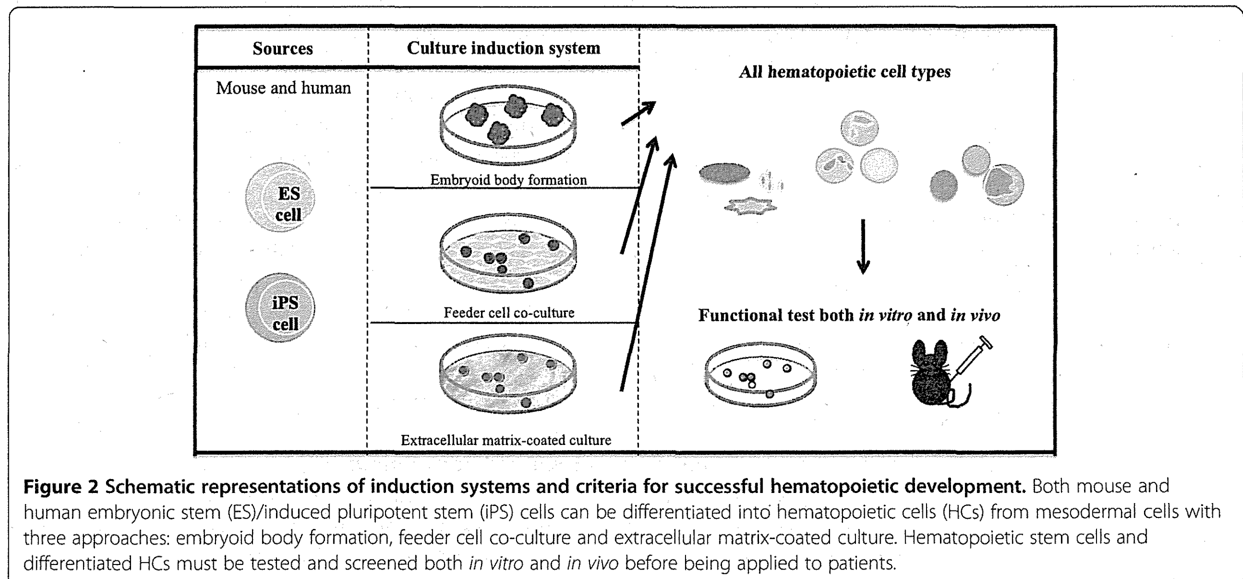

Figure 2 Schematic representations of induction systems and criteria for successful hematopoietic development. Both mouse and human embryonic stem (ES)/induced pluripotent stem (iPS) cells can be differentiated into hematopoietic cells (HCs) from mesodermal cells with three approaches: embryoid body formation, feeder cell co-culture and extracellular matrix-coated culture. Hematopoietic stem cells and differentiated HCs must be tested and screened both *in vitro* and *in vivo* before being applied to patients.

human iPS cells throughout a 21-day EB formation demonstrated a sequential gene expression from *BRACHYURY* (mesoderm) to *GATA-2* and *SCL* (hematopoietic commitment), followed by the emergence of $CD34^+CD45^+$ cells that denote hematopoietic lineages [32].

To improve the efficiency of EB formation using human ES cells, the modified spin-EB technique has been developed – in which a defined number of human ES cells are aggregated by centrifugation in low-attachment 96-well plates, and which could reproduce the hematopoietic differentiation potential with more than 90% of the wells able to form hematopoietic cells. This spin-EB method efficiently accelerates hematopoietic differentiation and approximately 500 hematopoietic progenitors are induced from single ES cells [33]. Human iPS cells also differentiate into hematopoietic cells by the spin-EB method. $CD34^+$ cells derived from them are $CD45^+$ (27 to 64%) and $CD43^+$ (36 to 60%) after 13 to 17 days of culture [34].

Feeder cell co-culture

Co-culture is a method of culturing a layer of feeder cells together with ES/iPS cells to give support to the growing cells (germ layer cells) towards development of hematopoietic lineages in appropriate culture medium. A well-known feeder cell layer, OP9 stromal cells are derived from newborn bone marrow of the mouse calvaria model, which reveals osteopetrosis due to lack of macrophage CSF [35]. In addition to OP9 co-cultures, stromal cells from the AGM region can be used to support hematopoiesis of both mouse and human ES cells in the culture system, suggesting that factors such as cytokines are secreted to regulate the early stem cell development and hematopoietic differentiation *in vitro* [36].

In mouse ES cells, the use of OP9 stromal cells could promote lymphohematopoietic differentiation by minimizing preferential differentiation towards monocyte–macrophage lineages [37]. AGM stromal cell co-cultures with mouse ES cells could induce $CD45^+$ hematopoietic cells, including cell differentiation from both Mac-1^+ myeloid cells and $B220^+$ B cells, suggesting that AGM stromal cell lines support hematopoietic differentiation. To improve the efficiency of hematopoietic differentiation, a two-step co-culture system was designed, first by co-culturing of mouse ES cells with OP9 cells followed by AGM stromal cells at defined time points. Approximately 90.6% of $CD45^+$ hematopoietic cells (23.6% in the OP9 system), 87% of Mac-1^+ cells (16% in the OP9 system) and 19.1% of c-Kit^+ cells (43% in the OP9 system) could be detected using this system [36]. Using a colony-forming assay, Krassowska and colleagues showed that ES cell-derived mesodermal cells could stimulate HPC production with 3.5-fold efficiency in primary AGM region co-cultures as compared with those derived from feeder-free culture. They also co-cultured differentiating ES cells on the dorsal aorta and mesenchyme-derived, urogenital ridge-derived and fetal liver-derived stromal cell lines that had previously been reported to support BM-derived HSCs [38]. The time-course of ES/iPS cell differentiation towards hematopoietic cells does not synchronize with regular hematopoietic differentiation, implying that hematopoietic differentiation does not occur via HSCs. In OP9 cell co-culture, mouse iPS cells initially formed *Brachyury*-expressing mesodermal cells and later co-cultured again onto fresh OP9 cells supplemented with hematopoietic cocktails were induced to form hematopoietic lineages, including erythroid and myeloid lineage cells but not lymphoid lineage cells [39].

