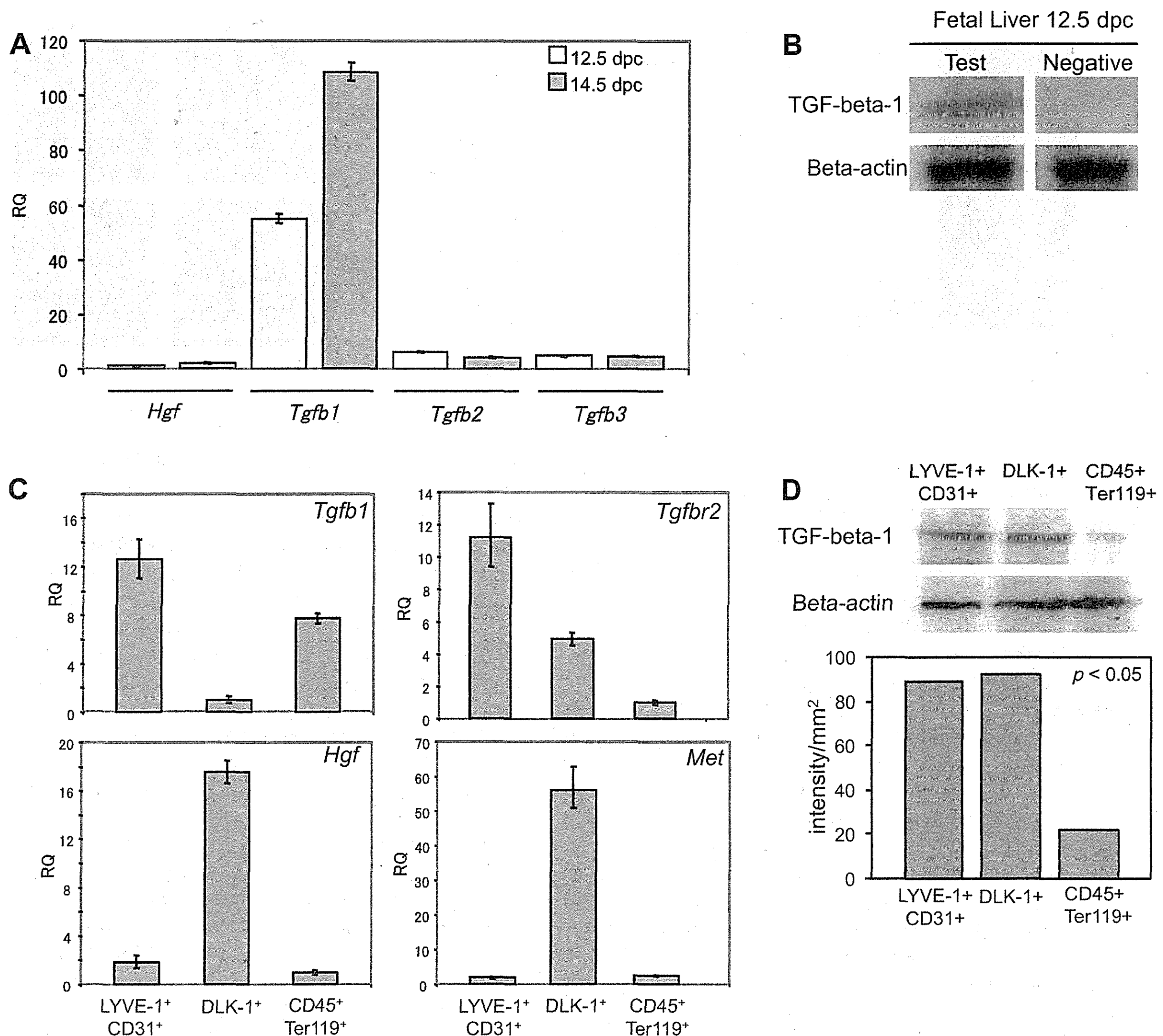

Fig. 4 – TGF-beta and HGF expression in fetal liver. (A) Expression of *Tgfb1*, *Tgfb2*, *Tgfb3* and *Hgf* was examined in fetal liver samples at 12.5 and 14.5 dpc. *Tgfb1* was highly expressed at both stages. (B) TGF-beta-1 protein was detected by Western blot analysis of fetal liver at 12.5 dpc. (C) Expression of *Tgfb1* and *Tgfbr2*, which encodes its receptor, and *Hgf* and *Met*, which encodes its receptor, was examined by real-time PCR in hepatoblasts, sinusoid endothelial cells and hematopoietic cells according to gates defined in Fig. 3C. *Tgfb1* was predominantly expressed in sinusoid endothelial cells and hematopoietic cells, whereas *Tgfbr2* was highly expressed both in sinusoid endothelial cells and hepatoblasts. Both *Hgf* and *Met* were highly expressed in hepatoblasts. (D) TGF-beta-1 expression was examined by Western blot analysis in each fraction of cells sorted from ICR mouse fetal liver at 12.5 dpc according to gate settings defined in Fig. 3C. Protein expression levels were normalized to Beta-actin and displayed as intensity per mm^2.

anti-TGF-beta-1 blocking antibody (Fig. S3). As shown in Fig. S4, some DLK-1 positive and negative cells were stained with anti-phosphorylated SMAD3 Ab in fetal liver at 10.5 dpc. Taken together, it is likely that TGF-beta-1 signaling in both hepatoblasts expressing DLK-1 and non-hepatoblasts was down-regulated after administration of anti-TGF-beta-1 blocking antibody.

To further investigate how TGF-beta-1 controls ECM production in hepatoblasts, we sorted hepatoblasts expressing DLK-1 and cultured them in the presence of TGF-beta-1. After 6 h of culture, *Vtn*, *Fn1* and *Tnc* expression was up-regulated (Fig. 5D). Taken together, these data strongly suggest that TGF-beta-1/TGFR-2 signaling promotes ECM production by hepatoblasts in fetal liver.

4. Discussion

Hepatoblasts were originally regarded as common progenitors of hepatocytes and biliary epithelial cells and thought to support liver construction through formation of a mesh-like structure (Tanimizu et al., 2003; Sasaki and Sonoda, 2000). Recently, it was reported that expression of SCF and EPO by

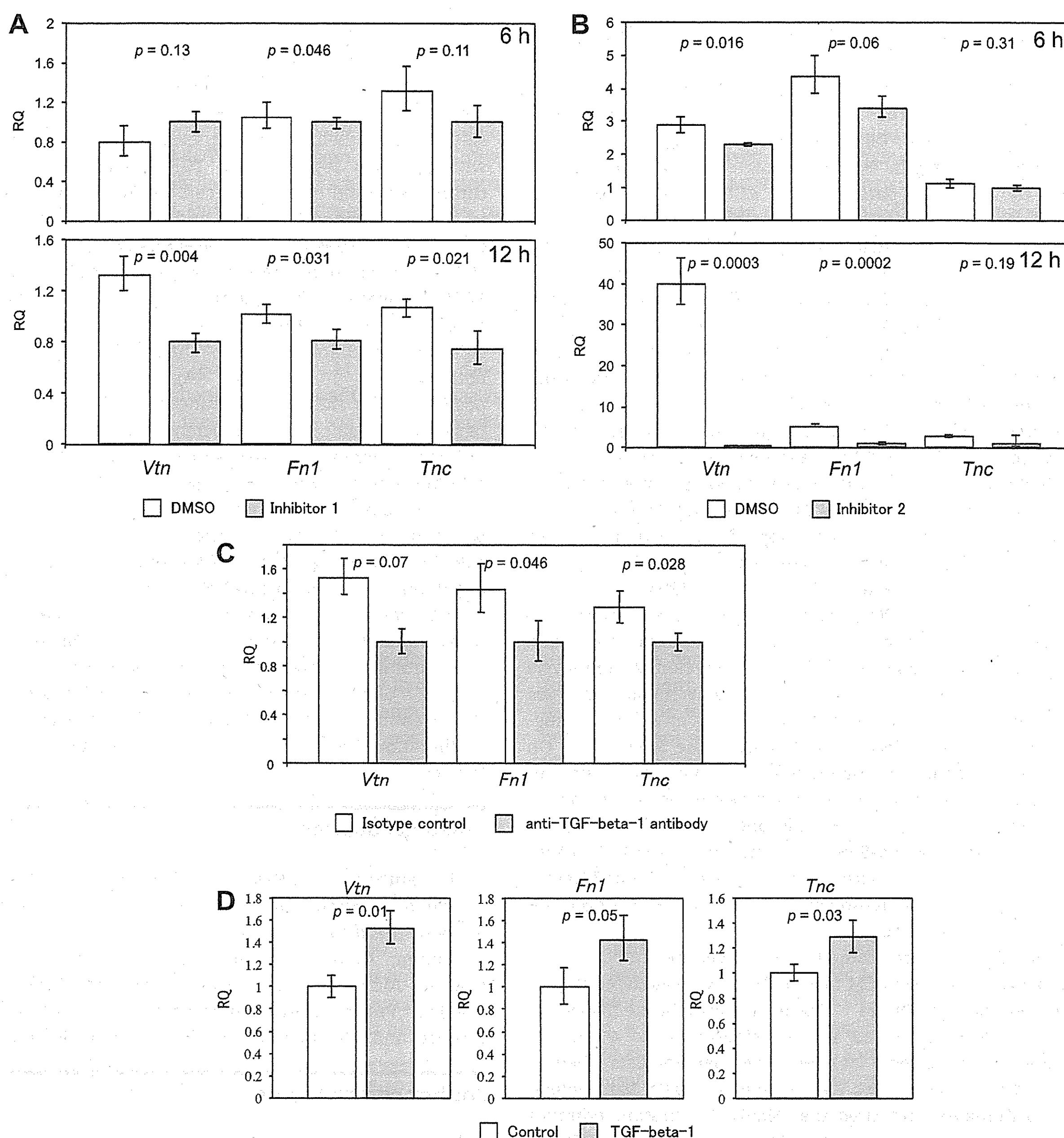

Fig. 5 – Functional analysis of the effect of TGF-beta-1/TGFR2 signaling on ECM production in fetal liver. (A) A TGF-beta receptor type-1 Kinase Inhibitor (inhibitor 1) was administered to ICR mouse embryos at 10.25 dpc by intra-cardiac injection, and embryos were then cultured in a whole embryo culture system. Left and right bars show control (DMSO injection) and sample (inhibitor injection) responses, respectively. Expression of *Vtn*, *Fn1* and *Tnc* genes was slightly down-regulated after 6 h and then more significantly so at 12 h of whole embryo culture compared to controls. (B) TGF-beta receptor type-1 Kinase Inhibitor II (inhibitor 2) was administered as in (A). Expression of *Vtn*, *Fn1* and *Tnc* was slightly and then significantly down-regulated relative to controls after 6 and 12 h of whole embryo culture, respectively. (C) Anti-TGF-beta-1 blocking antibody was administered as in (A). Left and right bars show control (Isotype IgG injection) and sample (blocking antibody injection), respectively. Expression of *Vtn*, *Fn1* and *Tnc* was down-regulated after 12 h of whole embryo culture. (D) Hepatoblasts expressing DLK-1 were sorted from 12.5 dpc fetal liver by flow cytometry and 10,000 cells were cultured with or without TGF-beta-1 (10 ng/mL). *Vtn*, *Fn1* and *Tnc* expression was up-regulated after 6 h of *in vitro* culture with TGF-beta-1.

hepatoblasts promotes HSC expansion and differentiation (Lodish et al., 2010; Sugiyama et al., 2011). ECMs act together with cytokines to maximize cytokine signaling in specific tissues (Hynes and Yamada, 1982; Humphries et al., 1989; Frisch and Ruoslahti, 1997; Taipale and Keski-Oja, 1997).

