafsdottir GH, Jonsson T, Alexandersen P, Feenstra B, Boyd HA, et al. Genome-wide association study identifies sequence variants on 6q21 associated with age at menarche. Nat Genet. 2009; 41:734–738. [PubMed: 19448622]

Szilvassy SJ, Ragland PL, Miller CL, Eaves CJ. The marrow homing efficiency of murine hematopoietic stem cells remains constant during ontogeny. Exp Hematol. 2003; 31:331–338. [PubMed: 12691921]

Takahashi K, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc. 2007; 2:3081–3089. [PubMed: 18079707]

Tamplin OJ, Durand EM, Carr LA, Childs SJ, Hagedorn EJ, Li P, Yzaguirre AD, Speck NA, Zon LI. Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. Cell. 2015; 160:241–252. [PubMed: 25594182]

Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nature immunology. 2003; 4:1238–1246. [PubMed: 14608381]

Urbach A, Yermalovich A, Zhang J, Spina CS, Zhu H, Perez-Atayde AR, Shukrun R, Charlton J, Sebire N, Mifsud W, et al. Lin28 sustains early renal progenitors and induces Wilms tumor. Genes Dev. 2014; 28:971–982. [PubMed: 24732380]

Vodyanik MA, Bork JA, Thomson JA, Slukvin II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood. 2005; 105:617–626. [PubMed: 15374881]

Wang LD, Rao TN, Rowe RG, Nguyen PT, Sullivan JL, Pearson DS, Doulatov S, Wu L, Lindsley RC, Zhu H, et al. The role of Lin28b in myeloid and mast cell differentiation and mast cell malignancy. Leukemia. 2015; 29:1320–1330. [PubMed: 25655194]

Widen E, Ripatti S, Cousminer DL, Surakka I, Lappalainen T, Jarvelin MR, Eriksson JG, Raitakari O, Salomaa V, Sovio U, et al. Distinct variants at LIN28B influence growth in height from birth to adulthood. Am J Hum Genet. 2010; 86:773–782. [PubMed: 20398887]

Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development. 1991; 111:259–267. [PubMed: 1893864]

Xie H, Xu J, Hsu JH, Nguyen M, Fujiwara Y, Peng C, Orkin SH. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. Cell Stem Cell. 2014; 14:68–80. [PubMed: 24239285]

Xu J, Shao Z, Glass K, Bauer DE, Pinello L, Van Handel B, Hou S, Stamatoyannopoulos JA, Mikkola HK, Yuan GC, et al. Combinatorial assembly of developmental stage-specific enhancers controls gene expression programs during human erythropoiesis. Dev Cell. 2012; 23:796–811. [PubMed: 23041383]

Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, Porayette P, Shelley WC, Conway SJ, Dorshkind K, Yoder MC. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. Proc Natl Acad Sci U S A. 2011; 108:1468–1473. [PubMed: 21209332]

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318:1917–1920. [PubMed: 18029452]

Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. Science. 2012; 335:1195–1200. [PubMed: 22345399]

Zhen F, Lan Y, Yan B, Zhang W, Wen Z. Hemogenic endothelium specification and hematopoietic stem cell maintenance employ distinct Scl isoforms. Development. 2013; 140:3977–3985. [PubMed: 24046317]

Zhou Y, Li YS, Bandi SR, Tang L, Shinton SA, Hayakawa K, Hardy RR. Lin28b promotes fetal B lymphopoiesis through the transcription factor Arid3a. J Exp Med. 2015; 212:569–580. [PubMed: 25753579]

