



Regulation of the embryonic erythropoietic niche: a future perspective

Ayako Yumine¹, Stuart T. Fraser^{1,2}, Daisuke Sugiyama¹

¹Department of Research and Development of Next Generation Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan, ²Disciplines of Physiology, Anatomy and Histology, School of Medical Sciences, University of Sydney, Sydney, Australia

p-ISSN 2287-979X / e-ISSN 2288-0011
<https://doi.org/10.5045/br.2017.52.1.10>
Blood Res 2017;52:10-7.

Received on March 1, 2017
Revised on March 10, 2017
Accepted on March 13, 2017

*This study was supported by Translational Research Network Program, JSPS KAKENHI grant number 26670468, Inter-university and faculty-level international exchange agreement promotional program and Subsidies for establishment of research centers.

Correspondence to

Daisuke Sugiyama, Ph.D.
Department of Research and Development of Next Generation Medicine, Faculty of Medical Sciences, Kyushu University, Station for Collaborative Research1 4F, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan
E-mail: sugiyama@jsd.med.kyushu-u.ac.jp

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Abstract

The production of red blood cells, termed erythropoiesis, occurs in two waves in the developing mouse embryo: first primitive erythropoiesis followed by definitive erythropoiesis. In the mouse embryo, both primitive and definitive erythropoiesis originates in the extra-embryonic yolk sac. The definitive wave then migrates to the fetal liver, fetal spleen and fetal bone marrow as these organs form. The fetal liver serves as the major organ for hematopoietic cell expansion and erythroid maturation after mid-gestation. The erythropoietic niche, which expresses critical cytokines such as stem cell factor (SCF), thrombopoietin (TPO) and the insulin-like growth factors IGF1 and IGF2, supports hematopoietic expansion in the fetal liver. Previously, our group demonstrated that $DLK1^+$ hepatoblasts support fetal liver hematopoiesis through erythropoietin and SCF release as well as extracellular matrix deposition. Loss of $DLK1^+$ hepatoblasts in *Map2k4*^{-/-} mouse embryos resulted in decreased numbers of hematopoietic cells in fetal liver. Genes encoding proteinases and peptidases were found to be highly expressed in $DLK1^+$ hepatoblasts. Capitalizing on this knowledge, and working on the assumption that these proteinases and peptidases are generating small, potentially biologically active peptides, we assessed a range of peptides for their ability to support erythropoiesis *in vitro*. We identified KS-13 (PCT/JP2010/067011) as an erythropoietic peptide—a peptide which enhances the production of red blood cells from progenitor cells. Here, we discuss the elements regulating embryonic erythropoiesis with special attention to niche cells, and demonstrate how this knowledge can be applied in the identification of niche-derived peptides with potential therapeutic capability.

Key Words Erythropoiesis, Embryo, Niche regulation, Cytokine peptide

INTRODUCTION

In adult mammals, hematopoiesis (the production of red blood cells, white blood cells and platelets) is normally restricted to the bone marrow. During mammalian embryonic development, hematopoiesis is not limited to one site but can be found in a range of locations which vary with the developmental age of the embryo or fetus [1-3]. This migration of hematopoietic stem cells (HSC) throughout the conceptus (the yolk sac, placenta and embryo/fetus combined) is dependent upon the embryonic stage of development and requires the formation of supportive microenvironments, termed hematopoietic niches. Ontogenetically, hematopoietic

activity can be identified in the extra-embryonic yolk sac; dorsal aorta at the level of the gonad-mesonephros (AGM); placenta; vitelline and omphalomesenteric arteries; fetal liver, spleen; skeletal muscle surrounding the developing long bones; and finally in the bone marrow [1, 2, 4-6]. Embryonic hematopoiesis can be divided into primitive (fetal) and definitive (adult-type) hematopoiesis. These first primitive hematopoietic cells originate from different populations of mesoderm which develop from the posterior midline region of the epiblast. Definitive (adult-type) hematopoietic cells are derived from the lateral plate mesoderm [7]. Both the primitive and definitive hematopoiesis are initiated in the yolk sac. Definitive hematopoiesis, however, later shifts to the fetal liver for expansion. Embryonic hematopoiesis can be