Based on integrin expression patterns, we hypothesized that their binding partners were ECM proteins. Following comparison of *integrin-alpha* in each hematopoietic cell fraction, we found that *Itga4* and *Itga6* are highly expressed in HSC and BFU-E fractions. It is reported that Integrin alpha-4 accelerates erythroid cell differentiation but does not affect HSC colonization in fetal liver (Yanai et al., 1994; Arroyo et al., 1999). Our data suggest that regulation of erythroid cell differentiation through Integrin alpha-4 occurs primarily at the level of HSCs and BFU-E in fetal liver. We found that *Itga5*, *Itga9* and *ItgaV* were moderately expressed in HSC and BFU-E fractions. Since hematopoietic cells derived from *Itga5*, *Itga9* or *ItgaV* single knockout mice can colonize fetal liver, it is likely that only Integrin beta-1 is specifically required for hematopoietic cell colonization of fetal liver and that various Integrin alpha can substitute for each other to some extent (Taverna et al., 1998; Bader et al., 1998; Huang et al., 2000). In fetal liver, *Vtn* was highly expressed among several ECM genes at both 12.5 and 14.5 dpc, and Vitronectin protein was detected at high levels in hepatoblasts at 12.5 dpc. *Vtn*-deficient mouse embryos reportedly show no abnormalities (Zheng et al., 1995), suggesting that other ECM factors compensate for its loss. On the other hand, *fibronectin*-deficient mice die early in embryogenesis probably due to abnormal mesoderm development (George et al., 1993). Fibronectin binds to Integrins alpha-4/beta-1, alpha-5/beta-1 and alpha-V/beta-1. These results and our gene expression data suggest that Integrin alpha-4/beta-1 and Fibronectin signaling is likely important for HSC differentiation.

Among cells comprising the fetal liver, hepatoblasts predominantly produce ECM factors. Here, we evaluated mouse embryos lacking $Map2k4^{-/-}$, the gene encoding the dual specificity mitogen-activated protein kinase kinase 4 protein. These mice reportedly lack fetal liver hepatoblasts (Nishina et al., 1997a, 1997b, 1999; Watanabe et al., 2002). Morphological examination indicated that $Map2k4^{-/-}$ mutant embryos developed normally except for their liver, which exhibited an unusual pouch-like morphology and contained hematopoietic cells. The hematopoietic activity of these cells is reportedly normal (Nishina et al., 1999). Although it is formally possible that MAPK signaling could directly impact ECM factor expression, our findings overall strongly suggest that down-regulation of Fibronectin and Vitronectin production is due to loss of hepatoblasts.

We also show that TGF-beta-1/TGFR-2 signaling functions in ECM production by fetal liver cells, predominantly hepatoblasts. TGF-beta-1/TGFR-2 signaling regulates diverse processes such as cell growth, differentiation, and apoptosis in adult animals and in embryogenesis (Shi and Massague, 2003; Watabe et al., 2003; Moustakas and Heldin, 2009). We found that sinusoid endothelial cells, hematopoietic cells and hepatoblasts express TGF-beta-1 mRNA and protein. Flow cytometry analysis showed that TGFR-2 was expressed in 1.8% of hematopoietic cells, 56.2% of hepatoblasts and 99% of sinusoid endothelial cells, suggesting that hepatoblasts and sinusoid endothelial cells are affected by TGF-beta-1/TGFR-2 signaling to a greater extent than hematopoietic cells. The observation that a high proportion of sinusoid endothelial cells expresses TGFR-2 is compatible with previous reports that TGF-beta-1/TGFR-2 signaling controls sinusoid endothelial cell differentiation in addition to normal angiogenesis (Shi and Massague, 2003; Watabe et al., 2003; Moustakas and Heldin, 2009; Yoshida et al., 2007). Interestingly, half of all hepatoblasts expressed TGFR-2, suggesting a functional separation of hepatoblasts based on TGFR-2 expression. Although expression levels of *Tgfb2 and Tgfb3* are lower than that of *Tgfb1* in fetal liver at both 12.5 and 14.5 dpc (Fig. 4A), we cannot exclude the possibility that these factors function in ECM production by hepatoblasts, since *Tgfb1*-deficient mouse embryos exhibit no gross embryonic abnormalities, suggesting a compensatory mechanism (Shull et al., 1992).

Taken together with data showing that TGF-beta-1 up-regulates *Vtn*, *Fn1* and *Tnc* expression *in vitro*, TGF-beta-1/TGFR-2 signaling likely regulates ECM-production in fetal liver hepatoblasts *in vivo*, facilitating interactions with Integrins. In embryos injected with either TGF-beta receptor type-1 Kinase Inhibitor or TGF-beta receptor type-1 Kinase Inhibitor II, the number of fetal liver cells was significantly decreased after 12 h of whole embryo culture (Fig. S5). It is also possible that treatment with TGF-beta-1 inhibitors may alter cell proliferation. That possibility will be investigated in future studies.

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Author contributions

D. S. designed and performed the research, analyzed data and wrote the paper. K. K. and C. M. performed research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mod.2012.09.003.

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stem cell research&therapy

REVIEW

Zebrafish erythropoiesis and the utility of fish as models of anemia

Kasem Kulkeaw and Daisuke Sugiyama* **{AU Query: are the following author initials correct for inclusion in the PubMed Database: 'Kulkeaw K, Sugiyama D'?}**

Abstract

Erythrocytes contain oxygen-carrying hemoglobin to all body cells. Impairments in the generation of erythrocytes, a process known as erythropoiesis, or in hemoglobin synthesis alter cell function because of decreased oxygen supply and lead to anemic diseases. Thus, understanding how erythropoiesis is regulated during embryogenesis and adulthood is important to develop novel therapies for anemia. The zebrafish, *Danio rerio*, provides a powerful model for such study. Their small size and the ability to generate a large number of embryos enable large-scale analysis, and their transparency facilitates the visualization of erythroid cell migration. Importantly, the high conservation of hematopoietic genes among vertebrates and the ability to successfully transplant hematopoietic cells into fish have enabled the establishment of models of human anemic diseases in fish. In this review, we summarize the current progress in our understanding of erythropoiesis on the basis of zebrafish studies and highlight fish models of human anemias. These analyses could enable the discovery of novel drugs as future therapies.

Introduction

Red blood cells, or erythrocytes, carry hemoglobin to supply oxygen to all tissues and organs. Approximately 2×10^{13} erythrocytes circulate throughout the whole body. In humans, more than 10^{11} new erythrocytes are generated daily from bone marrow (BM) through a process known as erythropoiesis [1]. In the BM, the hierarchy of erythropoiesis is topped by hematopoietic stem cells (HSCs), which first differentiate into common myeloid and common lymphoid progenitors. Common lymphoid and myeloid progenitors give rise to the adaptive and innate immune systems, respectively. Common myeloid progenitors differentiate into megakaryocyte/erythroid progenitors and granulocyte/monocyte progenitors. At the same time, common lymphoid progenitors differentiate into B lymphocytes, T lymphocytes, and natural killer cells. Megakaryocyte/erythroid progenitors later give rise to erythrocytes or thrombocytes (platelets), whereas granulocyte/monocyte progenitors give rise to granulocytes (neutrophils, eosinophils, and basophils), monocytes, and dendritic cells (Figure 1). Erythrocytes synthesize hemoglobin, which is composed of two β-globin subunits and two α-globin subunits that interact with an iron-containing heme moiety. Intrinsic transcription factors and extrinsic signaling molecules coordinately regulate erythroid differentiation and hemoglobin synthesis. Impaired erythrocyte production or hemoglobin synthesis results in anemia and decreases the oxygen supply throughout the body, a condition known as hypoxia. Much of what we know about human erythropoiesis and anemic diseases comes from studies using animal models such as *Xenopus*, zebrafish, chicks, and mice [2-5].

Zebrafish (*Danio rerio*) is a teleost freshwater fish widely distributed throughout tropical and subtropical areas of South Asia, including India, Nepal, Bangladesh, and Northern Burma [6]. Zebrafish are known worldwide as models for the study of development, cell biology, physiology, and genetics. *In vivo* analyses using zebrafish have some advantages over those using **{AU Query: OK?}** mice. First and foremost, zebrafish produce large numbers of small-sized embryos, permitting drug screening and functional analysis of specific genes on a large scale. Second, zebrafish have a short life span (42 to 66 months) [7] and develop rapidly, requiring 90 days to develop into adults [7], shortening periods required for experiments. Third, zebrafish embryos are transparent and develop outside a uterus, enabling researchers to view zebrafish development and genetically manipulate embryos under a microscope. Finally, many zebrafish

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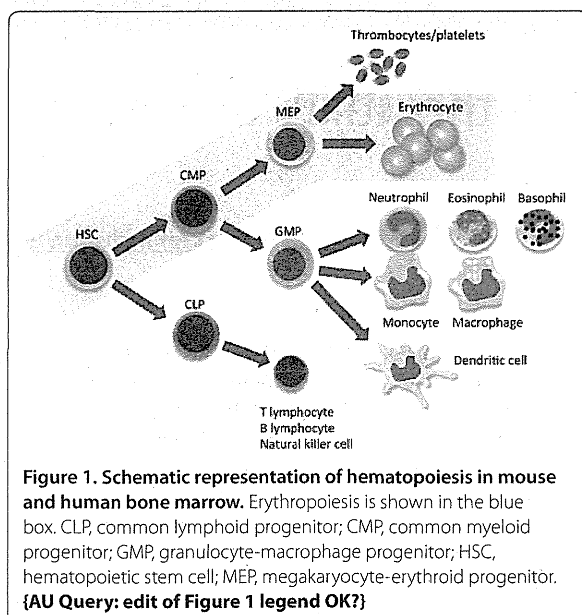

Figure 1. Schematic representation of hematopoiesis in mouse and human bone marrow. Erythropoiesis is shown in the blue box. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor. **{AU Query: edit of Figure 1 legend OK?}**

gene functions are conserved in mice and humans, enabling researchers to translate results obtained in zebrafish studies to mammalian contexts. Currently, through large-scale mutagenesis, several models of human anemic diseases have been established in zebrafish, enabling us to develop novel therapies in anemias.

