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

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

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

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

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

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Author contributions

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

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Appendix A. Supplementary data

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

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REVIEW

Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells

Wai Feng Lim[1,2*†], Tomoko Inoue-Yokoo[1,3†], Keai Sinn Tan[1,2], Mei I Lai[2] and Daisuke Sugiyama[1*]

Abstract

Pluripotent stem cells, both embryonic stem cells and induced pluripotent stem cells, are undifferentiated cells that can self-renew and potentially differentiate into all hematopoietic lineages, such as hematopoietic stem cells (HSCs), hematopoietic progenitor cells and mature hematopoietic cells in the presence of a suitable culture system. Establishment of pluripotent stem cells provides a comprehensive model to study early hematopoietic development and has emerged as a powerful research tool to explore regenerative medicine. Nowadays, HSC transplantation and hematopoietic cell transfusion have successfully cured some patients, especially in malignant hematological diseases. Owing to a shortage of donors and a limited number of the cells, hematopoietic cell induction from pluripotent stem cells has been regarded as an alternative source of HSCs and mature hematopoietic cells for intended therapeutic purposes. Pluripotent stem cells are therefore extensively utilized to facilitate better understanding in hematopoietic development by recapitulating embryonic development *in vivo*, in which efficient strategies can be easily designed and deployed for the generation of hematopoietic lineages *in vitro*. We hereby review the current progress of hematopoietic cell induction from embryonic stem/induced pluripotent stem cells.

Introduction

Hematopoietic stem cells (HSCs) lay the foundation of hematopoiesis to generate all functional hematopoietic lineages, including erythrocytes, leukocytes (neutrophils, basophils, eosinophils, lymphocytes, monocytes and macrophages) and platelets [1]. Perturbations in the hematopoietic system have been reported to cause numerous diseases such as anemia, leukemia, lymphomas and thrombocytopenia. Currently, HSC transplantation and hematopoietic cell transfusion are useful treatments for some hematological diseases, such as thalassemia and leukemia. However, some conventional sources of HSCs – such as cord blood, bone marrow and peripheral blood – are limited in usage, owing to the limited number of transplantable cells as well as inefficient strategies for expanding those cells. Additionally, problems such as shortage of human leukocyte antigen-matched donors, adverse effects from graft-versus-host disease, viral contamination and immunoreactions impede the utility of readily available HSCs. The use of alternative sources for these cells will therefore be a great advantage for regenerative medicine.

Pluripotent stem cells are one of the potential sources for HSCs and the *in vitro* model for further elucidating the regulatory mechanisms underlying embryonic hematopoietic development. Embryonic stem (ES) cells are pluripotent cells established from the inner cell mass of blastocyst-stage embryos, in both mouse and human [2,3], and are capable of giving rise to three germ layers after directed differentiation in culture [3,4]. However, manipulation of human ES cells raises some ethical issues and immunoreactions. Induced pluripotent stem (iPS) cell technology has made a groundbreaking discovery to circumvent the problems of ethical and practical issues in using ES cells [5]. It is of great importance to develop efficient and controllable induction strategies to drive hematopoietic differentiation from ES/iPS cells in culture prior to the realization of pluripotent cell-derived therapies. To review current progress of

* Correspondence: limwaifeng85@gmail.com; ds-mons@yb3.so-net.ne.jp
†Equal contributors
[1]Department of Advanced Medical Initiatives, Division of Hematopoietic Stem Cells, Advanced Medical Initiatives, Faculty of Medical Sciences, Kyushu University, Station for Collaborative Research 1, 4F, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan
[2]Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia
Full list of author information is available at the end of the article

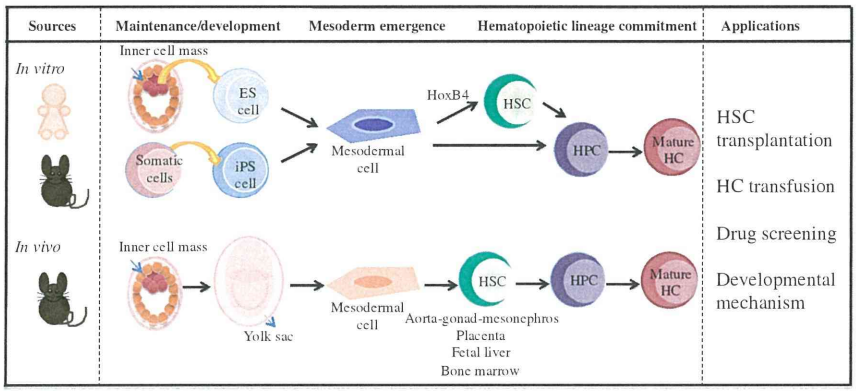

Figure 1 Schematic representations of hematopoietic development from *in vivo* and *in vitro* models. Both human and mouse *in vitro* models have been established for hematopoietic differentiation in a defined culture system from embryonic stem (ES) and adult cell-derived induced pluripotent stem (iPS) cells. For the *in vivo* model, the mouse inner cell mass undergoes differentiation, later forming the yolk sac, which generates mesodermal cells and induces hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and mature hematopoietic cells (HCs). Successfully generated HSCs from both *in vitro* and *in vivo* models might be applied to HSC transplantation for hematopoietic disorders. Further differentiation of HSC in a cytokine-defined culture system produces hematopoietic cells for hematopoietic cell transfusion. Thorough understanding of molecular mechanism on these models will be beneficial for both drug screening as well as the mechanism of hematopoiesis development.

