Table 1 Generation of hematopoietic lineage from pluripotent stem cells

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

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

Mesodermal Cells

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

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

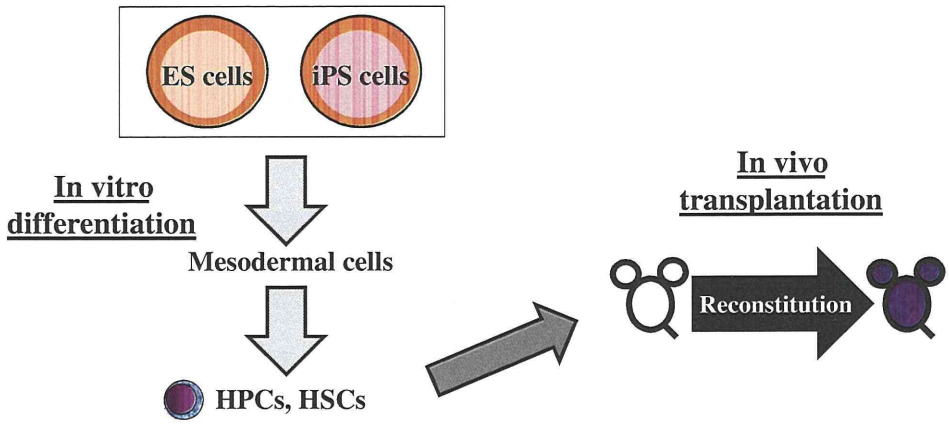
In mouse, a high percentage of $Flk1^+$ mesodermal cells are reportedly obtained from EBs around days 4 to 4.5 after ES cell differentiation [12, 13]. ES cells cultured on collagen-IV-coated dishes differentiate into $Flk1^+PDGFR\alpha^+$ immature mesodermal precursors, which then give rise to $Flk1^+PDGFR\alpha^-$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

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

Hematopoietic Stem (HSCs) and Progenitor (HPCs) Cells

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

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



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

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

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

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

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

finding suggests that other factors regulate HSC generation from human ES cells.

Importantly, Niwa et al. report that a novel serum-free monolayer culture system, independent of OP9 feeder cells and EB formation enables hematopoietic differentiation from both ES (KhES1) and iPS (201B7, 253 G4) cells. Using the method, the investigators generated $CD34^+CD45^+$ HPCs from human KhES1 between culture days 10 to 25 [17]. Tolar et al. reported hematopoietic differentiation of iPS cells from patients with mucopolysaccharidosis (MPS) type I, which is treated via HSC transplantation. An EB-mediated method generated HPCs expressing CD34 and CD45 and HSCs expressing *CD34* and *CD38* mRNA [20]. It is now necessary to determine whether HPCs derived from gene manipulation-free methods can have repopulation capacity in vivo.

Erythroid Cells

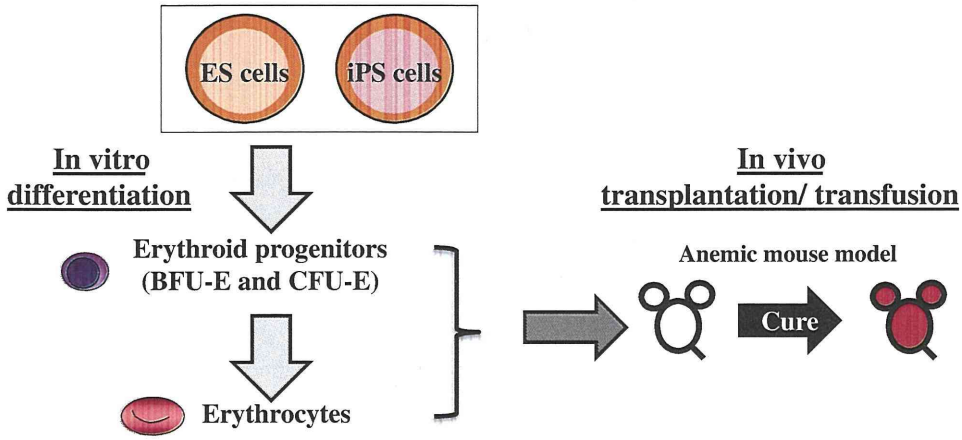
Red blood cells (RBCs), which are differentiated from erythroid progenitors (BFU-E, CFU-E) and erythroblasts, deliver oxygen (O_2) to the body tissues. Pluripotent cell-derived erythroid cells could be utilized as blood products in future clinical applications. It is not yet known whether erythroid cells derived from ES and iPS cells are definitive (EryD), enucleate, generate adult type of hemoglobin or carry oxygen (Fig. 3). In mouse, ES-derived cells co-cultured with OP9 cells contain primitive erythroid cells (EryP) within 8 days and erythroid progenitor cells (EryD) in 10 when cultured in methylcellulose semi-solid culture in the presence of Epo and IL-3. $Ter119^+$ cells were generated in the same condition after 14 days [28–30]. Within 5–6 days, EB culture-derived cells contain erythroid progenitor cells in methylcellulose culture in the presence of Epo and SCF, and those cells show a peak of *βH1 globin*, *βmajor globin* and *Gata1* gene expression within 7–8 days of culture [31].In addition to co-culture and EB formation methods, a three-step differentiation method is highly efficient in inducing erythroid cells from ES cells [32]. After 6 days of EB culture, ES-derived erythroid cultures (ES-EPs), which contain $CD71^+$c-$Kit^+Ter119^+$proerythroblasts and *βmajor globin*-expressing definitive erythroid cells, are induced with Epo, SCF, dexamethasone (Dex), insulin-like growth factor (IGF)-1. Following that, Epo, insulin, glucocorticoid receptor antagonist and transferrin were added to induce hemoglobinized, enucleated RBCs from ES-EP cells that also differentiate into $Ter119^+$ cells in vivo [32]. Hanna's group reported that after 6 days of culture on OP9 stromal cells, mouse autologous iPS cells, which were established from fibroblasts from a sickle cell anemia model, gave rise to c-Kit^+CD41^+ early HPCs in vitro and that HPCs derived by EB formation could rescue anemia in vivo after transplantation [25]. This work suggested that similar strategies are possible in humans.

Human ES cells co-cultured with mouse fetal liver-derived stromal cells (mFLSCs) generated erythroid progenitors after day 10, dominantly express adult type β-globin rather than embryonic type ε-globin, and gradually enucleate and show oxygen saturation around day 16 [33]. Lu et al. report that functional oxygen-carrying erythrocytes can be prepared on a large scale (10^{10}–10^{11} cells per 6-well plate of human ES cells) using four culture steps. They are: Step1; EB formation and hemangioblast precursor induction with BMP4, $VEGF_{165}$, bFGF, SCF, TPO and Flt-3 L (days −3.5 to 0); Step2; hemangioblast expansion with bFGF and tPTD-HoxB4 fusion protein (days 0 to10); Step3; erythroid cell differentiation and expansion with EPO (days 11 to 20); and Step4; enrichment of erythroid cells (day21) [34]. Human fibroblast-derived cell lines, such as hFib2-iPS5, 201B6, 201B7, 253G1 and 253G4, and the bone marrow mesenchymal stem cell-derived MSC-iPS1 cell line, all established from healthy donors, produced erythroid cells at around 30 days of culture via either EB formation or co-culture with OP9 stromal cells [17–19, 35]. On the other hand, two groups reported that human iPS cells from patients with hematological diseases also differentiated into erythroid cells. A combination method using EBs and OP9 cells produced BFU-E progenitor cells from dermal fibroblast-reprogrammed iPS cell lines, which were established from patients with Fanconi anemia (FA) [36]. Human peripheral blood-reprogrammed iPS cells established from patients with polycythemia vera (PV), a myeloproliferative disorder, also differentiate into CFU-E and $CD45^-$/CD235a $(GPA)^+$ mature erythroid cells after 2–3 weeks of EB formation, followed by culture of EB-derived $CD34^+CD45^+$ cells with SCF, IL-3. EPO [37].

Megakaryocytes and Platelets

Platelets, which are derived from fragmentation of precursor megakaryocytes, play a pivotal role in hemostasis by aggregation and adhesion to subendothelial tissue. In terms of regenerative medicine, pluripotent cell-derived platelets could be utilized as blood products in clinical settings. As an experimental tool, pluripotent cell-derived megakaryocytes could be useful to explore signaling pathways utilized in platelet formation, as a genetic approach has limited utility in studying anucleated platelets. One question is whether cells derived from ES and iPS cells would express surface antigen marker, such as CD41 andCD61, have the same signal pathway, exhibit the same morphology and have the same aggregation and adhesion capacity as megakaryocytes and platelets in vivo (Fig. 4).

In mice, Era et al. first reported that megakaryocytes derived from ES cells were observed 8 days after co-culture with OP9 cells and TPO [38].Co-culture of ES cells with OP9



Cell type	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Erythrocytes	Ter119	GPA	*Gata1* *Eklf* *βH1–globin* *β major-globin*	*GATA1* *EKLF* *β-GLOBIN*	1) Oxygen-carrying capacity 2) Improvement the anemic status by transplantation and transfusion (In vivo)

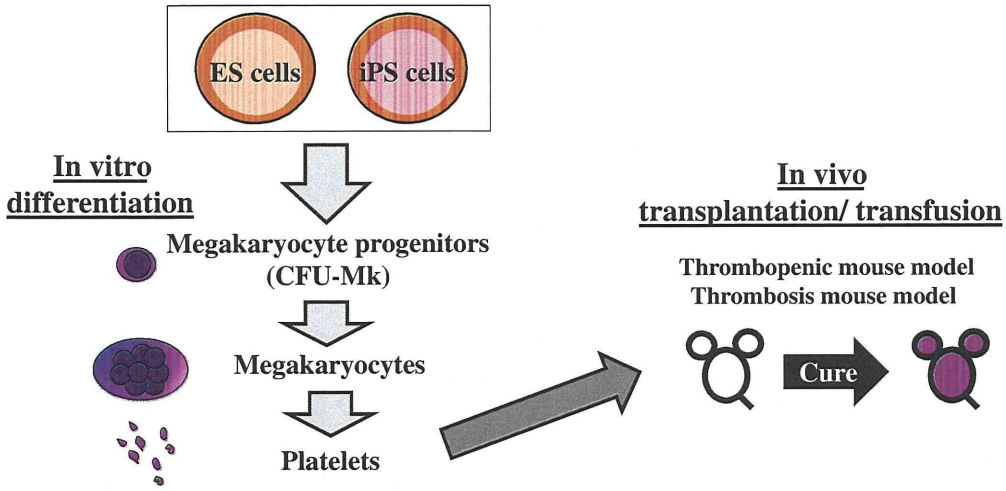
Fig. 3 Schematic diagram of pluripotent cell- derived erythrocytes. ES and iPS cells-derived erythrocytes are characterized by the expression of erythroid surface markers (Ter119 in mouse; Glycophorin A in human) and specific transcription factor genes (*Gata1, Eklf, βH1-globin* and *β−major globin* in mouse; *GATA1, EKLF* and *β-GLOBIN* in human). Hemoglobins in erythrocytes function as an oxygen-carrier and can be evaluated by plotting the oxygen equilibrium curves. To further investigate the function of erythrocytes in vivo, erythrocytes are transplanted/transfused into anemic mouse model. BFU-E; Burst Forming Unit-Erythroid, CFU-E; Colony Forming Unit-Erythroid, GPA; Glycophorin A

stromal cells for 8–12 days in the presence of TPO, IL-6 and IL-11 resulted in development of large, polyploid megakaryocytes, which produce proplatelets, could bound fibrinogen after exposure to platelet agonists, and exhibited integrin-mediated megakaryocyte signaling [39]. Fujimoto et al. confirmed primitive and definitive megakaryopoiesis from mouse ES cells with OP9 stromal cells in the presence of TPO. At the same time, $CD41^+$ platelets were generated in the culture supernatant via two waves of differentiation. ES-derived platelets of the definitive wave are similar in structure to those in peripheral blood and exhibit fibrinogen-binding ability and CD62P (P-selectin) expression after addition of AYPGFK, a platelet agonistpeptide [40].