divided into three stages or waves [8, 9]. The yolk sac exhibits both primitive and definitive hematopoietic activity [2, 10-12]. Primitive hematopoiesis is a transient wave of blood production which does not give rise to cells found in the adult and is distinct from definitive hematopoiesis occurring in the fetal liver. *In vitro* organ culture demonstrated that the AGM region, but not yolk sac, contains HSCs which are capable of long-term, multi-lineage repopulation of irradiated adult recipient animals [12]. Yolk sac cells isolated prior to embryonic day (E) 10.5 fail to engraft in adult recipient mice. However, cells expressing endothelial markers but lacking hematopoietic markers are capable of contributing long-term to the recipient hematopoietic system if injected into the newborn liver. Therefore, the waves of embryonic hematopoiesis are composed of one primitive and (at least) 2 definitive waves.

Primitive hematopoiesis is characterized by the production of red blood cells that express fetal hemoglobin as well as the production of primitive megakaryocytes and primitive macrophages [13, 14]. In contrast, during definitive hematopoiesis, red blood cells expressing adult hemoglobin (adult red blood cells) are generated along with lymphoid progenitors and multilineage hematopoietic stem cells [15]. Summaries of the main features of the niche in each hematopoietic site of the mouse conceptus are shown in Fig. 1 and described in greater detail below.

Yolk sac

The yolk sac is a membrane-like tissue surrounding the rodent fetus and is the first hematopoietic site. The human embryo, the yolk sac is a short-lived membrane that is gradually resorbed during development. In mouse yolk sac, structures called “blood islands” are formed at E7.5. Blood islands are composed of clusters of vascular endothelial cells and with developing hematopoietic cells forming in the lumens of these vascular structures. These include primitive erythroid cells, macrophages and fetal megakaryocytes. These blood cells are known to be derived from the mesoderm and their differentiation is promoted by Indian Hedgehog and Vascular Endothelial Growth Factor (VEGF) secreted from the visceral endoderm adjacent to mesoderm, which serves as a niche [16]. The primitive erythroid cells and definitive proerythroblasts enter the blood circulation at E9.0. By E15.5, almost all primitive erythrocytes are terminally mature and have enucleated. These mature primitive erythroblasts can be observed in the blood stream for up to 3 weeks after birth [17]. In the yolk sac, in addition to the primitive hematopoietic activity, definitive hematopoietic activity is observed. Adult-type red blood cells and myeloid progenitor cells appear at E8.5-11.5 [2]. In addition, highly proliferative potential colony forming cells (HPP-CFC), B lymphocyte precursor cells and HSCs are observed [18, 19]. The components of yolk sac hematopoietic