differentiation protocol from ES/iPS cells, we first summarize the knowledge of hematopoietic development during early mouse hematopoiesis followed by the manipulation of ES/iPS cells in hematopoietic cell induction (Figure 1).

Embryonic hematopoiesis

Studies of hematopoietic development during embryogenesis *in vivo* are important to gain insight into its underlying mechanisms, whereby accumulated knowledge will facilitate the induction of HSCs, hematopoietic progenitor cells (HPCs) and mature hematopoietic cells from pluripotent stem cells in culture. In mouse blastocyst, the inner cell mass at 3.5 days post coitum (dpc) comprises a population of cells – which can give rise to a derivative of three germ layers (endoderm, mesoderm and ectoderm) – that eventually develop into both intraembryonic and extraembryonic tissues as embryo develops [6]. The hematopoietic system that derives from the mesodermal germ layer can be classified into two waves. The first hematopoiesis (primitive hematopoiesis) begins to develop primitive erythroid and macrophage progenitors in the yolk sac (YS) blood islands at 7.0 dpc [7]. Para-aortic splanchnopleural regions that will develop into aorta–gonad–mesonephros (AGM) already possess hematopoietic precursors beginning at 8.5 dpc [8]. Before the establishment of circulation (8.0 dpc), both YS and para-aortic splanchnopleural-derived mesodermal cells acquire HSC activity after co-culturing with AGM-derived stromal cells [9]. After circulation commences, $CD34^{+}c\text{-}Kit^{+}$ cells derived from both YS and para-aortic splanchnopleura at 9.0 dpc were able to reconstitute the hematopoietic system in newborn recipient pups, but not in adult recipient mice [10]. These findings demonstrate that both YS and para-aortic splanchnopleura possess HSC potential that can contribute to definitive hematopoiesis under a favorable microenvironment.

The first definitive HSCs that can reconstitute the adult hematopoietic system appear in the AGM region at 10.5 dpc followed by the YS, placenta and liver, spanning from 11.0 to 11.5 dpc [11-13]. YS cells expressing *Runx1* at 7.5 dpc progressed into fetal lymphoid progenitors at 16.5 dpc in both fetal liver and thymus as well as adult HSCs in 9-month-old to 12-month-old mouse bone marrow [14]. In view of these results, both the YS and the AGM region contribute to HSC generation. However the extent of their contribution still remains unclear. To address this issue, YS–YS chimeric embryos were generated before blood circulation at 8.25 dpc, where no B-cell activity was detected, which is relevant to HSC activity in the early mouse embryo. As the chimeric embryos develop into 11.0 dpc equivalent in whole embryo culture, the grafted YS cells contributed to B-cell activity in the AGM region, but with low frequency [15]. This observation implies that the main source of HSCs is derived from the AGM region.

In addition to the YS and the AGM region, the placenta is another site for HSC generation. The placenta exchanges oxygen and nutrient between mother and fetus, and is formed around 9.0 dpc after fusion of chorion and allantois. A fluorescent-labeled allantois region at 8.25 dpc could be detected in the hematopoietic cell cluster after 42 hours of whole embryo culture, suggesting that allantois alone possesses HSC potential

and also proposing that the placenta is likely to be an ixndependent site of HSC generation, regardless of cells circulating from other hematopoietic organs [16]. Taken together, the YS, AGM region and placenta contribute to HSC generation prior to the HSC expansion in the fetal liver .

Gene and protein markers for mesodermal and hematopoietic lineages

During early embryonic development, *Brachyury* that is indispensable for mesodermal formation expresses transiently [17]. Subsequently tyrosine kinase *Flk1* for blood-island formation and vasculogenesis expresses in hemangioblasts, which is the common embryonic endothelial and hematopoietic precursor [18].