In humans, 17 days after human H1 ES-derived cells on mouse bone marrow S17 cell lines contained megakaryocyte progenitor cells (CFU-Mk) that expressed CD41 antigen [41]. Gaur et al. reported that human H9 ES-derived cells grown on OP9 cells also generate megakaryocytic cells after 15–17 days of culture with TPO. CD41-, CD42b- and von Willebrand factor (vWF)-expressing megakaryocytes were polyploid (2 N to 32 N) and responsive to integrin $\alpha_{IIb}\beta_3$ activation by agonists such as ADP and PAR1 [42]. Takayama et al. reported a modified method of OP9 co-culture useful to produce not only megakaryocytes but also functional platelets. Differentiated ES cells co-cultured on OP9 or 10 T1/2 cells (a mouse embryonic mesenchymal stem cell line) with VEGF for 14–15 days formed embryonic stem cell-derived sacs (ES-sacs), which consist of multiple cysts that retain properties of endothelial cells and express CD34, VE-cadherin, CD31, CD41a, and CD45. After 9–10 days culture of ES-sacs with TPO, IL-6, IL-11, SCF and heparin, cultures produced functional platelets [43]. The same group succeeded in inducing platelets through "iPS-sacs" from human iPS cell lines reprogrammed from healthy adult human dermal fibroblasts [44].

Macrophages

Macrophages, which are differentiated from monocytes, are amoeboid cells that function in both innate immunity and adaptive immunity, phagocytize bacteria, viruses and dead cells, and stimulate lymphocytes and other immune cells to respond to pathogens. Monocytes and macrophages play a critical role in initiation and progression of atherosclerotic lesions. Therefore, pluripotent cell-derived macrophages could be used in vitro as an experimental tool to clarify the mechanisms of atherosclerotic lesions. It is not known whether macrophages derived from pluripotent cells express



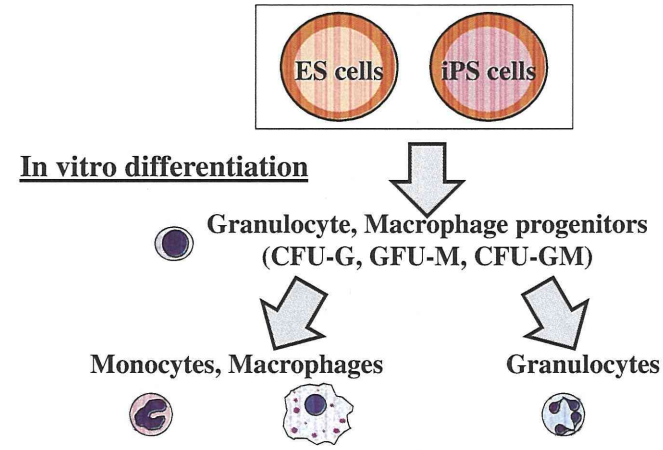
Cell types	Surface markers	Specific genes	Other characteristics
Megakaryocytes	CD41, CD42 CD61	*NF-E2* *FOG-1, Fli-1*	1) DNA ploidy 2) Aggregation and adhesion capacity
Platelets	CD41, CD42 CD61, CD62P	—	1) Aggregation and adhesion capacity 2) Thrombus formation to blood vessel injury (In vivo)

Fig. 4 Schematic diagram of pluripotent cell-derived megakaryocytes and platelets. ES and iPS cells-derived megakaryocytes are characterized by the expression of megakaryocyte surface markers (CD41, CD42 and CD61 in both mouse and human) and specific transcription factor genes (*NF-E2, FOG-1* and *Fli-1* in both mouse and human). DNA ploidy, a representative characteristic of megakaryocytes is examined in CD41 positive cells. Aggregation and adhesion capacity, another characteristic of megakaryocytes, are examined by fibrinogen binding assay and the integrin alpha II beta 3 signaling pathway. ES and iPS cells-derived platelets are characterized by the expression of surface markers (CD41, CD42, CD61 and CD62P in both mice and human). To further investigate the function of platelets in vivo, platelets are transplanted/transfused into thrombopenic mice and injury-induced mice models, in which the improvement of its status and hemostasis can be evaluated, respectively. CFU-Mk; Colony Forming Unit-Megakaryocyte

appropriate cell surface antigen markers, such as CD11b and F4/80, exhibit similar gene expression patterns as normal macrophages, or show phagocytotic activity (Fig. 5).

In mice, 13–15 days after EB formation by ES cells (CCEG2 and D3) in the presence of Epo, IL-1, IL-3, and M-CSF, HCs containing macrophages are generated [45]. Co-culture on OP9 cells with ES (D3) cells for 10 days also induced macrophage progenitors in the presence of IL-3 and Epo [28]. In these reports, macrophages were confirmed by morphologically. Lindmark et al. found that ES-derived macrophages are more similar to those in the peritoneum than mouse macrophage cell lines (J774.A1 and RAW264.7) by DNA microarray analysis [46]. After 10–12 days of culture, ES (J1) cells formed EBs in methylcellulose in the presence of CSF-1 and IL-3. After 3–5 days of liquid culture with CSF-1 and IL-3, differentiated cells expressed F4/80, FcγRII, scavenger receptor A (SR-A), CD36 and CD68, exhibited phagocytosis, and secreted TNF-α and IL-6 in response to an inflammatory stimulus [47], suggesting ES cell-derived macrophages could be an appropriate model to study atherosclerosis-related macrophages. Senju et al. reported that F4/80- and CD11b- expressing macrophages from the mouse iPS cell line 38C-2, which was established from MEFs with Oct3/4, Sox2, Klf4, and c-Myc, were generated via co-culture with OP9 cells for 6 days without cytokines and for the next 6 days with GM-CSF, followed by feeder free culture with M-CSF. iPS-derived macrophages also showed complement C5a-induced chemotaxis, phagocytic capacity and produced nitric oxide after stimulation with LPS and IFN-γ [48].

In humans, 17 days after human H1 ES-derived cells on mouse bone marrow S17 cell lines contained macrophage progenitor cells (CFU-M) that expressed CD15 antigen [35, 41]. Human ES cell lines, such as KhES-1, KhES-3, H1 and HES2, and human fibroblast-derived cell lines, such as hFib2-iPS5, 201B7 and 253G4 and the bone marrow mesenchymal stem cell-derived MSC-iPS1 line, all established from healthy donors, contained CFU-Ms and produced $CD14^+$ monocytes, $CD11b^+$ macrophages (at day14–22) and $CD13^+$ myeloid cells (at day 30) after culture via EB formation or co-culture with OP9 stromal cells in the presence of cytokines [17, 19, 35].



Cell types	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Monocytes Macrophages	CD11b, CD14, F4/80	CD11b, CD13, CD14, CD15, CD18	*MMP-3 MMP-9*	*PU.1*	1) Phagocytotic capacity 2) Cytokine production
Granulocytes	Gr-1	CD11b, CD13, CD16, CD33	*PU.1*	*PU.1, MPO, CEBPα Lactoferrin Gelatinase*	1) Superoxide production 2) Chemotactic capacity

Fig. 5 Schematic diagram of pluripotent cell-derived monocytes, macrophages and granulocytes. ES and iPS cells-derived monocytes and macrophages are characterized by the expression of surface markers (CD11b, CD14 and F4/80 in mouse; CD11b, CD13, CD14, CD15 and CD18 in human) and specific transcription factor genes (*Metalloproteinase (MMP)-3* and *MMP-9* in mouse; *PU.1* in human). A representative characteristic of macrophages is phagocytotic capacity, in which fluorescence-conjugated *Escheria coli* is administered and its incorporation into macrophages is evaluated by in vitro culture. Also, macrophages can produce cytokine (interleukine-6 and tumor necrosis factor) in response to inflammatory stimulus, such as lipopolysaccharide. ES and iPS cells-derived granulocytes are characterized by the expression of surface markers (Gr-1 in mouse; CD11b, CD13, CD16 and CD33 in human) and specific transcription factor genes (*PU.1* in mouse; *PU.1, Myeloperoxidase (MPO), CEBPα, Lactoferrin* and *Gelatinase* in human). The representative characteristics of granulocytes are superoxide production in response to phorbolmyristate acetate (PMA) stimuli, and chemotactic capacity in response to the chemo-attractant in vitro. CFU-G; Colony Forming Unit-Granulocyte, CFU-M; Colony Forming Unit-Macrophage, CFU-GM; Colony Forming Unit-Granulocyte Macrophage

Granulocytes

Granulocytes, which are derived from common myeloid progenitors and myeloblasts and account for approximately 60 % of leukocytes, are composed of neutrophils, basophils and eosinophils. They contain cytoplasmic granules and play a pivotal role in immunesystem by consuming bacteria and dead cells. Decreases in the number of neutrophils promote infection in several pathological situations, such as leukocyte function deficiencies or myelosuppression caused by chemotherapy. Granulocyte transfusion therapy is considered effective for infections unresponsive to conventional antimicrobial therapies in severely neutropenic cancer patients. Therefore, pluripotent cell-derived neutrophils could be used for such procedures. Thus it is important to determine whether neutrophils derived from pluripotent cells express appropriate markers and exhibit behaviors such as chemotaxis and phagocytosis (Fig. 5).

Leiber et al. reported that mouse CCE ES cell-derived neutrophils are generated after 8 days of EB culture, followed by 7 days of co-culture with OP9 cells and cytokines. The co-culture system is more effective in enhancing the number and culture period of neutrophils produced from ES cells compared to methods that do not employ OP9 stromal cells. Those neutrophils are positive for the surface marker Gr-1, have granules containing lactoferrin and gelatinases, and exhibit chemotactic responses and superoxide production [49].

Several groups have reported neutrophil induction from human ES cells. EB formation of human KhES cells grown in the presence of OP9 cells and cytokines gives rise to CD15-, CD11b- (both neutrophil and monocyte markers) and CD16- (mature neutrophil marker) positive cells at day13 similar to neutrophils found in peripheral blood. They also exhibit the oxidative burst function in killing microorganisms and phagocytotic activity in vitro [50]. Saeki et

al. developed feeder free culture systems for neutrophil induction. ES-derived neutrophils were generated after 3 days of "sphere formation" culture of KhES-3 ES cells on low-attachment dishes in the presence of cytokines, followed by culture on gelatin-coated dishes up to 40 days, results similar to those reported by Yokoyama's group [51]. Choi et al. succeeded in expanding neutrophils from human ES cells at least 60 times more efficiently than previously reported. Their culture system employed three steps, including induction of $CD34^+/CD45^+$ HPCs for 9 days on OP9 cells, expansion of HPCs for 2–8 days without OP9 cells, and differentiation of HPCs for 8 days on OP9 cells plus G-CSF. In addition, a similar method is reportedly useful for human iPS cell lines established from neonatal foreskin and adult fibroblasts [52]. Morishima et al. analyzed the differentiation process and function of iPS-derived neutrophil in greater detail. Expression of *PU.1*, *CEBPα*, *MPO*, *lactoferrin*, and *gelatinase* was confirmed during induction, as was chemotactic activity, phagocytotic activity, and MPO chlorination activity was observed in neutrophils derived from 201B6, 253G1, 253G4 iPS cell lines, all of which were established from healthy dermal fibroblasts [18].

Recently, Zou's group reported that culture on OP9 stromal cells of human autologous iPS cells established from bone marrow mesenchymal stem cells from a patient with X-linked chronic granulomatous disease (X-CGD) gave rise to oxidase-deficient neutrophils. They were also successful in rescuing oxidase-deficiency by gene modification using zinc finger nuclease-mediated safe harbor targeting [53]. These findings demonstrate that precise gene targeting may be applied to correct a disease-causing mutation in patient iPS cells.

