cells emerging onto the OP9 stromal layer, after the initial 6-day differentiation period, contain hemogenic progenitors [16].

Globin switching has been thoroughly investigated, both as a model of tissue- and temporal-specific transcriptional control and as a tool for drug discovery aimed at ameliorating the effects of fetal hemoglobin synthesis in patients with hemoglobinopathies [17]. In the present study, we specifically analyze the temporal pattern of globin switching in the α- and β-cluster of erythrocytes induced from primate ESCs in the OP9 coculture system. For this purpose, we separated VEGFR-2^{high} $CD34^{+}$ hemogenic progenitors and cultured them in the presence of appropriate cytokines. This system enables the sequential analysis of mature floating erythrocytes and immature erythroid clonogenic progenitors, both at the transcriptional and translational level, and may serve as a novel in vitro model for the globin switch in humans.

Materials and Methods

Cell Lines

The ESC line CMK6, established from cynomolgus monkey blastocysts, was maintained according to the procedure of Suemori et al. [9]. The green fluorescent protein (GFP)-transfected ESC subline [18] was used to exclude OP9 cells. The proportion of GFP^{+} ESC-derived cells within the culture was determined by FACS. OP9 stromal cells, kindly provided by Dr. Hiroaki Kodama, were maintained as reported previously [15].

Cytokines and Growth Factors

Recombinant human granulocyte cell-stimulating factor (G-CSF), erythropoietin (EPO), interleukin-3 (IL-3), stem cell factor (SCF), and thrombopoietin (TPO) were kindly provided by Kirin Brewery Co. (Tokyo, http://www.kirin.co.jp/english). Recombinant human VEGF was purchased from R&D Systems Inc. (Minneapolis, http://www.rndsystems.com).

Antibodies

Primary antibodies used in this study included mouse anti-human hemoglobin (Hb) γ- and -β mononuclear antibodies (mAbs) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, http://www.scbt.com), mouse anti-human CD34 (clone 563) from BD Pharmingen (San Diego, http://www.bdbiosciences.com/pharmingen), and rabbit anti-human Hb polyclonal antibodies from MP Biomedicals (Irvine, CA, http://www.mpbio.com). Mouse anti-human Hb-ε- and -ζ mAbs and mouse anti-human VEGFR-2 mAb were used according to previous reports [19–21]. Mouse anti-human Hb-α mAb was established in the laboratory of David H. K. Chui. All primary antibodies against human antigens used in this study cross-reacted with cynomolgus monkey compartments, as observed previously [15, 21, 22]. The secondary antibodies used included cyanine 3 (Cy3)-conjugated donkey anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, http://www.jacksonimmuno.com).

In Vitro Hematopoietic Differentiation of Primate ESCs

In vitro differentiation of ESCs and cell sorting were performed as reported previously [15, 16]. In brief, trypsin-treated undifferentiated ESCs were transferred to fresh confluent OP9 cells in six-well plates at a concentration of 1×10^4 cells per well and cultured in α-minimal essential medium (α-MEM) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 50 μM 2-mercaptoethanol (2-ME), and 20 ng/ml VEGF. On day 6, cells were harvested and sorted with phycoerythrin-conjugated CD34 and allophycocyanin-conjugated VEGFR-2 mAbs using a FACSVantage flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Nonviable cells were excluded from the analysis by propidium iodide costaining. The purity of isolated VEGFR-2^{high} $CD34^{+}$ cells was determined using FACS. Preparations that were 96%–98% pure were employed in the experiments. Sorted cells were transferred to fresh confluent OP9 cells in six-well plates at a concentration of 1×10^4 cells per well in α-MEM, 10% FCS, 50 μM 2-ME, and a mixture of 2 U/ml EPO, 20 ng/ml IL-3, 100 ng/ml SCF, and 10 ng/ml TPO. Floating hematopoietic cells (HCs) that emerged after cell sorting were processed every 3 days for May-Giemsa staining and immunostaining with anti-human Hb antibodies as described previously [15, 16]. Nuclei were labeled with Hoechst 33342. Fluorescence was detected and photographed with an AxioCam photomicroscope (Carl Zeiss GmbH, Jena, Germany, http://www.zeiss.com). In sequential analyses, data are presented as means ± SDs of triplicate wells. Representative results from one of three independent experiments are shown.

Colony-Forming Assays for Primitive and Definitive Cells

Colony-forming assays were performed every 6 days in semisolid medium consisting of α-MEM, 0.9% methylcellulose, 30% FCS, 10% bovine serum albumin, 50 μM 2-ME, and a mixture of 10 ng/ml G-CSF, 2 U/ml EPO, 20 ng/ml IL-3, 100 ng/ml SCF, and 10 ng/ml TPO. For colonies consisting of primitive cells, the medium was replaced with fresh semisolid medium [15]. For colonies consisting of definitive cells, we took advantage of the nature of OP9 stromal cells that adhered faster to culture dishes than ESC-derived cells [23]. After floating HCs were gently washed three times with phosphate-buffered saline (PBS), and the remaining adherent cells, including OP9 cells, were treated with 0.25% trypsin/EDTA. Trypsinized cells were transferred to a new culture dish and incubated for 30 minutes to allow OP9 cells to adhere. Floating cells obtained from the resulting culture supernatant after incubation were transferred (3×10^4 cells per well) to a new 35-mm Petri dish or to a fresh OP9 cell layer in a 35-mm culture dish. Colonies ($\geq$50 cells) were counted using an inverted microscope according to previously established criteria [15, 24, 25]. Data are presented as means ± SDs of triplicate wells. Representative results from one of three independent experiments are shown. After 7 days for primitive and 12 days for definitive cells, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, washed twice with PBS, and processed for May-Giemsa staining, immunostaining, and reverse transcription-polymerase chain reaction (RT-PCR) analysis. At least 10 individual colonies were analyzed by immunostaining and RT-PCR analysis.

RT-PCR for Globin Gene Expression

RNA isolation and RT-PCR were performed using the procedure of Umeda et al. [15]. Samples were initially denatured at 94°C for 5 minutes, followed by amplification cycles of

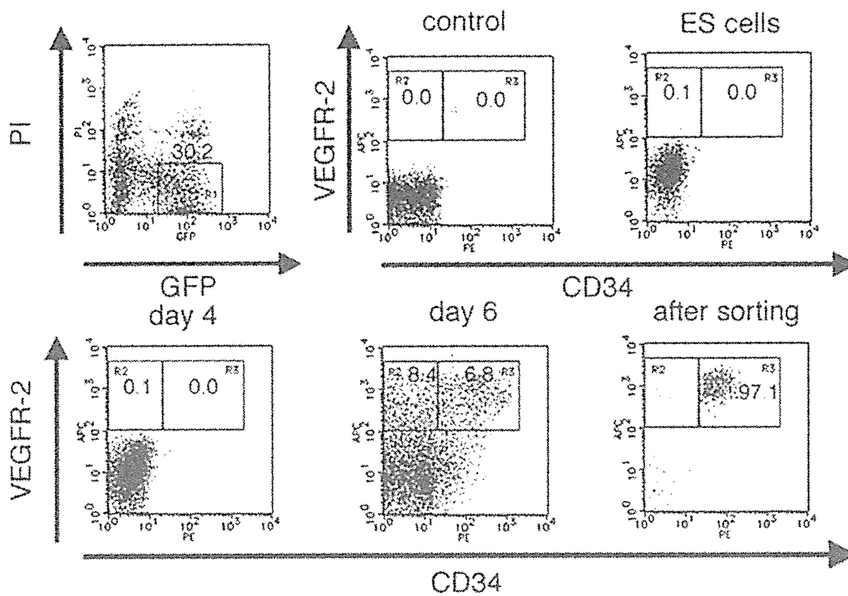


Figure 1. Fluoresence-activated cell sorting analysis and cell sorting using antibodies against VEGFR-2 and CD34. The amounts of VEGFR-2^{high} CD34^{-} (R2, upper left quadrant) or VEGFR-2^{high} CD34^{+} cells (R3, upper right quadrant) are shown as a percentage of the total GFP^{+} ES cells (R1). Abbreviations: APC, allophycocyanin; ES, embryonic stem; GFP, green fluorescent protein; PI, propidium iodide; VEGFR-2, vascular endothelial growth factor receptor-2.