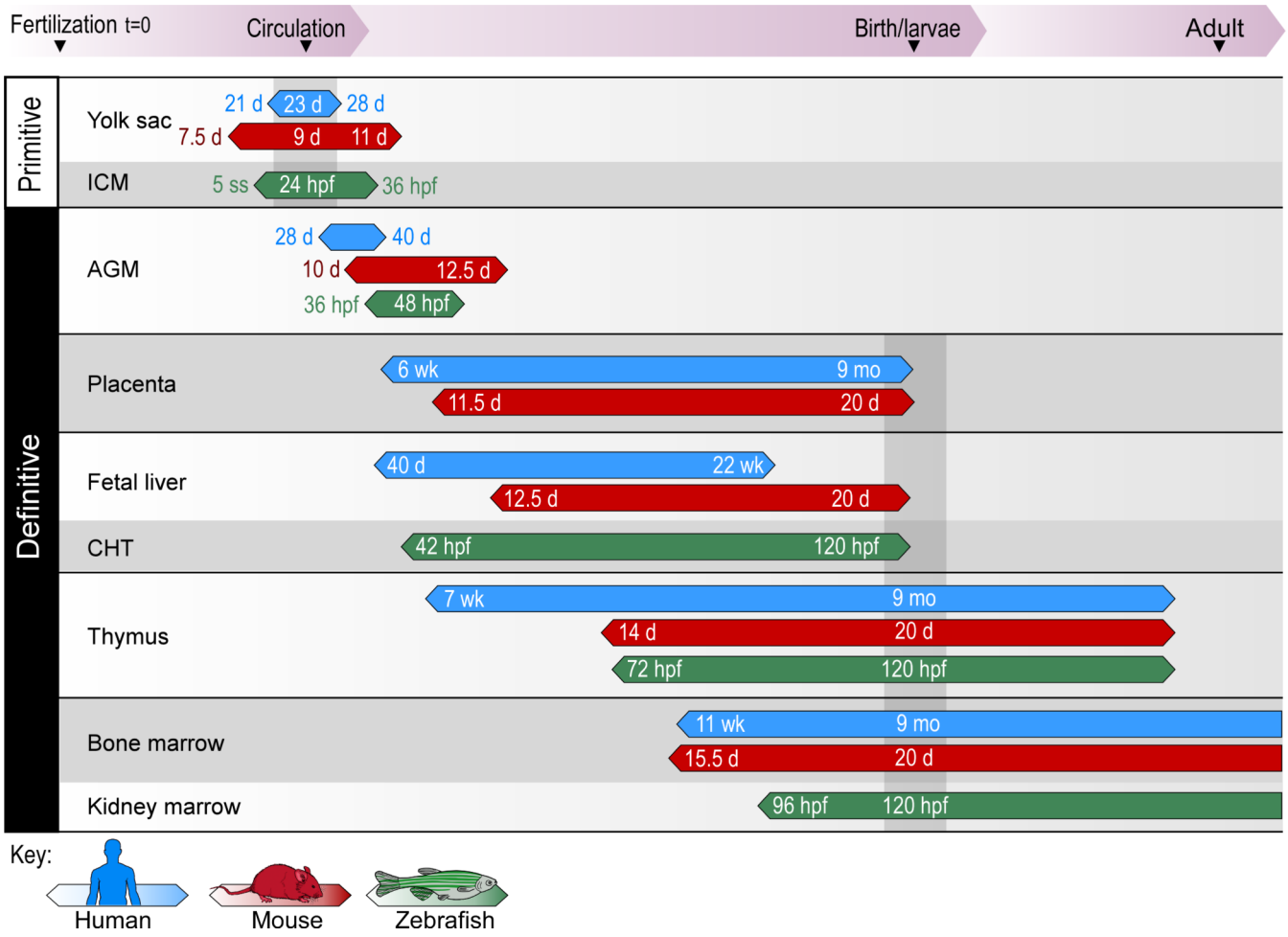

Figure 1. Timing of hematopoietic maturation across species

The relative timing of hematopoiesis at specific anatomic sites in the human (blue), mouse (red), and zebrafish (green) are shown. Although the pace of hematopoietic maturation varies in each organism, hematopoiesis matures in highly conserved patterns through analogous organs prenatally and postnatally, with primitive hematopoiesis occurring in extraembryonic mesoderm derived cells.