Lymphocytes

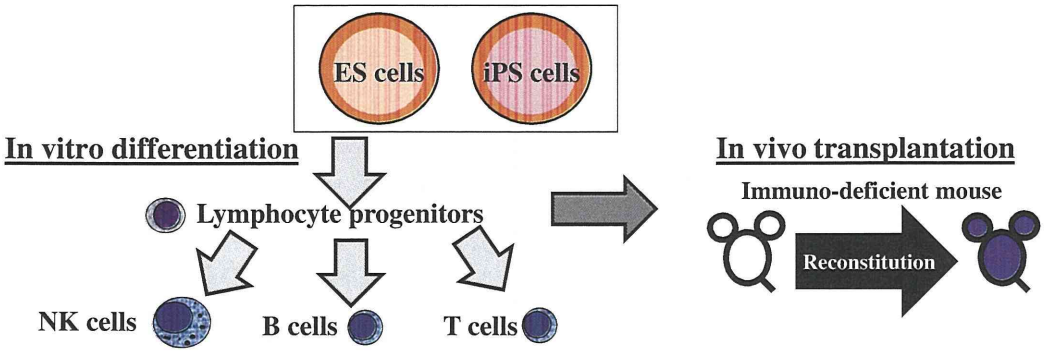
Lymphocytes, which consist of small lymphocytes (T cells and B cells) and large granular lymphocytes (NK cells), determine the specificity of immune response to infectious microorganisms and foreign substances. A decrease in lymphocyte number results in serious infections in several pathological situations, such as leukocyte function deficiencies or myelosuppression caused by chemotherapy. Immunotherapy for cancer is considered to be effective for cancer patients in clinical settings. Therefore, antitumor lymphocytes derived from pluripotent stem cells may be applicable to these approaches. It becomes the focus whether lymphocytes-derived from pluripotent cell express the surface antigen marker, achieve rearrangement of T cell antigen receptor (TCR) in T cell and immunoglobulin (Ig) in B cell and function such as cytotoxity (Fig. 6).

In mice, Nakano et al. reported that ES cells co-cultured on OP9 cells with IL-7 generated $B220^+$ early B cells, which were almost all IgM^- and achieved diversity-joining gene rearrangement, although a small portion of the hematopoietic cluster differentiated into mature IgM^+ cells expressing the complete μ chain mRNA [28]. B cell maturation from ES cells was enhanced in the presence of IL-7 and Flt3-L. Treatment with LPS stimulated B cell maturation and IgG secretion [54]. B cell differentiation from mouse iPS cells via the OP9 method revealed differences in outcomes according to iPS cell origin. MEF-derived iPS cells differentiated into B cells; however, B cell-derived iPS cells were relatively resistant to B cell lineage differentiation and showed defective Pax5 expression in differentiated cells [55].

Human H1 and H9 ES cell-derived $CD34^+$ cells co-cultured with MS-5 stromal cells in the presence of IL-3, IL-7, SCF and Flt3-L generated B cells positive for CD19 and for mRNA encoding the pre-B receptor complex (*VpreB*, *Igα*) [56]. Carpenter et al. first reported B cell lymphopoiesis from human iPS cells. $CD45^+CD19^+CD10^+$ cells-derived from iPS cells also expressed *Pax5*, *IL7αR*, *λ-like* and *VpreB receptor* mRNA. $CD45^+CD19^+$ cells exhibited multiple genomic D-J (H) rearrangements suggestive of pre-B-cell status [57].

T cell induction from ES cells has rarely been successful, due to difficulties in recapitulating the thymic stromal environment. de Pooter et al. reported that low population of $CD4^+$ and $CD8^+$ thymic subsets were generated from mouse ES cells as a result of co-culturing on OP9 cells, followed by $Flk1^+CD45^-$HPCs generated on fetal thymic organ cultures (FTOCs) [58]. They also developed modified OP9-DL1, which sustained mature T cells that expressed γδ and αβ TCRs, were positive for CD8, and produced IFN-γ in response to stimulation [59]. The OP9-DL1 method was also useful for mouse iPS differentiation. iPS-derived T cells secreted IL-2 and IFN-γ in response to in vitro stimulation and could also reconstitute T cell pools in Rag-deficient mice, suggesting that they follow a normal T cell differentiation program [60].

Galic et al. reported T-cell differentiation from human ES cells. Resulting differentiated H1 ES cells-derived progenitors grown in the presence of OP9 stromal cells for 10 to 19 days in vitro engrafted into human thymic tissues in immunocompromised mice. T cell development was observed in the conjoint organ 3–5 weeks after transplantation, indicating that human ES cells could be used to treat T cell disorders [61]. EB-derived T cell progenitors also generate to phenotypically and functionally normal cells of the T lineage when transferred into human thymic tissue implanted in immunodeficient mice [62]. Moreover, Timmermans et al. reported that during differentiation of ES cells on OP9 cells, two-dimensional structures form that strongly resemble blood islands, which arise during normal embryonic development and consist mostly of $CD34^+$ cells. Sequential culture of ES cell-derived cells on OP9-DL1 monolayers produced $CD34^{high}CD43^{low}$ cells that generated T cells. These hESC-derived T cells proliferated in response to



Cell types	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
T cells	CD4, 8 TCR	TCR, CD3 CD4, CD7, CD8, CD45	*Rag1 Hes1 Gata3*	*HES1 CD3 GATA3*	1) Cytokine production 2) T cell differentiation in transplanted mouse (In vivo)
B cells	B220 CD19	CD10 CD19 CD45	*IgM Pax5*	*IgM PAX5 λ-like*	1) Rearrangement of VDJ 2) Rearrangement of VJ
NK cells	CD94	CD45 CD56 CD94	*CD94, Ly49, NKR-P1, Granzyme A,B*	*CD122 ID2 ID3*	1) Cytotoxity 2) Cytokine production

Fig. 6 Schematic diagram of pluripotent cell-derived lymphocytes. ES and iPS cells-derived T cells are characterized by the expression of surface markers (CD4, CD8 and T cell receptor (TCR) in mouse; TCR, CD3, CD4, CD7, CD8 and CD45 in human) and specific transcription factor genes (*Rag1, Hes1* and *Gata3* in mouse; *HES1, CD3* and *GATA3* in human). A representative characteristic of T cells is cytokine (interleukin-2 and interferon-γ) production in response to anti-CD3 antibody and PMA in vitro. To further investigate the in vivo differentiation of pluripotent cells into T cells, pluripotent cells are transplanted into congenic mice in the mouse T cells and SCID-hu mice in human, respectively. ES and iPS cells-derived B cells are characterized by the expression of surface markers (B220 and CD19 in mouse; CD10, CD19 and CD45 in human) and specific transcription factor genes (*IgM* and *PAX5* in both mouse and human). A representative characteristic of B cells is VDJ and VJ gene rearrangement, which reveals early B cell commitment from pro-B cell to pre-B cells, and from pre-B cells to immature-B cells, respectively. Other characteristic of B cells is the protein expression of immunoglobulin chains such as μH chain composing pre-B receptor and L-chain for IgM, respectively. ES and iPS cells-derived NK cells are characterized by the expression of surface markers (CD94 in mouse; CD45, CD56 and CD94 in human) and specific transcription factor genes (*CD94, Ly49, NKR-P1, Granzyme A* and *Granzyme B* in mouse; *CD122, Inhibitor of DNA binding (ID) 2* and *ID3* in human). The representative characteristics of NK cells are tested by cytotoxity assay and cytokine (interleukine-6 and tumor necrosis factor alpha) production in vitro, which reveals the abilities to kill the tumor cells and to initiate the immune reaction, respectively

PHA stimulation, suggesting that hESCs can give rise to functional T cells [63]. Nevertheless, $CD34^+CD45^+$ cells from hESCs did not produce T cells on either OP9-DL1 monolayers or in FTOC cultures [64].

Mouse ES cells co-cultured with OP9 cells in the presence of Flt3-L, IL-15, IL-6, IL-7 and SCF generated NK cells, which were positive for CD94/NKG2 receptors and functioned to kill certain tumor lines and MHC class-I-deficient lymphoblasts [65]. Natural killer T (NKT) cells are a heterogeneous group of T cells that share properties of both T and NK cells. The OP9-DL1 method was also useful for NKT cell differentiation of mouse iPS cells established from MEFs and adult splenic NKT cells. iPS-derived NKT cells secreted the Th1 cytokine IFN-γ in response to in vitro stimulation and could recapitulate the adjuvant effect and suppress tumor growth in vivo [66].

Ni et al. reported NK cell generation from human H9 ES and BJ1-iPS12 iPS cells established from healthy adult human dermal fibroblasts. Among $CD45^+CD56^+$ cells generated, $CD117^-CD94^+$cells were mature NK cells with cytotoxic activity in vitro. In addition, both ES cell- and iPS cell-derived NK cells inhibited HIV-1 infection of a CEM cell line and of human primary $CD4^+$ T cells via killing through direct lysis, antibody-dependent cellular cytotoxicity, and production of chemokines and cytokines [67].

Perspective

Basic researchers have established methods for HSC and mature HC differentiation from ES and iPS cells that could

be useful for potential transplantation and transfusion therapy. Time will be required to standardize these methods, since individual researchers use diverse materials and methods, resulting in varied differentiation capability among ES and iPS cells, depending on cell line, passage number, methylation status and cell origin. To minimize clinical risks, special attention to potential tumorigenicity of manipulated cells must be paid. Although culture conditions, such as use of feeder-free cultures or serum-free media have already been improved, it remains necessary to shorten culture periods for iPS cell establishment and differentiation of the desired cell lineage induction from ES or iPS cells. Recently, "direct conversion" from human fibroblasts to HPCs and mature HCs was reported without establishing iPS cells [68]. In that case, ectopic expression of OCT4 plus treatment with a specific cytokine was effective to induce the $CD45^{+}$ cells, which had in vivo engraftment capacity. This method represents a new approach for autologous cell-transplant therapies that avoids difficulties involved with using human pluripotent stem cells. Thus, some issues remain to be resolved before ES and iPS cells can be applied to regenerative medicine.