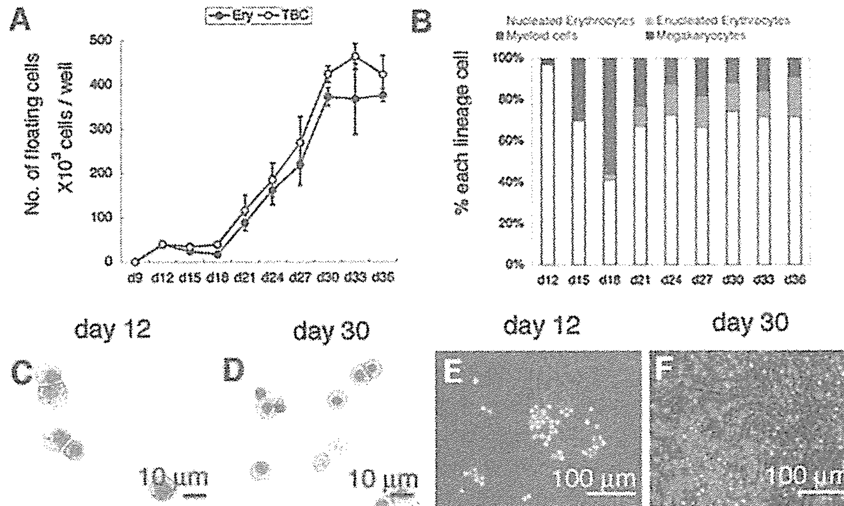


Figure 2. Two-wave erythropoiesis generated from VEGFR-2^{high} CD34^{+} cells. **(A):** Sequential analysis of the number of floating erythrocytes and total blood cells. **(B):** Sequential analysis of the proportion of each cell lineage. **(C, D):** May-Giemsa staining of floating erythrocytes (×400). **(E, F):** Micrographs of an adherent hematopoietic cell cluster (×100). Abbreviations: d, day; Ery, erythrocyte; TBC, total blood count; VEGFR-2, vascular endothelial growth factor receptor-2.

94°C for 1 minute (denaturing), 60°C for 1 minute (annealing), and 72°C for 1 minute (extension), and a final extension at 94°C for 7 minutes. The following primers were used for RT-PCR: ε-globin (359 base pairs [bp]), forward 5′-TGC ATT TTT ACT GCT GAG GAG A-3′, reverse 5′-TGC CAA AGT GAG TAG CCA GAA TAA-3′; γ-globin (221 bp), forward 5′-GGC AAC CTG TCC TCT GCC TC-3′, reverse 5′-GAA ATA GAT TGC CAA AAC AG-3′; β-globin (183 bp), forward 5′-CTC ATG GCA AGA AAG TGC TTG-3′, reverse 5-AAT TCT TTG CCA AAG TGA TGG G-3′; ζ-globin (327 bp), forward 5′-CCG CCA TGT CTC TGA CCA A-3′, reverse 5′-GCT CGC TCA GCT TGG ACA GGG-3′; α-globin (395 bp), forward 5′-CCG ACA AGA CCA ACG TCA AGG-3′, reverse 5′-AGG TCG AAG TGC GGG AAG TA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (360 bp), forward 5′-CAC CAG GGC TGC TTT TAA CTC TG-3′, reverse 5′-ATG GTT CAC ACC CAT GAC GAA C-3′. The PCRs consisted of 35 cycles for floating HCs and 40 cycles for individual erythroid colonies. cDNAs from cynomolgus monkey bone marrow or human erythroleukemia K562 cells were used as positive controls. For semiquantitative comparisons, samples were normalized by dilution to produce equivalent signals for GAPDH.

The quantitative RT-PCR assay of globin transcripts was performed using gene-specific double-fluorescent-labeled probesin an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The florescent reporter and quencher were 6-carboxyfluorescein (FAM) and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA), respectively. The following primers and probes were used for real-time PCR: ε-globin, forward 5′-TGG CAA GGA GTT CAC CCC T-3′, reverse 5′-AAT GGC GAC AGC AGA CAC C-3′, probe 5′-FAM-TGC AGG CTG CCT GGC AGA AGC-TAMRA-3′; γ-globin, forward 5′-TGG CAA GAA GGT GAC TTC-3′, reverse 5′-TCA CTC AGC TGG GCA AAG-3′, probe 5′-FAM-TGG GAG ATG CCA TAA AGA ACC TGG-TAMRA-3′; β-globin, forward 5′-CAA GAA AGT GCT

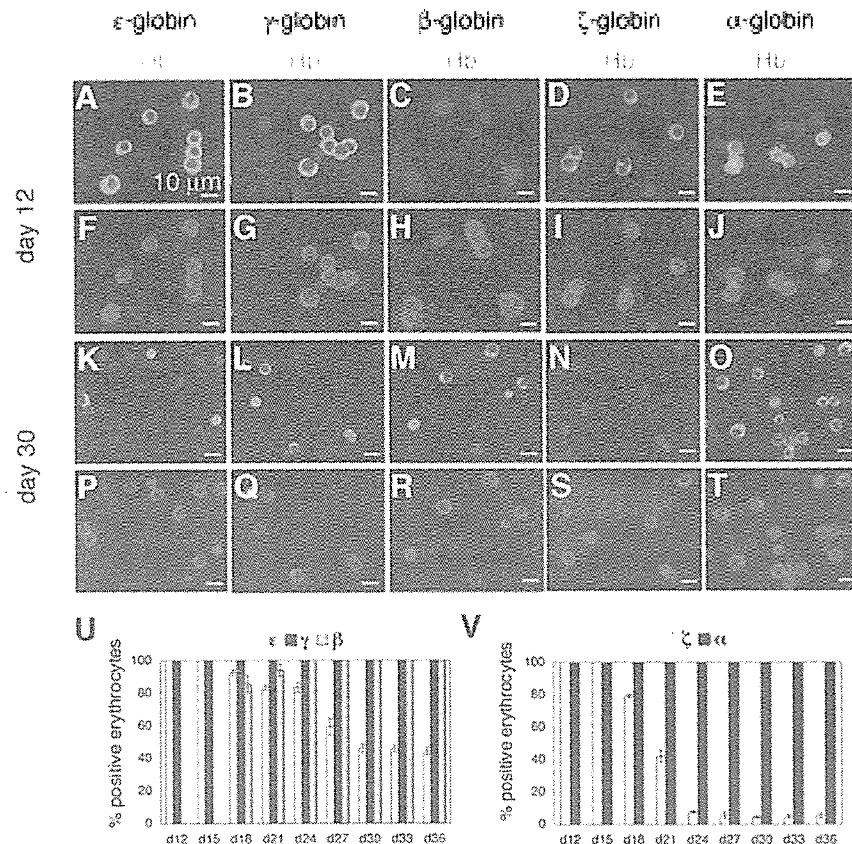


Figure 3. Immunostaining analysis of globin gene expression in floating erythrocytes. **(A–T):** Immunostaining of erythrocytes (×400). Red (cyanine 3) indicates globin-type stains, and green (fluorescein isothiocyanate) indicates hemoglobin. **(U, V):** Sequential analysis of the proportion of erythrocytes stained with ε-, γ-, and β-globin monoclonal antibodies (mAbs) **(U)** and ζ- and α-globin mAbs **(V)**. Abbreviation: Hb, hemoglobin.

TGG TGC CT-3′, reverse 5′-GCA AAG GTG CCC TTG AGG T-3′, probe 5-FAM-TAG TGA TGG CCT GGC TCA CCT GGA C-TAMRA-3′; ζ-globin, forward 5′-GGA CCC TCA TTG TGT CCA TGT-3′, reverse 5′-TGC GGG TAG CTG AGG AAG AG-3′, probe 5′-FAM-TCC ACT CAG GCC GAC AC-TAMRA-3′; α-globin, forward 5′-TCC CCA CCA CCA AGA CCT AC-3′; reverse 5′-CCT AAC CTG GGC AGA GCC-3′, probe 5′-FAM-TCC CCA CTT CGA CCT GAG CCA-TAMRA-3′; 18S rRNA, forward 5′-AGT CCC TGC CCT TTG TAC ACA-3′, reverse 5′-GAT CCG AGG GCC TCA CTA AAC-3′, probe 5′-FAM-CGC CCG TCG CTA CTA CCG ATT GG-TAMRA3′. The ε- and β-globin-specific primers and probes have been described previously [26, 27]. For all samples, the globin expression levels normalized to the housekeeping gene, 18S rRNA, were determined using the comparative threshold cycle (C_T) method [28, 29]. Briefly, cDNA was mixed with primers and the PCR master mix (Applied Biosystems) and amplified in an ABI PRISM 7900HT instrument (Applied Biosystems). The C_T value (the cycle number at which the emitted fluorescence exceeded an automatically determined threshold) for each globin gene was normalized against the corresponding rRNA C_T value and plotted against the log quantities of target. The efficiency of the reaction (E) was calculated from the slope of the dilution curve using the following equation: $E = 10^{1/-S} - 1$, in which E = PCR efficiency and S = slope [28]. Data are presented as means ± SDs of triplicate wells. Representative results from one of three independent experiments are shown.