Transcription factors characterized to be involved in the hematopoietic lineages are shown below. In the transition of mesodermal to hematopoietic lineages, *Scl* is indispensable for the development of all hematopoietic lineages, in which a homozygous mutant $Scl^{-/-}$ model showed undetectable hematopoietic lineages in both *in vivo* and *in vitro* studies [19]. *Runx1* plays an important role in governing definitive hematopoiesis but not primitive hematopoiesis through the observation of blast colony-forming cells, which are both hematopoietic and endothelial cell precursors from ES cells and equivalent of hemangioblast, using a $Runx1^{-/-}$ ES cell culture model [20]. The GATA family of transcription factors, especially GATA-1 and GATA-2, are differentially regulated at multiple steps from the early development of hemangioblast to hematopoietic commitment [21].

Surface markers of hematopoietic lineages are briefly described. Cells expressing endothelial markers, such as CD34, PECAM-1 (CD31) and VE-cadherin, are closely associated with both hematopoietic and endothelial cell commitment [22]. Expression of CD41 initiates the primitive and definitive hematopoiesis, whereas HPCs are highly dependent on c-Kit expression [23]. Additionally, CD34 as a marker for HSCs and CD45 as a marker for pan-leukocyte and/or maturation of HSCs are utilized respectively [24,25].

Differentiation of embryonic stem/induced pluripotent stem cells and hematopoietic cell induction

Mouse ES/iPS cells can be maintained and propagated indefinitely on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) and/or leukemia inhibitory factor in culture. On the contrary, human ES cells achieve an undifferentiated state on a layer of MEFs and/or basic fibroblast growth factor. After removal of leukemia inhibitory factor and basic fibroblast growth factor, these ES cells can differentiate into any kind of cell depending on appropriate culture conditions. One of the ultimate goals of *in vitro* differentiation of ES/iPS cells is to generate HSCs with the acquisition of long-term reconstitution capacity.

By recapitulating mouse embryogenesis, *in vitro* differentiation of ES/iPS cells toward hematopoietic development has been technically approached: through formation of a cystic-like structure that exhibits three germ layers that are able to generate mesodermal cells (embryoid body (EB) formation); through feeder cell co-culture that can provide ES/iPS cells an intimate cell contact with secreting factor(s) that promote cell proliferation and differentiation (feeder cell co-culture); and through an extracellular matrix-coated dish that supports differentiation of ES/iPS cells (Figure 2).

Embryoid body formation

EBs are three-dimensional cell aggregates formed in suspension culture and they mimic the spatial organization of the embryo through enhancing cell–cell interactions to form three germ layers [3,4]. There are a number of approaches to EB formation, such as methylcellulose or suspension culture medium either using a bacterial-grade dish, a methylcellulose-coated plate, a low-adherent micro-well plate or the hanging drops method. To specifically induce a hematopoietic lineage, single-cell suspension of EBs are directed into methylcellulose culture medium that functions to support hematopoietic development in the presence of hematopoietic cytokines, such as stem cell factor (SCF), granulocyte colony-stimulating factor (CSF), macrophage CSF, granulocyte–macrophage CSF, erythropoietin (Epo) and interleukins (ILs) such as IL-1, IL-3, IL-4 and IL-6 [26]. In the absence of cytokines in culture, EB cells themselves reportedly possess the capability to secrete proteins having macrophage CSF, IL-3 and Epo activity in order to support the early development of erythroid and macrophage precursors [27].

Using mouse ES cells, 75% of blast colonies were generated from EBs of methylcellulose culture consisting of bipotential precursors (hematopoietic and endothelial cells) as blast colony-forming cells at day 2.5, and then decreased at day 4 (<15%) [28]. In mouse ES cells with GFP targeted to the *Brachyury*, EB formation at day 2.5 in suspension culture gave rise to $GFP^{-}Flk1^{-}$, $GFP^{+}Flk1^{-}$ and $GFP^{+}Flk1^{+}$ distinct populations, which are comparable with the developmental progression ranging from pre-mesoderm to pre-hemangioblast mesoderm to the hemangioblast, respectively [29].

EBs derived from human ES cells formed three embryonic germ layers that can be identified using the following markers: ζ-globin, neurofilament 68Kd and α-fetoprotein [30]. By differentiating human ES cells through EBs at day 3.0 to 4.0, blast colony-forming cells were generated in liquid culture and expressed *FLK1*, *BRACHYURY* and *SCL* genes [31]. Differentiation of