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References

1. Dzierzak, E., & Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology, 9*, 129–136.
2. Wang, L. D., & Wagers, A. J. (2011). Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nature Reviews Molecular Cell Biology, 12*, 643–655.
3. Mizuochi, C., Fraser, S. T., Biasch, K., Horio, Y., Kikushige, Y., Tani, K., Akashi, K., Tavian, M., & Sugiyama, D. (2012). Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis. *PLoS One*; in press.
4. Sasaki, T., Mizuochi, C., Horio, Y., Nakao, K., Akashi, K., & Sugiyama, D. (2010). Regulation of hematopoietic cell clusters in the placental niche through SCF/Kit signaling in embryonic mouse. *Development, 137*, 3941–3952.
5. Sugiyama, D., Kulkeaw, K., Mizuochi, C., Horio, Y., & Okayama, S. (2011). Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production. *Biochemical and Biophysical Research Communications, 410*, 301–306.
6. Sugiyama, D., Inoue-Yokoo, T., Fraser, S. T., Kulkeaw, K., Mizuochi, C., & Horio, Y. (2011). Embryonic regulation of the mouse hematopoietic niche. *Scientific World Journal, 11*, 1770–1780.
7. Evans, M. J., & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature, 292*, 154–156.
8. Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*, 663–676.
9. Orr-Urtreger, A., Bedford, M. T., Do, M. S., Eisenbach, L., & Lonai, P. (1992). Developmental expression of the alpha receptor for platelet-derived growth factor, which is deleted in the embryonic lethal Patch mutation. *Development, 115*, 289–303.
10. Takakura, N., Yoshida, H., Ogura, Y., Kataoka, H., & Nishikawa, S. (1997). PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5. *Journal of Histochemistry and Cytochemistry, 45*, 883–893.
11. Kabrun, N., Buhring, H. J., Choi, K., Ullrich, A., Risau, W., & Keller, G. (1997). Flk-1 expression defines a population of early embryonic hematopoietic precursors. *Development, 124*, 2039–2048.
12. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., & Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development, 125*, 725–732.
13. Fehling, H. J., Lacaud, G., Kubo, A., et al. (2003). Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development, 130*, 4217–4227.
14. Sakurai, H., Era, T., Jakt, L. M., Okada, M., Nakai, S., & Nishikawa, S. (2006). In vitro modeling of paraxial and lateral mesoderm differentiation reveals early reversibility. *Stem Cells, 24*, 575–586.
15. Kulkeaw, K., Horio, Y., Mizuochi, C., Ogawa, M., & Sugiyama, D. (2010). Variation in hematopoietic potential of induced pluripotent stem cell lines. *Stem Cell Reviews, 6*, 381–389.
16. Inoue, T., Kulkeaw, K., Okayama, S., Tani, K., & Sugiyama, D. (2011). Variation in mesodermal and hematopoietic potential of adult skin-derived induced pluripotent stem cell lines in mice. *Stem Cell Reviews, 7*, 958–968.
17. Niwa, A., Heike, T., Umeda, K., et al. (2011). A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors. *PLoS One, 6*, e22261.
18. Morishima, T., Watanabe, K., Niwa, A., et al. (2011). Neutrophil differentiation from human-induced pluripotent stem cells. *Journal of Cellular Physiology, 226*, 1283–1291.
19. Grigoriadis, A. E., Kennedy, M., Bozec, A., et al. (2010). Directed differentiation of hematopoietic precursors and functional osteoclasts from human ES and iPS cells. *Blood, 115*, 2769–2776.
20. Tolar, J., Park, I. H., Xia, L., et al. (2011). Hematopoietic differentiation of induced pluripotent stem cells from patients with mucopolysaccharidosis type I (Hurler syndrome). *Blood, 117*, 839–847.
21. Burt, R. K., Verda, L., Kim, D. A., Oyama, Y., Luo, K., & Link, C. (2004). Embryonic stem cells as an alternate marrow donor source: engraftment without graft-versus-host disease. *The Journal of Experimental Medicine, 199*, 895–904.
22. Kyba, M., Perlingeiro, R. C., & Daley, G. Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell, 109*, 29–37.
23. Wang, Y., Yates, F., Naveiras, O., Ernst, P., & Daley, G. Q. (2005). Embryonic stem cell-derived hematopoietic stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 19081–19086.
24. Zhang, X. B., Beard, B. C., Trobridge, G. D., et al. (2008). High incidence of leukemia in large animals after stem cell gene therapy with a HOXB4-expressing retroviral vector. *The Journal of Clinical Investigation, 118*, 1502–1510.
25. Hanna, J., Wernig, M., Markoulaki, S., et al. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science, 318*, 1920–1923.
26. Lin, J., Fernandez, I., & Roy, K. (2011). Development of feeder-free culture systems for generation of ckit+sca1+ progenitors from mouse iPS cells. *Stem Cell Reviews, 7*, 736–747.
27. Wang, L., Menendez, P., Shojaei, F., et al. (2005). Generation of hematopoietic repopulating cells from human embryonic stem

cells independent of ectopic HOXB4 expression. *The Journal of Experimental Medicine, 201*, 1603–1614.
28. Nakano, T., Kodama, H., & Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science, 265*, 1098–1101.
29. Nakano, T., Kodama, H., & Honjo, T. (1996). In vitro development of primitive and definitive erythrocytes from different precursors. *Science, 272*, 722–724.
30. Motoyama, N., Kimura, T., Takahashi, T., Watanabe, T., & Nakano, T. (1999). bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. *The Journal of Experimental Medicine, 189*, 1691–1698.
31. Keller, G., Kennedy, M., Papayannopoulou, T., & Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Molecular and Cellular Biology, 13*, 473–486.
32. Carotta, S., Pilat, S., Mairhofer, A., et al. (2004). Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. *Blood, 104*, 1873–1880.
33. Ma, F., Ebihara, Y., Umeda, K., et al. (2008). Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 13087–13092.
34. Lu, S. J., Feng, Q., Park, J. S., et al. (2008). Biologic properties and enucleation of red blood cells from human embryonic stem cells. *Blood, 112*, 4475–4484.
35. Lengerke, C., Grauer, M., Niebuhr, N. I., et al. (2009). Hematopoietic development from human induced pluripotent stem cells. *Annals of the New York Academy of Sciences, 1176*, 219–227.
36. Raya, A., Rodriguez-Piza, I., Guenechea, G., et al. (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature, 460*, 53–59.
37. Ye, Z., Zhan, H., Mali, P., et al. (2009). Human-induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood, 114*, 5473–5480.
38. Era, T., Takagi, T., Takahashi, T., Bories, J. C., & Nakano, T. (2000). Characterization of hematopoietic lineage-specific gene expression by ES cell in vitro differentiation induction system. *Blood, 95*, 870–878.
39. Eto, K., Murphy, R., Kerrigan, S. W., et al. (2002). Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 12819–12824.
40. Fujimoto, T. T., Kohata, S., Suzuki, H., Miyazaki, H., & Fujimura, K. (2003). Production of functional platelets by differentiated embryonic stem (ES) cells in vitro. *Blood, 102*, 4044–4051.
41. Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R., & Thomson, J. A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 10716–10721.
42. Gaur, M., Kamata, T., Wang, S., Moran, B., Shattil, S. J., & Leavitt, A. D. (2006). Megakaryocytes derived from human embryonic stem cells: a genetically tractable system to study megakaryocytopoiesis and integrin function. *Journal of Thrombosis and Haemostasis, 4*, 436–442.
43. Takayama, N., Nishikii, H., Usui, J., et al. (2008). Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood, 111*, 5298–5306.
44. Takayama, N., Nishimura, S., Nakamura, S., et al. (2010). Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *The Journal of Experimental Medicine, 207*, 2817–2830.
45. Wiles, M. V., & Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development, 111*, 259–267.
46. Lindmark, H., Rosengren, B., Hurt-Camejo, E., & Bruder, C. E. (2004). Gene expression profiling shows that macrophages derived from mouse embryonic stem cells is an improved in vitro model for studies of vascular disease. *Experimental Cell Research, 300*, 335–344.
47. Moore, K. J., Fabunmi, R. P., Andersson, L. P., & Freeman, M. W. (1998). In vitro-differentiated embryonic stem cell macrophages: a model system for studying atherosclerosis-associated macrophage functions. *Arteriosclerosis, Thrombosis, and Vascular Biology, 18*, 1647–1654.
48. Senju, S., Haruta, M., Matsunaga, Y., et al. (2009). Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. *Stem Cells, 27*, 1021–1031.
49. Lieber, J. G., Webb, S., Suratt, B. T., et al. (2004). The in vitro production and characterization of neutrophils from embryonic stem cells. *Blood, 103*, 852–859.
50. Yokoyama, Y., Suzuki, T., Sakata-Yanagimoto, M., et al. (2009). Derivation of functional mature neutrophils from human embryonic stem cells. *Blood, 113*, 6584–6592.
51. Saeki, K., Nakahara, M., Matsuyama, S., et al. (2009). A feeder-free and efficient production of functional neutrophils from human embryonic stem cells. *Stem Cells, 27*, 59–67.
52. Choi, K. D., Vodyanik, M. A., & Slukvin, I. I. (2009). Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. *The Journal of Clinical Investigation, 119*, 2818–2829.
53. Zou, J., Sweeney, C. L., Chou, B. K., et al. (2011). Oxidase-deficient neutrophils from X-linked chronic granulomatous disease iPS cells: functional correction by zinc finger nuclease-mediated safe harbor targeting. *Blood, 117*, 5561–5572.
54. Cho, S. K., Webber, T. D., Carlyle, J. R., Nakano, T., Lewis, S. M., & Zuniga-Pflucker, J. C. (1999). Functional characterization of B lymphocytes generated in vitro from embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 9797–9802.
55. Wada, H., Kojo, S., Kusama, C., et al. (2011). Successful differentiation to T cells, but unsuccessful B-cell generation, from B-cell-derived induced pluripotent stem cells. *International Immunology, 23*, 65–74.
56. Vodyanik, M. A., Bork, J. A., Thomson, J. A., & Slukvin, I. I. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood, 105*, 617–626.
57. Carpenter, L., Malladi, R., Yang, C. T., et al. (2011). Human induced pluripotent stem cells are capable of B-cell lymphopoiesis. *Blood, 117, 4008–4011.*
58. de Pooter, R. F., Cho, S. K., Carlyle, J. R., & Zuniga-Pflucker, J. C. (2003). In vitro generation of T lymphocytes from embryonic stem cell-derived prehematopoietic progenitors. *Blood, 102*, 1649–1653.
59. Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S., & Zuniga-Pflucker, J. C. (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nature Immunology, 5*, 410–417.
60. Lei, F., Haque, R., Weiler, L., Vrana, K. E., & Song, J. (2009). T lineage differentiation from induced pluripotent stem cells. *Cellular Immunology, 260*, 1–5.
61. Galic, Z., Kitchen, S. G., Kacena, A., et al. (2006). T lineage differentiation from human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 11742–11747.
62. Galic, Z., Kitchen, S. G., Subramanian, A., et al. (2009). Generation of T lineage cells from human embryonic stem cells in a feeder free system. *Stem Cells, 27*, 100–107.
63. Timmermans, F., Velghe, I., Vanwalleghem, L., et al. (2009). Generation of T cells from human embryonic stem cell-derived hematopoietic zones. *Journal of Immunology, 182*, 6879–6888.
64. Martin, C. H., Woll, P. S., Ni, Z., Zuniga-Pflucker, J. C., & Kaufman, D. S. (2008). Differences in lymphocyte developmental potential

between human embryonic stem cell and umbilical cord blood-derived hematopoietic progenitor cells. *Blood, 112*, 2730–2737.
65. Lian, R. H., Maeda, M., Lohwasser, S., et al. (2002). Orderly and nonstochastic acquisition of CD94/NKG2 receptors by developing NK cells derived from embryonic stem cells in vitro. *Journal of Immunology, 168*, 4980–4987.
66. Watarai, H., Fujii, S., Yamada, D., et al. (2010). Murine induced pluripotent stem cells can be derived from and differentiate into natural killer T cells. *The Journal of Clinical Investigation, 120*, 2610–2618.
67. Ni, Z., Knorr, D. A., Clouser, C. L., et al. (2011). Human pluripotent stem cells produce natural killer cells that mediate anti-HIV-1 activity by utilizing diverse cellular mechanisms. *Journal of Virology, 85*, 43–50.
68. Szabo, E., Rampalli, S., Risueno, R. M., et al. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature, 468, 521–526.*

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Research Article

Ectopic expression of Hmgn2 antagonizes mouse erythroid differentiation *in vitro*

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Abstract

Hmgn2 (high mobility group nucleosomal 2), a ubiquitous nucleosome-binding protein that unfolds chromatin fibres and enhances DNA replication, reportedly regulates differentiation of epithelial and mesenchymal cells. To investigate how Hmgn2 regulates HC (haemopoietic cell) differentiation, we quantified *Hmgn2* expression in HCs of mouse FL (fetal liver) during erythroid differentiation. *Hmgn2* expression levels were >10-fold higher in immature erythroid progenitors than in mature erythroid cells, suggesting that Hmgn2 antagonizes erythroid differentiation. To address this issue, *Hmgn2* were transfected into both Friend erythroleukaemia cells and FL HCs. There was a 3.3-fold decrease in relatively mature c-Kit$^+$/CD71$^+$ erythroid cells, a 2.9-fold increase in immature c-Kit$^+$ CD71$^-$ erythroid cells in transfected Friend cells, a 1.1-fold decrease in relatively mature CD71$^+$/Ter119$^+$ erythroid cells, and a 1.7-fold increase in relatively immature c-Kit$^+$/CD71$^+$ erythroid cells in FL HCs accompanied by down-regulation of genes encoding the erythroid transcription factors, Gata1 and Klf1. Two days after *Hmgn2* transfection of Friend erythroleukaemia cells, the number of S-phase cells increased, whereas the number of cells in G_1 decreased, while that of mitotic cells remained unchanged. We conclude that ectopic expression of Hmgn2 antagonizes mouse erythroid differentiation *in vitro*, which may be due to enhancement of DNA replication and/or blocking entry of mitosis at S-phase.