Results

Seeding VEGFR-2^{high} CD34$^+$ Hemogenic Progenitors in the OP9 Coculture System Leads to Two-Wave Erythropoiesis

In a previous report, our group showed that the numbers of HCs that develop from ESCs increase if, after the initial 6-day VEGF treatment, the whole cultures are replated onto a new confluent OP9 cell layer [15]. However, this induced hematopoiesis is not sufficient for quantitative analysis of globin expression, primarily due to the concomitant development of other lineages. Therefore, we employed a two-step culture system to efficiently induce erythropoiesis by purifying and reseeding hemogenic progenitors. For FACS analysis, VEGFR-2 and CD34 were employed as key markers of early hematopoietic progenitors [30–33]. As shown in Figure 1, undifferentiated ESCs were all negative for CD34, whereas approximately 80% of the cells expressed VEGFR-2 at low levels. VEGFR-2^{low} cells gradually decreased during culturing, whereas VEGFR-2^{high} cells first detected on day 6 and half of these were CD34$^+$. We purified the VEGFR-2^{high} CD34$^+$ cell fraction, which possessed greater hemogenic potential, consistent with previous data [16], and reseeded on a new OP9 feeder layer in the presence of EPO, IL-3, SCF, and TPO, all of which enhance erythroid lineage development [23, 34, 35]. Reanalysis of sorted cells confirmed purity of 96%–98%.

Sequential analyses demonstrated that floating HCs, mostly large nucleated erythrocytes, were generated on day 9 with maximum levels on day 12, 6 days after cell sorting, but

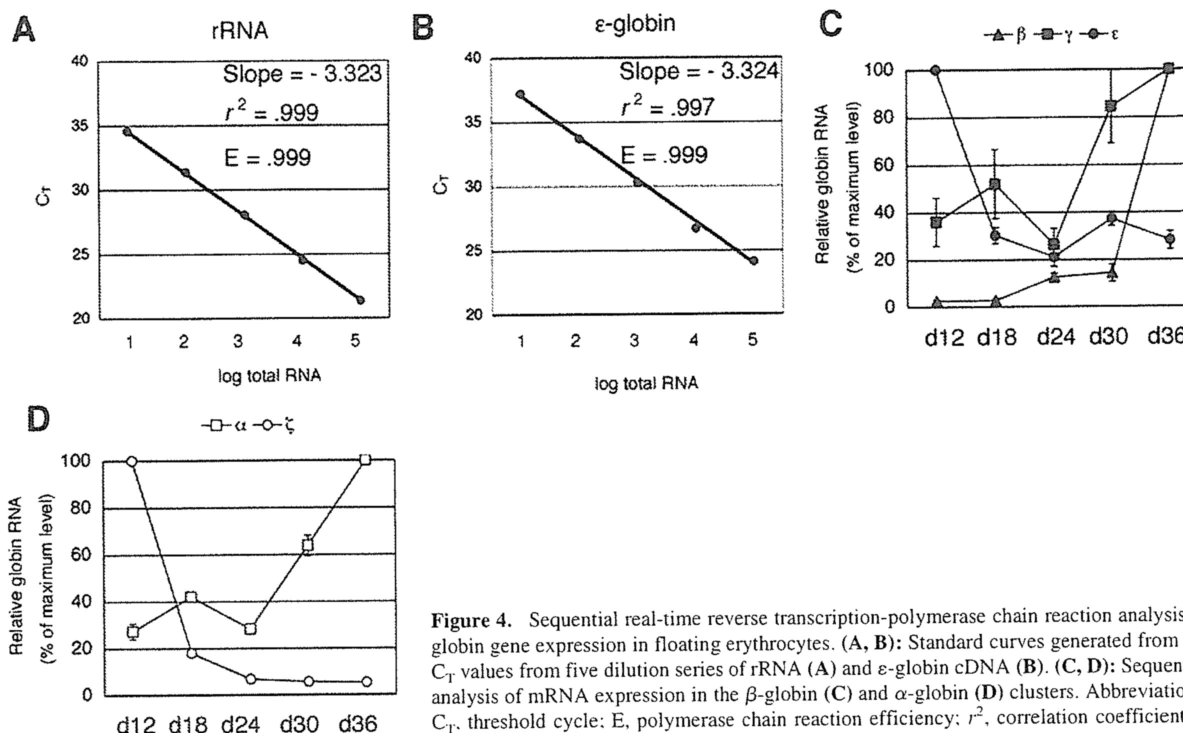


Figure 4. Sequential real-time reverse transcription-polymerase chain reaction analysis of globin gene expression in floating erythrocytes. **(A, B):** Standard curves generated from the C_T values from five dilution series of rRNA **(A)** and ε-globin cDNA **(B)**. **(C, D):** Sequential analysis of mRNA expression in the β-globin **(C)** and α-globin **(D)** clusters. Abbreviations: C_T, threshold cycle; E, polymerase chain reaction efficiency; r^2, correlation coefficient.

gradually decreased thereafter (Fig. 2A, 2C). Subsequently, small nucleated and enucleated erythrocytes appeared on day 18 (Fig. 2D). As the floating cells increased in number, a second wave of hematopoiesis, approximately 90% of which involved small erythrocytes, developed and 20%–25% of the total cells were enucleated erythrocytes on day 21 onwards (Fig. 2A, 2B). In contrast, adherent HCs were generated beginning on day 8 and maximizing on day 12 (Fig. 2E). The adherent fraction contains more progenitors than the floating fraction in the OP9 coculture system [15, 23]. The clusters decreased on day 18 and regrew all over the stromal layer by day 20 and thereafter (Fig. 2F). Thus, with a two-step system using OP9 stromal cells, two-wave hematopoiesis with a relatively high proportion of floating erythrocytes was induced from primate ESCs, along with the development of immature adherent hematopoietic progenitors.

Globin Switches Occur in Parallel with Transition from Primitive to Definitive Erythropoiesis

Primitive erythrocytes are relatively large and nucleated, whereas in definitive erythropoiesis, erythroid progenitors mature sequentially and eventually lose their nuclei [36]. To determine the mechanism of erythropoietic transition in this culture system, sequential immunostaining analyses of temporal expression patterns of embryonic, fetal, and adult Hbs in floating erythrocytes were performed. Cy3 detection of erythrocytes stained with ε-, γ-, β-, ζ-, and α-globin mAbs and FITC detection of erythrocytes stained with Hb polyclonal Ab, which reacts with embryonic, fetal, and adult erythrocytes, were used to detect all erythrocytes in the total HCs. Until day 15, all floating erythrocytes were positive for ε-, γ-, ζ- and α-globins, but not β-globin expression (Fig. 3A–3J). In contrast, the second wave of erythrocytes was positive for γ-, β-, and α-globin, but one-half and less than 10% of the total erythrocytes expressed ε- and ζ-globin, respectively (Fig. 3K–3V). Notably, the proportion of total enucleated erythrocytes that were positive for ε- and ζ-globin was equivalent to the proportion of total nucleated cells (data not shown). Thus, expression of β-globin, but not embryonic ε- and ζ-globins, defines the switch in parallel with the transition from primitive (EryP) to definitive (EryD) erythrocytes during culturing.