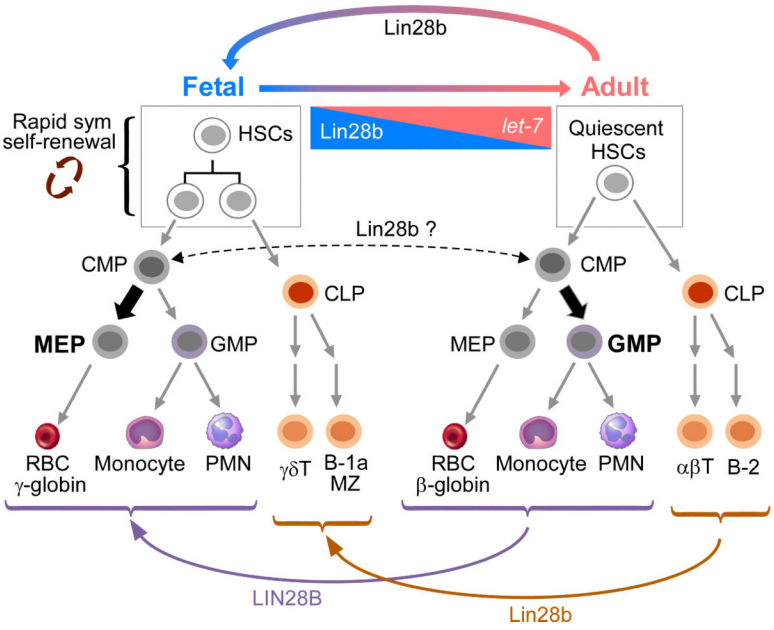

Figure 2. LIN28 and let-7 in developmental timing and hematopoiesis

Maturation of definitive hematopoiesis from the fetal to adult stage is depicted. Hematopoiesis shifts from a fetal state characterized by rapid HSC self-renewal, fetal-specific lymphoid output, erythroid dominant myeloerythropoiesis, and, in humans, fetal globin expression, to the adult stage characterized by HSC quiescence, predominance of granulopoiesis, adult lymphopoiesis, and adult globin expression in humans. *Lin28b* is downregulated during maturation, allowing for increase in *let-7* microRNAs. Effects of ectopic *Lin28b*/*LIN28B* expression on the timing of hematopoietic maturation are shown by arrows connecting adult and fetal hematopoietic populations. The broken arrow hypothesizes a possible effect of *Lin28b* modulation of granulopoiesis and erythropoiesis. Abbreviations: RBC, red blood cell; PMN, polymorphonuclear neutrophil; CMP, common myeloid progenitor; MEP, megakaryocyte erythroid progenitor; GMP, granulocyte macrophage progenitor; CLP, common lymphoid progenitor; MZ, marginal zone.