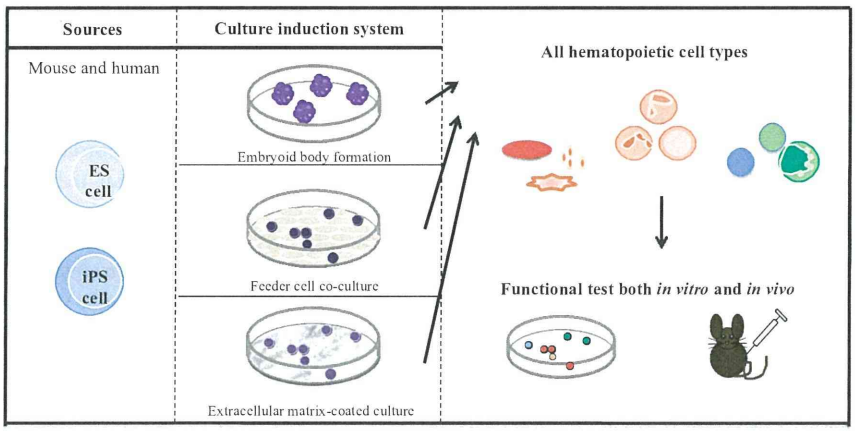

Figure 2 Schematic representations of induction systems and criteria for successful hematopoietic development. Both mouse and human embryonic stem (ES)/induced pluripotent stem (iPS) cells can be differentiated into hematopoietic cells (HCs) from mesodermal cells with three approaches: embryoid body 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 $Mac\text{-}1^+$ 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 $Mac\text{-}1^+$ cells (16% in the OP9 system) and 19.1% of $c\text{-}Kit^+$ 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 ($Sca\text{-}1^+$, 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 ($B220^-Mac\text{-}1^-$ $JORO75^-Ter119^-$ (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.

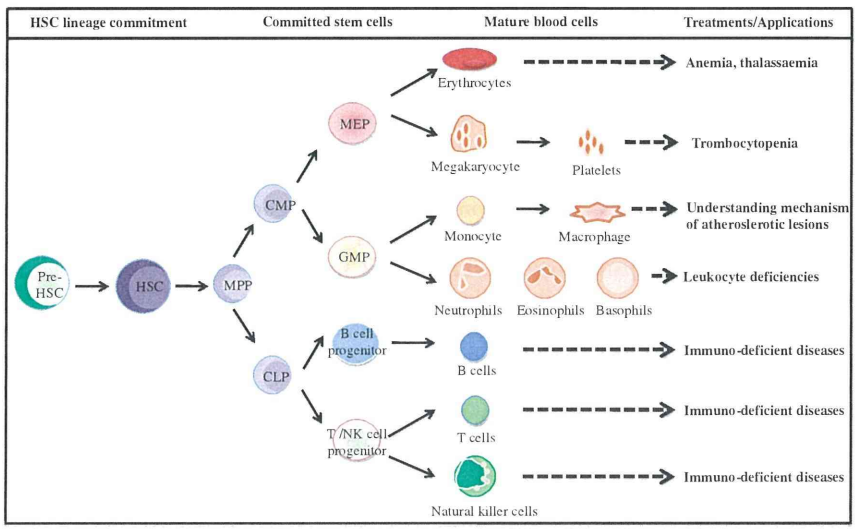

Figure 3 Schematic representations of each hematopoietic cell lineage with respect to their applications and disease-treatment potentials. After pre-hematopoietic stem cells (HSCs) commit to mature HSCs, multipotent progenitor (MPP) cells are generated with the potential to further differentiate into 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, $c\text{-}Kit^+CD41^+$ 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 co-culture 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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Author details

[1]Department of Advanced Medical Initiatives, Division of Hematopoietic Stem Cells, Advanced Medical Initiatives, Faculty of Medical Sciences, Kyushu University, Station for Collaborative Research 1, 4F, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan. [2]Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia. [3]Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan.

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Metabolism:

Deficiency of Nicotinamide Mononucleotide Adenylyltransferase 3 (Nmnat3) Causes Hemolytic Anemia by Altering the Glycolytic Flow in Mature Erythrocytes

Keisuke Hikosaka, Masashi Ikutani, Masayuki Shito, Kohei Kazuma, Maryam Gulshan, Yoshinori Nagai, Kiyoshi Takatsu, Katsuhiro Konno, Kazuyuki Tobe, Hitoshi Kanno and Takashi Nakagawa

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Deficiency of Nicotinamide Mononucleotide Adenylyltransferase 3 (Nmnat3) Causes Hemolytic Anemia by Altering the Glycolytic Flow in Mature Erythrocytes

Keisuke Hikosaka[1], Masashi Ikutani[2], Masayuki Shito[5], Kohei Kazuma[4], Maryam Gulshan[1,3], Yoshinori Nagai[2,8], Kiyoshi Takatsu[2,7], Katsuhiro Konno[4], Kazuyuki Tobe[3], Hitoshi Kanno[5,6] and Takashi Nakagawa[1*]

[1]Frontier Research Core for Life Sciences; [2]Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research; [3]The First Department of Internal Medicine, Graduate School of Medicine and Pharmaceutical Science for Research; [4]Institute of Natural Medicine, University of Toyama, Toyama 930-0194, Japan; [5]Department of Transfusion Medicine and Cell Processing; [6]Department of Advanced Biomedical Engineering and Science, Graduate School of Medicine, Tokyo Women's Medical University, Tokyo 162-8666, Japan; [7]Toyama Prefectural Institute for Pharmaceutical Research, Toyama 939-0363, Japan; [8]JST, PRESTO, Saitama 332-0012, Japan