Human ES/iPS cells co-cultured with OP9 cells generated HPCs defined by $CD34^+CD43^+$ expression and evaluated by the clonogenic progenitor cell assay [40]. Using a co-culture system with mouse fetal liver-derived stromal cells, human ES cells could generate the erythroid progenitors as determined by colony-forming cells, followed by enucleated erythrocytes with oxygen-carrying capacity [41]. To examine the properties among stromal cells, primary cells and cell lines derived from the mouse AGM region and fetal liver were compared in supporting hematopoietic differentiation from human ES cells. Eighteen days after co-culturing, primary cells derived from the AGM region exhibited the highest number of both $CD34^+$ and $CD45^+$ cells among the cells, suggesting that cell lines probably lose their ability to support during the passages [42].

Extracellular matrix-coated dish

Dishes coated with extracellular matrixes, such as collagen and fibronectin, are used as monolayer cultures to differentiate ES/iPS cells. In mouse ES cells, the use of collagen IV-coated dishes directs the generation of E-cadherin$^-$Flk1$^+$VE-cadherin$^-$-expressing mesodermal cells and further accelerate differentiation into hemangioblast (VE-cadherin$^+$Flk1$^+$CD45$^-$), HPCs (c-Kit$^+$CD45$^+$) and mature hematopoietic cells (c-Kit$^-$CD45$^+$ or Ter119$^+$) [43].

In human ES/iPS cells, matrixes such as human fibronectin, human collagen IV and mouse collagen IV are utilized to generate hematopoietic progenitors ($CD43^+CD34^+$), which could give rise to several lineages of differentiated blood cells including erythroid cells ($CD71^+CD235a^+$), megakaryocytes ($CD41a^+CD42b^+$), neutrophils ($CD15^+CD66b^+$), macrophages ($CD14^+CD68^+$) and dendritic cells (HLA-DR$^+$CD1a$^+$) phenotypically [44]. Human ES/iPS cells could differentiate into mesodermal cells in the presence of the major matrix components such as laminin, collagen I, entactin and heparin-sulfate proteoglycan as well as growth factors and several other undefined compounds. These mesodermal cells were able to induce hematopoietic cells after substitution with hematopoietic cocktail culture medium [45].

These studies suggested that monolayer culture system could commit hematopoietic potential in differentiating ES/iPS cells. Combination of this system with xenogeneic-free culture has been widely considered a more suitable approach for future clinical applications.

Embryonic stem/induced pluripotent stem cell-derived hematopoietic lineages

As mechanisms of hematopoietic development in both mouse and human are being established, manipulation of ES/iPS cells for hematopoietic differentiation is also improving. ES/iPS cells possess embryonic natures and are likely to be differentiated into primitive hematopoietic cells. Exogenous cytokines, feeder cells and extracellular matrix-coated dishes enable ES/iPS cells to differentiate into definitive hematopoietic cells in addition to primitive types. In the following section, induction of hematopoietic cell types from both mouse and human ES/iPS cells is described (Figure 3).

Hematopoietic stem cells and hematopoietic progenitor cells

HSCs are the cells that possess self-renewing capacity to maintain the stem cell pool and multipotency to differentiate into all lineages of mature hematopoietic cells, while HPCs do not self-renew and their differentiation capacity is limited only to certain types of cells. HSCs/HPCs can be defined by the surface marker expressions, which enables us to investigate the molecular mechanisms underlying HSC/HPC development. Mouse-derived HSCs/HPCs are commonly defined by lineage marker (Lin$^-$, a cocktail of lineage markers including B220, Ter119, Mac-1, Gr-1, CD3, CD4 and CD8), stem cell antigen (Sca-1$^+$, a putative marker of stem and progenitor cells) [46], c-Kit$^+$ (a receptor for SCF) [47], $CD34^{low}$ [48] and Tie-2$^+$ (a tyrosine kinase receptor expressed in endothelial and hematopoietic cells) [49]. However, unlike in mouse, CD34 is the representative marker for human HSCs [50].

In mouse ES cells, after co-culturing them with the bone marrow stromal cell line with a combination of IL-3, IL-6, fetal calf serum and cell-free supernatants of fetal liver stromal cell line culture, the PgP-1$^+$ (CD44, phagocytic glycoprotein-1) Lin$^-$ cells (B220$^-$Mac-1$^-$ JORO75$^-$Ter119$^-$ (JORO75 is a marker of T-cell progenitors)) are generated and transplanted to repopulate the lymphoid, myeloid and erythroid lineages of primary adult irradiated mice 15 to 18 weeks post-transplantation. The PgP-1$^+$Lin$^-$ cells also possess self-renewal potential as examined in secondary adult irradiated mice after 16 to 20 weeks post-transplantation [51].

Primitive HPCs were generated from EB-derived mouse ES cells and injected into lethally irradiated adult mice to reconstitute the hematopoiesis system, showing a survival rate of more than 3 weeks and subsequently lymphoid and myeloid engraftment from $CD45^+$ ES-derived cells was detected 12 weeks post-transplantation [52]. Another group demonstrated the acquisition of long-term multilineage capacity in lethally irradiated mice after injection with c-Kit$^+$CD45$^+$ HSCs, which was derived from mouse ES cells in methylcellulose culture supplemented with SCF, IL-3, IL-6 and fetal bovine serum [53]. However, the reproducibility of these results still remains elusive, suggesting that those successful attempts were highly dependent on the individual culture condition setup as well as the cellular phenotype of HSCs/HPCs that are being investigated.