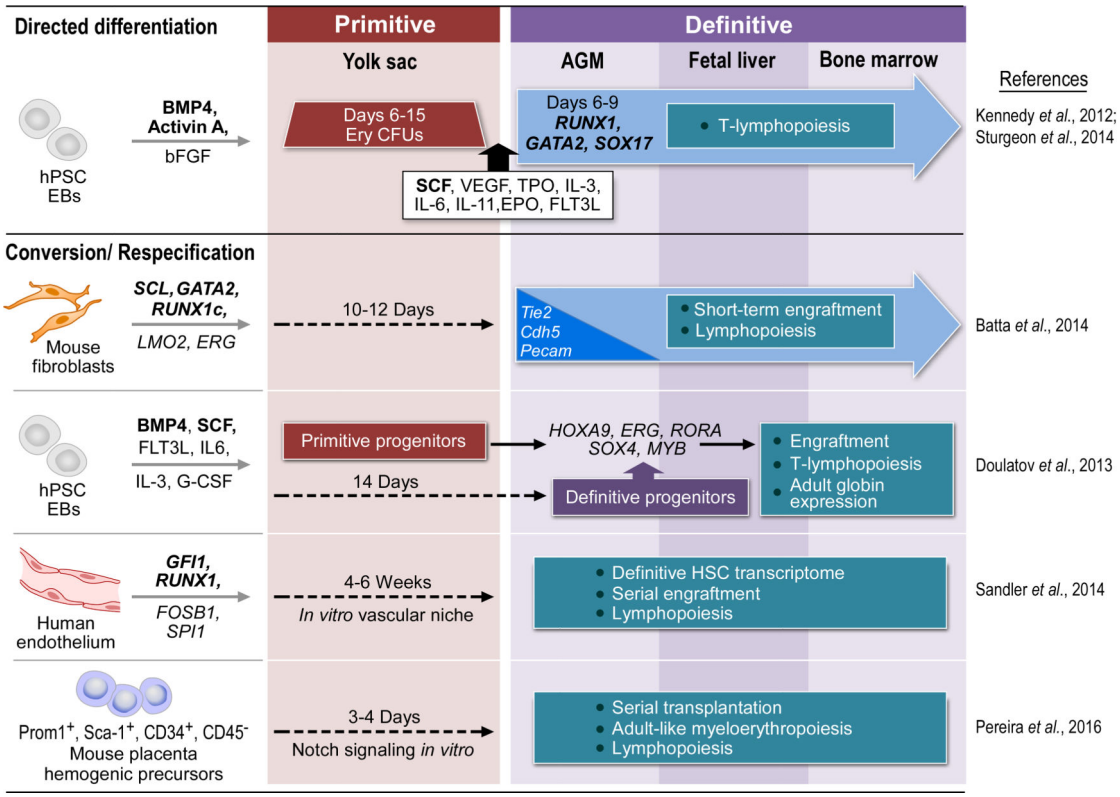

Figure 3. Examples of hematopoietic maturation in blood derivation strategies

This diagram highlights several key examples of blood derivation strategies showing the relative timing of developmental events, and classification of derived cells at stages of normal hematopoietic maturation. Examples of strategies using morphogen directed differentiation and conversion/respecification are depicted. The strategies employed by the Keller group typify morphogen directed differentiation approaches to hemogenic cell induction (Kennedy et al., 2012; Sturgeon et al., 2014). Sequential exposure of PSC EBs to morphogen cocktails initially specifies mesoderm fate, followed by hematopoietic fate induction by hematopoietic cytokines (Kennedy et al., 2012; Sturgeon et al., 2014). Though several studies have succeeded in reprogramming somatic cells to hematopoietic lineages (Pereira et al., 2013; Riddell et al., 2014), we present one example (Batta et al., 2014) whereby mouse fibroblasts were reprogrammed by expression of hematopoietic transcription factors, eventually producing cells with short-term engraftment *in vivo*. PSC reprogramming is an approach also used by multiple groups to generate hematopoietic cells (Doulatov et al., 2013; Elcheva et al., 2014). Here, we summarize the strategy used by Doulatov and colleagues, where hematopoietic progenitors generated by morphogen directed differentiation were respecified to engraftable definitive progenitors, and lineage output analyzed *in vivo* (Doulatov et al., 2013). Finally, two distinct approaches leveraging understanding of EHT are shown (Pereira et al., 2016; Sandler et al., 2014). Sandler and colleagues reprogrammed human endothelial cells with transcription factors that promoted an EHT-like process, generating multilineage engraftable MPP-like cells (Sandler et al., 2014). Pereira and colleages isolated mouse placental hemogenic precursors based on an immunophenotype identified on endothelia-like cells generated in their prior somatic cell

reprogramming (Pereira et al., 2013), showing that these cells could be induced to engraft and undergo multilineage hematopoiesis (Pereira et al., 2016). Known stage-specific hematopoietic regulators are shown in bold. Abbreviations: hPSC, human pluripotent stem cell; EBs, embryoid bodies.