Next, quantitative real-time PCR analysis of globin genes in floating erythrocytes was performed to confirm the globin switch at the mRNA level. cDNA obtained from adult bone marrow or floating erythrocytes on day 30 was used as a standard for estimating the linear ranges and amplification efficiencies of the globin and ribosomal RNA systems. The reaction efficiency (E) was calculated from the slope of the dilution curve [27, 28], which was confirmed to be between 0.993 and 0.999 for each transcript. The linear regression equation, correlation (r^2), and E are illustrated in Figure 4A and 4B. In the β-globin cluster, embryonic ε-globin gradually decreased on day 18 but was subsequently detected thereafter, whereas fetal γ-globin was constantly expressed during culture. Notably, adult β-globin had an approximately 30-fold increase in expression on day 36, compared with its expression on day 12 (ΔC_T [globin − rRNA] was −14.86 ± 0.34 on day 12 and −9.52 ± 0.41 on day 36), although its expression was far below than that of γ-globin (Fig. 4C). In the α-globin cluster, ζ- and α-globin genes were coexpressed on day 12 and α-globin was expressed constantly during subsequent culturing, whereas the ζ-globin level decreased from day 18 onwards (Fig. 4D). Early definitive erythrocytes in the fetal liver express large amounts of fetal hemoglobin and a small amount of adult hemoglobin [37]. Collectively, analyses at the transcriptional and translational levels revealed that this culture system

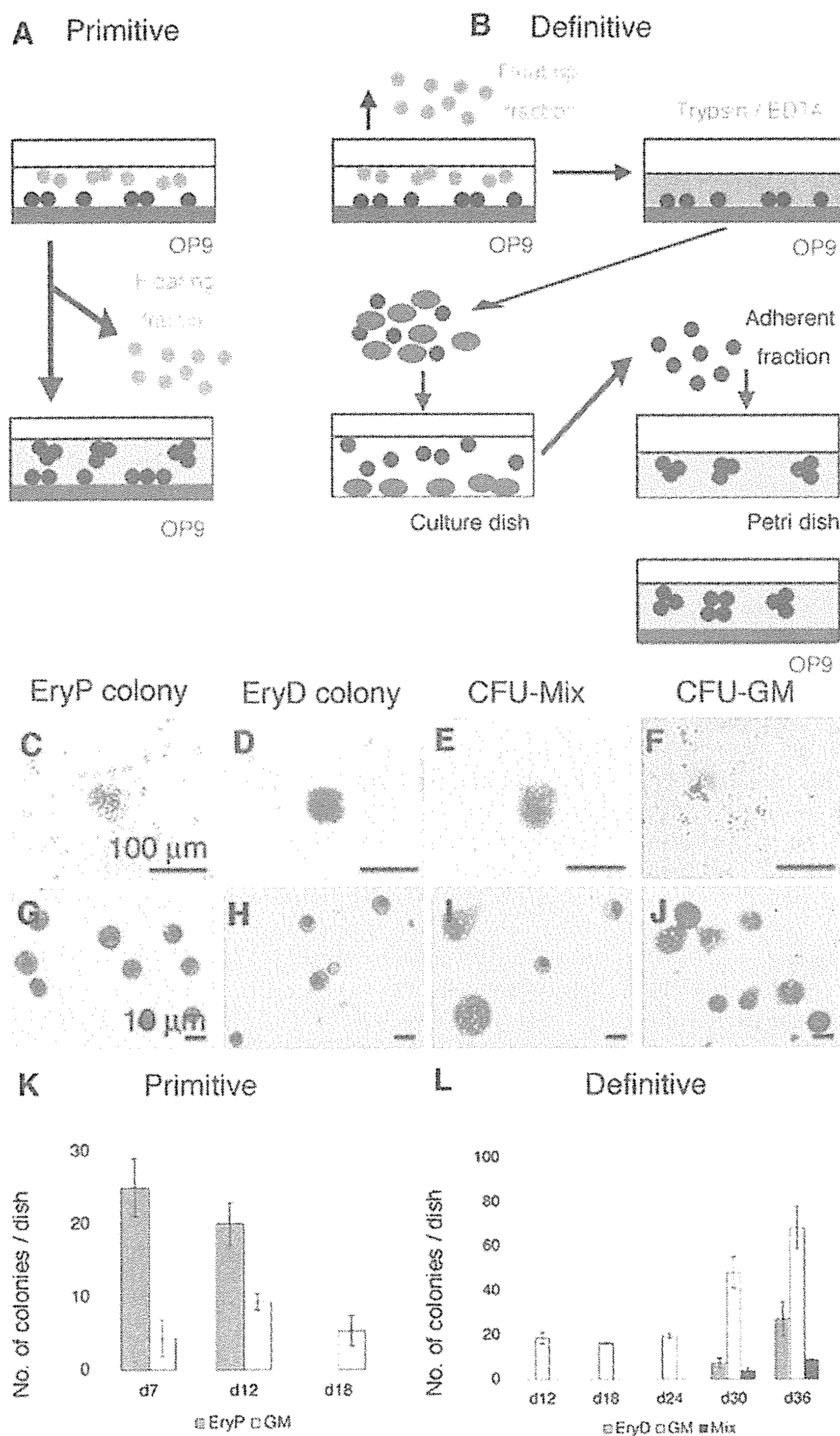


Figure 5. Development of primitive and definitive hematopoietic colonies. **(A, B):** Schematic representation of colony-forming assays. **(C–F):** Light micrographs of colonies (×100). **(G–J):** May-Giemsa staining of colonies (×400). **(K, L):** Sequential analysis of colony-forming assays. Abbreviations: CFU-GM, colony-forming unit granulocyte/macrophage colonies; CFU-Mix, mixed colony-forming unit; EryD, definitive erythroid; EryP, primitive erythroid.

recapitulates transition from embryonic to fetal/adult globin expression during sequential development of erythrocytes in the yolk sac and early fetal liver.

Sequential Development of Primitive and Definitive Erythroid Colonies

To evaluate the mechanism of globin switching at the clonogenic progenitor level, we performed methylcellulose colony-forming assays for both primitive and definitive colonies, as depicted schematically in Figure 5A, 5B. The phenotypes of colony-forming cells included EryP (Fig. 5C, 5G), EryD (Fig. 5D, 5H), mixed colony-forming unit (CFU-Mix) (Fig. 5E, 5I), and colony-forming unit granulocyte-macrophage (CFU-GM) colonies (Fig. 5F, 5J). Sequential colony-forming assays revealed EryP colonies until day 18 and the initial development of EryD and CFU-Mix colonies on day 30 (Fig.

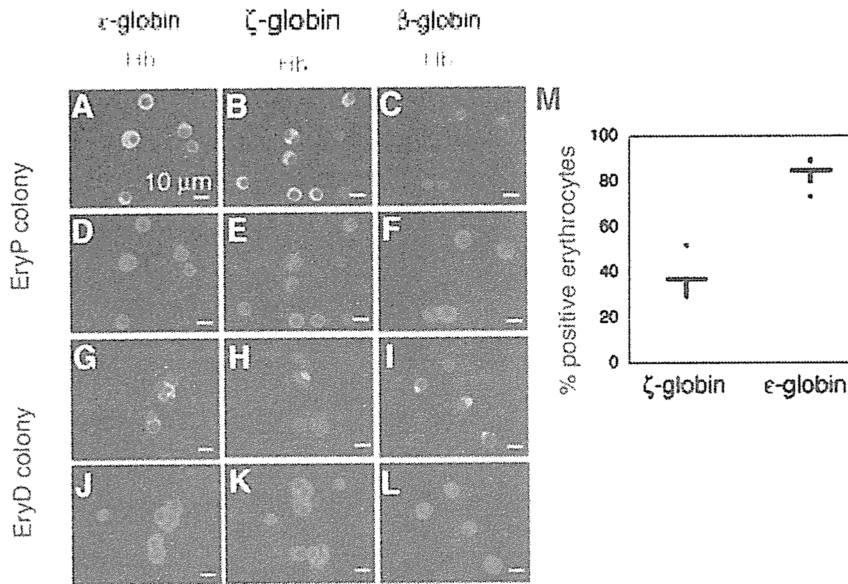


Figure 6. Immunostaining analysis of erythrocytes from primitive and definitive erythroid colonies. **(A–L):** Immunostaining of erythrocytes obtained from EryP **(A–F)** and EryD **(G–L)** colonies (×400). Staining is as in Figure 3. **(M):** Proportion of ε- or ζ-globin-positive erythrocytes in individual EryD colonies. Data are presented as the mean of 10 individual colonies. Abbreviations: EryD, definitive erythroid; EryP, primitive erythroid; Hb, hemoglobin.

5K, 5L). Immunostaining experiments showed that all of the erythrocytes in individual EryP colonies were positive for ε-, γ-, ζ-, and α-globin, but not β-globin (Fig. 6A–6F and data not shown), consistent with previous reports [15]. In contrast, erythrocytes in individual EryD colonies were positive for γ-, α-, and β-globin, although β-globin was expressed at low levels (Fig. 6I, 6L and data not shown). Analysis with embryonic globin mAbs revealed ε- and ζ-globin expression in 83.7% ± 6.5% and 35.0% ± 6.5%, respectively, of the erythrocytes in individual EryD colonies (Fig. 6G, 6H, 6J, 6K, 6M). These results indicate that β-globin is exclusively expressed in EryD, but not EryP, colonies and that the decline in embryonic globin expression occurs in individual definitive erythroid progenitors.