1. Development of zebrafish erythropoiesis

Like the generation of other blood cell types, zebrafish erythropoiesis takes place in the mesodermal germ layer and is classified into two sequential waves: primitive and definitive. The primitive wave generates erythrocytes and macrophages during embryonic development, whereas the definitive wave produces definitive HSCs, which can differentiate into every blood cell type (namely, erythrocytes, granulocytes, lymphocytes, and platelets), and maintains homeostasis throughout the zebrafish lifetime (Figure 2). To understand how erythropoiesis develops embryonically and is maintained in the adult, we also discuss the origin of erythrocytes from HSCs.

1.1. Primitive erythropoiesis

The fertilized egg, or zygote, {AU Query: OK?} divides and forms three germ layers: ectoderm, mesoderm, and endoderm; this process is known as gastrulation. Mesoderm gives rise to muscle, notochord, hematopoietic cells, pronephros, and blood vessels. Mesoderm is divided into dorsal and ventral mesoderm. Dorsal mesoderm develops into the notochord, whereas ventral mesoderm gives rise to hematopoietic cells, the pronephros, and blood vessels. Primitive hematopoiesis intraembryonically starts in ventral mesoderm-derived tissue, known as the intermediate cell mass (ICM) (Figures 2 and 3). The ICM is located between the somites and yolk sac and consists of anterior and posterior ICM (Figure 3). At the two-somite stage, which is equivalent to 10 to 11 hours post fertilization (hpf), genes encoding transcription factors required for hematopoietic cell specification, such as T-cell acute lymphocytic leukemia 1 (*tal1*), GATA-binding protein 2a (*gata2a*), **{AU Query: per journal style, abbreviations of gene names should be in italics; please confirm whether this rule is applied correctly throughout the text}** and LIM domain only 2 (*lmo2*), and vasculogenesis, such as ets variant gene 2 (*etv2*), are co-expressed in both the anterior and posterior ICM, implying the existence of a common ancestor of hematopoietic and endothelial cells, known as the hemangioblast [8-10] (Figure 4). These genes are highly conserved among vertebrates [10-13]. Among proteins encoded by these genes, tal1, previously known as stem cell leukemia (scl), is a basic helix-loop-helix transcription factor required for both primitive and definitive hematopoiesis as well as endothelial cell differentiation, whereas gata2a is a zinc finger transcription factor functioning in proliferation and maintenance of hematopoietic progenitor cells (HPCs). Zebrafish lmo2 is an LIM domain transcription factor that interacts with tal1 and gata2a, forming a DNA-binding complex, which activates the transcription of both hematopoietic and endothelial genes [10]. Zebrafish etv2, previously known as ets1-related protein (etsrp), is an E-twenty six (ets) domain-containing factor that activates transcription of endothelial-specific genes essential for vasculogenesis [11]. The posterior ICM expresses GATA-binding protein 1a (*gata1a*), which encodes an erythroid-specific transcription factor, and spleen focus-forming virus (*SFFV*) proviral integration oncogene spi1 (*spi1*), which encodes a myeloid-specific transcription factor, whereas the anterior ICM expresses only *spi1* [12,13]. These studies suggest that the ICM has been committed to erythroid and myeloid lineage. Between 12 and 24 hpf, the anterior ICM develops into myeloid cells (macrophages), whereas the posterior ICM develops primarily into erythroid and some myeloid cells (Figure 3).

At 20 to 24 hpf, primitive erythroid cells (proerythroblasts and erythroblasts) expressing *gata1a* are present in the posterior ICM [14] (Figure 2). Similar to the mammalian yolk sac, primitive erythroid cells are surrounded by endothelial cells [15]. After the onset of blood circulation at 24 hpf, *gata1a*$^+$ primitive erythroid cells migrate throughout the embryo and differentiate into mature erythrocytes expressing aminolevulinate, delta-, synthetase 2 (*alas2*), which is an enzyme required for heme synthesis, and embryonic globin genes [16]. Unlike

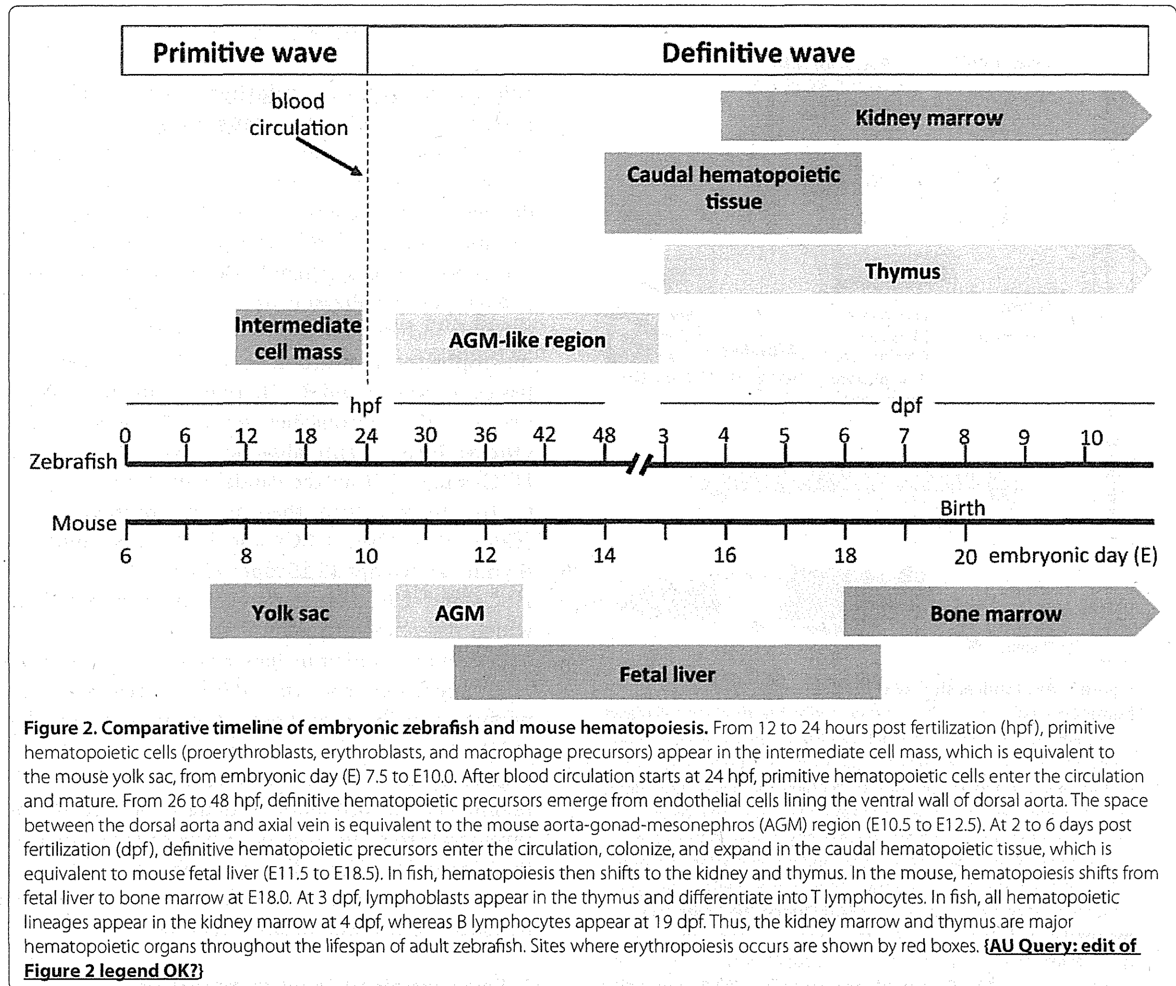

Figure 2. Comparative timeline of embryonic zebrafish and mouse hematopoiesis. From 12 to 24 hours post fertilization (hpf), primitive hematopoietic cells (proerythroblasts, erythroblasts, and macrophage precursors) appear in the intermediate cell mass, which is equivalent to the mouse yolk sac, from embryonic day (E) 7.5 to E10.0. After blood circulation starts at 24 hpf, primitive hematopoietic cells enter the circulation and mature. From 26 to 48 hpf, definitive hematopoietic precursors emerge from endothelial cells lining the ventral wall of dorsal aorta. The space between the dorsal aorta and axial vein is equivalent to the mouse aorta-gonad-mesonephros (AGM) region (E10.5 to E12.5). At 2 to 6 days post fertilization (dpf), definitive hematopoietic precursors enter the circulation, colonize, and expand in the caudal hematopoietic tissue, which is equivalent to mouse fetal liver (E11.5 to E18.5). In fish, hematopoiesis then shifts to the kidney and thymus. In the mouse, hematopoiesis shifts from fetal liver to bone marrow at E18.0. At 3 dpf, lymphoblasts appear in the thymus and differentiate into T lymphocytes. In fish, all hematopoietic lineages appear in the kidney marrow at 4 dpf, whereas B lymphocytes appear at 19 dpf. Thus, the kidney marrow and thymus are major hematopoietic organs throughout the lifespan of adult zebrafish. Sites where erythropoiesis occurs are shown by red boxes. **{AU Query: edit of Figure 2 legend OK?}**

similar cells in humans and mice, zebrafish erythrocytes are nucleated and oval in shape (Figure 4). Primitive erythropoiesis accounts for all circulating erythrocytes for the first 4 days after fertilization [15].

In zebrafish, primitive erythropoiesis is regulated intrinsically by transcription factors expressed in erythroid cells and extrinsically by erythropoietin secreted from surrounding cells and tissues. Zebrafish gata1a, a zinc finger transcription factor, activates the expression of erythroid-specific genes functioning in hemoglobin synthesis, iron utilization, and cell membrane stabilization. Gata1a also suppresses the expression of myeloid-specific genes [14]. As in the mouse, a nonsense mutation in the gene encoding zebrafish gata1a results in a lack of circulating erythrocytes at 26 hpf [17], suggesting a conservation of gata1a function among vertebrates. Unlike in the mouse, zebrafish primitive erythropoiesis depends on the extrinsic factor erythropoietin (epo). *Epo* and *epo* receptor mRNA is detected in the ICM during 16 to 24 hpf. Knockdown of *epo receptor* impairs primitive erythropoiesis in fish [18].

1.2. Definitive erythropoiesis

In mice, definitive HSCs are defined as having the ability to reconstitute all blood cell types in lethally irradiated adult mice. Definitive HSCs are first detected in the aorta-gonad-mesonephros (AGM) region [19] and umbilical vessels [20] at mouse embryonic day 10 or 11. Definitive HSCs enter the blood circulation and colonize fetal liver, the first site of definitive hematopoiesis, where they expand and differentiate into erythroid and myeloid cells.