Running Title: Nmnat3 deficiency causes hemolytic anemia

Address Correspondence to: Takashi Nakagawa; Frontier Research Core for Life Sciences, University of Toyama; 2630 Sugitani, Toyama, Toyama 930-0194, Japan; Phone: +81-76-415-8849; FAX: +81-76-415-8849; E-mail: nakagawa@med.u-toyama.ac.jp

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Background: Nmnat3 is considered a mitochondria-localized NAD synthesis enzyme. However, its physiological function *in vivo* remains unclear.
Results: Loss of Nmnat3 results in drastic depletion of the NAD pool and stalls the glycolytic flow in mature erythrocytes.
Conclusion: Nmnat3 deficiency causes splenomegaly and hemolytic anemia in mice.
Significance: This report reveals the essential role of Nmnat3 in mature erythrocytes.

ABSTRACT

NAD biosynthesis is of substantial interest owing to its important roles in regulating various biological processes. Nicotinamide mononucleotide adenylyltransferase 3 (Nmnat3) is considered a mitochondria-localized NAD synthesis enzyme involved in *de novo* and salvage pathways. Although the biochemical properties of Nmnat3 are well documented, its physiological function *in vivo* remains unclear. In this study, we demonstrated that Nmnat3 was localized in the cytoplasm of mature erythrocytes and critically regulated their NAD pool. Deficiency of Nmnat3 in mice caused splenomegaly and hemolytic anemia, which was associated with the findings that Nmnat3-deficient erythrocytes had markedly lower ATP levels and shortened lifespans. However, NAD level in other tissues were not apparently affected by the deficiency of Nmnat3. LC-MS/MS based metabolomics revealed that the glycolysis pathway in Nmnat3-deficient erythrocytes was blocked at glyceraldehyde 3-phosphate dehydrogenase (GAPDH) step because of the shortage of co-enzyme NAD. Stable isotope tracer analysis further demonstrated that deficiency of Nmnat3 resulted in glycolysis stall and a shift to pentose phosphate pathway. Our

findings indicate the critical roles of Nmnat3 in maintenance of the NAD pool in mature erythrocytes and the physiological impacts at its absence in mice.

Nicotinamide adenine dinucleotide (NAD) is an important coenzyme involved in numerous metabolic enzymatic reactions including glycolysis, β-oxidation, and the tricarboxylic acid (TCA) cycle (1). NAD also serves as a substrate for poly (ADP-ribose) polymerases (PARPs) and the class III NAD-dependent deacetylases (sirtuins), and plays key roles in diverse biological processes in response to cellular stresses, including genotoxic and nutrient stress (2). The NAD level in the cell is vital for facilitating sirtuin functions as energy sensors: upon fasting or calorie restriction (3). In particular, sirtuins deacetylate central metabolic enzymes and directly control their enzymatic activities (4,5). In addition, nuclear sirtuins can indirectly regulate central metabolic pathways by deacetylating diverse transcriptional factors and cofactors including FOXO, PPAR, and PGC1α (6-8). Recent studies indicate that NAD metabolism also regulates various biological processes via NAD-dependent deacetylase SIRT1 (9-11). However, there is no direct evidence that NAD metabolism can regulate metabolic enzymes that requires NAD as a coenzyme.

In organisms, NAD can be synthesized via a *de novo* synthesis pathway or a salvage pathway (12). In the salvage pathway, Nicotinamide phosphoribosyltransferase (Nampt) converts nicotinamide (NAM) and phosphoribosyl pyrophosphate (PRPP) to nicotinamide mononucleotide (NMN). Then, nicotinamide mononucleotide adenylyltransferase (Nmnat) transfers the adenylyl moiety from ATP to NMN for generating NAD (12). In mammals, there are three Nmnat isoforms (Nmnat1–3) with different subcellular localizations and tissue distributions (13,14). Nmnat1, Nmnat2, and Nmnat3 are considered to be localized in the nucleus, Golgi apparatus, and mitochondria, respectively. Nmnat1 has the most robust enzymatic activity of the three isoforms (13). A recent genetic study revealed that *Nmnat1* gene mutations cause Leber congenital amaurosis, a rare hereditary blindness (15-18). *Nmnat1* is also identified as a fusion gene with *Ube4b* in Wallerian degeneration slow (WldS) mice, which exhibit drastic delays in injured axonal clearance (19). Indeed, Nmnat1 overexpression in mice is protective against sciatic nerve injury (20). Although Nmnat1 has important roles in central and peripheral nervous system, its protein expression level is relatively low compared with that of Nmnat2 (21). Several papers have suggested the implication of Nmnat2 in axonal cell survival and protection (22,23). Nmnat1 and Nmnat2 also have essential roles in axonal growth and survival during embryogenesis as their deficiency in mice results in embryonic lethal. (21,24,25).