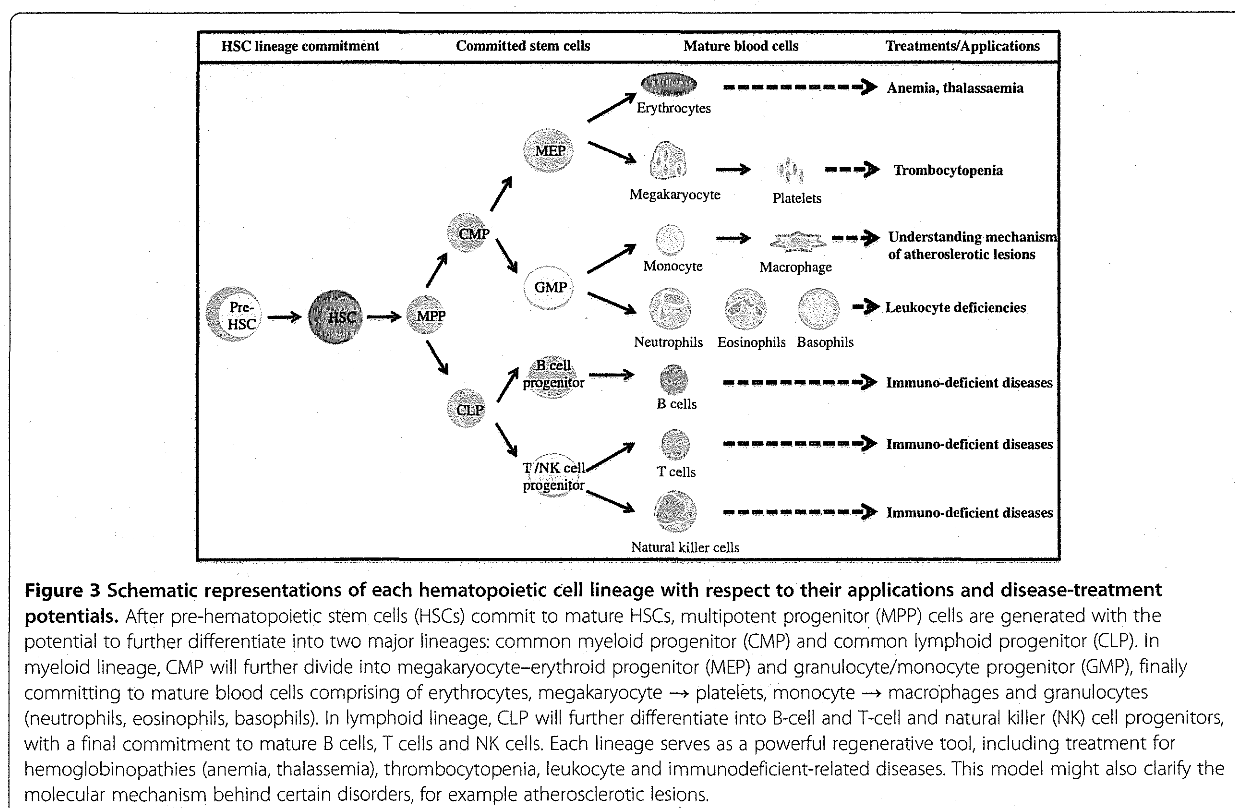

Figure 3 Schematic representations of each hematopoietic cell lineage with respect to their applications and disease-treatment potentials. After pre-hematopoietic stem cells (HSCs) commit to mature HSCs, multipotent progenitor (MPP) cells are generated with the potential to further differentiate into two major lineages: common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). In myeloid lineage, CMP will further divide into megakaryocyte–erythroid progenitor (MEP) and granulocyte/monocyte progenitor (GMP), finally committing to mature blood cells comprising of erythrocytes, megakaryocyte → platelets, monocyte → macrophages and granulocytes (neutrophils, eosinophils, basophils). In lymphoid lineage, CLP will further differentiate into B-cell and T-cell and natural killer (NK) cell progenitors, with a final commitment to mature B cells, T cells and NK cells. Each lineage serves as a powerful regenerative tool, including treatment for hemoglobinopathies (anemia, thalassemia), thrombocytopenia, leukocyte and immunodeficient-related diseases. This model might also clarify the molecular mechanism behind certain disorders, for example atherosclerotic lesions.

In addition, ectopic expression of certain genes into ES/iPS cells has been used to induce development of HSCs/HPCs in an *in vitro* system. *HoxB4* (a homeobox transcription factor) was retrovirus-transduced into EB-derived mouse ES cells followed by co-cultures in OP9 stromal cells to induce hematopoietic development. This has resulted in a population of definitive HSCs that could repopulate lymphoid and myeloid cell lineages in both primary and secondary transplanted irradiated adult mice [54]. On the other hand, Wang's group exploited the fact that ectopic expression of Cdx4 (a family of caudal-related homeobox-containing transcription factors) in mouse ES cells induced mesodermal specification together with increased HPC production. With this notion, they also observed the synergistic effect of *HoxB4* and *Cdx4* on HPC production after EB-derived ES cells grown on OP9 stromal cells, and those cultured cells could support the engraftment of all hematopoietic lineages in irradiated adult mice [55]. EB-derived mouse ES cells are able to generate c-Kit$^+$CD41$^+$CD45$^-$ cells without HoxB4 regulation, but their ability to reconstitute adult hematopoiesis is only seen after those cells undergo maturation in OP9 co-cultures in the presence of HoxB4, which was measured by total chimerism 16 weeks post-transplantation of fractionated co-cultured cells, such as CD41$^+$ cells, c-Kit$^+$ cells, CD34$^+$ cells and CD45$^-$ cells [56], suggesting that HoxB4 probably regulates HSC maturation through upregulating the CD34 surface marker expression.