In zebrafish, an AGM-like region exists along the trunk in the space between the dorsal aorta and the underlying axial vein [21-24] (Figure 3). Cell-tracking and time-lapse imaging analyses indicate that HSCs and HPCs originate

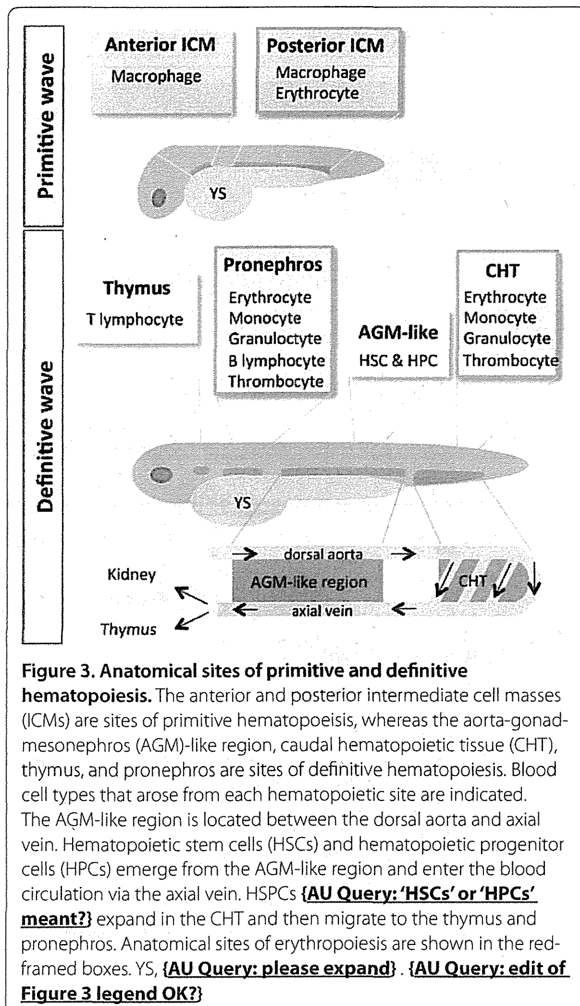

Figure 3. Anatomical sites of primitive and definitive hematopoiesis. The anterior and posterior intermediate cell masses (ICMs) are sites of primitive hematopoeisis, whereas the aorta-gonad-mesonephros (AGM)-like region, caudal hematopoietic tissue (CHT), thymus, and pronephros are sites of definitive hematopoiesis. Blood cell types that arose from each hematopoietic site are indicated. The AGM-like region is located between the dorsal aorta and axial vein. Hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) emerge from the AGM-like region and enter the blood circulation via the axial vein. HSPCs **{AU Query: 'HSCs' or 'HPCs' meant?}** expand in the CHT and then migrate to the thymus and pronephros. Anatomical sites of erythropoiesis are shown in the red-framed boxes. YS, **{AU Query: please expand}**. **{AU Query: edit of Figure 3 legend OK?}**

directly from the ventral wall of dorsal aorta (VDA) at 26 hpf (Figure 2) [24,25]. The morphology of endothelial cells lining the VDA changes from a long flat shape to a round shape, and the cells egress into the subaortic space and enter the blood circulation via the axial vein (Figure 3), a process called endothelial-to-hematopoietic transition [24]. **{AU Query: edit of previous two sentences OK?}** As in mammals, runt-related transcription factor 1 (runx1) is critical for the emergence of hematopoietic cells from endothelial cells [26]. At 24 hpf, *runx1*-expressing HSCs and HPCs are observed in the VDA [27]. Later, at 26 hpf, dorsal aorta-derived *runx1*$^+$ HSCs and HPCs start to express the transcription factor *cmyb* [28], which is required for HSC migration and differentiation but not for the endothelial-to-hematopoietic **{AU Query: OK?}** transition in zebrafish [29]. Later, *cmyb*$^+$ HSCs and HPCs express integrin alpha 2b (*itga2b*), also known as *CD41* [30]. As in the mouse, CD41 is the earliest HSC and HPC surface marker seen in endothelial cells lining on **{AU Query: is 'endothelial cell lining on' or 'endothelial cells lining' meant instead of 'endothelial cells lining on'?}** the dorsal aorta. Dorsal aorta-derived CD41$^+$ HSCs and HPCs enter the blood circulation via the axial vein rather than the dorsal aorta and colonize caudal hematopoietic tissue by 48 hpf (Figure 3) [30]. This tissue, also known as the caudal vein plexus, is highly vascularized. From 48 hpf to 7 days post-fertilization (dpf), *cmyb*$^+$ HSCs and HPCs expand and differentiate into erythrocytes, monocyte/macrophages, and thrombocytes in caudal hematopoietic tissue (Figures 2 and 3). Therefore, caudal hematopoietic tissue exhibits properties similar to those of the **{AU Query: OK?}** mammalian fetal liver. Next, HSCs and HPCs migrate from the caudal hematopoietic tissue first to the thymus and then to the pronephros [21-23] (Figure 2). CD41$^+$ HSCs and HPCs first appear in the thymus at 54 hpf (2.25 dpf) [23], and by 4 dpf, *cmyb*$^+$ HSCs and HPCs appear in the pronephros (Figure 2), which later develops into the kidney and functions equivalently to mammalian BM [21,22]. Two routes of HSC migration from the AGM-like region to the pronephros have been proposed: the first to caudal hematopoietic tissue and pronephros via the circulation (Figure 3) and the second directly from the AGM-like region via the pronephric tubules [30]. At 4 dpf, only myeloerythroid lineages have progressively expanded in the kidney [15]. By 7 dpf, erythroblasts are found in the kidney [15], where they later become the major definitive hematopoietic organ of adult zebrafish.

2. Erythropoiesis in adult zebrafish

In mammals, adult erythropoiesis is maintained primarily in the BM. HSCs differentiate into erythroid progenitor cells and later erythroblasts. Subsequently, erythroblasts undergo terminal differentiation into mature erythrocytes. Mature erythrocytes are spherical and biconcave with a typical size of 7 to 8 μm. As in embryos, cell-extrinsic cues, such as erythropoietin, and cell-intrinsic cues, such as erythroid-specific transcription factors Gata1 and Kruppel-like factor 1 (Klf1), coordinately regulate erythropoiesis in the BM [31].

Unlike in mammals, zebrafish erythropoiesis is maintained in the interstitium of the anterior and posterior kidney [2]. In adult kidney marrow, common myeloid progenitors differentiate into megakaryocyte/erythroid progenitors expressing the transcription factor gata1a, an ortholog of mouse Gata1. The megakaryocyte/erythroid progenitors differentiate into erythroblasts. Erythroblasts later differentiate into mature erythrocytes, which are elliptical and nucleated cells with a typical size of 7×10 μm [32].

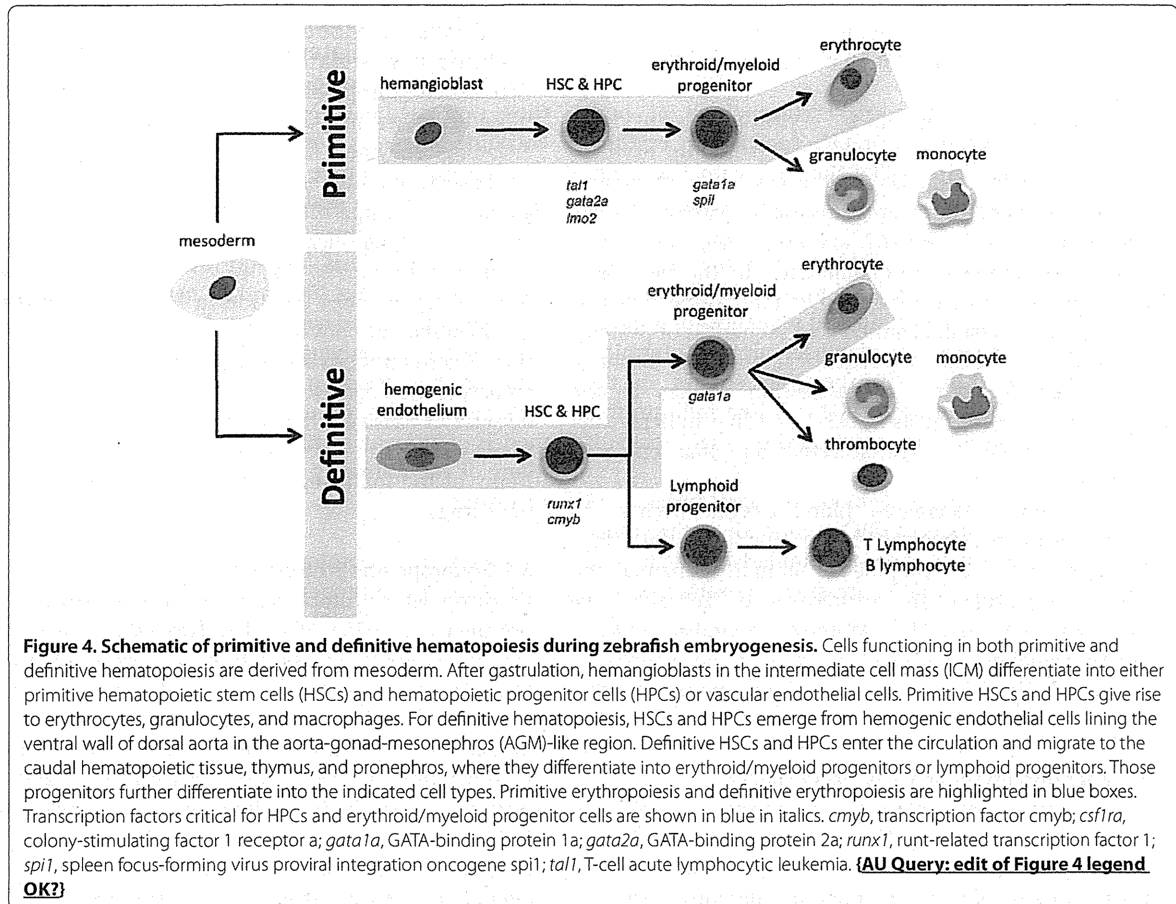

Figure 4. Schematic of primitive and definitive hematopoiesis during zebrafish embryogenesis. Cells functioning in both primitive and definitive hematopoiesis are derived from mesoderm. After gastrulation, hemangioblasts in the intermediate cell mass (ICM) differentiate into either primitive hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) or vascular endothelial cells. Primitive HSCs and HPCs give rise to erythrocytes, granulocytes, and macrophages. For definitive hematopoiesis, HSCs and HPCs emerge from hemogenic endothelial cells lining the ventral wall of dorsal aorta in the aorta-gonad-mesonephros (AGM)-like region. Definitive HSCs and HPCs enter the circulation and migrate to the caudal hematopoietic tissue, thymus, and pronephros, where they differentiate into erythroid/myeloid progenitors or lymphoid progenitors. Those progenitors further differentiate into the indicated cell types. Primitive erythropoiesis and definitive erythropoiesis are highlighted in blue boxes. Transcription factors critical for HPCs and erythroid/myeloid progenitor cells are shown in blue in italics. *cmyb*, transcription factor cmyb; *csf1ra*, colony-stimulating factor 1 receptor a; *gata1a*, GATA-binding protein 1a; *gata2a*, GATA-binding protein 2a; *runx1*, runt-related transcription factor 1; *spi1*, spleen focus-forming virus proviral integration oncogene spi1; *tal1*, T-cell acute lymphocytic leukemia. **{AU Query: edit of Figure 4 legend OK?}**

Although kidney marrow erythrocytes can be fractionated from blood cell mixtures by flow cytometry based on forward and side scatter (which reveal cell size and granularity, respectively), their surface markers have not been fully identified [33]. Although some potentially useful antibodies cross-react among species, it remains a challenge to analyze erythroid cells by using antibody-based techniques, owing to a shortage of reagents.