Keywords: erythroleukaemia cell; erythroid differentiation; fetal liver; Hmgn2

1. Introduction

Hmgn (high mobility group nucleosomal) binding proteins constitute a family of ubiquitous nuclear proteins comprising Hmgn1–Hmgn4 and NSBP1 (nucleosomal binding protein 1). Hmgn1 and Hmgn2 are widely expressed, whereas the remaining family members have a more tissue-specific expression pattern (Bustin 1999, 2001). Nuclear Hmgn1 and Hmgn2, which are 53% homologous, constitute approximately 10% of all nuclear protein in mice (Bustin and Reeves, 1996). Both bind to nucleosomes as a homodimer. The NBD (nucleosome-binding domain) binds to the 147 bp nucleosome core particle rather than to a specific DNA sequence (Shirakawa et al., 2000; Ueda et al., 2008), while the CHUD (chromatin-unfolding domain) reduces compactness of the chromatin fibre (Bustin, 2001). Hmgn2 facilitates accessibility of transcription factors and the replication machinery to chromatin, thereby enhancing transcription (Trieschmann et al., 1995a, 1995b) and DNA replication (Vestner et al., 1998). In addition to nucleosome binding, Hmgn2 also modulates DNA binding of homeodomain transcription factors (Amen et al., 2008). At the 2-cell stage in mouse embryos, *Hmgn2* knockdown by antisense oligonucleotide delays cell division (Mohamed et al., 2001). *Hmgn2* expression is also down-regulated during differentiation of osteoblasts in mouse embryos (Shakoori et al., 1993). However, Hmgn2 is highly expressed in cells undergoing differentiation, such as the basal layer of epithelial cells in *Xenopus laevis* (Korner et al., 2003) and mesenchymal-to-epithelial transitions in the mouse kidney (Lehtonen and Lehtonen, 2001).

Haematopoiesis is the process in which HSCs (haemopoietic stem cells) are generated, differentiate into specific progenitors and mature into various blood cell types, such as erythrocytes, megakaryocytes, lymphocytes, neutrophils and macrophages (Weissman, 2000). Erythropoiesis is the process by which a large number of enucleated erythrocytes are produced from HSCs (McGrath and Palis, 2008). During embryogenesis, the FL (fetal liver) is a major organ of HSC expansion and erythrocyte production (Ema and Nakauchi, 2000; Sugiyama and Tsuji, 2006). Erythropoiesis shifts to BM (bone marrow) shortly before birth (Dzierzak et al., 1998). During erythroid differentiation, erythroblasts lose their capacity to proliferate and leave the cell cycle (Buttitta and Edgar, 2007). However, molecular mechanisms underlying erythroid differentiation have not been fully elucidated.

We have focused on the role of Hmgn2 in erythroid differentiation in mice. By assessing the differentiation status of erythroid cells by flowcytometry and gene expression after

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Abbreviations: AcGFP, *Aequorea coerulescens* green fluorescent protein; α-MEM, α-minimum essential medium; APC; antigen-presenting cell; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; CHUD, chromatin-unfolding domain; dpc, day post coitum; EPO, erythropoietin; FL, fetal liver; GFP, green fluorescent protein; HC, haemopoietic cell; Hmgn, high mobility group nucleosomal; HSC, haemopoietic stem cell; IL-3, interleukin 3; IRES, internal ribosome entry site; MNC, mononuclear cell; NBD, nucleosome-binding domain; NLS, nuclear localization signal; PI, propidium iodide; PBSBA, BSA in PBS; RQ, relative quantity; RT–PCR, reverse transcription–PCR; SCF, stem cell factor; TBS-T, TBS containing 0.1% Tween-20; TPO, thrombopoietin; WB, Western blot.

ectopic expression of *Hmgn2* by electroporation, Hmgn2 is shown to antagonize erythroid differentiation of erythroleukaemia cells and mouse FL cells *in vitro*.

2. Materials and methods

2.1. Animals and cell lines

C57BL6J mice were purchased from Nihon SLC (Hamamatsu). Noon on the day of the plug was defined as 0.5 dpc (day post coitum). To analyse their cells, pregnant mice were killed and their embryos dissected out. Animals were handled according to the Guidelines for the Care and Use of Laboratory Animals of Kyushu University. This study was approved by Animal Care and Use Committee, Kyushu University (Approval ID: A21-068-0). The Friend erythroleukaemia cell line, F5-5.fl, was purchased from the RIKEN Bio-Resource Center. Cells were maintained in RPMI 1640 (Wako Pure Chemical Industries) containing 10% FBS and 10 units/ml penicillin and 10 mg/ml streptomycin (Sigma–Aldrich). Cells were passaged every 3–4 days.

2.2. Construction of an *Hmgn2* vector

A primer set was designed from the known mouse mRNA sequence (accession no. NM_016957). Full-length *Hmgn2* was amplified from mouse FL cDNA and ligated into the pIRES2-AcGFP (*Aequorea coerulescens* green fluorescent protein) vector (Clontech). *Hmgn2* was located upstream of an IRES (internal ribosome entry site), which was flanked downstream by cDNA encoding AcGFP (Supplementary Figure S2B available at http://www.cellbiolint.org/cbi/vvv/cbivvvppppadd.htm). *Hmgn2* sequence was confirmed by DNA sequencing. Endotoxin-free plasmid was prepared using the QIAGEN® Plasmid Midi kit (QIAGEN). The construct was introduced into $CaCl_2$-competent DH5α *E. coli* by the heat shock method.

2.3. Preparation of FL MNCs (mononuclear cells)

At 12.5 dpc, embryos blood cells were collected by disassociating FL on a 40 μm nylon mesh and washed once with PBS. MNCs were obtained after centrifugation with lympholyte solution (CEDARLANE Laboratories). MNCs were washed twice in PBS and cultured for 24 h in α-MEM (α-minimum essential medium) containing 10% FBS, 20 ng/ml each of SCF (stem cell factor) and TPO (thrombopoietin) (PEPROTECH) and 10 units/ml penicillin and 10 mg/ml streptomycin (Sigma–Aldrich). Floating cells were used for electroporation.

2.4. Electroporation and cell differentiation

Friend erythroleukaemia cells were collected after 24 h culture and washed twice with PBS. Cells were resuspended in Gene Pulser® electroporation buffer (Bio-Rad) at 5×10^6 cells/ml. The cell suspension (400 μl) was mixed with 8 μg of endotoxin-free pIRES2-*Hmgn2-AcGFP* or pIRES2-*AcGFP* (mock), transferred into an electroporation cuvette (0.4 cm gap), and placed on ice for 5 min. Cells were electroporated using a Gene Pulser MXCell™ Electroporator (Bio-Rad) at optimized conditions (200 volts, 2000 μF, 1000 Ω and 20 ms). They were cultured in RPMI 1640 containing 10% FBS without antibiotics for 48 h. GFP (green fluorescent protein)-expressing cells were collected using a FACS Aria cell sorter (BD Biosciences) and cultured in RPMI 1640 containing 10% FBS, 10 units/ml penicillin and 10 mg/ml streptomycin (Sigma–Aldrich) for 6 days.

For FL MNCs, cells were collected by gently pipetting and washing twice with PBS. Cells were electroporated as above. Optimal conditions for FL cells were 300 volts, 2000 μF, 1000Ω and 20 ms. Cells were cultured with α-MEM containing 10% FBS, 20 ng/ml each of SCF and TPO (PEPROTECH) without antibiotics for 48 h. GFP^+ $CD71^+$/$Ter119^-$ cells were collected using a FACS Aria cell sorter (BD Biosciences) and cultured in α-MEM containing 10% FBS, 20 ng/ml each of SCF, IL-3 (interleukin 3) and EPO (erythropoietin) (PEPROTECH) with 10 units/ml penicillin and 10 mg/ml streptomycin.

2.5. Flow cytometry and cell sorting

To isolate HSCs and erythroid cells, cells were stained with a FITC-conjugated anti-mouse CD71 antibody (BD Biosciences), a PE-conjugated anti-mouse Sca-1 antibody (BD Biosciences), an APC (antigen-presenting cell)-conjugated anti-mouse c-Kit (CD117) antibody (BD Biosciences), a PE-Cy7-conjugated anti-mouse CD45 antibody (eBioscience) and an APC-Cy7-conjugated anti-mouse Ter119 antibody (eBioscience) (Hattangadi et al., 2010; Inoue et al., 2011). To analyse differentiation of erythroleukaemia and erythroid cells from FL, cells were stained with an APC-conjugated anti-mouse c-Kit (CD117) antibody (BD Biosciences), a PE-conjugated anti-mouse CD71 antibody (eBiosciences), and an APC-Cy7-conjugated anti-mouse Ter119 antibody (eBioscience). Dead cells were excluded by PI (propidium iodide) staining (Life Technologies). Cells were sorted in using a FACS Aria cell sorter (BD). Data files were analysed with FlowJo software (Tree Star Inc.).

2.6. Real-time PCR

RNA was extracted from sorted cells using RNAqueous-4PCR™ and RiboPure™ kits (Life Technologies). mRNA was reverse-transcribed using a high-capacity RNA-to-cDNA kit (Life Technologies). The quality of cDNA synthesis was checked by amplifying mouse β-actin in PCR. Thirty thermal cycles were as follows: denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. Gene expression levels were evaluated using RT–PCR (reverse transcription–PCR) with a TaqMan® Gene Expression Master Mix and StepOnePlus™ RT–PCR machine (Life Technologies). All probes were from TaqMan® Gene Expression Assays (Life Technologies). Samples were taken in triplicate. mRNA levels were normalized to β-actin and the RQ (relative quantity) of expression was compared with a reference sample. Statistical comparisons of RQ values were calculated using a *t* test.

2.7. Antibody staining and confocal microscopy

HSCs and erythroid cells were isolated from FL at 12.5 dpc by flowcytometry as above. They were cytocentrifuged on to glass slides (Matsunami) using a Shandon Cytospin® 3 cytocentrifuge (Thermo Electron Corporation) at 450 rev./min for 7 min. After drying at 25°C, cells were fixed in 1% PFA (paraformaldehyde) in PBS at 4°C for 30 min and washed in PBS 3 times. After blocking with 1% BSA in PBS, cells were stained with a rabbit anti-mouse Hmgn2 polyclonal antibody (Chemicon International) overnight at 4°C. After three washes in PBS, cells were stained with Alexa Fluor® 488 goat anti-rabbit IgG (1:300) (Life Technologies) and TOTO®-3 iodide (642/660, Life Technologies). Samples were examined under a FV-1000 laser scanning confocal microscope (Olympus).

2.8. WB (Western blot) analysis

Protein was extracted from the sorted cells by using Qproteome® Mammalian Protein Prep Kit (Qiagen) and was quantified by using Quick Start™ Bradford Dye Reagent (Bio-Rad Laboratories). Then 5 μg of protein was run on 15% SDS-polyacrylamide gels concurrently with a pre-stained protein marker (Precision Plus Protein™ Standards, Bio-Rad Laboratories), using Laemmle buffer. Gels were trans-blotted on to a PVDF membrane (Immobilon®-P Transfer Membrane, Millipore Billerica). The PVDF membrane was blocked in 5% non-fat dried skimmed milk powder in TBS-T (TBS containing 0.1% Tween-20) at 25°C for 1 h, washed with TBS-T and reacted with 1:1000 rabbit anti-mouse Hmgn2 polyclonal antibody (Chemicon International) overnight at 4°C. The membrane was thoroughly washed and incubated in a solution of 1:1000 goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugate (R&D Systems) at 25°C for 1 h. After washing, signals were visualized by soaking the membrane in substrate solution (Amersham™ ECL® Plus Western Blotting Detection System, GE Healthcare). Images were captured using ChemiDoc XRS (Bio-Rad Laboratories) and data were analysed by Quantity One ver. 4.6.7 (Bio-Rad Laboratories) and displayed as intensity per mm^2.