Although the production of HSCs/HPCs is possible using a HoxB4-expressing approach, problems such as an inhibitory effect on lymphoid cell differentiation due to long-term constitutive HoxB4 expression [54] as well as the risk of leukemogenesis in dogs and monkeys after transducing with *HOXB4*-expressing retroviral vector [57] have been reported. To alleviate these adverse effects, EB-derived CD41$^+$c-Kit$^+$ cells derived from ES/iPS cells were transduced with adenoviral vector of human HOXB4 and then co-cultured on OP9 stromal cells together with hematopoietic cytokines (SCF, fms-like tyrosine kinase 3 receptor ligand (Flt-3 ligand), thrombopoietin, IL-3 and IL-6) and fetal bovine serum. Although human HOXB4 expression was transiently expressed (expression level decreased 6 days post-transduction), those transduced CD41$^+$c-Kit$^+$ cells could proliferate up to 20 days with traceable CD45$^+$, CD41$^+$ and Sca-1$^+$, indicating the existence of immature hematopoietic cells with hematopoietic differentiation potential in a colony-forming assay [58]. However, whether these HPCs possess *in vivo* repopulation capacity remains an ongoing experiment. Nevertheless, with the use of iPS cell technologies, the humanized sickle

cell anemic mouse has been successfully treated with the injection of *HoxB4*-induced mouse iPS cells (from tail tip fibroblast of this mouse), whereby the human sickle globin gene has been corrected through gene-specific targeting beforehand [59]. This finding suggests a valuable tool to approach human gene therapy.

In humans, ES cell-derived hematopoietic cells are engrafted successfully by direct femoral injection but not intravenous transplant (due to cell aggregation in response to rodent serum), enforce expression of *HOXB4* in human ES-derived hematopoietic cells, but confer no effect in promoting repopulation capacity [60], suggesting that different mechanisms control HSC regulation between human and mouse ES cells. After human ES cell co-cultures with mouse fetal liver stromal cells, the derivation of multipotential HPCs from the cobblestone-like $CD34^+$ cell populations displayed hematopoietic potential in a colony-forming assay [61]; however, the reconstitution capacity remains uninvestigated. On the other hand, Ji and colleagues used an OP9 stromal cell co-culture with human ES cells; although they found that OP9 stromal cells could prevent $CD45^+CD34^+$ HPCs from apoptosis, those HPCs failed to engraft both adult and newborn NOD/SCID mice [62], suggesting that this model did not improve the repopulating capacity in HPCs, unlike in the mouse model. In OP9-free and serum-free culture conditions, ES/iPS cell differentiation on extracellular matrix-coated dishes could cultivate HSCs/HPCs, defined as $CD34^+CD45^+$ cells that could support myeloid, erythroid and megakaryocyte cell lineages in the presence of respective hematopoietic cytokines [45]. As an effort to overcome the shortage of HSC/HPC sources for transplantation, several attempts in generating them from ES/iPS cells have been reported; however, some of their *in vivo* reconstitution capacity still remains to be determined.

Mature hematopoietic cells

In this section, we review the current progress in lineage-restricted mature hematopoietic cells of pluripotent stem cell differentiation in culture.

Erythrocytes

Erythrocytes are differentiated sequentially from HSCs, BFU-E, CFU-E (erythroid progenitors) and later into erythroblasts and reticulocytes (immature erythrocytes) through a process known as erythropoiesis. Erythropoiesis involves a multistep regulation of cytokines such as Epo.

After EB formation of mouse ES cells, erythroid cells expressing *βH1 globin*, *βmajor globin* and *Gata1* are generated in the presence of Epo and SCF in methylcellulose culture medium [63]. Co-culture of ES cells with OP9 stromal cells in the presence of Epo and IL-3 could generate erythroid cells that are characterized by Ter119 (glycophorin A-associated protein) [64]. By co-culturing of mouse iPS cells with OP9 stromal cells, c-Kit^+CD41^+ HPCs were generated and transplanted into sickle cell anemia mice in which the anemic status was ameliorated, suggesting that HPCs derived from iPS cells differentiated into erythrocytes *in vivo* [59].

Human ES-derived erythrocytes principally express CD235a (glycophorin A) and both embryonic and fetal globins such as ξ-globin and γ-globin by EB formation and extracellular matrix culture [65]. Continuous coculture of human ES cells with fetal liver-derived stromal cells enables the induction of adult type *β-globin*, while suppressing ξ-globin expression [41]. On the other hand, Olivier and colleagues reported a three-step culture system to obtain large-scale production of erythroid cells, in which they co-cultured human ES cells with immortalized human liver cells, followed by culture of $CD34^+$ cells with cytokines, and subsequently co-culturing with mouse bone marrow stromal cells. Consequently, ES-derived $CD34^+$ cells could amplify the erythroid cells efficiently (5,000-fold to 10,000-fold in number), which are hemoglobinized and expressed embryonic-globin and fetal-globin, but not β-globin [66].

In human iPS cells, EB-derived $CD34^+CD45^+$ HPCs established from patients with polycythemia vera could produce both CFU-E and $CD235a^+CD45^-$ erythroid lineage cells in the presence of SCF, IL-3 and Epo [34]. Both human embryonic and fetal mesenchymal-reprogrammed iPS cells recapitulate early human erythropoiesis that are characterized by embryonic ($\zeta_2\varepsilon_2$ and $\alpha_2\varepsilon_2$) and fetal ($\alpha_2\gamma_2$) hemoglobin, by co-culturing iPS cells with human fetal liver-derived feeder layer, followed by liquid culture in the presence of IL-3, SCF, Epo, bone morphogenic protein 4 and insulin-like growth factor-1 [67].

Granulocytes

Granulocytes can be classified into neutrophils, basophils and eosinophils, which are differentiated from HPCs. Granulocyte differentiation antigen 1 (Gr-1) is commonly used as a marker for granulocytes. Both CD15 and CD11b are neutrophil and monocyte markers while CD16 is the mature neutrophil marker.

In mouse, $Gr\text{-}1^+$ neutrophils are generated from EB-derived ES cells and after 4 to 20 days co-culture with OP9 cells in the presence of granulocyte CSF, granulocyte–macrophage CSF and IL-6. These neutrophils comprise granules of lactoferrin and gelatinase, and exhibit chemotactic responses and superoxide production [68].