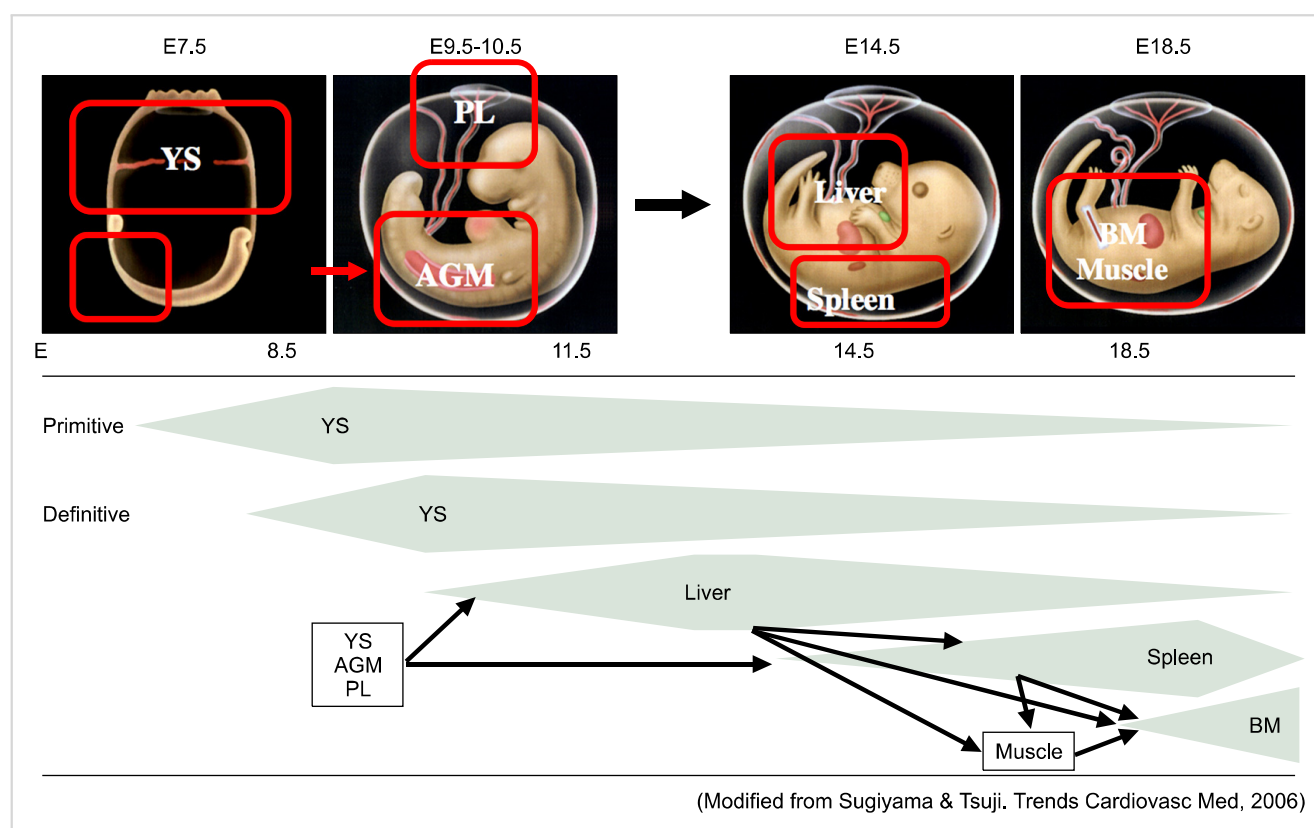


Fig. 1. Erythroid cell development in the mouse embryo. The sites for primitive and definitive erythropoiesis are shown. Arrows reveal presumable pathway of hematopoietic homing.

Abbreviations: AGM, Aorta-Gonad-Mesonephros; BM, Bone Marrow; E, Embryonic day; PL, Placenta; YS, Yolk Sac.

niche which specifically support definitive hematopoiesis are still being elucidated. Yolk sac vascular endothelial cells secrete growth factors such as TGF β 1 and angiopoietin-1 [20]. The visceral endodermal layer also produces growth factors that support hematopoiesis. Conditional deletion of VEGF in the visceral endoderm compartment leads to vascular defects, hematopoietic defects and embryonic lethality [21]. Transcriptome analysis of the maturing yolk sac revealed changes of expression of genes previously only observed in the gastric epithelium. This includes Gastrin-2, a small peptide normally produced by gastric epithelial cells, which we found was strongly expressed by visceral endodermal cells at E12.5 when hematopoietic activity in the yolk sac is coming to an end [22].

In the past, a bipotent progenitor termed the “hemangioblast” has been suggested to have the ability to differentiate into both blood cells and vascular endothelial cells. Although it is difficult to prove their existence, since in primary yolk sac contains multi-potential blast colony forming cells (BL-CFC), further investigation is needed [23].

Aorta-gonad-mesonephros region

In the aorta-gonad-mesonephros region (AGM region), derived from the para-aortic-splanchnopleural (p-Sp) region containing mesoderm, during E8.5-11.5, a variety of blood cells including HSCs, lymphoid progenitor cells, myeloid progenitor cells and adult-type erythroid progenitor cells are produced, as part of the definitive hematopoietic program [24-26]. The AGM region contains three arteries: the aorta, the omphalomesenteric artery and the vitelline artery [27]. These arteries contain intra-aortic clusters (IACs) which are found attached to the vascular endothelium and consist of hematopoietic stem and progenitor cells expressing c-Kit, CD31 and CD34. The IACs appear at E9.0 in omphalomesenteric artery and peak in frequency at E10.5 [27-29]. The AGM region is composed of numerous cell types acting as a hematopoietic niche, secreting hematopoietic factors and