2.9. Cell cycle analysis

Cells were washed twice with PBS containing 2% FBS and resuspended in 300 μl PBS. They were permeabilized by adding 700 μl 100% ethanol precooled to −20°C. After mixing by gentle inversion, the cell suspension was placed on ice overnight. Cells were collected by centrifugation at 4000 rev./min at 4°C for 2 min. To eliminate RNA, cells were incubated with 50 μl RNase A solution (100 μg/ml) at 37°C for 30 min. To stain DNA, cells were resuspended in 450 μl of staining solution containing 2 μg/ml PI in 100 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% Nonidet P40, and incubated at 25°C for 30 min in the dark. Cells were analysed using a FACS Aria cell sorter (BD). The percentages of cells in G_0+G_1 and in S+G_2 phases were calculated using the Watson Pragmatic method and FlowJo software.

2.10. Mitotic analysis by flow cytometry

Mitotic cell status was analysed as per Taylor (2004). Briefly, cells were washed twice in PBS, fixed in 70% ethanol at −20°C and resuspended in 400 μl PBS. Then 1 ml of cold ethanol (precooled to −20°C) was added to the cell suspension, while gently vortexing the ethanol. The cells were fixed for at least 1 h at −20°C, transferred to a 1.5 ml Eppendorf tube and centrifuged at 3800 rev./min at 4°C for 7 min. After removal of supernatant by aspiration, the cell pellet was resuspended with 1 ml of PBS, centrifuged at 3800 rev./min at 4°C for 7 min, and the supernatant removed as above. The cell pellet was resuspended with 1.4 ml of PBS+0.25% Triton X-100 and incubated on ice for 15 min before being centrifuged at 3800 rev./min at 4°C for 7 min before the supernatant was removed. The cell pellet was resuspended in 100 μl 1% PBSBA (BSA in PBS) containing rabbit anti-phosphorylated histone H3 serine 10 IgG (Abcam) and incubated for 2 h at 25°C. Tubes were rocked gently for entire period. Then 1.3 ml of PBSBA was added, mixed by inverting and centrifuged at 3800 rev./min at 4°C for 7 min. After removal of the supernatant, the cell pellet was resuspended in 100 μl PBSBA containing Alexa Fluor® 647 goat anti-rabbit IgG (1:200) (Life Technologies) and incubated for 30 min at 25°C in the dark with gentle mixing as above. The cell suspension was washed once with 1.3 ml of PBSBA and centrifuged at 3800 rev./min at 4°C for 7 min. The cell pellet was resuspended in 500 μl of PBS containing 100 μg/ml DNase-free RNase A and incubated at 37°C for 30 min. After cooling on ice for a few seconds, 100 μl PI solution was added and incubated on ice for 15 min in the dark. Cells were analysed by flow cytometry.

2.11. Statistics

Student's *t* test in Microsoft Office Excel 2007 program was used to test a statistically significant differences ($P<0.05$) in the data.

3. Results

3.1. Expression of Hmgn2 in mouse FL during erythroid differentiation

Erythroid cell populations ranging from uncommitted HSCs to mature erythrocytes were sorted by flowcytometry from mouse FL at 12.5 dpc based on expression of the cell surface molecules CD45 (common leucocyte antigen), Sca-1 (stem cell antigen-1), c-Kit (CD117, stem cell factor receptor), CD71 (transferrin receptor) and Ter119 (glycophorin) (Supplementary Figure S1A available at http://www.cellbiolint.org/cbi/vvv/cbivvvppppadd.htm). Erythroid cell populations were identified as follows: (i) the $CD45^+/Sca\text{-}1^+/c\text{-}Kit^+$ fraction defined HSCs; (ii) the $Sca\text{-}1^-/c\text{-}Kit^+/CD71^-/Ter119^-$ fraction defined BFU-E (burst forming unit-erythroid); (iii) the $Sca\text{-}1^-/c\text{-}Kit^+/CD71^+/Ter119^-$ fraction defined committed erythroid progenitors or CFU-E (colony forming unit-erythroid); (iv) the $Sca\text{-}1^-/c\text{-}Kit^-/CD71^+/Ter119^+$ fraction defined proerythroblasts;

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and (v) the Sca-1$^-$/c-Kit$^-$/CD71$^-$/Ter119$^+$ fraction defined reticulocytes and erythrocytes (Supplementary Figures S1A and S1B).

To quantify *Hmgn2* expression levels during erythroid differentiation, RT–PCR analysis was carried out on each sorted population. *Hmgn2* expression levels increased slightly as HSCs differentiated into BFU-E (~1.4-fold, Figure 1A), but gradually decreased at later stages of erythroid cell differentiation (Figure 1A). *Hmgn2* expression in BFU-E and CFU-E was 14- and 12-fold higher than in mature erythrocytes, respectively ($P<0.05$; Figure 1A). Examining the expression of Hmgn2 protein by immunohistochemistry, single cell suspensions of HSCs, BFU-E, CFU-E, proerythroblasts and erythrocytes at 12.5 dpc were prepared, spun on to slides, stained with antibody to Hmgn2 and observed by confocal microscopy. Hmgn2 protein was expressed in all fractions (Figure 1B). To quantify the amount of Hmgn2 protein in each faction, WB analysis was used after loading the same amount of protein. Compatible with the immunohistochemistry data, Hmgn2 protein was detected in all fractions, the highest expression level being in the BFU-E fraction (Figure 1C).

3.2. Hmgn2 expression antagonizes erythroleukaemia cell differentiation

To investigate the function of Hmgn2 in erythroid differentiation, *Hmgn2* was ectopically expressed in Friend erythroleukaemia cells. Full-length mouse *Hmgn2* was cloned into an expression plasmid. The deduced amino acid sequence of Hmgn2 indicated 2 NLSs (nuclear localization signals; NLS1 and NLS2) in addition to the NBD and CHUD (Supplementary Figure S2A). Friend erythroleukaemia cells were transfected with plasmids with and without (mock) *Hmgn2* cDNA by electroporation. Two days later, expression levels of *Gata1* and *Klf1* that encode erythroid transcription factors, were measured in transfected cells, identified by their GFP staining. Expression of *Gata1* and *Klf1* decreased in GFP$^+$ *Hmgn2*-transfected cells compared with mock controls ($P<0.05$; Figure 2A). However, expression levels of both *Gata1* and *Klf1* did not differ significantly between *Hmgn2*-transfected and mock control cells at day 8 of culture ($P>0.05$; Figure 2A). By flow-cytometry, 69% c-Kit$^+$/CD71$^+$ cells (equivalent to CFU-E) and 4.2% c-Kit$^+$/CD71$^-$ cells (equivalent to BFU-E) were observed in mock controls at day 8 of culture, whereas 21% c-Kit$^+$/CD71$^+$ cells and 12.6% c-Kit$^+$/CD71$^-$ cells were observed in *Hmgn2*-transfected cells, suggesting that ectopic expression of *Hmgn2* suppressed erythroid differentiation (Figure 2B). Moreover, the percentage of CD71$^+$/Ter119$^+$ cells (equivalent to proerythroblasts) in *Hmgn2*-transfected cells was lower than that seen in mock controls (0.16% versus 1.4%; Figure 2B). Thus ectopic expression of *Hmgn2* inhibited erythroid differentiation of Friend erythroleukaemia cells.

3.3. Hmgn2 misexpression increases the number of erythroleukaemia cells at S-phase of the cell cycle

To investigate how Hmgn2 regulates differentiation of Friend erythroleukaemia cells, the cell cycle status of *Hmgn2*-transfected

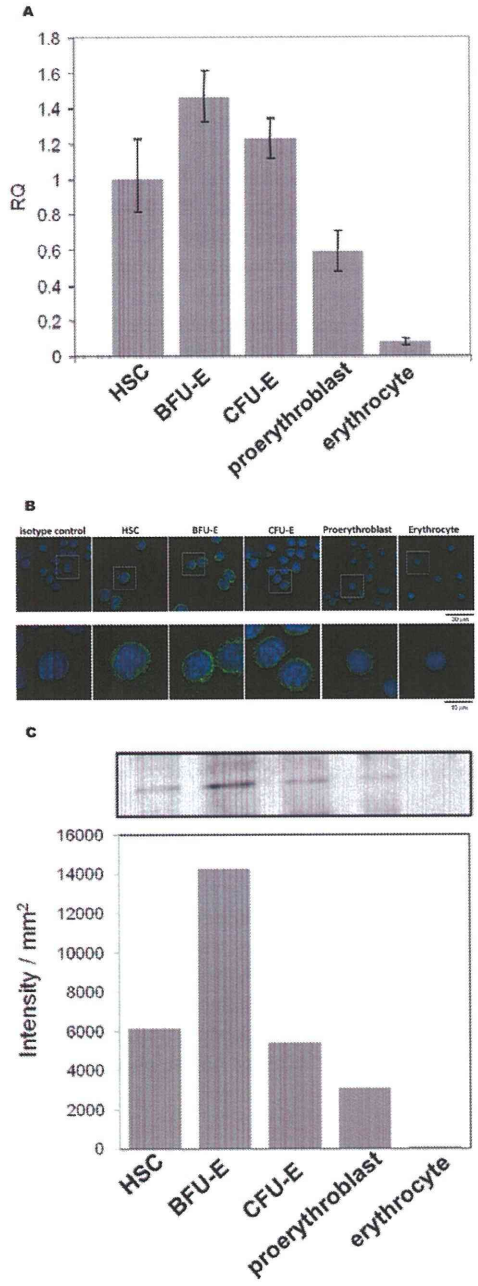

Figure 1 Expression of *Hmgn2* during erythrocyte differentiation in mouse FL
(**A**) Expression of *Hmgn2* in each population of erythroid cells described in Supplementary Figure S1(A) and S1(B) was quantified by RT–PCR. The *Y*-axis represents the RQ of gene expression after normalization with β-actin. (**B**) Confocal images of HSCs, BFU-E, CFU-E, proerythroblasts and erythrocytes expressing Hmgn2. Single cells were prepared from mouse FL at 12.5 dpc. Hmgn2 (green) and TOTO®-3 (blue) are shown. Lower panel shows higher magnification of the cells in boxes of upper panel. Scale bars in upper and lower panels reveal 30 and 10 μm respectively. (**C**) WB analysis of Hmgn2 protein in HSCs, BFU-E, CFU-E, proerythroblasts and erythrocytes. Equal amount of protein was trans-blotted on to PVDF membrane and reacted with anti-Hmgn2 antibody. Intensity of protein bands was quantified by Quantity One ver. 4.6.7 and displayed as intensity per mm^2.