In human ES cells, neutrophils expressing $CD15^+$, $CD11b^+$ and $CD16^+$ exhibiting equivalent phenotype to those in peripheral blood are produced from the EBs after culture in OP9 cells with SCF, Flt-3 ligand, IL-6, IL-6 receptor, thrombopoietin, IL-3 and subsequently

with granulocyte CSF, and exhibited oxidative burst function and phagocytic activity *in vitro* [69]. Culture of human iPS cells established from bone marrow mesenchymal stem cells (from a patient with X-linked chronic granulomatous disease) on OP9 stromal cells produced oxidase-deficient neutrophils. They were also successful in rescuing oxidase deficiency by gene modification using zinc finger nuclease-mediated safe harbor targeting [70]. This finding demonstrated that precise gene targeting might be applied to correct a disease-causing mutation in patient iPS cells.

Lymphocytes

Several kind of lymphocytes play important roles in regulating immune response, such as T lymphocytes/T cells (CD3, CD4, CD8), B lymphocytes/B cells (CD10, CD19) and natural killer cells (CD56, CD94), which can be stimulated from mouse and human pluripotent stem cells.

In mice, mature $CD8^+$ T cells expressing γδ and αβ T-cell receptors were generated from ES cells after co-culture on OP9-expressing Notch ligand, delta like 1 (OP9-DL1). Additionally, T-cell progenitors generated after stimulation by Flt-3 ligand and IL-7 were capable of reconstituting the T-cell compartments in sublethally irradiated *Rag2*$^{-/-}$ mice [71]. OP9-DL1 co-cultures with both iPS cell lines derived from murine splenic B cells and MEF also resulted in T-cell development with traceable CD44 and CD24 in addition to CD4 and CD8 markers, but are not committed to the $CD19^+$ B-cell lineage in the presence of Flt-3 ligand and IL-7 [72]. In the presence of Flt-3 ligand, IL-15, IL-6, IL-7 and SCF, co-cultures of mouse ES cells with OP9 stromal cells generated natural killer cells with CD94/NKG2 receptors to combat certain tumor cell lines and major histocompatibility complex (MHC) class I-deficient lymphoblasts [73].

Co-culturing of human ES cells with OP9 cells induced $CD34^{high}CD43^{low}$ cells, and subsequent culture of $CD34^{high}CD43^{low}$ cells in OP9-DL1 cells in the presence of Flt-3 ligand, IL-7 and SCF generate functional T cells that have a response to phytohemagglutinin stimulation [74]. After 10-day co-cultures of OP9 stromal cells with iPS cells derived from adult human dermal fibroblasts, $CD34^+$ cells were harvested and subsequently co-cultured on MS-5 stromal cells for another 21 days in the presence of SCF, Flt-3 ligand, IL-7 and IL-3, which were then capable of generating $CD45^+CD19^+CD10^+$ pre-B cells [75]. Additionally, using human pluripotent stem cells, Ni and colleagues demonstrated that the generation of $CD45^+CD56^+$ and $CD117^-CD94^+$ natural killer cells can inhibit HIV-1 infection [76], a possible potential to treat immunologic diseases in humans.

Monocytes and macrophages

Macrophages are differentiated from monocytes and function to regulate both innate and adaptive immunity to combat foreign particles including pathogens by stimulating the response of immune cells, such as lymphocytes. The putative surface markers for macrophages are CD11b (Mac-1), CD14 (ligand receptor of lipopolysaccharide), CD115 (colony-stimulating factor 1 receptor) and F4/80 (a highly glycosylated proteoglycan extracellular antigen).

In mice, after EB formation of CCEG2 and D3 ES cell lines, the generated HPCs drive the development of macrophage that expresses F4/80 marker in the presence of Epo, IL-1, IL-3 and macrophage CSF [27]. From bone marrow-derived iPS cells, macrophages were generated after co-culture with OP9 stromal cells and further induced differentiation in the presence of fetal calf serum and macrophage CSF. These iPS-derived macrophages showed similar expression of F4/80 and CD11b surface markers and phagocytic capacity with those bone marrow-derived macrophages [77].

In humans, co-culture of ES cells with S17 cells, a mouse bone marrow-derived stromal cell, were able to generate CD15-expressing macrophage progenitor cells [78]. After differentiating human ES cells by EB formation, monocytes and macrophages were induced in culture medium containing macrophage CSF and IL-3 [79]. EB formation of bone marrow mesenchymal stem cell-reprogrammed iPS cells reportedly produce monocyte–macrophage lineage cells after stimulation with cytokine cocktails including macrophage CSF and RANKL, evidenced by surface marker expression such as CD14, CD18, CD11b and CD115 [80].

Megakaryocytes and platelets

Platelets are particles fragmented from megakaryocytes that function in modulating hemostasis and vascular repair through cell aggregation and adhesion. CD41, also known as αIIb integrin, is reportedly expressed on megakaryocytes and platelets. This is a platelet glycoprotein relating to a complex of receptors such as fibronectin, fibrinogen, von Willebrand factor and thrombin that regulate platelet aggregation and attachment to extracellular matrix.

In mice, ES-derived megakaryocytes were formed after OP9 stromal cell co-cultures with thrombopoietin [81]. The proplatelets, which fragment into blood platelets, were developed from ES cells with integrin $\alpha IIb\beta_3$-mediated signaling after OP9 stromal cell co-cultures followed by supplementation of thrombopoietin, IL-6 and IL-11 [82].

In humans, $CD41^+$ megakaryocyte progenitors were generated after co-culturing the ES cells with S17 cells [78]. $CD41a^+/CD42b^+$ megakaryocyte lineage cells could

be generated from human ES cells after co-cultures with OP9 stromal cells [83]. Platelets were induced from cell populations that expressed CD34, VE-cadherin, CD31, CD41a and CD45 surface antigen markers from both human ES cells and adult fibroblast-derived iPS cells after OP9 co-cultures [78].