Nmnat3 has been considered to be localized in mitochondria and to have a pivotal role in regulation of mitochondrial NAD level (13,26). Despite the importance of NAD metabolism in mitochondria, the role of Nmnat3 *in vivo* is still unclear. Nmnat3 overexpression confers robust protection against axon injury *in vitro* and *in vivo* as well as WldS chimeric protein (27,28). Although the significance of Nmnat3 in axonal protection is evident, the molecular mechanism is still unknown, as for the case of Nmnat1.

It has long been suspected if red blood cells (RBCs) possess NAD synthesis activity (29,30). Several reports have indicated that RBCs show Nmnat-like activity (31-34), but the actual identity of responsible enzymes has been uncertain. Magni's group, employing a Nmnat discrimination assay, found Nmnat3-specific activity in human RBCs (35). However, given that RBCs have no mitochondria in cells, it has not been clarified whether Nmnat3 has a physiological function in RBCs. In this study, we found that Nmnat3-deficient mice exhibited splenomegaly and hemolytic anemia resulting from a glycolysis pathway blockade at glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Our findings revealed unexpected roles of Nmnat3 in the maintenance of the NAD pool in mature erythrocytes and their lifespan regulation.

EXPERIMENTAL PROCEDURES

Generation of Nmnat3 gene trap mice - Nmnat3 gene-trap heterozygous frozen embryo was

obtained from TG Resource Bank (Transgenic Inc. Japan) and embryonic transfer was performed to get gene-trap heterozygous mice. To determine the precise inserted location of gene-trap cassette in the *Nmnat3* gene, the genomic walking was carried out using GenomeWalker Universal Kit (Takara, Japan). After confirming the accurate insertion point of gene-trap cassette, we designed primer sets for genotyping as shown in Table 1. Nmnat3 gene-trap homozygous (Nmnat3$^{gt/gt}$) mice were obtained by crossing heterozygous mice. Mice were maintained under controlled temperature and standard light conditions (12h:12h light-dark cycle) and were allowed free access to water and food. All animal experiments were approved by the Animal Experiment Committee at University of Toyama and were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals at University of Toyama, which were based on international policies.

Real-time quantitative PCR (qPCR) - Total RNAs were extracted from mice tissues using TRI Reagent (Molecular Research Center, Inc.). cDNA was prepared using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) according to the supplier's protocol. Real-time PCR was carried out using THUNDERBIRD SYBR qPCR Mix (Toyobo) on Thermal Cycler Dice Real Time System II (Takara). Quantification was done by Delta Delta Ct method, and *Rpl13a* or *B2m* gene was used as a reference gene. Primers used in qPCR are listed in Table 1.

Preparation of mature erythrocytes - Mature erythrocytes were separated by a Percoll gradient method according to a standard protocol (36). Briefly, Percoll solution of densities at 1.096 and 1.058 g/ml were prepared. 2 ml of Percoll solution (density, 1.096 g/ml) was added at the bottom, followed by 1 ml of Percoll (density, 1.058 g/ml) layered on top. Finally, 1 ml of whole blood was applied onto the gradient, followed by centrifuge at 250 x *g* for 30 min. Mature erythrocytes packed in the bottom of layer were collected carefully and washed with ice-cold saline.

Western blotting experiments - Mature erythrocytes isolated by a Percoll gradient were lysed with NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, and 50 mM Tris-HCl, pH 7.4), and were subjected to Western blotting. For fractionation experiments, hemolysed samples were used as cytoplasm fraction and ghost was used as membrane fraction. Whole blood was centrifuged at 1,000 x *g* for 5 min at room temperature, and then plasma and buffy coat were discarded. Erythrocytes were lysed with 5 mM sodium phosphate buffer (pH 8.0) on ice for 30 min and centrifuged at 20,000 x *g* for 20 min at 4 °C. The supernatant was used as hemolysed sample. The pellet was washed with 5 mM sodium phosphate buffer (pH 8.0) twice and used as ghost fraction. Antibodies used for Western blotting experiments included Nmnat1 (Novus), Nmnat2 (Sigma), GAPDH (Sigma), PFK (Abcam), β-actin (Cell Signaling), Nampt (Bethyl), NADK (Santa Cruz), β-tubulin (Cell Signaling), Tom20 (Santa Cruz) and acetyl-lysine (Abcam and Cell Signaling). Anti-mouse Nmnat3 rat monoclonal antibody (clone R88) was raised against His-tagged full-length mouse Nmnat3 recombinant protein and spleen cells were used to make hybridoma cells by polyethylene glycol method. For Western blotting, hybridoma supernatant was used at 100 times dilution. HRP-conjugated secondary antibodies were obtained from Millipore. PVDF membrane (Millipore) was used for blotting and signals were detected by LAS4000 mini digital imager (GE Health Care).