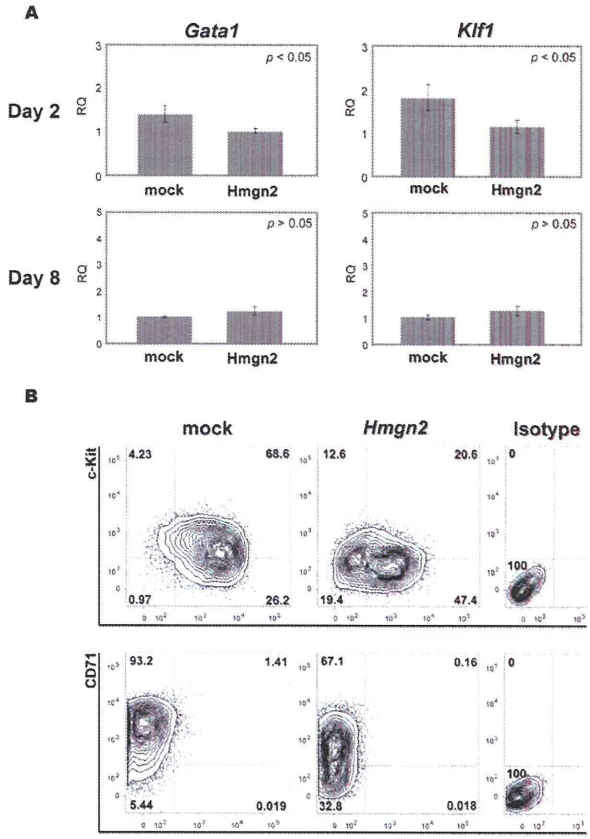

Figure 2 Ectopic expression of *Hmgn2* alters phenotypes of Friend erythroleukaemia cells

(**A**) Expression of transcription factor genes *Gata1* and *Klf1* that regulate erythroid cell differentiation. Gene expression levels in GFP$^+$ cells were quantified by real time-PCR. The *Y*-axis represents the RQ of gene expression after normalization with β-actin. Two days after electroporation, *Gata1* and *Klf1* levels in *Hmgn2*-transfected cells were down-regulated ($P<0.05$) (see Supplementary Figure S2C for experimental design). We observed no significant difference in level of *Gata1* and *Klf1* at day 8 of culture ($P>0.05$). (**B**) Flowcytometric analysis for surface markers of Friend erythroleukaemia cells. Surface markers of *Hmgn2*-transfected cells were analysed at day 8 of culture. The percentage of the c-Kit$^+$/CD71$^+$ population in *Hmgn2*-transfected cells (20.6%) was decreased compared with that seen in mock controls (68.6%), whereas the percentage of the c-Kit$^-$/CD71 population in *Hmgn2*-transfected cells (12.6%) was higher than that seen in mock-transfected cells (4.23%).

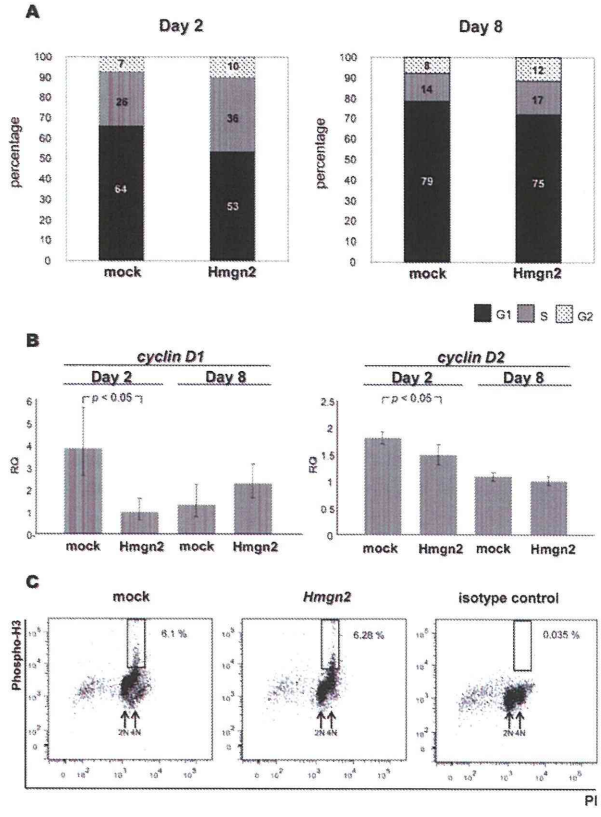

Figure 3 *Hmgn2* misexpression increases in the number of S-phase cells and down-regulates G_1-specific genes

(**A**) The percentage of cells in G_1, S and G_2 phases. Sorted GFP$^+$ cells at day 2 of culture and all cells at day 8 of culture were fixed and stained with PI. DNA content was quantified by flowcytometry. The Watson Pragmatic method was used to calculate cell percentages. (**B**) Expression of the G_1 phase-specific genes *cyclin D1* and *cyclin D2*. Gene expression was measured at days 2 and 8 of culture. The *Y*-axis represents the RQ of gene expression after normalization with β-actin. (**C**) The percentage of mitotic status of *Hmgn2*-transfected cells. Sorted GFP$^+$ cells at day 2 of culture were fixed and stained with anti-phosphorylated histone H3 at Ser^{10} (phospho-H3) and PI (DNA content). Mitotic cells were defined as the cells that have 4N content of DNA and high level of histone H3 phosphorylation.

cells by flowcytometry was analysed, with evaluation of expression levels of the G_1 phase-specific genes, *cyclin D1* and *cyclin D2*. Two days after transfection with Friend erythroleukaemia cells with *Hmgn2*, 36% of GFP$^+$ cells were in S-phase, whereas 26% of mock controls were in S-phase ($P<0.05$; Figure 3A and Supplementary Figure S3 available at http://www.cellbiolint.org/cbi/vvv/cbivvvppppadd.htm). There was no significant difference in the percentage of cells in S-phase at day 8 of culture between *Hmgn2*-transfected and mock control cells (14 and 17%, respectively, $P>0.05$; Figure 3A). Two days after transfection, expression levels of both *cyclin D1* and *cyclin D2* assessed by real-time PCR were down-regulated in *Hmgn2*-transfected cells ($P<0.05$; Figure 3B). At day 8 of culture, there was no difference in expression levels of *cyclin D1* and *cyclin D2* between *Hmgn2*-transfected and mock control cells (Figure 3B).

To investigate mitosis in transfected cells, flow cytometric analysis was carried out of *Hmgn2*-transfected cells using anti-phosphorylated histone H3 at Ser^{10} (phospho-H3), an indicator of late G_2 to anaphase in the cells with 4N content of DNA. There was no significant difference at day 2 of culture in the percentage of mitotic cells between *Hmgn2*-transfected cells and mock control (6.3 and 6.1% respectively $P>0.05$; Figure 3C).

3.4. Hmgn2 expression antagonizes erythroid cell differentiation in FL

Hmgn2 expression in FL was down-regulated during erythroid differentiation (Figure 1A). To investigate whether Hmgn2 functions in erythroid differentiation in FL as well as erythroleukaemia cells, *Hmgn2* was ectopically expressed in MNCs isolated from FL at 12.5 dpc. Two days after transfection by electroporation, CD71$^+$/Ter119$^-$ cells expressing Hmgn2, as indicated by GFP

positivity, were sorted and cultured in the presence of SCF, IL-3 and EPO for 7 days. Flowcytometry showed that the percentage of c-Kit$^+$/CD71$^+$ cells in *Hmgn2*-transfected cells (26%) was higher than that seen in mock controls (15.2%), while the percentage of CD71$^+$/Ter119$^+$ cells in *Hmgn2*-transfected cells (68.4%) was lower than in mock controls (73.6%; Figure 4A). *Gata1* expression levels were 4.2-fold lower in *Hmgn2*-transfected cells than in mock controls, and *Klf1* expression was undetectable in *Hmgn2*-transfected cells (Figure 4B). These results indicate that ectopic expression of *Hmgn2* inhibits erythroid differentiation in mouse FL.

4. Discussion

Hmgn2 is ubiquitously expressed and down-regulated during the differentiation of several cell types in vertebrates (Crippa et al., 1991; Shakoori et al., 1993; Lehtonen and Lehtonen, 2001).

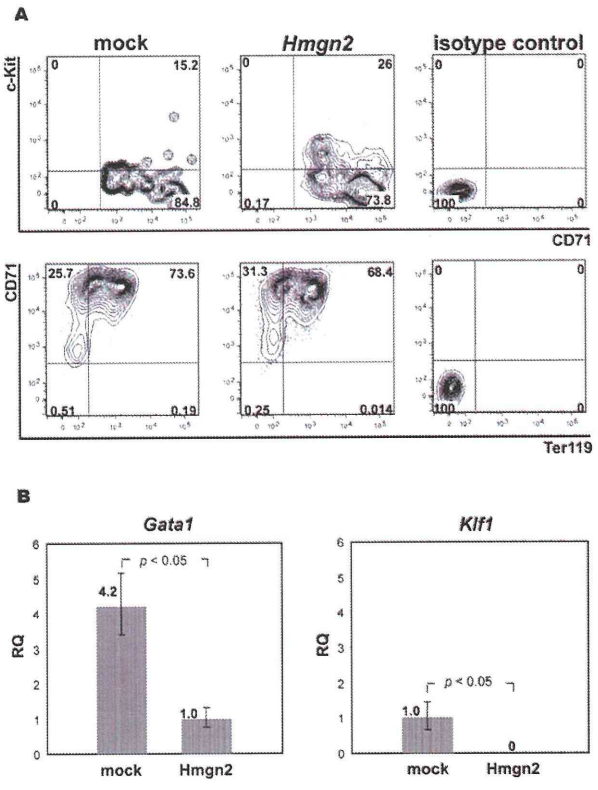

Figure 4 Ectopic expression of *Hmgn2* suppresses differentiation of erythroid progenitor cells by down-regulating *Gata1*

(**A**) Flow cytometric analysis of erythroid cells of FL MNCs transfected with *Hmgn2*/GFP expression vector. Two days after electroporation, GFP$^+$ CD71$^+$/Ter119$^-$ cells were sorted and cultured in the presence of SCF, IL-3 and EPO for 7 days. The percentage of c-Kit$^+$/CD71$^+$ cells in *Hmgn2*-transfected cells (26%) was higher than that seen in mock-transfected cells (15.2%), whereas the percentage of CD71$^+$/Ter119$^+$ cells in *Hmgn2*-transfected cells (68.4%) was lower than that seen in mock-transfected cells (73.6%). (**B**) Expression levels of *Gata1* and *Klf1* in erythroid cells were determined by RT–PCR. Two days after electroporation, *Gata1* was down-regulated in *Hmgn2*-transfected CD71$^+$/Ter119$^-$ cells compared with mock controls. *Klf1* expression was not detectable in these cells. Levels of gene expression were normalized to β-actin.

Relevant to HC (haemopoietic cell) development, injection of Hmgn2 protein into *Xenopus* embryos delayed the expression of mesoderm-specific genes (Korner et al., 2003). *Hmgn2* is also reportedly down-regulated during differentiation of erythroid cells in chicken embryos (Crippa et al., 1991) and in osteoblasts of mouse embryos (Shakoori et al., 1993). However, the role of Hmgn2 in mammalian HC development has remained unclear. The pattern of Hmgn2 gene and protein expression in mice suggests a function in erythroid differentiation (Figure 1). In mouse FL, *Hmgn2* was down-regulated during differentiation of BFU-E into reticulocytes and mature erythrocytes, when globin synthesis and enucleation occurs. Hmgn2 protein was expressed in HSCs and all stages of differentiated erythroid cells, while its expression level was down-regulated as BFU-E differentiates into mature erythrocyte. Hmgn2 therefore may regulate erythroid differentiation regardless of differentiation stage.