As in mammals, zebrafish erythrocytes contain hemoglobin. Human and mouse α-globin and β-globin genes are located on separate chromosomes and arranged in order of embryonic and adult expression. The change from embryonic to adult globin expression is known as globin switching [34]. By contrast, zebrafish α-globin and β-globin genes are located on the same chromomsome and found in embryonic and adult clusters separated by non-coding genomic DNA [35]. The embryonic cluster consists of hemoglobin alpha embryonic-1 (*hbae1*) and hemoglobin beta embryonic-1.1 (*hbbe1.1*), whereas the adult cluster contains hemoglobin alpha adult-1 (*hbaa1*) and beta adult-1 globin (*ba1*). Moreover, in the adult cluster, globin genes are oriented in a head-to-head pattern: 3'-5' in the case of *α-globin* and 5'-3' in the case of *β-globin* genes. Thus, they are transcribed in the opposite direction [36]. High-performance liquid chromatography analysis shows that adult zebrafish erythrocytes in peripheral blood contain three major α-globin and two β-globin proteins [36]. As in mammals, globin switching also occurs during zebrafish development at a stage 10 dpf **{AU Query: OK?}** [36]. As in embryos, adult zebrafish erythropoiesis is regulated by extrinsic and intrinsic cues.

2.1. Zebrafish erythropoietin

Erythropoietin (Epo) is a glycoprotein crucial for survival and proliferation of erythroid progenitor cells. In mammals, there is only one Epo gene and its protein **{AU Query: insert 'is' here?}** primarily produced from kidney and BM [37]. Binding of EPO to its receptor activates Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5) signaling pathway, which upregulates anti-apoptotic genes and promotes cell survival [31].

Mammalian EPO is not required for primitive erythropoiesis but is indispensable for definitive erythropoiesis [38,39].

Unlike the case in mice, erythropoiesis of both primitive and definitive zebrafish depends on erythropoietin signaling [18]. Unlike the case with the mammalian *Epo* gene, there are three splice variants of *epo* gene in fish: *epo-L1*, *epo-L2*, and *epo-S* [40]. *epo-L1* and *epo-L2* are expressed predominantly in the heart and liver, whereas *epo-S* is expressed in adult kidney marrow [40,41]. The C-terminal amino acid sequences of proteins encoded by these genes are identical, but the N-terminal signal peptides differ. Misexpression studies in the monkey kidney fibroblast COS-1 cell line indicate that epo-L1 and epo-L2 are secreted but that epo-S is cytosolic [40].

In mammals, decreased blood oxygen because of anemia or hypoxia induces EPO production in the kidney and accelerates erythropoiesis. Similarly, anemia and hypoxia upregulate the expression of zebrafish *epo* mRNA in the heart [18]. Moreover, zebrafish erythropoietin signaling requires stat5.1 protein, an ortholog of human STAT5 [18]. These observations demonstrate that epo/epor function is highly conserved among vertebrates.

2.2. Zebrafish erythroid transcription factors

In the mouse, Gata1 regulates transcription of erythropoietic genes, including Klf1. Gata1 knockout mice die during gestation because of severe anemia [42]. Like Gata1, mouse Klf1 is essential for definitive erythropoiesis in fetal liver [43]. Klf1 reportedly regulates the expression of several erythroid-specific genes encoding (a) globin, (b) enzymes for heme biosynthesis, and (c) erythroid membrane and cytoskeletal proteins [44].

Little is known about the role of gata1a in adult erythropoiesis because of the lethal phenotype of null mutants at an early stage of development [17]. In addition, no functional ortholog of mouse Klf1 has been identified in zebrafish. Although the zebrafish *klfd* gene is expressed in both primitive and definitive erythropoietic organs [45] and the amino acid sequence of zebrafish klfd is similar to that of mouse Klf1 [45], there is currently no direct evidence that klfd functions in definitive erythropoiesis. Zebrafish *klf4* knockdown using antisense morpholino oligonucleotides downregulates the expression of embryonic *β-globin* and genes involved in heme biosynthesis, but no defect is seen in definitive erythropoiesis [46]. Therefore, it is unlikely that klf4 is the ortholog of mouse Klf1. Thus, owing to the lack of a model, the roles of gata1a and klfd in adult erythropoiesis have not been clarified. These outcomes prompted us to establish transient and reversible downregulation of both transcription factors in adult zebrafish, as discussed in section 3.8.

3. Zebrafish as models of human erythropoiesis-related diseases

Zebrafish erythropoietic genes are functionally similar to those expressed in mice and humans and include genes encoding enzymes for heme biosynthesis, structural erythrocyte membrane proteins, epo/epo receptor, and globin [47]. Large-scale mutagenesis of zebrafish has enabled the identification of genes regulating hematopoiesis/erythropoiesis [32,48]. The human homologs of some of these genes function in hematological diseases [3]. Zebrafish are advantageous for evaluating the function of genes underlying erythropoietic disease since fish embryos are resistant to severe anemic conditions because of passive diffusion of oxygen into the fish. Fish models of human anemias (Table 1) are described below. Many of the following mutants could be useful to test new drugs.

3.1. Erythropoietic protoporphyria

Erythropoietic protoporphyria occurs worldwide, has a prevalence of 1:75,000 in The Netherlands [49], and is caused by ferrochelatase deficiency. Ferrochelatase catalyzes the formation of heme by transferring iron to protoporphyrin, a heme intermediate. Mutations in the human ferrochelatase gene promote protoporphyrin accumulation in the skin, erythrocytes, and liver, resulting in sensitivity to light exposed to **{AU Query: OK?}** the skin or even erythrocytes and skin burning and itching [49]. Among patients, 20% to 60% also exhibit anemia due to decreased heme synthesis and light-dependent erythrocyte lysis. Some patients (1% to 4%) show liver disease due to the accumulation of free protoporphyrin released from lysed erythrocytes [49]. Although the avoidance of sun exposure and treatment with light-protective substances such as β-carotene and melanin in skin can ameliorate symptoms, no curative treatment is yet available [49].

The zebrafish mutant *dracula* exhibits a point mutation in the ferrochelatase gene, creating an in-frame stop codon and expression of a dysfunctional enzyme. *Dracula* fish manifest autofluorescent erythrocytes, light-dependent hemolysis, and liver malfunction, similar to conditions seen in humans [50]. Owing to the transparency of fish embryos and protoporphyrin autofluorescence, protoporphyrin accumulation can be monitored microscopically in various organs of an intact fish, an analysis impossible in humans and mice, making *dracula* mutants a suitable model for human erythropoietic protoporphyria.

3.2. Hemolytic anemia

Abnormality of erythroid cell membrane leads to massive erythrocyte destruction in the spleen, a condition **{AU Query: OK?}** known as hemolytic anemia. Human hereditary elliptocytosis is characterized by elliptical

Table 1. Zebrafish mutant strains relevant to human erythropoiesis-related diseases

Human disease	Mutant	Gene	Function	Reference
Erythropoietic protoporphyria	*dracula*	Ferrochelatase	Enzyme functioning in heme synthesis	[50]
Hemolytic anemia	*merlot*	Erythrocyte membrane protein 4.1R	Structural protein of erythrocyte membrane	[54]
			Stabilizes β-spectrin/actin interaction	
Congenital dyserythropoietic anemia type II (HEMPAS)	*retsina*	Solute carrier family 4 (anion exchanger 1a or band 3)	Chloride exchanger	[56]
Hereditary spherocytosis	*riesling*	β-spectrin	Structural protein of erythrocyte membrane	[59]
Congenital sideroblastic anemia	*sauternes*	Aminolevulinate synthetase 2 (alas2)	Enzyme functioning in heme synthesis	[63]
Hypochromic anemia (resembles thalassemia)	*zinfandel*	Globin	Oxygen transport	[35]
Type IV hemochromatosis	*weissherbst*	Ferroportin 1	Iron transport	[65]

HEMPAS, **{AU Query: please expand}**. **{AU Query: edit of Table 1 OK?}**

erythrocytes, in which abnormal cell membranes lead to hemolytic anemia. Human hereditary elliptocytosis occurs worldwide but is prevalent in West Africa [51]. Often patients show no symptoms, and only 10% have mild to severe anemia [51]. In human hereditary elliptocytosis, many patients harbor point mutations in the gene encoding protein 4.1R, a major component of the erythrocyte cytoskeleton that maintains biconcave morphology. These mutations promote decreased protein expression or impair protein interaction with other cytoskeletal proteins [52]. Owing to massive hemolysis, patients with hereditary elliptocytosis have complications such as cardiomegaly, splenomegaly, and gallstones. Only supportive treatments, such as folate therapy, blood transfusion, splenectomy, and gallstone removal, are currently available [51,53]. Although HSC transplantation is one curative therapy, novel drugs are needed to antagonize hemolysis.

The zebrafish *merlot* mutant exhibits severe hemolytic anemia due to mutation in the gene encoding 4.1R protein. Unlike mammalian erythrocytes, wild-type mature zebrafish erythrocytes exhibit both spherical and elliptical morphologies. *Merlot* mutants show spiculated erythrocyte membranes, resulting in hemolytic anemia and conditions such as cardiomegaly and splenomegaly, phenotypes similar to those seen in humans [54].