controlling the development and differentiation of blood cells. Among those cells, vascular endothelial cells express *Jag1* gene and secrete stem cell factor (SCF) protein [30, 31]. Mesenchymal cells express *Tpo* and *Hedgehog* (Dhh, Ihh, Shh) genes and secrete BMP4 and IL3 proteins [25, 32]. Mesonephric cells, which go on to form the kidney, secrete CSF1 protein, and IACs express receptor genes for these hematopoietic factors (Notch1, Ptc1, Bmpr2, Alk3, Alk6) and proteins including KIT, MPL, IL-3Ra, CSF1R [24, 30]. IACs are reportedly derived from vascular endothelial cells incorporating Ac-LDL and migrate to liver [33].

Placenta

The placenta functions to exchange oxygen, carbon dioxide and nutrients between the mother and the fetus. The placenta also functions as a hematopoietic organ. The placenta is formed from the fusion of the allantois at fetus side and the chorion which exists in the place where the placenta will be formed later, and the umbilical cord is also formed. The allantois in the mouse is a site of definitive erythropoiesis [34].

In the mouse placenta, hematopoietic activity of adult type erythroid progenitors and myeloid progenitors is detected from E10 and lasts until E17 [35]. The HPP-CFC and HSC activities are identified around E12.5 in placenta. Thus, it suggests that placenta plays a role in producing HSPCs and supplying them to the fetal liver as well as the AGM region. Similar to AGM region, IACs are also observed in placenta and peak at E11.5 [31]. Utilizing laser-micro-dissection technology, we previously collected niche cells surrounding IACs and investigated the gene expression of various hematopoietic factors by these supporting cells (Fig. 2A). Expression of *Scf* gene was detected, whereas *Tpo*, *Flt3l*, *Il3*, *Il6*, *Il11*, *Csf2*, *Csf3* and *Epo* were not expressed in the placenta. In addition, measurement of SCF protein amount in the cells fractionated by flow cytometry and immunohistochemical analysis revealed that vascular endothelial cells highly express SCF at protein level [31]. When

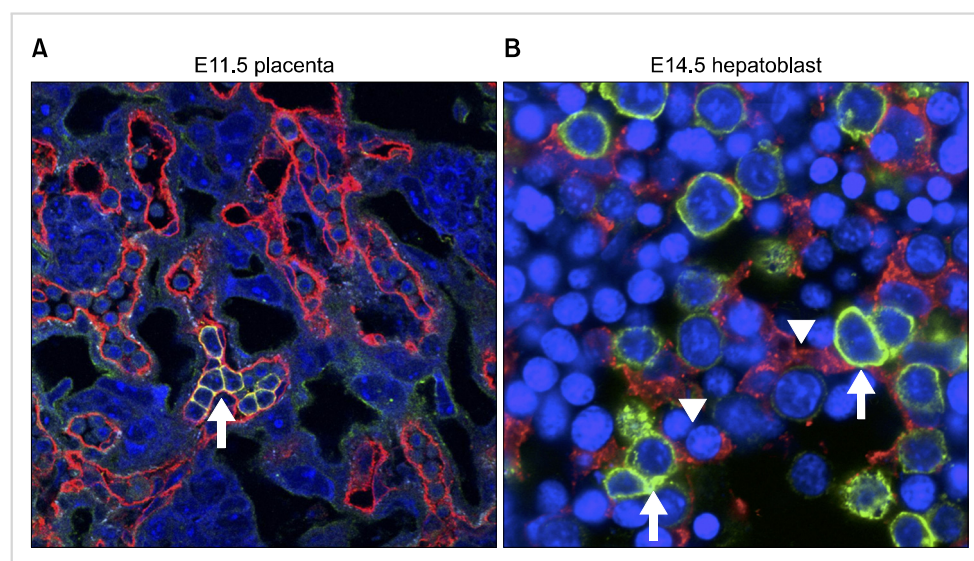


Fig. 2. Embryonic hematopoietic niche. (A) Hematopoietic stem cells (arrow) are surrounded by the endothelial cells. Green: KIT, Red: CD31. (B) Hematopoietic stem/progenitor cells (arrows) are surrounded by hepatoblasts (arrow heads). Green: KIT, Red: DLK1.