Friend erythroleukaemia cells are characterized as cells expressing c-Kit and CD71 (Friend 1957; Moreau-Gachelin 2008). Decrease in the number of c-Kit$^+$/CD71$^+$ cells (equivalent to CFU-E) and increase in c-Kit$^+$/CD71$^-$ cells (equivalent to BFU-E), in addition to down-regulation of *Gata1* and *Klf1* after transfection with *Hmgn2*, strongly suggest that Hmgn2 suppresses erythroid differentiation of Friend erythroleukaemia cells. During erythroid differentiation, Gata1 induces expression of CD71 and controls *Klf1* expression (Welch et al., 2004). Decrease in the number of c-Kit$^+$/CD71$^+$ cells and down-regulation of *Klf1* could be due to *Gata1* down-regulation (Figure 2A). We also showed that ectopic expression of *Hmgn2* in CD71$^+$/Ter119$^-$ FL MNCs suppresses their differentiation, indicated by the low percentage of CD71$^+$/Ter119$^+$ cells (CFU-E) and the high percentage of c-Kit$^+$/CD71$^-$ cells (BFU-E), accompanied by *Gata1* and *Klf1* down-regulation. These results are compatible with those seen in Friend erythroleukaemia cells and suggest that Hmgn2 suppresses erythroid differentiation in mice. Friend erythroleukaemia cells are comprised of c-Kit$^+$/CD71$^+$ and c-Kit$^-$/CD71$^+$ cells, equivalent to CFU-E and relatively mature cells (proerythroblasts and erythrocytes) respectively (Friend 1957; Moreau-Gachelin 2008; Supplementary Figure S4 available at http://www.cellbiolint.org/cbi/vvv/cbivvvpppadd.htm). Decrease of c-Kit$^+$/CD71$^+$ cells and increases of c-Kit$^+$/CD71$^-$, c-Kit$^-$/CD71$^+$ and c-Kit$^-$/CD71$^-$ cells were observed after transfection with *Hmgn2*, implying that differentiation arrest occurred at CFU-E. In mouse FL, expression level of *Hmgn2* declined from CFU-E to proerythroblast ($P<0.05$; Figure 1A). Hmgn2 may therefore function particularly at CFU-E during erythroid differentiation.

To investigate how Hmgn2 down-regulates erythroid differentiation, we analysed cell cycle status and related gene expression in *Hmgn2*-misexpressing cells. Following *Hmgn2* transfection at 2 and 8 days of culture, the percentage of Friend erythroleukaemia cells in S-phase increased, whereas the percentage of cells in G_1 decreased (Figure 3A). Cyclins D1 and D2 are G_1 phase-specific cyclins predominantly expressed in human, chicken and mouse erythroid cells (Dolznig et al., 1995; Dai et al., 2000). Observation of down-regulation of *cyclin D1* and *cyclin D2* genes 2 days after *Hmgn2* transfection is compatible with a low percentage of cells in G_1 and a high percentage in S-phase (Yang et al., 2006). Hmgn2 reportedly enhances DNA

replication of M13 DNA containing SV40 origin *in vitro* (Vestner et al., 1998). The data suggests that Hmgn2 does not function in mitosis of Friend erythroleukaemia cells (Figure 3C). It will be necessary to investigate whether Hmgn2 functions in mitosis during erythroid differentiation.

During erythroid differentiation, erythroblasts loss their proliferation capability and exit the cell cycle (Buttitta and Edgar, 2007) and condense chromatin. However, we do not know what is the key regulator underlying these molecular mechanisms. Our novel finding is that Hmgn2 is involved in erythroid differentiation. Therefore, further study of Hmgn2 should enable us to investigate the role of DNA replication and cell mitosis in erythroid differentiation. In conclusion, Hmgn2 appears to reduce compaction of chromatin fibres and facilitates accessibility of DNA polymerase to nucleosomes, enhancing DNA synthesis (Vestner et al., 1998; Bustin, 2001). An increase in the number of Friend erythroleukaemia cells in S-phase and a decrease of those in G_1 after *Hmgn2* misexpression might be due to enhanced DNA replication and/or blocking entry of mitosis in S-phase that promotes down-regulation of erythroid differentiation.

5. Conclusion

We have shown that ectopic expression of *Hmgn2* altered differentiation of mouse erythroid cells *in vitro*. In Friend erythroleukaemia cells, a decrease in relatively mature c-Kit$^+$/CD71$^+$ erythroid cells and an increase in immature c-Kit$^+$ CD71$^-$ erythroid cells occurred, whereas in FL HCs there was also a decrease in relatively mature CD71$^+$/Ter119$^+$ erythroid cells and an increase in relatively immature c-Kit$^+$/CD71$^+$ erythroid cells. An increase in the number of S-phase cells and a decrease in the number of G_1-phase cells in erythroleukaemia suggests that Hmgn2 antagonizes mouse erythroid differentiation may be due to enhancement of DNA replication and/or blocking entry of mitosis in S-phase.

Author Contribution

Kasem Kulkeaw performed the research and wrote the manuscript. Tomoko Inoue, Chiyo Mizuochi, Yuka Horio and Yasushi Ishihama performed the research. Daisuke Sugiyama designed and performed the research and wrote the manuscript.

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References

Amen M, Espinoza HM, Cox C, Liang X, Wang J, Link TM et al. Chromatin-associated HMG-17 is a major regulator of homeodomain transcription factor activity modulated by Wnt/b-catenin signaling. Nucleic Acids Res 2008;36:462–76.

Bustin M. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol 1999;19:5237–46.

Bustin M. Chromatin unfolding and activation by HMGN(*) chromosomal proteins. Trends Biochem Sci 2001;26:431–7.

Bustin M, Reeves R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acids Res Mol Biol 1996;54:35–100.

Buttitta LA, Edgar BA. Mechanisms controlling cell cycle exit upon terminal differentiation. Curr Opin Cell Biol 2007;19:697–704.

Crippa MP, Nickol JM, Bustin M. Differentiation-dependent alteration in the chromatin structure of chromosomal protein HMG-17 gene during erythropoiesis. J Mol Biol 1991;217:75–84.

Dai MS, Mantel CR, Xia ZB, Broxmeyer HE, Lu L. An expansion phase precedes terminal erythroid differentiation of hematopoietic progenitor cells from cord blood *in vitro* and is associated with up-regulation of cyclin E and cyclin-dependent kinase 2. Blood 2000;96:3985–7.

Dolznig H, Bartunek P, Nasmyth K, Mullner EW, Beug, H. Terminal differentiation of normal chicken erythroid progenitors: shortening of G_1 correlates with loss of D-cyclin/cdk4 expression and altered cell size control. Cell Growth Diff 1995;6:1341–52.

Dzierzak E, Medvinsky A, De Bruijn M. Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. Immuno Today 1998;19:228–36.

Ema H, Nakauchi H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. Blood 2000;95:2284–8.

Friend C. Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 1957;105:307–18.

Hattangadi SM, Burke KA, Lodish HF. Homeodomain-interacting protein kinase 2 plays an important role in normal terminal erythroid differentiation. Blood 2010;115:4853–61.

Inoue T, Sugiyama D, Kurita R, Oikawa T, Kulkeaw K, Kawano H et al. APOA-1 is a novel marker of erythroid cell maturation from hematopoietic stem cells in mice and humans. Stem Cell Rev 2011;7:43–52.

Korner U, Bustin M, Scheer U, Hock R. Developmental role of HMGN proteins in Xenopus laevis. Mech Dev 2003;120:1177–92.

Lehtonen S, Lehtonen E. HMG-17 is an early marker of inductive interactions in the developing mouse kidney. Differentiation 2001;67:154–63.

McGrath K, Palis J. Ontogeny of erythropoiesis in the mammalian embryo. Curr Top Dev Biol 2008;82:1–22.

Mohamed OA, Bustin M, Clarke HJ. High-mobility group proteins 14 and 17 maintain the timing of early embryonic development in the mouse. Dev Biol 2001;229:237–49.

Moreau-Gachelin F. Multi-stage Friend murine erythroleukemia: molecular insights into oncogenic cooperation. Retrovirology 2008;5:99.

Shakoori AR, Owen TA, Shalhoub V, Stein JL, Bustin M, Stein GS et al. Differential expression of the chromosomal high mobility group proteins 14 and 17 during the onset of differentiation in mammalian osteoblasts and promyelocytic leukemia cells. J Cell Biochem 1993;51:479–87.

Shirakawa H, Herrera JE, Bustin M, Postnikov Y. Targeting of high mobility group-14/-17 proteins in chromatin is independent of DNA sequence. J Biol Chem 2000;275:37937–44.

Sugiyama D, Tsuji K. Definitive hematopoiesis from endothelial cells in the mouse embryo; a simple guide. Trends Cardiovasc Med 2006;16:45–9.
Taylor WR. FACS-based detection of phosphorylated histone H3 for the quantitation of mitotic cells. Methods Mol Biol 2004;281:293–9.
Trieschmann L, Alfonso PJ, Crippa MP, Wolffe AP, Bustin M. Incorporation of chromosomal proteins HMG-14/-17 into nascent nucleosomes induces an extended chromatin conformation and enhances the utilization of active transcription complexes. EMBO J 1995a;14:1478–89.
Trieschmann L, Postnikov Y, Rickers A, Bustin M. Modular structure of chromosomal proteins HMG-14/-17: Definition of a transcriptional activation domain distinct from the nucleosomal binding domain. Mol Cell Biol 1995b;15:6663–9.
Ueda T, Catez F, Gerlitz G, Bustin M. Delineation of the protein module that anchors HMGN proteins to nucleosomes in the chromatin of living cells. Mol Cell Biol 2008;28:2872–83.
Vestner B, Bustin M, Gruss C. Stimulation of replication efficiency of a chromatin template by chromosomal protein HMG-17. J Biol Chem 1998;273:9409–14.
Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell 2000;100:157–68.
Welch JJ, Watts JA, Vakoc CR, Yao Y, Wang H, Hardison RC et al. Global regulation of erythroid gene expression by transcription factor GATA-1. Blood 2004;104:3136–47.
Yang K, Hitomi M, Stacey DW. Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell. Cell Div 2006;1:32.

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Variation in Mesodermal and Hematopoietic Potential of Adult Skin-derived Induced Pluripotent Stem Cell Lines in Mice

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Abstract Induced pluripotent stem cells (iPSCs) are a promising tool for regenerative medicine. Use of iPSC lines for future hematotherapy will require examination of their hematopoietic potential. Adult skin fibroblast somatic cells constitute a source of iPSCs that can be accessed clinically without ethical issues. Here, we used different methods to compare mesodermal and hematopoietic potential by embryoid body formation of five iPSC lines established from adult mouse tail-tip fibroblasts (TTFs). We observed variation in proliferation and in expression of genes (*Brachyury, Tbx1, Gata1, Klf1, Csf1r*) and proteins (Flk1, Ter119 and CD45) among TTF-derived lines. 256H18 iPSCs showed highest proliferation and most efficient differentiation into mesodermal and hematopoietic cells, while expression levels of the pluripotency genes *Oct3/4, Sox2, Klf4* and *Nanog* were lowest among lines analyzed. By contrast, the 212B2 line, transduced with *c-Myc*, showed lowest proliferation and differentiation potential, although expression levels of *Oct3/4, Sox2* and *Klf4* were highest. Overall, we find that mesodermal and hematopoietic potential varies among iPSCs from an identical tissue source and that *c-Myc* expression likely underlies these differences.

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Introduction

Hematopoietic stem cells (HSCs) are already in use for transplantation therapy for hematological diseases. However, problems associated with HSC transplantation remain, such as a shortage of donors or immunoreactivity caused by HLA mismatching (rejection and graft versus host disease (GVHD)). To overcome these issues, induced pluripotent stem cells (iPSCs) could serve to generate autologous HSCs without the need for a donor. iPSCs have been established from various somatic cells by forced expression of defined factors [1, 2]. These cells display properties similar to embryonic stem cells (ESCs) in terms of differentiation capacity into various cell types, teratoma formation in immuno-deficient mice, and generation of chimeric mice with germ line transmission [3]. Therefore, iPSC technology could enable us to generate cells for clinical purposes without an embryonic source or a donor [4, 5]. Generally, *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* are retrovirally transduced into somatic cells to initiate reprogramming and establish iPSC lines. Since both expression of the *c-Myc* oncogene and retroviral infection are associated with malignancy, investigators have devised reprogramming protocols lacking *c-Myc* transduction [6] or avoiding use of non-integrated viral vectors [7] or plasmids [8] for gene delivery. Various novel approaches, including combining transcription factors and reporters, have been employed to reprogram various types of somatic cells, such as mouse embryonic fibroblasts (MEFs), adult fibroblasts, pancreatic β-cells, hepatocytes, gastric epithelial cells, B cells, and $CD34^+$ cord blood cells