3.3. Congenital dyserythropoietic anemia type II

Human congenital dyserythropoietic anemia type II is an erythroid-specific abnormality in cell division, leading to multinuclear erythroblasts, erythroblast apoptosis (dyserythropoiesis), and anemia. The condition varies from mild to severe: approximately 15% of patients require blood transfusions during infancy and early childhood but not thereafter [55]. Splenomegaly occurs in 50% to 60% of patients, and gallstones are frequently observed. In the BM, 10% to 45% of erythroblasts are bi- and **{AU Query: is 'or' meant instead of 'and'?}** multi-nucleated [55]. Dyserythropoiesis is caused by a mutation in the anion exchanger protein band 3, which is present in the human erythrocyte membrane [55]. The zebrafish *retsina* mutant exhibits erythroid-specific defects in cell division because of mutation in the fish ortholog of the band 3 gene. These defects resemble those of **{AU Query: OK?}** the human disease [56]. Severely affected anemic patients require blood transfusion and HSC transplantation [57].

3.4. Hereditary spherocytosis

Human hereditary spherocytosis is a hemolytic anemia common in Caucasians and has a prevalence of 1:2,000 to 1:5,000 [53]. Hereditary spherocytosis is characterized by abnormal erythrocyte morphology. Normally, the shape of human erythrocytes is a biconcave disk. In hereditary spherocytosis, the erythrocytes exhibit a spherical shape, leading to their massive destruction in the spleen. Mutation in the gene encoding the cytoskeletal protein spectrin has been identified as a **{AU Query: is 'the' meant instead of 'a'?}** cause of human hereditary spherocytosis. Erythroid spectrin stabilizes membrane bilayers [58]. Anemia ranging from mild (blood transfusion-independent) to severe (blood transfusion-dependent) is the main clinical feature of this condition. Patients also exhibit hyperbilirubinemia, causing jaundice, and splenomegaly. The zebrafish *riesling* carries a mutant *β-spectrin* gene and exhibits anemia due to erythrocyte hemolysis, similar to conditions seen in humans [59]. Zebrafish β-spectrin shares 62.3% identity with the human ortholog. In addition to **{AU Query: word missing here? ('undergoing' or 'exhibiting'?)}** abnormal cell morphology-induced hemolysis, zebrafish *riesling* erythrocytes undergo apoptosis, which had not been observed in human hereditary spherocytosis. Thus, analysis of the zebrafish *riesling* mutant has revealed a novel mechanism of erythrocyte hemolysis [59].

3.5. Congenital sideroblastic anemia

Human congenital sideroblastic anemia is characterized by iron deposition in mitochondria of erythroblasts in the BM and is caused by a mutation in the gene encoding δ-aminolevulinate synthase, or ALAS2, which catalyzes the first step of heme biosynthesis. A lack of heme promotes increases in free iron levels and subsequent iron deposition in erythroblast mitochondria, causing insufficient production of mature erythrocytes [60]. Generally, patients have symptoms of anemia, such as skin paleness, fatigue, dizziness, and enlargement of the spleen and liver. In addition to a decrease of mature erythrocytes, hypochromic microcytic erythrocytes are observed in the patients. Heme reportedly promotes gene expression of *β-globin* through binding Batch1, a transcriptional repressor of *β-globin* gene [61,62]. Therefore, decreased intracellular heme because of mutated ALAS2 may contribute to hypochromic microcytic anemia. Mutation in the zebrafish gene *sauternes*, which encodes alas2 protein, results in a condition similar to hypochromic microcytic anemia in humans [63]. *Sauternes* mutant embryos show delayed erythrocyte maturation and decreased β-globin expression. These mutants represent the first animal model to allow the investigation of mechanisms underlying heme-induced globin synthesis.

3.6. Hypochromic anemia

Hypochromic anemia is a general term for anemia in which erythrocytes look paler and smaller than they do normally. In humans, hypochromic anemia results from reduction in either globin synthesis (as occurs in thalassemia) or iron absorption (as occurs in iron deficiency) or from vitamin B_6 deficiency. In mammals and fish, different globin subtypes are expressed in embryonic and adult stages. In adult mammals, embryonic globin synthesis is suppressed whereas adult globin synthesis is activated [34]. Thalassemic patients show reduced adult globin synthesis and re-activate fetal globin expression, which can ameliorate anemia severity. The zebrafish *zinfandel* mutant exhibits hypochromic microcytic anemia due to defective embryonic globin production. This condition is rescued in adult fish once adult globin is produced [35].

3.7. Type IV hemochromatosis (iron overload)

Type IV hemochromatosis, or iron overload, is characterized by increased intestinal iron absorption and progressive iron deposition in various tissues, resulting in hepatic cirrhosis, arthritis, cardiomyopathy, diabetes, hypopituitarism, and **{AU Query: is 'or' meant instead of 'and'?}** hyperpigmentation [64]. Its primary cause is mutation in genes encoding membrane proteins functioning in iron transportation, such as hemochromatosis protein, the transferrin receptor, and hemojuvelin. Analysis of zebrafish *weissherbst* mutants revealed mutations in a novel iron transporter, named ferroportin 1 [65]—mutations later identified in patients with type IV hemochromatosis [66,67].

3.8. Other anemias

As noted, most zebrafish mutant lines with defects in erythropoiesis were generated by mutagenesis by using ethylnitrosourea [32,48]. These pioneering studies provided useful animal models of anemia and identified numerous genes underlying human anemias. Nonetheless, such screens take time and are expensive, prompting us to establish a novel, simpler anemic model.

It is known that temperature regulates hematopoiesis [68]. High temperature increases the number of hematopoietic cells in the BM of rats and the nine-banded armadillo (*Dasypus novemcinctus*), whereas low temperature has opposite effects [68]. However, in nature, zebrafish survive in a wide range of temperatures, from 6°C in winter to 38°C in summer [6], making them useful to investigate the effect of temperature on hematopoiesis. Previously, we established a novel reversible anemic model by keeping zebrafish at 17°C (cold zebrafish) [41]. By comparison with fish kept at a higher temperature (26.5°C), 'cold' zebrafish appear paler starting at the first week of cold exposure (Figure 5a, right panel). The kidney marrow of cold zebrafish shows abnormally structured renal tubules and erythrocyte clusters (Figure 5b, right panel). We examined the expression of genes essential for HSC maintenance, erythropoiesis, and myelopoiesis by real-time polymerase chain reaction (PCR). Interestingly, only HSC-regulated genes (*runx1*, *cmyb*, *gata2a*, and *tal1*) (data not shown) and erythropoietic genes (*gata1a*, *klfd*, *epo*, *epor*, *hbaa1*, and *ba1*) (Figure 5c) were downregulated the first week of exposure. These genes were expressed at generally lower levels by the second week, suggestive of anemia. However, expression of *colony-stimulating factor 1a*, which is required for macrophage differentiation, and of *colony-stimulating factor 3* (granulocyte), which is required for granulocyte differentiation, was unchanged [41]. These results suggest that cold exposure specifically suppresses erythropoiesis. Moreover, cold-induced anemia was reversed when fish were returned to 26.5°C conditions, implying that phenotypes are directly caused by temperature [41].

To further identify novel genes regulating erythropoiesis mediated by cold exposure, we assessed global changes in gene expression in the kidney marrow by DNA microarray (unpublished data). Although decreased temperature suppressed enzymatic activity and resulted in a global decrease in gene expression, we observed both increased and decreased gene expression in the kidney marrow of cold zebrafish, as confirmed by real-time PCR.

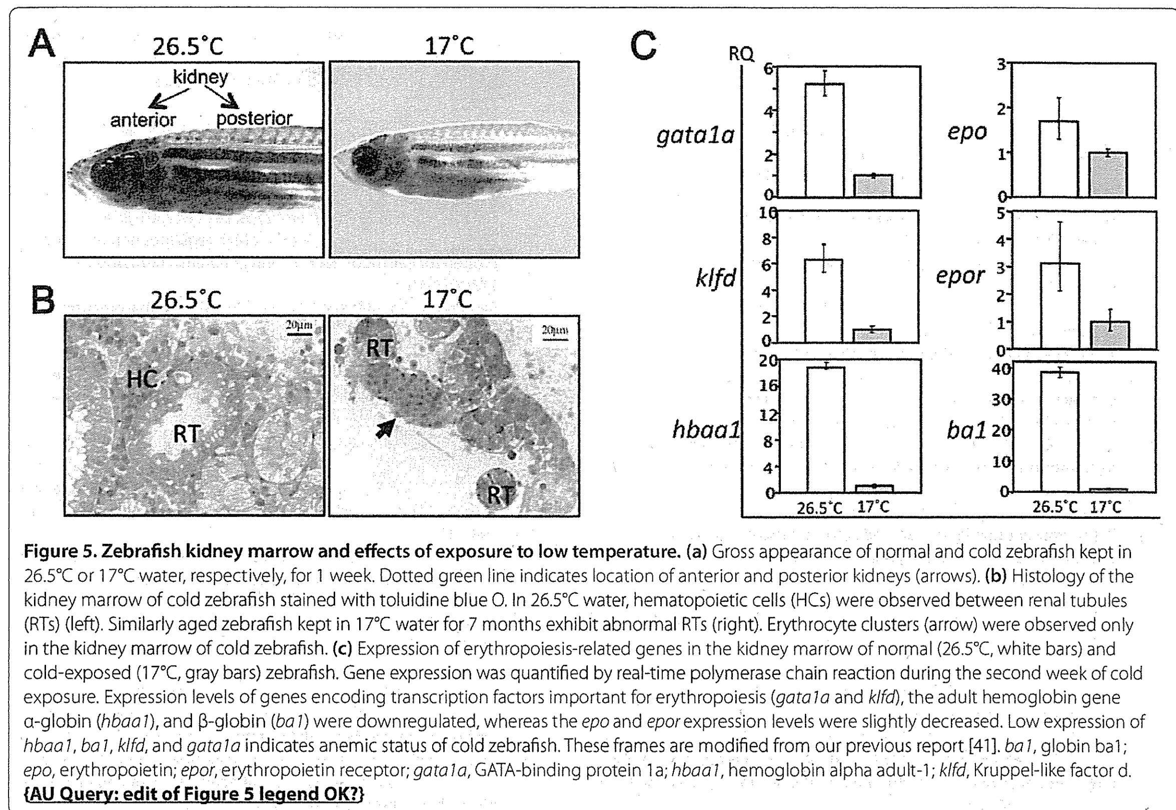

Figure 5. Zebrafish kidney marrow and effects of exposure to low temperature. (a) Gross appearance of normal and cold zebrafish kept in 26.5°C or 17°C water, respectively, for 1 week. Dotted green line indicates location of anterior and posterior kidneys (arrows). **(b)** Histology of the kidney marrow of cold zebrafish stained with toluidine blue O. In 26.5°C water, hematopoietic cells (HCs) were observed between renal tubules (RTs) (left). Similarly aged zebrafish kept in 17°C water for 7 months exhibit abnormal RTs (right). Erythrocyte clusters (arrow) were observed only in the kidney marrow of cold zebrafish. **(c)** Expression of erythropoiesis-related genes in the kidney marrow of normal (26.5°C, white bars) and cold-exposed (17°C, gray bars) zebrafish. Gene expression was quantified by real-time polymerase chain reaction during the second week of cold exposure. Expression levels of genes encoding transcription factors important for erythropoiesis (*gata1a* and *klfd*), the adult hemoglobin gene α-globin (*hbaa1*), and β-globin (*ba1*) were downregulated, whereas the *epo* and *epor* expression levels were slightly decreased. Low expression of *hbaa1*, *ba1*, *klfd*, and *gata1a* indicates anemic status of cold zebrafish. These frames are modified from our previous report [41]. *ba1*, globin ba1; *epo*, erythropoietin; *epor*, erythropoietin receptor; *gata1a*, GATA-binding protein 1a; *hbaa1*, hemoglobin alpha adult-1; *klfd*, Kruppel-like factor d. **{AU Query: edit of Figure 5 legend OK?}**

We are now undertaking loss-of-function analysis of candidate factors by using antisense morpholino oligonucleotides and small interference RNAs in fish, and we are conducting gain-of-function analysis in mouse and human cell lines.