neutralizing antibody against c-Kit was injected into embryos, hematopoietic genes such as *Runx1*, *Myb* and *Gata2* were partially down-regulated in HSPCs, implying the existence of regulatory mechanisms other than SCF/c-Kit [31]. Platelet-Derived Growth Factor (PDGF)- β secreted by vascular endothelial cells down-regulates the gene expression of Epo, which is crucial for erythropoiesis. Therefore, it is likely that PDGF- β secreted from endothelial cells regulates erythropoiesis indirectly [36]. Since trophoblasts do not express EPO during normal hematopoiesis, this regulation might occur under conditions with altered PDGF- β /PDGFR signaling.

Fetal liver

The fetal liver (FL) acts as a site for HSPC expansion as well as a site of differentiation of myeloid, lymphoid, definitive erythroid and megakaryocyte progenitors. HSPCs generated in the yolk sac, AGM region and placenta are thought to migrate and home to FL from E9.5 [37–42]. Hematopoietic activity in FL begins shortly after liver development commences at E10.5 and is active at E11.5. From E12.5 until E16.5, HSCs increase the number of HSPCs in FL through self-renewal and also differentiating into progenitors [43]. These progenitors then differentiate into various types of mature blood cells, including vast numbers of adult-type erythrocytes.

The FL also contains non-hematopoietic cells, such as developing hepatoblasts (hepatocyte progenitor cells), sinusoidal angioblasts and endothelial cells, and hepatic stellate cells. Although the size and weight of the liver increases along with the developmental stage, the proportion of hematopoietic cells reaches to the peak (74%) at E13–14. The liver then shifts to a more hepatic function and hematopoietic activity decreases [44]. Oval hematopoietic foci, approximately 35–70 \times 20–40 μ m in size, were observed by Sasaki and Sonoda [43]. These microenvironments contain supportive hepatoblasts and also possess an erythroblastic island at the center. The erythroblastic islands (EBIs) are multicellular clusters of maturing erythroid progenitors surrounding a supportive central macrophage. The central macrophage supplies iron which is required for the hemoglobin synthesis of erythroblasts, phagocytes and removes aged erythrocytes [45]. The central macrophage also engulfs and destroys erythroid nuclei, expelled during enucleation. Thus, the erythroblastic island is also regarded as a critical hematopoietic niche.

Hepatoblasts differentiate into hepatic cells and biliary epithelial cells and express Delta-like 1 homolog (DLK1) [39]. DLK1⁺ hepatoblasts can be found adjacent to the HSPCs expressing the stem cell factor receptor c-Kit (Fig. 2B). Previously, we investigated the gene expression of hematopoietic factors in fractionated FL stromal populations including DLK1⁺ hepatoblasts, sinusoidal endothelial cells expressing Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE1)⁺ and CD31⁺, and CD45⁺/Ter119⁺ hematopoietic cells. In the FL at E12.5 and E14.5, genes encoding the cytokines Scf, Tpo, Flt3l and Epo were highly expressed;

however, the expression of Il3 which promotes erythropoiesis was not detected. The DLK1⁺ hepatoblasts expressed high levels of Scf, Tpo and Epo. LYVE1⁺/CD31⁺ sinusoidal endothelial cells expressed high levels of Scf whereas CD45⁺/Ter119⁺ hematopoietic cells expressed high levels of Flt3l. In addition, protein expression of SCF was detected in DLK1⁺ hepatoblasts and LYVE1⁺/CD31⁺ sinusoidal endothelial cells. These observations suggest that through the relatively uniform secretion of SCF, HSPC expressing the appropriate receptors can proliferate and differentiate. The expression of EPO is restricted to the DLK1⁺ hepatoblasts and its expression is 9.1-fold higher than that of SCF. Since the erythroblastic island is found in close proximity to hepatoblasts, this niche structure and expression pattern of hematopoietic factors is highly supportive of erythropoiesis. Evidence supporting a role of hepatoblasts comes from *Map2k4*-deficient mice which lack hepatoblasts [39]. In *Map2k4*-deficient animals, the frequency of hematopoietic cell is reduced to half of the number observed in wild type mouse embryos. The Ki-67⁺ proliferating hematopoietic cells are also reduced in *Map2k4*-deficient mouse embryos [39]. Hepatoblasts play a role in supporting the proliferation of hematopoietic cells. At E15.5, membrane-bound SCF⁺/DLK1⁺ hepatoblasts express *Tpo*, *Scf*, *Angptl3* and *Igf2* genes, indicating that hepatoblast is also important for maintenance and proliferation of HSC [46].