OP9 Stromal Cell-Derived Factors Enhance β-Globin Gene Expression in Definitive Erythroid Colonies

To determine whether erythrocytes in EryD colonies expressed less β-globin than floating definitive erythrocytes generated from the OP9 stromal layer, we examined the effect of OP9 stromal cells on definitive colony-forming assays. In semisolid medium with OP9 cells, EryD and CFU-Mix colonies developed earlier and increased with time (Fig. 7A, 7B). Immunostaining analyses revealed increased β-globin in EryD colonies with OP9 stromal cells (Fig. 7C–7F). Single-colony RT-PCR data additionally showed that both EryP and EryD colonies expressed embryonic (ε and ζ) as wells as fetal (γ and α) or adult (β) globin genes. These results are consistent with the globin expression patterns in erythroid colonies from human yolk sac or fetal liver [38]. Low β-globin expression was observed in EryD colonies in the absence of OP9 cells. In the presence of OP9 cells, however, β-globin expression was upregulated in individual EryD colonies, whereas ε- and ζ-globin levels were not affected (Fig. 7G). Thus, the microenvironment created by OP9 stromal cells enhanced not only the proliferation of definitive erythroid progenitors, but also β-globin expression.

Discussion

Hematopoiesis during embryogenesis is a dynamic process notable for the sequential emergence of distinct populations of blood-cell progenitors [1, 2]. Because the hematopoietic development of primates is distinct from that of mice, including the pattern of globin switching during the shift of hematopoietic sites [39], in vitro and in vivo studies on primate hematopoietic development should be performed using primate-derived materials. However, experiments on human embryos or fetuses, particularly for genetic manipulation, have ethical limitations. Immortalized cell lines also have critical disadvantages, in that they always reflect restricted aspects of the in vivo differentiation processes and sometimes behave differently from their physiological counterparts [40]. In this regard, ESCs serve as an alternative tool for analysis of hematopoietic development and differentiation, because the developmental process induced during differentiation is reproduced in a similar manner as that in early embryos. Furthermore, genetic manipulation of ESCs provides a system that facilitates the evaluation of embryonically lethal genetic changes in vivo. Globin switches in murine ESCs have been extensively investigated using various culture systems [34, 41, 42], whereas there have been few reports about erythropoietic differentiation in primate ESCs. Qiu et al. [43] reported the mature primitive erythropoietic differentiation of human ESCs (hESCs) by coculture with immortalized human liver cells. Zambidis et al. [44] demonstrated the transition from primitive into definitive erythropoiesis in hESCs by a colony-forming assay, which did not allow for the investigation of erythroid proliferation and maturation into terminal differentiation.

The OP9 coculture system is preferred for the analysis of transition because mature HCs can be easily and repeatedly obtained as floating cells in culture medium in the presence of protease treatment [34]. Thus, this culture system is a powerful experimental tool for elucidating the regulation of murine hematopoietic development and differentiation [23, 35]. We previously adapted this culture system for hematopoietic differentiation of primate ESCs for analysis of sequential development of primitive and definitive erythropoiesis in vitro. However, the low efficiency of mesodermal differentiation hampered the

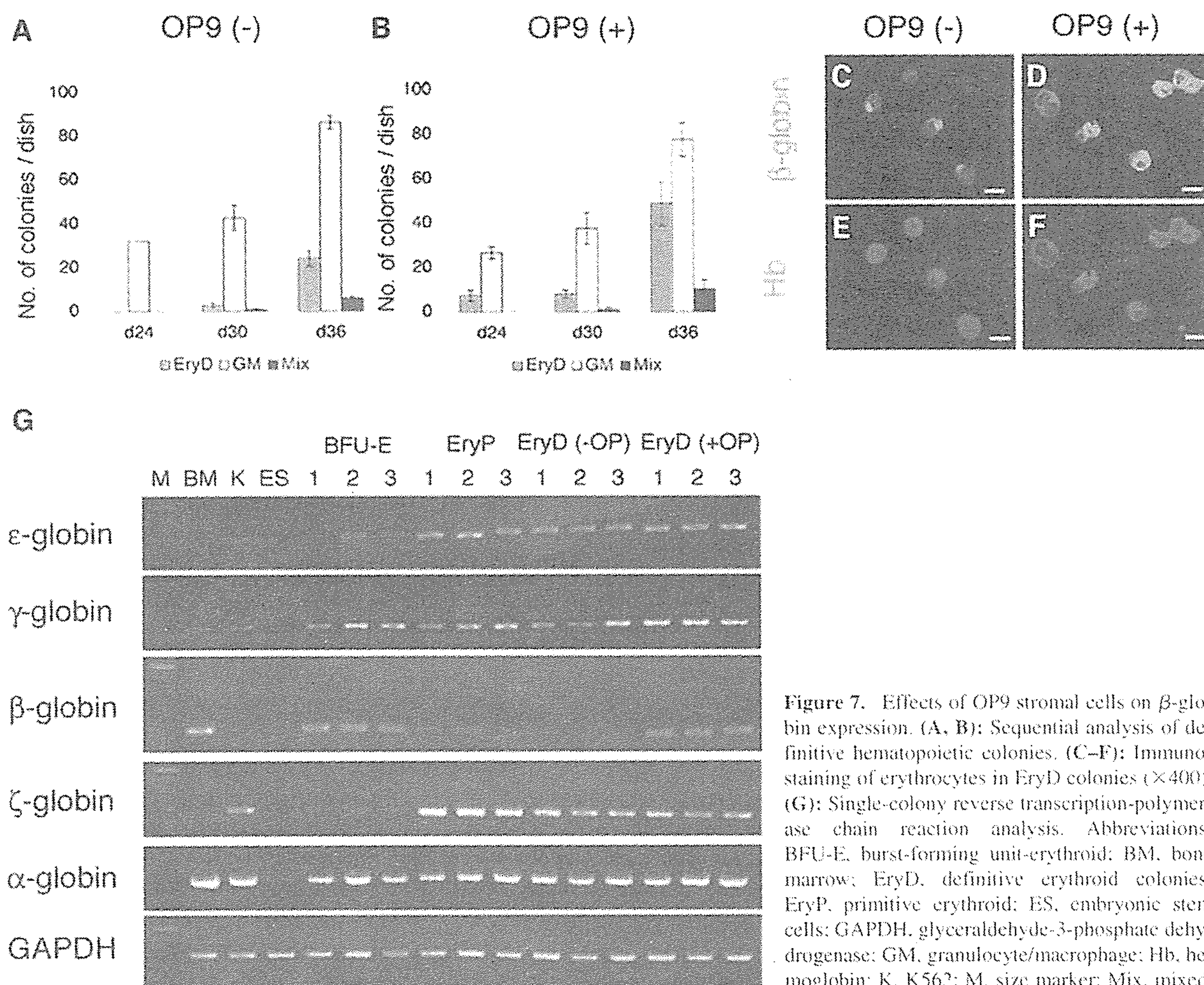


Figure 7. Effects of OP9 stromal cells on β-globin expression. **(A, B):** Sequential analysis of definitive hematopoietic colonies. **(C–F):** Immunostaining of erythrocytes in EryD colonies (×400). **(G):** Single-colony reverse transcription-polymerase chain reaction analysis. Abbreviations: BFU-E, burst-forming unit-erythroid; BM, bone marrow; EryD, definitive erythroid colonies; EryP, primitive erythroid; ES, embryonic stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM, granulocyte/macrophage; Hb, hemoglobin; K, K562; M, size marker; Mix, mixed.

quantitative analysis [15]. The existence of hemogenic progenitors during early mesodermal differentiation in this coculture system was additionally demonstrated by serial FACS analysis with VEGFR-2 and CD34 mAbs [16] because VEGFR-2 and CD34 are expressed at this stage [30–33]. To induce abundant erythropoietic production, we established a two-step culture system using a combination of previously reported methods [15, 16]: (a) an initial 6-day culture to generate VEGFR-2^{high} $CD34^{+}$ cells in the presence of VEGF (hemogenic progenitor differentiation), and (b) purification and further culturing of VEGFR-2^{high} $CD34^{+}$ cells in the presence of EPO, IL-3, SCF, and TPO (erythroid lineage differentiation). With this culture system, we have successfully achieved effective and sustained production of mature erythrocytes, which allows the quantitative analyses of erythropoiesis corresponding to that from yolk sac to the early fetal liver stage in vivo.