4. Conclusions

Defects in erythropoiesis result in various anemic diseases. To gain an understanding of these diseases, it is necessary to determine how erythropoiesis is regulated in normal conditions as well as to establish *in vivo* models. We propose that, in addition to several anemic mutants, our cold zebrafish model is a useful tool to explore novel genes functioning in erythropoiesis. **{AU Query: edit of previous sentence OK?}** Given the advantages of zebrafish models, it is feasible to reach these objectives. Knowledge of the etiology and molecular mechanisms underlying these conditions will lead to the development of novel therapies.

This article is part of a thematic series on *Stem cell research in the Asia-Pacific* edited by Oscar Lee, Songtao Shi, Yufang Shi, and Ying Jin. Other articles in the series can be found online at http://stemcellres.com/series/asiapacific.

Abbreviations

AGM, aorta-gonad-mesonephros; *ba1*, globin ba1; BM, bone marrow; *cmyb*, transcription factor cmyb; dpf, days post-fertilization; epo, erythropoietin; *etv2*, ets variant gene 2; *gata1a*, GATA-binding protein 1a; *gata2a*, GATA-binding protein 2a; *hbaa1*, hemoglobin alpha adult-1; HPC, hematopoietic progenitor cell; hpf, hours post fertilization; HSC, hematopoietic stem cell; ICM, intermediate cell mass; klf, Kruppel-like factor; *lmo2*, LIM domain only 2; PCR, polymerase chain reaction; *runx1*, runt-related transcription factor 1; *spi1*, spleen focus-forming virus proviral integration oncogene spi1; stat5.1, signal transducer and the activator of transcription 5.1; *tal1*, T-cell acute lymphocytic leukemia; *VDA*, ventral wall of dorsal aorta. **{AU Query: edit of this section OK? (per journal style, this section includes only those abbreviations that are used more than once in the main text)}**

Competing interests

The authors declare that they have no competing interests.

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Mesodermal and Hematopoietic Differentiation from ES and iPS Cells

Tomoko Inoue-Yokoo · Kenzaburo Tani · Daisuke Sugiyama

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Abstract Embryonic stem (ES) and induced pluripotent stem (iPS) cells can differentiate into any type of tissue when grown in a suitable culture environment and are considered valuable tools for regenerative medicine. In the field of hematology, generation of hematopoietic stem cells (HSCs) and mature hematopoietic cells (HCs) from ES and iPS cells through mesodermal cells, the ancestors of HCs, can facilitate transplantation and transfusion therapy. Several studies report generation of functional HCs from both mouse and human ES and iPS cells. This approach will likely be applied to individual patient-derived iPS cells for regenerative medicine approaches and drug screening in the future. Here, we summarize current studies of HC-generation from ES and iPS cells.

Keywords Mesoderm induction · Hematopoietic cell differentiation · ES cell · iPS cell

Abbreviations

ES	Embryonic stem
iPS	Induced pluripotent stem
HSC	Hematopoietic stem cell
HPC	Hematopoietic progenitor cell
HC	Hematopoietic cell

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Introduction

Hematopoiesis is the process by which mature and functional hematopoietic cells (HCs), such as leukocytes (granulocytes, macrophages, lymphocytes), erythrocytes and platelets, are generated from hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) to maintain homeostasis. Hematopoiesis is controlled intrinsically via transcription factors and small RNAs [1–3] and extrinsically through growth factors and extracellular matrices secreted from niche cells surrounding HCs [4–6]. Faulty regulation of hematopoiesis leads to hematological diseases, such as anemia, leukemia and lymphoma, in which HSC transplantation and/or transfusion of erythrocytes or platelets are dependent on disease status. Regardless of the HSC source (patient cells, donor cells, or cord blood cells), transplantation is a promising therapy for some hematological diseases. However, problems remain in HSC transplantation, such as donor shortages, viral contamination and graft-versus host disease. To overcome these problems, HSC generation from other cells is a possible alternative to expansion of cord blood HSCs. Mature HCs, such as erythrocytes and platelets, are obtained primarily from donors and transfused into patients with hematological diseases and under surgical operation. Likewise, use of HSCs is also associated with problems, such as shortage of donors, viral infection and rejection. Overall, the ability to generate mature HCs from other cells would guarantee a continuous supply of cells and ensure safe and efficient transfusion therapy.

To address these issues, in vitro HC generation from other cells, particularly from embryonic stem (ES) cells [7] and induced pluripotent stem (iPS) cells [8], has been attempted. ES cells are established from the inner cell mass of a blastocyst, maintain their pluripotent and undifferentiated status in vitro and differentiate into any cell type in appropriate culture conditions. Furthermore, ES cells can have pluripotency in vivo and form teratoma in immunodeficient mice, and can be used to generate chimeric mice in vivo. To understand molecular mechanisms underlying developmental processes, ES cells are frequently utilized, since they mimic in vivo development in vitro (Fig. 1). iPS cells, on the other hand are created by ectopic expression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) in somatic cells, such as fibroblasts, hepatocytes, gastric epithelial cells, pancreatic cells, B cells and $CD34^+$ cord blood cells, and exhibit properties of pluripotent ES cells. iPS cells established from a patient's somatic cells could function as useful tools for regenerative medicine and drug screening by manipulating lineage specific differentiation in vitro (Fig. 1).

Here we summarize current studies relevant to generation of HCs and mesodermal cells from ES and iPS cells in both mice and humans (Table 1).

General Induction Methods

Several methods to differentiate mesodermal cells and hematopoietic cells from ES and iPS cells have been reported. They include (i) embryoid body (EB) formation, (ii) co-culture with feeder cells, and (iii) growth in extra cellular matrix-coated dishes. In the first method, undifferentiated ES and iPS cell colonies are separated into small cell pieces enzymatically or by physical dissection, following by EB formation in suspension in a culture dish or hanging drop. EBs are spherical cell aggregates that proliferate and differentiate into all three germ layers (i). Alternatively, ES or iPS cells can be seeded on OP9 stromal cells, which are established from the newborn calvaria of op-/op- mice and support HCs differentiation (ii), or seeded on collagen IV-coated dishes, which promote cell proliferation and form

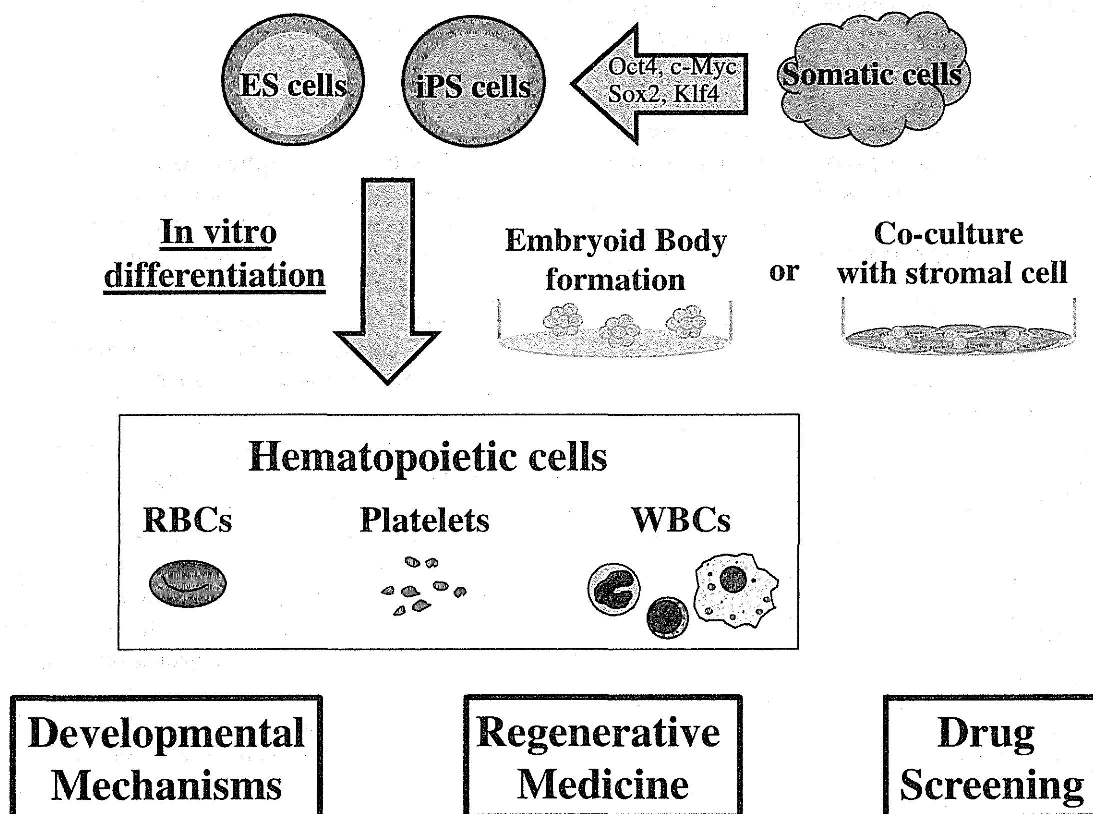


Fig. 1 Generation of hematopoietic cells from pluripotent stem cells in vitro. Embryonic stem (ES) and induced pluripotent stem (iPS) cells have been differentiated into hematopoietic cells (HCs) through mesodermal cells, ancestors of HCs, by two methods: embryoid body (EB) formation and co-culture with stromal cells in vitro. These cells are potentially applicable to regenerative medicine and drug screening by manipulating their lineage specific differentiation in vitro

Table 1 Generation of hematopoietic lineage from pluripotent stem cells

Species	Mouse		Human	
Pluripotent stem cell	ES	iPS	ES	iPS
Mesoderm	12–14	15, 16	17–20	17–20
HPC, HSC	21–23	25, 26	17, 20, 27	17, 20
Erythroid cell	28–32	25	33, 34	17-19, 35–37
Megakaryocyte	38, 39	–	41–43	44
Platelet	40	–	43	44
Macrophage	28, 43–47	48	17, 19, 35, 41	17, 19, 35
Neutrophil	49	–	18, 50, 51	52, 53
Lymphocyte	28, 54, 58, 59, 65	55, 60, 66	56, 61-63, 67	55, 67

an attachment scaffold (iii). In all cases, cell fate, directivity differentiation pattern for mesoderm and HCs differentiation is controlled by cytokines.