Integrin β 1, also known as CD29, is highly expressed on the surface of FL hematopoietic cells. Functionally, Integrin β 1 is essential for FL hematopoiesis. In chimeric mouse embryos created from stem cells lacking or possessing both copies of the gene encoding β 1-integrin, all of the FL hematopoietic cells were β 1-integrin⁺: no FL hematopoietic cell were derived from the β 1-integrin-deficient progenitors [47]. Integrins are composed of α - and β -chain and form dimers to adhere with extracellular matrix. Therefore, using the DLK1⁺ hepatoblasts, LYVE1⁺/CD31⁺ sinusoidal endothelial cells, and CD45⁺/Ter119⁺ hematopoietic cells collected from FL by utilizing flow cytometry, the expression of genes encoding components of the extracellular matrix was investigated [48]. Based on the combination of α - and β -chain, we further screened possible adhesion partner genes (*Lama1*, *Lamb1*, *Lamb2*, *Lamb3*, *Lamc1*, *Fnl*, *Vtn* and *Tnc*). These entire extracellular matrix genes are expressed in the FL at E12.5 and E14.5, and *Vtn* and *Fnl* were highly expressed in particular. DLK1⁺ hepatoblasts highly expressed all of these extracellular matrix genes. In addition, fibronectin and vitronectin protein were highly expressed in DLK1⁺ hepatoblasts at E12.5. Since the expression of fibronectin and vitronectin proteins were decreased in the *Map2k4* null mutant mouse embryos, it appears that the hepatoblast is a critical source of extracellular matrix consolidating the hematopoietic microenvironment and promoting differentiation. Therefore, it is suggested that hepatoblasts not only keep hematopoietic cells in the liver through extracellular matrix, but also regulate the differentiation of HSPCs. A portion of membrane bound SCF⁺ hepatoblasts express Chemokine (C-X-C motif) ligand 12 (CXCL12) protein and may be in-

volved in the migration and homing of hematopoietic cells [39]. Self-renewal potential of HSCs is maintained when Protein C Receptor, Endothelial (EPCR)⁺ HSCs are co-cultured with LYVE1⁺ sinusoidal endothelial cells [49].

Fetal spleen

The spleen is one of the hematopoietic organs which bridges hematopoiesis between the fetal liver and bone marrow. The spleen serves as a location for adult type erythropoiesis; however, little is known about the fetal spleen as a hematopoietic niche. Therefore, we analyzed erythropoiesis in the fetal spleen utilizing flow cytometry and found that the number of erythroid cells in spleen increases 18.6-fold from E16.5 to E19.5 [49]. Mesenchymal cells expressing CD51 promote erythropoiesis through the secretion of the hematopoietic factors including SCF and IGF1. In the fetal spleen at E16.5, SCF and IGF1 are highly expressed, whereas the expression of EPO, which is the most relevant factor for erythropoiesis, is not detected. EPO is expressed in the FL at the same developmental stage. Expression of EPO is detected in the spleen at E19.5, which indicates that adult-type erythropoiesis in mouse embryo shifts from liver to spleen as the embryo develops.

Skeletal muscle/Bone marrow

HSPCs proliferate in fetal liver and spleen, and then home to the fetal bone marrow at E19.5, just before birth to initiate adult-type bone marrow hematopoiesis. Discrepancy between vascularization of fetal bone marrow (E17.5) and HSPCs suggests that HSCs remain in the FL or other tissues [50]. Through detailed immunohistochemical observation of c-Kit expression from E14.5 to E19.5, we found that c-Kit⁺ HSPCs accumulate in the skeletal muscle tissue near the long bones [5]. We tracked the migration of muscle HSPCs utilizing exo-utero surgery and found that muscle HSPCs derive from FL and a subset of these HSPC migrate into the bone marrow. However, the physiological meaning regarding the role of muscle as a hematopoietic niche remains unclear and further analysis is ongoing.