In this coculture system, two-wave erythropoiesis develops sequentially. Our data show that, morphologically, the appearance of EryP precedes that of EryD. Sequential RT-PCR analyses reveal low β-globin in EryP, but increased expression, both at the RNA and protein level, in EryD. Thus, we propose that β-globin is the most sensitive and distinctive marker of EryD, consistent with earlier data from human embryos [3]. In contrast, ε- and ζ-globin expression decrease but are still detectable in EryD. It is unlikely that remnants of embryonic globin expression are solely due to coexisting EryP, because erythrocytes generated after day 18 are positive for β-globin exclusively and a proportion of enucleated erythrocytes also expressed ε- or ζ-globin. This finding is supported by a previous report that embryonic globin expression is still detected in erythrocytes, even after birth [45].

Recently, Chang et al. reported the development of definitive-like erythroid cells from hESCs by embryoid body (EB) formation [46]. Their observation that the erythroid cells coexpress high levels of embryonic and fetal globins is in agreement with our own study. In contrast, the erythroid cells in the report of Chang et al. are all nucleated and do not express adult globin even in the presence of OP9 stromal cells, whereas the definitive erythroid cells in our study contain enucleated ones and express adult globin. These differences may be partially due to differences in the culture conditions (the EB and OP9 coculture system) and/or the ESCs that were used for the induction of differentiation.

The sequential development of immature adherent hematopoietic progenitors can be induced in this coculture system, and the clonogenic potential of such progenitors alters during culture. EryP colonies and a few CFU-GM colonies develop initially, whereas EryD and CFU-Mix, as well as numerous CFU-GM colonies, appear subsequently. This finding is consis-

tent with a previous report on human yolk sac and fetal liver hematopoiesis that demonstrated the rapid expansion of clonogenic cells, along with shifting hematopoietic sites [47]. EryP colonies develop only in the presence of OP9 stromal cells, whereas EryD and CFU-Mix colonies appear irrespective of OP9 stromal cells. Immunostaining analysis shows that all erythrocytes in individual EryP colonies express embryonic ε- and ζ-globins, as well as fetal γ- and α-globins. In contrast, individual EryD colonies express embryonic (ε and ζ) as well as fetal (γ and α) or adult (β) globin genes. Single-colony RT-PCR analysis shows the same expression pattern, consistent with the data from hESCs [44]. Notably, ε- or ζ-globin expression is restricted to a subset of the total cells within individual EryD colonies, strongly suggesting that the ε-to-γ (or ζ-to-α) switch occurs in a single definitive erythroid progenitor during the early stage. The results strongly suggest that the definitive erythroid progenitors encode the same regulatory programs of globin gene expression that control the γ-to-β switch in fetal and adult progenitor-derived erythroid colonies [17]. The decline of embryonic globin expression in floating EryD, compared with that in EryD colonies, may also correspond to the decreasing proportion of fetal globin synthesis during fetal, neonatal, and adult erythroid cell maturation [47, 48].

In colony-forming assays with an OP9 stromal layer, EryD and CFU-Mix colonies are more abundant. These results are consistent with the finding that the microenvironment created by stromal cells is essential for the rapid expansion of erythrocytes in murine fetal liver [49]. Additionally, β-globin gene expression in each EryD is upregulated in the presence of OP9 stromal cells. It would be interesting to determine whether stromal regulation of erythropoiesis occurs through cell-cell contact, extracellular matrices, or factors secreted by stromal cells.

Summary

We have described a culture system for producing enriched mature erythrocytes and immature erythroid progenitors from primate ESCs, which reflects the transition from yolk sac to early fetal liver-stage erythropoiesis in vivo. This differentiation induction method should facilitate the analysis of the regulatory mechanisms of human globin switching and the establishment of a system for transfusion medicine and drug discovery for hemoglobinopathies.

Acknowledgments

We thank Tanabe Seiyaku Co. Ltd. (Osaka, Japan) for help with primate ESC preparation and Prof. Ogawa for critical reading of the manuscript. This work was supported by grants from the Science Research on Priority Areas and the Creative Science Research programs. It was also supported by the Japan Society for the Promotion of Science, by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

Disclosures

The authors indicate no potential conflicts of interest.

References

1 Dzierzak E. Medvinsky A. Mouse embryonic hematopoiesis. Trend Genet 1995:11:359–366.

2 Xu MJ. Matsuoka S. Yang FC et al. Evidence for the presence of murines primitive megakarycytopoiesis in the early yolk sac. Blood 2001:97: 2016–2022.

3 Peschle C. Migliaccio AR. Migliaccio G et al. Embryonic—fetal Hb switch in humans: Studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. Proc Natl Acad Sci U S A 1984: 81:2416–2420.

4 Peschle C. Mavilio F. Care A et al. Haemoglobin switching in human embryos: Asynchrony of $\zeta\rightarrow\alpha$ and $\varepsilon\rightarrow\gamma$ globin switches in primitive and definitive erythropoietic lineage. Nature 1985:313:235–238.

5 Stamatoyannopoulos G. Constantoulakis P. Brice M et al. Coexpression of embryonic. fetal. and adult globins in erythroid cells of human embryos: Relevance to the cell-lineage models of globin switching. Dev Biol 1987:123:191–197.

6 Thomson JA. Kalishman J. Golos TG et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 1995:92:7844–7848.

7 Thomson JA. Kalishman J. Golos TG et al. Pluripotent cell lines derived from common marmoset (*Callithrix jacchu*) blastocysts. Biol Reprod 1996:55:254–259.

8 Thomson JA. Itskovitz-Eldor J. Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998:282:1145–1147.

9 Suemori H. Tada T. Torii R et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 2001:222:273–279.

10 Odorico JS. Kaufman DS. Thomson JA. Mutilineage differentiation from human embryonic stem cell lines. STEM CELLS 2001:19:192–204.

11 Nakatsuji N. Suemori H. Embryonic stem cell lines of nonhuman primates. ScientificWorldJournal 2002:2:762–773.

12 Kaufman DS. Hanson ET. Lewis RL et al. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2001:98:10716–10721.

13 Li F. Lu S. Vida L et al. Bone morphogenetic protein 4 induces efficient hematopoietic differentiation of rhesus monkey embryonic stem cells in vitro. Blood 2001:98:335–342.

14 Chadwick K. Wang L. Li L et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood 2003:102:906–915.

15 Umeda K. Heike T. Yoshimoto M et al. Development of primitive and definitive hematopoiesis from nonhuman primate embryonic stem cells in vitro. Development 2004:131:1869–1879.

16 Umeda K. Heike T. Yoshimoto M et al. Identification and characterization of hemoangiogenic progenitors during cynomolgus monkey embryonic stem cell differentiation. STEM CELLS 2006:24:1348–1358.

17 Stamatoyannopoulos G. Kurnit DM. Papayannopoulou T. Stochastic expression of fetal hemoglobin in adult erythroid cells. Proc Natl Acad Sci U S A 1981:78:7005–7009.

18 Furuya M. Yasuchika K. Mizutani K et al. Electroporation of cynomolgus monkey embryonic stem cells. Genesis 2003:37:180–187.

19 Chui DHK. Mentzer WC. Patterson M et al. Human embryonic ζ-globin chains in fetal and newborn blood. Blood 1989:74:1409–1414.

20 Luo HY. Liang XL. Frye C et al. Embryonic hemoglobins are expressed in definitive cells. Blood 1999:94:359–361.

21 Sawano A. Iwai S. Sakurai Y et al. Flt-1. vascular endothelial growth factor receptor 1. is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood 2001:97:785–791.

22 Sone M. Itoh H. Yamashita J et al. Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. Circulation 2003:107:2085–2088.

23 Suwabe N. Takahashi S. T. Nakano T et al. GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. Blood 1998:92:4108–4118.

24 Nakahata T. Ogawa M. Hemopoietic colony-forming cells in umbilical cord blood with extensive capacity to generate mono- and multipotential hemopoietic progenitors. J Clin Invest 1982:70:1324–1328.

25 Tajima S. Tsuji K. Ebihara Y et al. Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human CD34^{+} cells. J Exp Med 1996;184:1357–1364.

26 Jimenez DF. Tarantal AF. Quantitative analysis of male fetal DNA in maternal serum of gravid rhesus monkeys (*Macaca mulatta*). Pediatr Res 2003;53:18–23.

27 Fibach E. Bianchi N. Borgatti M et al. Mithramycin induces fetal hemoglobin production in normal and thalassemic human erythroid precursor cells. Blood 2003:102:1276–1281.