Variation of hematopoietic differentiation in embryonic stem/induced pluripotent stem cells

In hematopoietic differentiation from mouse ES cells, CCE and D3 cell lines have been frequently used due to their high capability for hematopoietic differentiation, suggesting variation of hematopoietic potential among ES cell lines [37,39,43]. Previously, our group compared the differentiation potential of iPS cells derived from MEFs and adult somatic cells (hepatocytes and gastric epithelial cells). Among them, we found that MEF-derived iPS cells were more efficient in generating Flk1-expressing mesodermal cells and hematopoietic cells compared with adult-derived iPS cells [84]. In addition, we observed that each iPS cell line exhibits different mesodermal and hematopoietic potentials, although those iPS cell lines are derived from the same origin of tail tip fibroblasts [85]. Other groups also demonstrated that variation of mesodermal and hematopoietic potential is observed among mouse iPS cell lines depending on the origins of the cells [39,86].

In humans, hematopoietic potential was examined in several ES and iPS cells. *In vitro* differentiation capabilities of myeloid and erythroid cells are not identical among human ES and iPS cells based on surface marker expression of CD45 (myeloid) and CD235 (erythroid) by flow cytometry [45,87]. Concerning the HPC potential, variation of HPC generation was observed based on CD34 expression by flow cytometry and *in vitro* colony formation by culture [45,67]. Taken together, such variation should be considered in hematopoietic differentiation for clinical purpose.

Conclusion

For clinical use of hematopoietic cells derived from ES/iPS cells, benefits and risks for patients should be considered. For transplantation and transfusion of hematopoietic cells, infections, rejection and donor risks have been addressed. Although ES/iPS technology potentially improves these issues, other problems such as cost of the products, efficiency of hematopoietic differentiation and quality of differentiated cells emerge as research progresses. Recently, the reprogramming capacity of hematopoietic cells at different stages of differentiation was compared, and HSCs/HPCs could be reprogrammed into iPS cells more frequently than mature lymphoid and myeloid cells [88]. Taken together with the variation of hematopoietic potential among iPS cells, it will be necessary to choose appropriate cells for reprogramming and to standardize the methods in iPS cell generation and differentiation for future regenerative medicine.

Note: 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; CSF: Colony-stimulating factor; dpc: Days post coitum; EB: Embryoid body; Epo: Erythropoietin; ES: Embryonic stem; GFP: Green fluorescent protein; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; IL: Interleukin; iPS: Induced pluripotent stem; MEF: Mouse embryonic fibroblast; SCF: Stem cell factor; YS: Yolk sac.

Competing interests

The authors declare that they have no competing interests.

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REVIEW

Zebrafish erythropoiesis and the utility of fish as models of anemia

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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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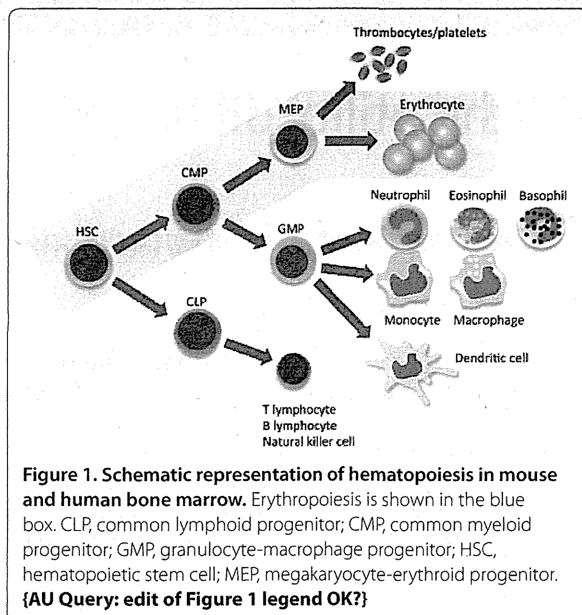

Figure 1. Schematic representation of hematopoiesis in mouse and human bone marrow. Erythropoiesis is shown in the blue box. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor.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