Table 1

Comparison of stage-specific markers of hematopoietic cells across vertebrate species.

	Human	**Mouse**	**Zebrafish**
Primitive	Erythroid cells are nucleated, macrocytic, and express embryonic ζ-, ε- and α-globin.	Yolk sac primitive progenitors are CD41-lo c-kit-lo. Erythroid cells express embryonic ζ-, εy- and α-globin.	Mesoderm derived erythromyeloid precursors express scl, lmo2, and gata2. Myeloid cells express spi1 (pu.1), mpeg, mpx, and mfap4. Erythroid cells express nucleated, express gatal, globin βe3, and marked by LCR-GFP. Endothelial cells express kdrl and fli1.
Definitive			
Extraembryonic	CD34+ CD38- HSPCs are present throughout gestation in placenta.	Yolk sac definitive EMP are c-kit-hi; CD41+ CD16/32+, and express definitive β-globin. Placental and umbilical artery hemogenic precursors are Sca-1+ CD34+ CD45-Prom1+. Onset of lymphoid potential in HSPCs.	
AGM	Aortic HSCs are CD34+ VE-cadherin+ CD45+ C-KIT+ THY-1 + Endoglin+ CD38-/lo CD45RA-, expressing *RUNX1.*	Hemogenic precursor cells are Sca-1+, CD34+, CD45-, Prom1+, and possess lymphoid potential. Erythroid cells acquire definitive β-globin expression.	Gata2b and sclβ are expressed in hemogenic endothelium prior to runx1 expression and HSPC emergence. Runx1 and cd41 are earlier HSPC markers, whereas c-myb is expressed in a more progenitor population. CD41-GFP^{lo} marks developing stem cells (in addition to other cells), and runx1-GFP (driven by mouse enhancer) marks stem cells weakly just before egress and migration.
Fetal Liver	HSCs express CD34+, CD38-lo/-, CD90+, GPI-80+. Myeloid progenitors express CD34+CD38+CD10-CD135+CD45RA-.	HSCs are enriched in Lin- Sca-1+ c-kit+ CD150+ CD48-CD11b+ fraction and rapidly cycling. Lymphoid cells are characterized by T-cell Vγ3 expression; γδ innate-like T cells; B-1a CD19+ B220-lo CD5+; MZ B-cells B220+ CD1d+ CD23-. Myeloid cells express CD11b, CD11c, F4/80, Gr-1, Ly6C, and Ly6G.	HSPCs are marked by runx1-GFP (under mouse enhancer) and CD41-GFP^{lo}. Erythroid cells express remain nucleated, express gatal, globin αal and βal, and marked by LCR-GFP. Thrombocytes express cd41^{hi}. Macrophages express mpeg, mfap4. Neutrophils express mpx, lyz. Eosinophils express gata2a.
Aault marrow	HSCs express CD34+ CD38- Thy1+ CD45RA-. Erythroid cells acquire adult β-globin expression.	HSCs become quiescent and are present in Lin-, Sca-1+, c-kit+, CD150+, CD34-, CD11b- fraction. Lymphoid cells are characterized by B-2 cells, which are CD19+ B220-hi CD5-; and Fo B- cells which are B220+ CD1d- CD23+.	Lymphoid cells (B and T cell) are marked by rag2 and ikaros. T cells express lck. HSPCs are marked by runx1-GFP (under mouse enhancer) and CD41-GFP^{lo}.

The spatiotemporal expression of genes and regulators in primitive and definitive hematopoiesis listed provide a sample of markers used to differentiate hematopoietic populations during developmental stages. In zebrafish, gene reporter lines and markers are shown due to the limited availability of antibodies that can be used. AGM, aorta-gonad mesonephros. HSCs, hematopoietic stem cells. HSPCs, hematopoietic stem/progenitor cells. EMP, erythro-myeloid progenitor.