Histological and cytological staining - All staining solutions were purchased from Muto Pure Chemicals (Japan). For HE staining, spleen was embedded in OCT (Tissue-Tek) using dry ice/acetone bath. Serial sections at 10 μm thickness were made on a cryostat (Leica CM3050S) and tissue sections were placed on the MAS coated slide glass (Matsunami, Japan). After the fixation with 4% paraformaldehyde, sections were stained with hematoxylin and eosin. For May-Giemsa staining, air-dried whole blood smear was made on MAS coated slide glass. Blood smear slides were fixed and stained with May Grunwald staining solution and Giemsa solution. For New Methylene Blue staining, two volume of blood and one volume of New Methylene Blue staining solution were mixed and incubated for 15 min at room temperature. Sample slides were observed using

BX61 microscope equipped with 100× oil-immersion lens (Olympus, Japan).

Scanning electron microscope (SEM) analysis - Blood samples were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4 °C. After the samples were washed with 0.1 M phosphate buffer two times, the post fixation was carried out using 1% Osmium Tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C. After the wash with 0.1 M phosphate buffer, samples were gradually dehydrated using 50%, 70%, 80%, 90%, 99% (v/v ethanol/water), and 100% ethanol for 5 min in each step. Ethanol was replaced by t-butyl alcohol and the samples were frozen at 4 °C. After vacuum drying, the surface of samples was coated with platinum/palladium. Samples were observed by field emission scanning electron microscope (FE-SEM) S-4500 (Hitachi, Japan).

Flow cytometer analysis and cell sorting experiments - Bone marrow cells were harvested from femur bones. The single-cell suspension of splenocytes and bone marrow cells were prepared by passing through a 70 μm cell-strainer (BD). Peripheral blood was obtained by tail bleeding. Ter119 and CD71 expression were examined using anti-Ter119-PE and anti-CD71-FITC conjugated antibodies (BD; 1:200 dilution). Flow-activated cell sorting (FACS) analysis was performed using a FACS Canto II Flow Cytometer (BD). Each erythroblasts population was obtained from splenocytes by FACS Aria Cell Sorter (BD).

In vivo erythrocyte lifespan assay - *In vivo* erythrocyte lifespan assay was described elsewhere (37). Briefly, 12 weeks old mice were biotinylated by tail vein injection of 3 mg EZ-Link Sulfo-NHS-biotin (Thermo) dissolved in 0.2 mL phosphate-buffered saline. After 4 h circulation, blood was drawn from the tail vein and labeled with phycoerythrin (PE)-conjugated streptavidin (1:500). The biotinylated erythrocyte was measured using FACS Canto II Flow Cytometer and calculated as 100% biotinylated erythrocyte at Day 0. Subsequently, blood was drawn at Day 3, 5, 10, 15, and 20 and the percentage of biotinylated erythrocyte was determined.

Nmnat activity and discrimination Assay - Hemolysed samples were prepared as described above. Each sample was normalized by hemoglobin concentration at 20 μg/μl, and then hemoglobin was depleted using HemogloBind (Biotech Support Group LCC.). After depletion of hemoglobin, samples were dialyzed against dialysis buffer (25 mM Tris-Cl pH 7.8, 100 mM NaCl and 10% glycerol) to remove interfering endogenous metal ions and metabolites. The condition of discrimination assay was determined by previous report (38). The total 100 μl of reaction mixture contains 10 μl of dialyzed sample, 1 mM ATP and 1 mM NMN in final concentration. Metal ions were used at 0.05 mM for $MgCl_2$ or 5 mM for $CoCl_2$ in final concentration. The reaction was terminated by adding 200 μl 0.5N perchloric acid (PCA) at 0, 10, 20, and 30 min. After centrifugation, supernatant was collected and the formed NAD amount was measured by LC-MS/MS method described below.

Enzymatic activity assay of erythrocytes metabolic enzymes - Heparinized whole blood was washed three times with ice-cold saline and passed through a column of α-cellulose and microcrystalline cellulose to deplete leukocytes and platelets. Enzyme activities in RBCs were measured by the methods recommended by International Committee for Standardization in Hematology (39).