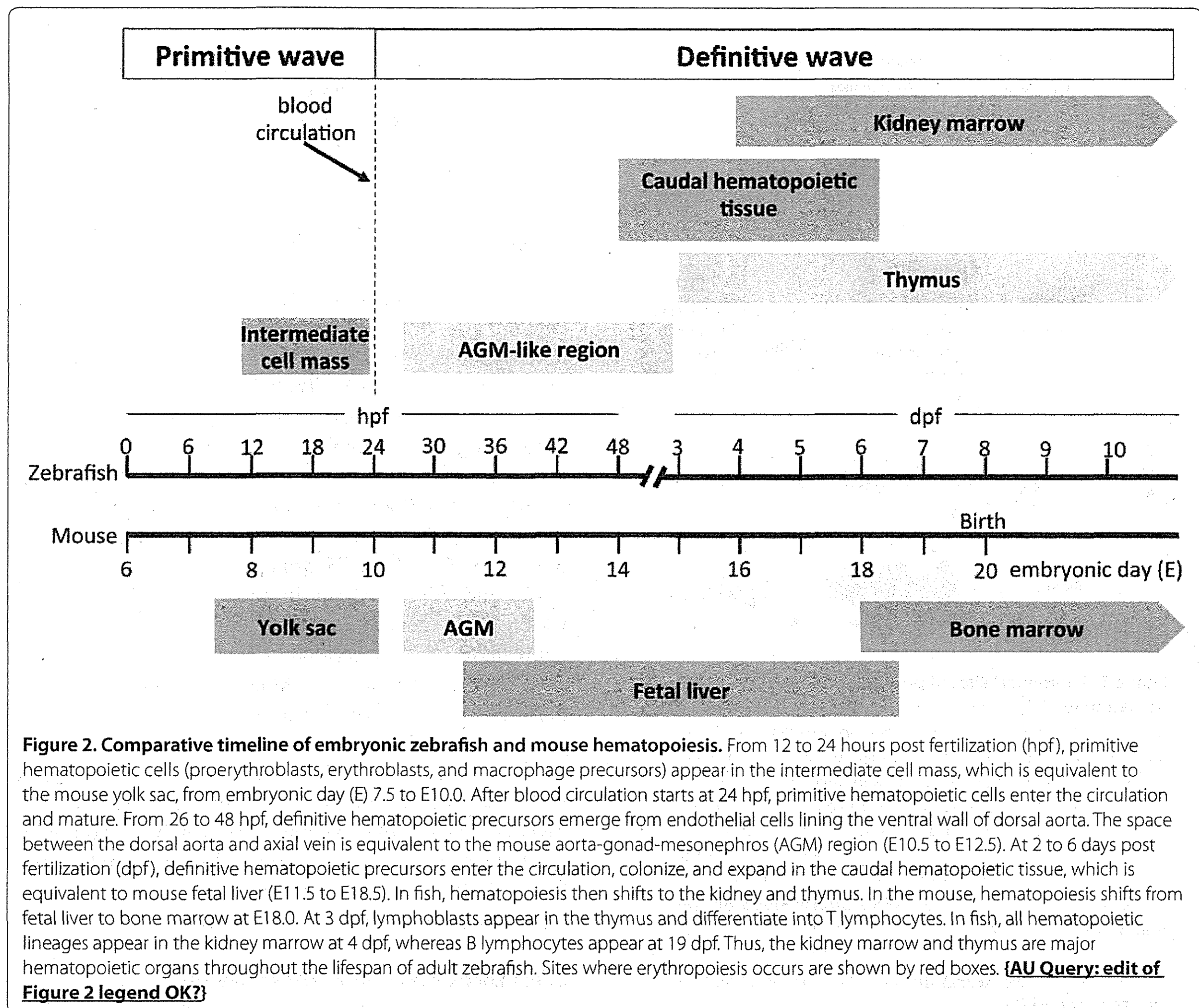

Figure 2. Comparative timeline of embryonic zebrafish and mouse hematopoiesis. From 12 to 24 hours post fertilization (hpf), primitive hematopoietic cells (proerythroblasts, erythroblasts, and macrophage precursors) appear in the intermediate cell mass, which is equivalent to the mouse yolk sac, from 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

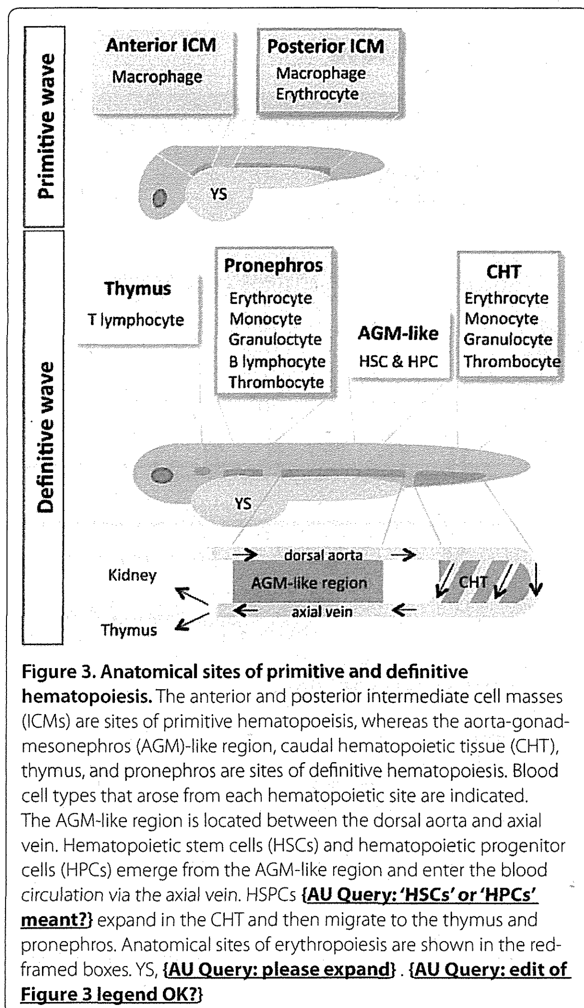

Figure 3. Anatomical sites of primitive and definitive hematopoiesis. The anterior and posterior intermediate cell masses (ICMs) are sites of primitive hematopoeisis, whereas the aorta-gonad-mesonephros (AGM)-like region, caudal hematopoietic tissue (CHT), thymus, and pronephros are sites of definitive 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].