Ex vivo expansion of human HSC

Perhaps the greatest example of bioengineering we have is the mammalian embryo, which develops from a sperm fused to an oocyte and within weeks will give rise to a complex organism. What knowledge can we obtain from developmental biology, and in particular, from analyzing hematopoietic development in the embryo which is of clinical relevance? One of the greatest challenges of modern hematology is the *ex vivo* expansion of human cord blood HSC as this has the potential to secure the safety of donors and solve the donor shortage in HSC transplantation for hematopoietic diseases [51, 52]. Various methods have been reported to improve the *ex vivo* expansion of human cord blood derived HSCs. In the early attempts, many researchers tried to identify the combinations of cytokines to support the commitment of lineage cells to maintain and expand HSC [52-54]. Stem cell factor (SCF), FMS-related tyrosine kinase

3 (FLT3/FLT2) ligand, Interleukin (IL)-3, IL-6, soluble IL-6 receptor, granulocyte-colony stimulating factor (G-CSF) and thrombopoietin (TPO) were used in combination to expand HSCs [55-58]. However, those expanded cells could not achieve long-term reconstitution in some patients, and large-scale indefinite *ex vivo* expansion of HSC is currently not feasible for clinical application [59-61].

As a product from our research in fetal liver hematopoiesis, we identified a bioactive peptide KS-13 (PCT/JP2010/067011) which promotes the proliferation of HSCs *ex vivo*. The KS-13 was derived from the extracellular domain of DLK1⁺, which expressed on HSC niche such as hematoblasts. We have further developed modified-KS-13 (2014-070968) to enhance uptake into cells. By combining these peptides with existing HSC culture protocol, we confirmed that KS-13 and modified-KS-13 increased the number of CD34⁺/CD38⁻ HSCs over 50-fold within several days. Transplantation of higher numbers of CD34⁺ HSCs reportedly improves clinical outcomes [62-64], and our results showed that peptides might provide new aspect to ameliorate current method for *ex vivo* HSC expansion.

Pluripotent stem cells, both embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, are potential sources for HSCs [65-68]. Pluripotent stem cells are also an excellent *in vitro* model to study the mechanisms which underlay embryonic hematopoietic development. The ES/iPS technology potentially ameliorates significant technical hurdles that allotransplantation faces including rejection, infection and risks to donors. However, new technical challenges have arisen including difficulties in differentiating reconstituting hematopoietic stem cells from iPS and the cost of generating such cells *in vitro*.

Conclusion and future prospective

While the investigation of the processes controlling blood production in the mammalian embryo and fetus may appear esoteric, there is a direct link to clinical relevance. Although tremendous effort has been made to expand HSC *ex vivo*, this has proven difficult. HSC *ex vivo* expansion promises to improve the safety and efficacy of HSC in clinical use. However, current protocols do not allow for the long-term *ex vivo* expansion of HSC while maintaining long-term reconstituting activity. HSC are useful not only for clinical transplantation but promise to be useful in the treatment of genetic hematological diseases such as sickle cell anemia and beta-thalassemia. If HSC can be expanded *ex vivo*, the genetic lesion causing the hematological pathology can be corrected *in vitro* and autologously transplanted. The HSC lacking the genetic lesion can then home to the bone marrow and expand, generating healthy mature blood cells. Finally, pluripotent stem cells such as ES and iPS cells are yet to be generated in clinically useful quantities for long-term reconstitution. Perhaps the missing factor is a signal generated from the embryonic hematopoietic niches. Differentiation of patient-specific iPS into clinically effective quantities of HSC *in vitro* would have a profound effect on clinical hematology and oncology. Thus, resolving the molecules

required in the very first stages of blood cell production will be of great utility to clinical medicine in the near future.

ACKNOWLEDGMENTS

We thank Dr. Nakanishi, director of Center for Clinical and Translational Research, Kyushu university, for supports. We also thank Ms. Takahara and MS. Tomoi for technical supports.

Authors' Disclosures of Potential Conflicts of Interest

Dr. Sugiyama holds shares of Science Lustre Co., Ltd. which sells KS-13 peptide.

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