Metabolites extraction for LC-MS/MS measurement - The metabolites of erythrocytes were extracted using PCA for NAD related metabolites except for NADH (40) or methanol for glycolysis related metabolites (41). NADH was extracted with ammonium acetate/ acetonitrile (ACN)/ chloroform (42). For PCA extraction, 400 μl of whole blood was centrifuged at 1,000 x *g* for 5 min at room temperature, and then plasma and buffy coat were discarded. Packed erythrocytes were washed with ice-cold saline twice and cell numbers were counted by automated cell counter (Invitrogen). Then, 400 μl of 0.5 N PCA was added to the packed $5x10^8$ erythrocytes. After the vortex, precipitated proteins were separated by centrifugation at 15,000 x *g* for 10 min at 4 °C. Right before the measurement, supernatant was neutralized with equal volume of 1 M ammonium formate and filtered with 0.45 μm Millex filter unit (Millipore). For

methanol extraction, 400 µl of whole blood was used for preparing packed erythrocytes. After washing with ice-cold saline twice, $5x10^8$ erythrocytes were lysed with 350 µl of LC-MS grade pure water followed by addition of 150 µl of 100% methanol. Then, samples were boiled at 95 °C for 1 min and kept on ice for 10 min. After centrifugation, collected supernatants were filtered with 0.45 µm Millex filter unit. NAD related metabolites extraction from whole blood was performed by direct PCA addition followed by the same procedure as RBC samples.

Metabolites measurement by LC-MS/MS - Metabolites level was determined by Agilent 6460 Triple Quad mass spectrometer coupled to Agilent 1290 HPLC system. Chromatographic conditions are used as previously described with modifications (40,43). NAD related metabolites were separated on Waters Atlantis T3 column (2.1 x 150 mm, 3 µm) with 10 µl volume injection and at a flow rate of 150 µl/min using 5 mM ammonium formate for mobile phase A and 100% methanol for mobile phase B. The setting for gradients was as following: 0-10 min, 0-70%B; 10-15 min, 70%B; 15-20 min, 0%B. MS was operated in positive ESI MRM mode. Absolute amount of NAD was calculated from NAD standard curve by MassHunter Quantitative analysis software (Agilent). Glycolysis related metabolites were separated on Waters XBridge Amide column (2.1 x 150 mm, 3.5 µm) with 10 µl volume injection and at a flow rate of 300 µl/min using 20 mM ammonium acetate pH 9.0 (H_2O:ACN=95:5) for mobile phase A and 100% ACN for mobile phase B. The setting for gradients was as following: 0-5 min, 85-42%B; 5-16 min, 42-2%B; 16-21 min, 2%B; 21-30 min, 85%B. MS was operated in negative ESI MRM mode. All solvents used for these experiments were LC-MS grade or HPLC grade purchased from Wako pure chemicals (Osaka, Japan).

13C-Glucose labeling and tracer analysis - Whole blood was centrifuged at 1,000 x *g* for 5 min at room temperature and plasma and buffy coat were removed. Packed erythrocytes were washed with 1 ml of physiological saline once. 2.5×10^8 cells/tube were pre-incubated with normal RPMI 1640 medium (Nacalai Tesque, Japan) at 37 °C for 30 min, then medium was changed to glucose-free RPMI 1640 medium (Nacalai Tesque) supplemented with 2.0 g/l [U-13C]-glucose or [1,2-13C]-glucose (Cambridge Isotope Inc.). The samples were collected at 0, 30, 60, 90, and 120 min after medium change. Metabolites were extracted by methanol described above.

ATP measurement - Whole blood ATP level was measured using Blood ATP measuring kit (TOYO INK, Japan), which is based on a renilla luciferase-ATP-luciferin luminescent reaction. Luminescence was measured by Varioskan Flash (Thermo) after Luciferin/Luciferase solution was injected to sample according to manufacture's instruction.

Peripheral blood cell count - For peripheral blood cell count, whole blood was collected from inferior vena cave under anesthesia. Whole blood was immediately transferred to a EDTA-2K containing tube. Peripheral blood cell count was carried out at the FALCO Bio Systems veterinary medicine laboratories (Kyoto, Japan). Reticulocyte and white blood cell subpopulations were counted by flow cytometer method at the FALCO Bio Systems veterinary medicine laboratories.

Statistic Analysis - Analysis was performed using an unpaired or paired Student's t-test, and significant differences are indicated by single asterisk (*) when $p < 0.05$, double asterisk (**) when $p < 0.01$ and triple asterisk (***) when $p<0.005$.

RESULTS

Nmnat3 is localized in cytoplasm of mature erythrocytes

The Nmnat3 protein expression pattern of wild-type mice in various tissues was examined by Western blotting, using a rat monoclonal antibody raised against the mouse Nmnat3 full-length recombinant protein. Although Nmnat3 is known to be important in mitochondrial NAD metabolism, Nmnat3 protein expression showed marked variation in the tissues (Fig. 1A). RBC showed the highest expression level among the tissues examined, and heart, skeletal muscle, and liver showed relatively lower amounts of Nmnat3 (Fig. 1A). Nmnat3 was reported as a mitochondrial protein by several previous researchers (13,26,28,44).