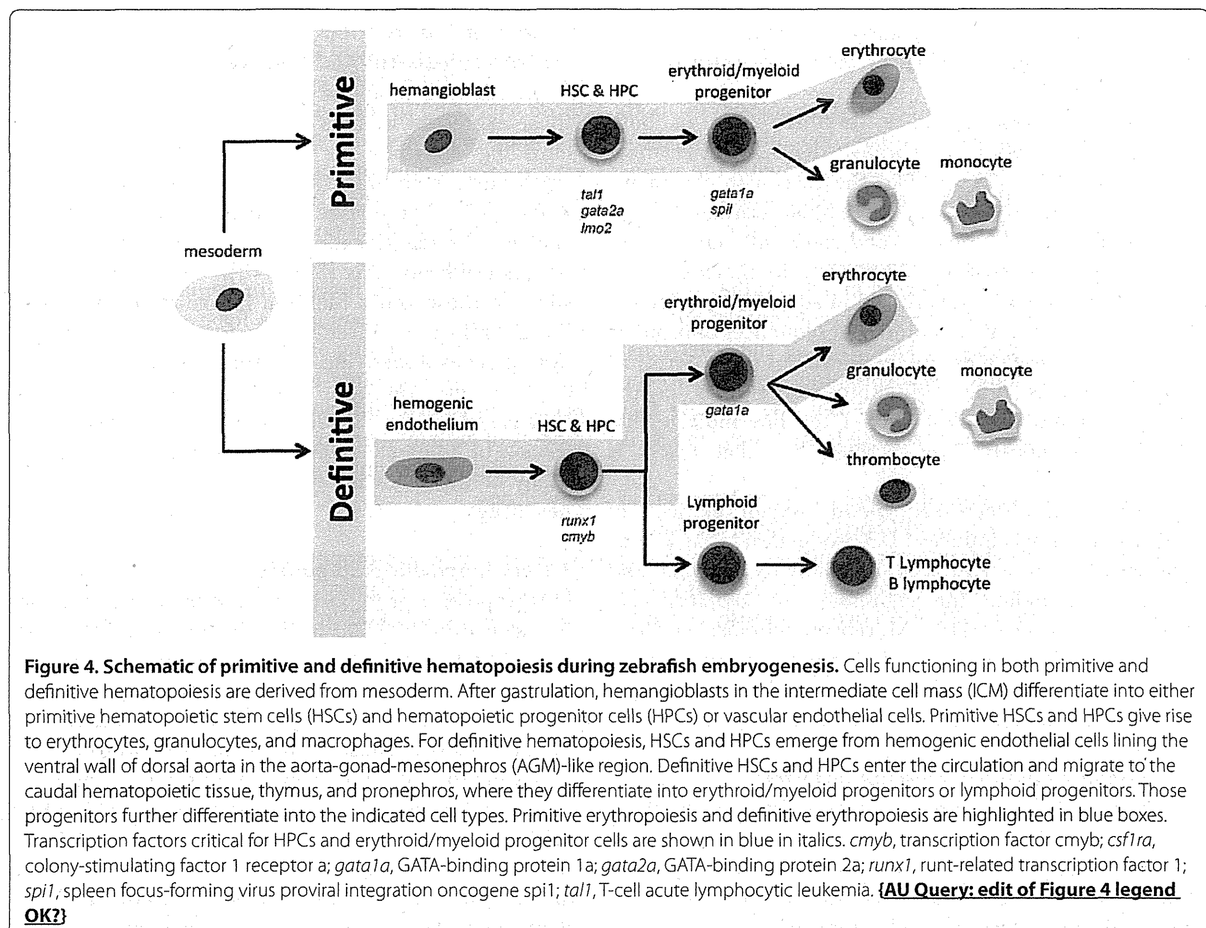

Figure 4. Schematic of primitive and definitive hematopoiesis during zebrafish embryogenesis. Cells functioning in both primitive and definitive hematopoiesis are derived from mesoderm. After gastrulation, hemangioblasts in the intermediate cell mass (ICM) differentiate into either primitive hematopoietic 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.

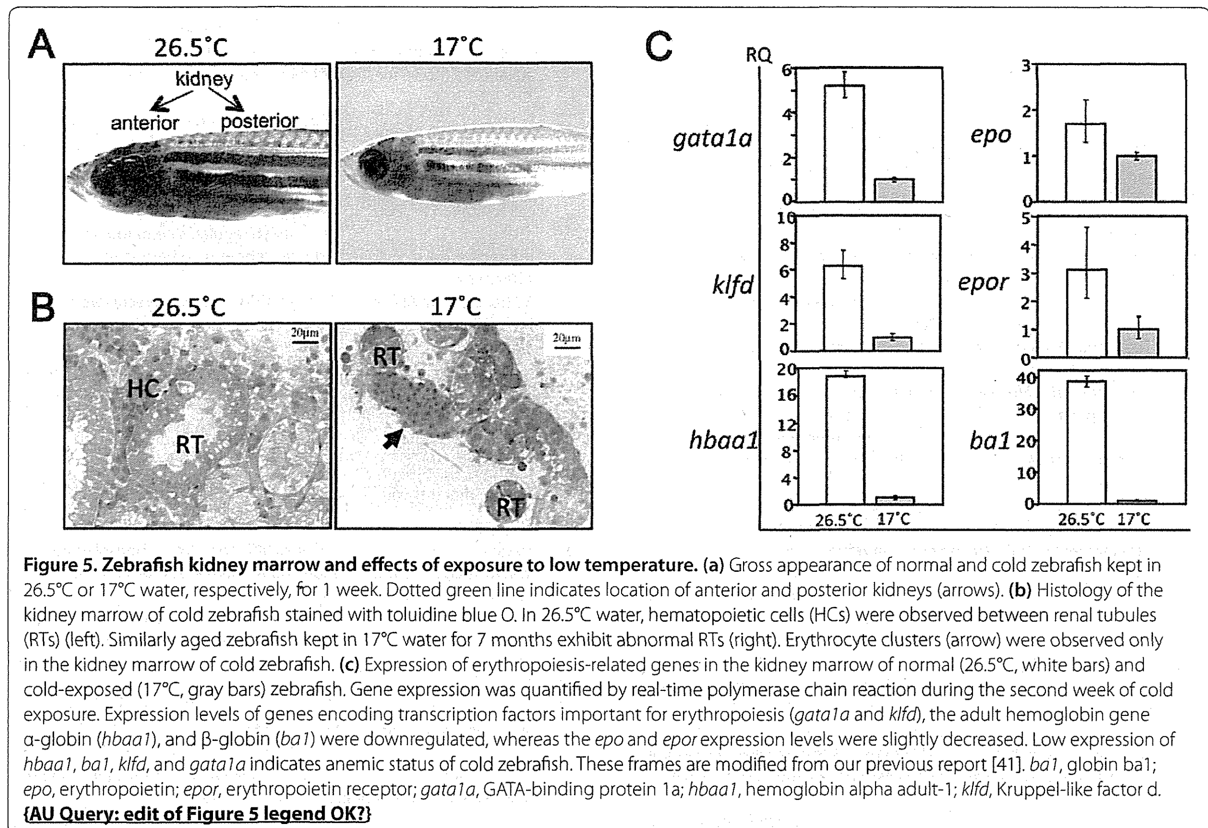

Figure 5. Zebrafish kidney marrow and effects of exposure to low temperature. (a) Gross appearance of normal and cold zebrafish kept in 26.5°C or 17°C water, respectively, for 1 week. Dotted green line indicates location of anterior and posterior kidneys (arrows). **(b)** Histology of the kidney marrow 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.

Acknowledgments

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