28 Klein D. Janda P. Steinborn R et al. Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: Influence of mismatches on quantification. Electrophoresis 1999;20:291–299.

29 Mullen AC. Hutchins AS. High FA et al. Hix is induced by and genetically interacts with T-bet to promote heritable $T_{H}1$ gene induction. Nat Immunol 2002;3:652–658.

30 Shalaby F. Rossant J. Yamaguchi TP et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995:376:62–66.

31 Shalaby F. Ho J. Stanford WL et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 1997:89:981–990.

32 Tavian M. Coulombel L. Luton D et al. Aorta-associated CD34^{+} hematopoietic cells in the early human embryo. Blood 1996:87:67–72.

33 Marshall CJ. Moore RL. Thorogood P et al. Detailed characterization of the human aorta-gonad-mesonephros region reveals morphological polarity resembling a hematopoietic stromal layer. Dev Dyn 1999:215: 139–147.

34 Nakano T. Kodama H. Honjo T. In vitro development of primitive and definitive erythrocytes from different precursors. Science 1996:272: 722–724.

35 Era T. Takagi T. Takahashi T et al. Characterization of hematopoietic lineage-specific gene expression by ES cell in vitro differentiation induction system. Blood 2000:95:870–878.

36 Gregory CJ. Eaves AC. The stages of erythropoietic cell differentiation distinguished by a number of physical and biologic properties. Blood 1978:51:527–537.

37 Stamatoyannopoulos G. Grosvled F. Hemoglobin Switching: The Molecular Basis of Blood Disease. Philadelphia: W.B. Saunders. 2001: 135–182.

38 Stamatoyannopoulos G. Control of globin gene expression during development and erythroid differentiation. Exp Hematol 2005:33:259–271.

39 Tomhon C. Zhu W. Millinoff D et al. Evolution of a fetal expression pattern via cis changes near the γ globin gene. J Biol Chem 1997:272: 14062–14066.

40 Rutherford T. Clegg JB. Higgs DR et al. Embryonic erythroid differentiation in the human leukemic cell line K562. Proc Natl Acad Sci U S A 1981:78:348–352.

41 Lindenbaum MH. Grosveld F. An in vitro globin gene switching model based on differentiated embryonic stem cells. Genes Dev 1990:4: 2075–2085.

42 Trimborn T. Gribnau J. Gorsveld F et al. Mechanisms of developmental control of transcription in the murine α- and β- globin loci. Genes Dev 1999:13:112–124.

43 Qiu C. Hanson E. Olivier E et al. Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver recapitulates the globin switch that occurs early in development. Exp Hematol 2005:33:1450–1458.

44 Zambidis ET. Peault B. Park TS et al. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hemato-endothelial. primitive. and definitive stages resembling human yolk sac development. Blood 2005:106:860–870.

45 Chui DHK. Wong SC. Enkin MW et al. Proportion of fetal hemoglobin synthesis decreases during erythroid cell maturation. Proc Natl Acad Sci U S A 1980:77:2757–2761.

46 Chang KH. Nelson AM. Cao H et al. Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin. Blood 2006: 108:1515–1523.

47 Migliaccio G. Migliaccio AR. Petti S et al. Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac → liver transition. J Clin Invest 1986:78:51–60.

48 Papayannopoulou T. Kalmantis T. Stamatoyannopoulos G. Cellular regulation of hemoglobin switching: Evidence for inverse relationship between fetal hemoglobin synthesis and degree of maturity of human erythroid cells. Proc Natl Acad Sci U S A 1979:76:6420–6424.

49 Ohneda O. Yanai N. Obinata M. Microenvironment created by stromal cells is essential for a rapid expansion of erythroid cells in mouse fetal liver. Development 1990:110:379–384.

α4-Integrin⁺ endothelium derived from primate embryonic stem cells generates primitive and definitive hematopoietic cells

Gen Shinoda,[1] Katsutsugu Umeda,[1] Toshio Heike,[1] Masato Arai,[1] Akira Niwa,[1] Feng Ma,[1] Hirofumi Suemori,[2] Hong Yuan Luo,[3] David H. K. Chui,[3] Ryuzo Torii,[4] Masabumi Shibuya,[5] Norio Nakatsuji,[6] and Tatsutoshi Nakahata[1]

[1]Department of Pediatrics, Graduate School of Medicine, [2]Laboratory of Embryonic Stem Cell Research, Stem Cell Research Center, Institute for Frontier Medical Sciences, and [6]Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Japan; [3]Department of Medicine, Boston University School of Medicine, MA; [4]Research Center for Animal Life Science, Shiga University of Medical Science, Japan; [5]Division of Genetics, Institute of Medical Science, University of Tokyo, Japan

The mechanism of commencement of hematopoiesis in blood islands of the yolk sac and the aorta-gonad-mesonephros (AGM) region during primate embryogenesis remains elusive. In this study, we demonstrated that VE-cadherin⁺CD45⁻ endothelial cells derived from nonhuman primate embryonic stem cells are able to generate primitive and definitive hematopoietic cells sequentially, as revealed by immunostaining of floating erythrocytes and colony-forming assay in cultures. Single bipotential progenitors for hematopoietic and endothelial lineages are included in this endothelial cell population. Furthermore, hemogenic activity of these endothelial cells is observed exclusively in the α4-integrin⁺ subpopulation; bipotential progenitors are 4-fold enriched in this subpopulation. The kinetics of this hemogenic subpopulation is similar to that of hemogenic endothelial cells previously reported in the yolk sac and the AGM region in vivo in that they emerge for only a limited time. We suggest that VE-cadherin⁺CD45⁻α4-integrin⁺ endothelial cells are involved in primitive and definitive hematopoiesis during primate embryogenesis, though VE-cadherin⁻CD45⁻α4-integrin⁺ cells are the primary sources for primitive hematopoiesis. (Blood. 2007;109: 2406-2415)

© 2007 by The American Society of Hematology

Introduction

During mammalian embryogenesis, hematopoietic system development undergoes 2 distinct phases, primitive hematopoiesis and definitive hematopoiesis. The phases are distinguished from each other by 2 characteristics. First, primitive hematopoiesis originates exclusively in the extraembryonic yolk sac and is transient, whereas definitive hematopoiesis occurs in the intraembryonic aorta-gonad-mesonephros (AGM) region, shifts to the liver, spleen, and bone marrow, and persists for life. Evidence also indicates that the yolk sac serves as a source of initial definitive hematopoietic progenitors in humans, as it does in mice.[1,2] Second, in primates, primitive erythrocytes are larger and primarily synthesize embryonic globin chains (ζ, ϵ), whereas definitive erythrocytes are smaller and synthesize fetal/adult globin chains (α, γ, and β).[3,4] Because the embryonic–fetal globin switch (ζ → α and ϵ → γ) occurs gradually in the fetal liver, embryonic and fetal globins are expressed during the transition from primitive to definitive hematopoiesis. However, adult β-globin is predominantly expressed in definitive erythrocytes and is only marginally expressed, if at all, in primitive erythrocytes.

The existence of the hemangioblast, the common precursor of hematopoietic and endothelial lineages, has been discussed for many years. Histologically, hematopoietic and endothelial cells develop from the same clusters of mesoderm in yolk sac blood islands.[5,6] In addition to the shared expression of several markers, gene-targeting experiments on vascular endothelial growth factor receptor-2 (VEGFR-2) disclose a common developmental pathway between both cell types.[7,8] Furthermore, a single common precursor generates both cell types during in vitro differentiation of mouse embryonic stem cells (ESCs).[9] Recent evidence shows that intraembryonic hematopoiesis originates from the ventral endothelial walls of the dorsal aorta and the umbilical and vitelline arteries, challenging the concept of common progenitors.[10-16] Endothelial cells capable of generating hematopoietic cells are designated "hemogenic endothelium."[17,18] Earlier studies using mouse embryos demonstrate that endothelial cells in the yolk sac are able to generate hematopoietic cells, which is also suggested by some reports on human embryos.[12,15,16] The embryos were used, however, at the stage after vascular connection between the yolk sac and the embryo proper. Hence, though it is established that definitive hematopoiesis in the AGM region originates at least in part in endothelial cells, the origin of primitive/definitive hematopoiesis in the yolk sac is still unclear.