Mesodermal Cells

HCs are mesodermal in origin. In mammalian embryogenesis, the three germ layers, ectoderm, endoderm and mesoderm, are formed via spatiotemporal signals. Formation of the primitive streak (PS), the structure that establishes bilateral symmetry, marks the beginning of gastrulation and the emergence of mesodermal precursors. Mesoderm, derived from interaction between the endoderm and ectoderm, forms paraxial, intermediate, and lateral tissues during the mid- to late-streak stage. Among precursors in the axial, paraxial, intermediate and lateral mesoderm, blood vessels and HCs are generated from the lateral mesoderm. Therefore, the appearance of lateral mesoderm is one of indicator to hematopoietic differentiation.

One question is whether ES and iPS cell-derived cells express the surface antigen markers, such as E-cadherin (E-cad), a marker of both ectoderm and endoderm), platelet-derived endothelial growth factor receptor (PDGFRα), and Flk1 (also known as VEGF receptor 2 and a marker lateral mesoderm) [9–11] and transcription factors, such as *Tbx6* and *Brachyury* (a marker pan mesoderm) (Fig. 2).

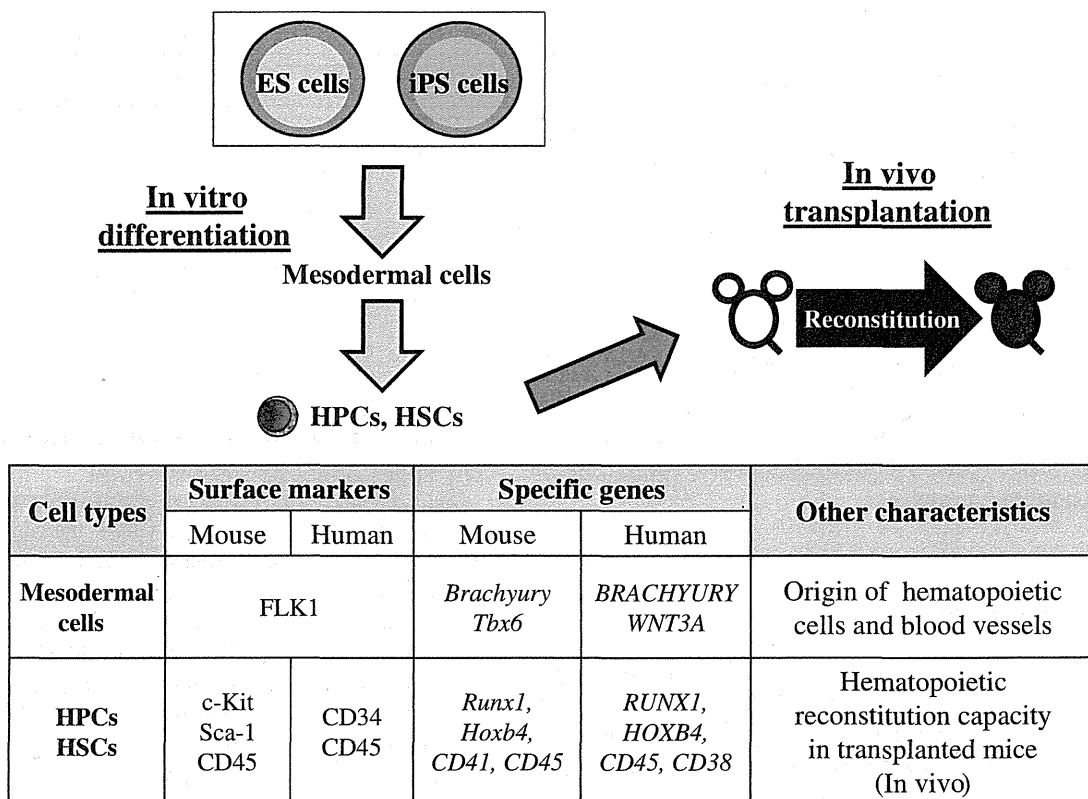
In mouse, a high percentage of Flk1$^+$ mesodermal cells are reportedly obtained from EBs around days 4 to 4.5 after ES cell differentiation [12, 13]. ES cells cultured on collagen-IV-coated dishes differentiate into Flk1$^+$PDGFRα^+ immature mesodermal precursors, which then give rise to Flk1$^+$PDGFRα^- cells, which are precursor of endothelial and HCs [14]. Flk1$^+$ mesodermal cells have been generated from several kinds of mouse iPS cells, such as mouse embryonic fibroblasts (MEFs), tail tip fibroblasts (TTFs), hepatocytes, and gastric epithelial cells. Among them, MEF-derived iPS cells exhibit the highest proportion of E-cad$^-$Flk1$^+$ cells [15]. Mesodermal potential as evaluated by the presence of E-cad$^-$Flk1$^+$ cells and expression of *Brachyury, Flk1*, and *Tbx6* mRNA vary among several iPS cell lines derived from identical TTFs. The level of ectopically expressed and remain of c-Myc likely underlies the differences [16].

Some groups have reported mesodermal differentiation from both human ES and iPS cells. Flk1$^+$CD34$^+$ mesodermal progenitors were generated from KhES1, KhES3 ES cells (KhES1, KhES3) and iPS cells (201B7, 253 G4; derived from dermal fibroblasts) co-cultured with OP9 cells and cytokines [17]. Morishima et al. report that ES (KhES3) and iPS (201B6, 253 G1, 253 G4; derived from dermal fibroblasts) cells generated Flk1$^+$ cells that contained hemangioblasts and Flk1^{high}CD34$^+$ cells with hematopoietic potential [18]. *BRACHYURY* and *WNT3A* mRNA expression was also confirmed in ES-, iPS-, and patient iPS-derived mesodermal cells by others [19, 20].

Hematopoietic Stem (HSCs) and Progenitor (HPCs) Cells

HSCs, which top the hematopoietic hierarchy, have self-renewal capacity and multipotency, and differentiate first into the progenitors of each hematopoietic lineage, which then mature into functional cells, including leukocytes, erythrocytes and platelets. As HSCs are used for transplantation, pluripotent cell-derived HSCs could serve as a source for future clinical applications. Questions remain as to whether HPCs and HSCs derived from pluripotent cells express c-Kit, Sca-1, CD45 in mouse, or CD34 and CD45 in humans in vitro, and whether these precursors have hematopoietic repopulation capacity in vivo (Fig. 2).

In mice, Burt et al. reported a differentiation method that did not require gene modification. EB formation of ES cells treated with SCF, IL-3 and IL-6 with serum for 7–10 days yielded CD45$^+$c-Kit$^+$HPCs with long term (for a maximum 20 weeks) repopulation capacity, as measured by chimerism and differentiation into lymphoid and myeloid lineages after transplantation into irradiated mice [21]. However, this approach is not widespread, suggesting that success may depend on serum they used. Therefore, establishment of serum-independent culture condition would be needed to get reproducible result.



Cell types	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Mesodermal cells	FLK1		*Brachyury* *Tbx6*	*BRACHYURY* *WNT3A*	Origin of hematopoietic cells and blood vessels
HPCs HSCs	c-Kit Sca-1 CD45	CD34 CD45	*Runx1, Hoxb4, CD41, CD45*	*RUNX1, HOXB4, CD45, CD38*	Hematopoietic reconstitution capacity in transplanted mice (In vivo)

Fig. 2 Schematic diagram of pluripotent cell-derived mesodermal cells, HPCs and HSCs. ES and iPS cells- derived mesodermal cells are characterized by the expression of FLK1 (also known as VEGF receptor 2) and specific transcription factor genes (*Brachyury* and *Tbx6* in mouse; *BRACHYURY* and *WNT3A* in human). Hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs)-derived from pluripotent cells are characterized by the expression of hematopoietic surface markers (c-Kit, Sca-1 and CD45 in mouse; CD34 and CD45 in human) and specific transcription factor genes (*Runx1, Hoxb4, CD41* and *CD45* in mouse; *RUNX1, HOXB4, CD45* and *CD38* in human). In vivo transplantation assay enables us to evaluate the potential of both HPCs and HSCs. Extensive long-term reconstitution with multipotency and self-renewal capacities of all hematopoietic lineages can be observed in HSCs, whereas short and/or relatively long-term reconstituting in HPCs can give rise to certain lineages

On the other hand, to promote the development and expansion of ES cell-derived HSCs, genetically-modified methods have been reported. Kyba et al. reported that forced expression of HoxB4 in immature ES cell-derived HPCs conferred definitive hematopoietic potential in mouse ES cells [22]. Temporal induction of HoxB4 during the course of EB formation increased the number of immature HPCs in vitro. In addition, HoxB4-induced ES-derived HPCs engrafted and produced lymphoid and myeloid cells in both primary (for a maximum 12 weeks) and secondary (for a maximum of 20 weeks) transplanted mice [22]. Combined ectopic expression of HoxB4 and Cdx4, which is also homeobox transcription factor, resulted in more efficient reconstitution than HoxB4 alone [23]. Unlike the case in mouse, a high incidence of leukemia occurred after transducing a *HOXB4*-expressing retroviral vector in large animals, such as dog and monkey [24], suggesting that care should be taken using gene-manipulation methods for HSC transplantation.

The HoxB4-constitutive transduction method is also effective to establish mouse iPS cells from fibroblasts in a sickle cell anemia model. Resulting iPS cell-derived progenitors grown in the presence of OP9 stromal cells reconstituted the hematopoietic system after transplantation into irradiated mice [25]. Lin et al. has reported a hematopoietic differentiation culture method for iPS cells established from MEFs, which lacks a feeder cell layer or gene manipulation. After 7 days of culture with "conditioned medium", EBs prepared from culture of OP9-DL1 cells (modified OP9 cells expressing the delta-like 1 (DL1) Notch ligand) generated c-Kit^{+}Sca1^{+}HPCs that could differentiate along the myeloid lineage [26].It has not yet been examined whether HPCs derived from this method could reconstitute in vivo.

Unlike the case in mouse, ectopic expression of HoxB4 temporarily enhanced generation and proliferation of human HPCs in vitro but did not promote a significant increase in the number of HSCs in vitro and had no effect on repopulating capacity of HSCs in immuno-deficient mice [27]. This