The aims of this study were to investigate the relationship between hemogenic endothelium and primitive/definitive hematopoiesis in primates and to identify markers of the hemogenic endothelium. Analyses using primate materials are necessary because a number of differences occur in hematopoietic development between mice and primates (human and monkey). These studies are difficult to perform because of the poor availability of primate embryos and the ethical limitations involved in their use. Recently established primate ESC lines[19-22] are promising alternative tools in developmental biology and regenerative medicine. We previously showed the development of hematopoietic and

Submitted June 26, 2006; accepted October 25, 2006. Prepublished online as *Blood* First Edition Paper, November 7, 2006; DOI 10.1182/blood-2006-06-031039.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2007 by The American Society of Hematology

endothelial cells when cynomolgus monkey ESCs were cocultured with OP9 stromal cells, which was enhanced by exogenous vascular endothelial growth factor (VEGF).[23,24] In our coculture system, the transition from primitive to definitive hematopoiesis was induced, as confirmed by globin switching.[25] Here, we examined the hematopoietic potential of endothelial cells in our coculture system and demonstrated that isolated VE-cadherin$^+$CD45$^-$ endothelial cells generated primitive and definitive hematopoietic cells based on morphologic and globin expression analyses. We used α4-integrin, an effective marker of the hemogenic population among endothelial cells in mouse embryos and in in vitro differentiating ESCs,[26] as a candidate marker of hemogenic endothelial cells in primates. Our data show that the capacity for primitive and definitive hematopoiesis resides exclusively in the α4-integrin$^+$ subpopulation among ESC-derived endothelial cells, though VE-cadherin$^-$CD45$^-$α4-integrin$^+$ cells are primary sources for primitive hematopoiesis.

Materials and methods

Maintenance of cell lines

The ESC line CMK6, established from cynomolgus monkey blastocysts, was maintained as described .[19] The GFP-transfected ESC subline[27] was applied to exclude OP9 cells in some experiments. OP9 stromal cells, a kind gift from Dr Hiroaki Kodama, were maintained as reported previously.[24]

Antibodies

Primary antibodies used in this study included mouse anti–human CD34-phycoerythrin (PE), CD41a-allophycocyanin (APC), α4-integrin-PE, endothelial nitric oxide synthase (eNOS) monoclonal antibodies (mAbs; BD PharMingen, San Diego, CA), mouse anti–human CD31-PE (eBioscience, San Diego, CA), rabbit anti–human von Willebrand factor (VWF; Nichirei, Tokyo, Japan), mouse anti–human CD45 and CD41 mAbs (Dako, Kyoto, Japan), mouse anti–human VE-cadherin mAb (Immunotech, Marseille, France), mouse anti–human β-globin and γ-globin mAbs (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–human hemoglobin (Hb) polyclonal Ab (Cappel, Aurora, OH), and their corresponding IgG1 isotype controls (BD PharMingen and Dako). Mouse anti–human VE-cadherin mAb (BD PharMingen) and its corresponding IgG1 isotype control were labeled with Alexa Fluor 647 monoclonal antibody labeling kit (Invitrogen, Carlsbad CA). Mouse anti–human ε-globin and ζ-globin and mouse anti–human VEGFR-2 mAbs were used, as reported previously.[28-30] Mouse anti–human α-globin mAb was established in the laboratory of D.H.K.C. All primary antibodies against human antigens used in this study cross-reacted with cynomolgus monkey compartments.[23,24] Secondary Abs included Cy3-conjugated, horseradish peroxidase (HRP)–conjugated, or alkaline phosphatase (ALP)–conjugated donkey anti–mouse IgG, fluorescein isothiocyanate (FITC)–conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), PE-conjugated goat anti–mouse IgG (Dako), and FITC- or APC-conjugated goat anti–mouse IgG (BD PharMingen).

In vitro differentiation of primate ESCs

Initial differentiation of ESCs and cell sorting were based on earlier experiments.[23,24] Briefly, trypsin-treated undifferentiated ESCs were transferred onto OP9 cells and cultured in the presence of 20 ng/mL VEGF (R&D Systems, Minneapolis, MN).

For hematopoietic differentiation, cells sorted on day 10 were cultured in α-MEM (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS; Sigma, St Louis, MO), 50 μM 2-mercaptoethanol (ME), and a mixture of 10 ng/mL G-CSF, 2 U/mL EPO, 20 ng/mL IL-3, 100 ng/mL SCF, and 10 ng/mL TPO (hematopoietic cytokine mixture; all were provided by Kirin Brewery, Tokyo, Japan). For endothelial differentiation, sorted cells were cultured on OP9 cells in α-MEM containing 10% FCS, 50 μM 2-ME, and 20 ng/mL VEGF or were seeded onto type I collagen-coated plates in medium (CS-C Complete Medium; Cell Systems, Kirkland, WA) supplemented with 20 ng/mL VEGF.

Fluorescence-activated cell sorter analysis and cell sorting

Staining procedures, FACS analysis, and cell sorting were performed as described earlier.[23,24] For multicolor staining, single-cell suspensions were initially stained with unconjugated anti–CD45 mAb or its corresponding IgG1 isotype control, followed by FITC-, PE-, or APC-conjugated goat anti–mouse IgG. The cells were washed twice, incubated with robust mouse IgG to prevent redundant secondary Abs from reacting with other mouse mAbs, and stained with fluorochrome-conjugated mAbs, including CD34, VE-cadherin, and α4-integrin. Dead cells were excluded by propidium iodide (PI) staining. Samples were analyzed with the use of FACSCalibur and Cell Quest software (Becton Dickinson, San Jose, CA) or were sorted on a FACSVantage SE (Becton Dickinson).

Immunochemistry and acetylated low-density lipoprotein (Ac-LDL) uptake

May-Giemsa staining, immunostaining of floating erythrocytes and endothelial colonies, and DiI-Ac-LDL incorporation assay were performed as described previously.[23,24] VE-cadherin$^+$CD45$^-$ cells isolated on day 10 or their progeny were cytospun onto glass slides, fixed, and permeabilized in a staining procedure similar to that for hemoglobin.[24] Cells were initially stained with anti–VE-cadherin mAb and Cy3-conjugated donkey anti–mouse IgG, followed by double staining with various Abs using the Vector MOM kit (Vector Laboratories, Burlingame, CA), visualized with the TSA fluorescence systems kit (PerkinElmer Life Sciences, Boston, WA), and counterstained with Hoechst 33342. Fluorescence was detected on an Olympus IX70 microscope (Olympus, Tokyo, Japan) that was equipped with 4 ×/0.13 NA, 10 ×/0.30 NA, and 20 ×/0.40 NA objectives, and images were obtained with an AxioCam photomicroscope and AxioVision software version 3.0.6 SP4 (Carl Zeiss Vision, Hallbergmoos, Germany). Images were processed using Adobe Photoshop 6.0 (Adobe Systems, San Diego, CA).

Colony-forming assays for primitive and definitive cells

Colony-forming assays were performed as described elsewhere.[24,31] Briefly, for colonies consisting of primitive cells, sorted cells in each subpopulation were reseeded on OP9 layers, and the medium was replaced with methylcellulose-containing medium supplemented with 30% FCS and hematopoietic cytokine mixture on the following day. For colonies composed of definitive cells, we initially trypsinized cells and allowed OP9 stromal cells to adhere to culture dishes to exclude OP9. Resultant floating fractions were transferred to new Petri dishes with methylcellulose-containing medium supplemented with 30% FCS and hematopoietic cytokine mixture and were cultured at 37°C, 5% CO_2, in a humidified incubator. Colonies were scored using an inverted microscope[24,32,33] after 7 days for primitive cells and 14 days for definitive cells. Colonies were selected for cytospin and further staining. All assays were performed at a concentration of 0.5-2 × 10^4 cells/mL in duplicate or triplicate.

Single-cell deposition assay for hematopoietic and endothelial differentiation

Single-cell deposition assay was performed as described earlier.[23] Briefly, single-sorted cells were deposited in individual wells of 96-well plates with confluent OP9 layers and were cultured in α-MEM containing 10% FCS, 50 μM 2-ME, and hematopoietic cytokine mixture for 7 days. Each well was initially stained with a mixture of anti-CD45, anti-CD41, and anti–γ-globin mAbs, followed by HRP-conjugated donkey anti–mouse IgG for hematopoietic lineage detection, and each was double stained with anti–VE-cadherin mAb using the Vector MOM kit (Vector), followed by ALP-conjugated donkey anti–mouse IgG for endothelial lineage detection.

Reverse transcription–polymerase chain reaction

We performed RNA isolation and RT-PCR according to previously established protocols.[23,24] Samples were initially denatured at 94°C for 5 minutes, followed by 35 to 40 amplification reactions consisting of 94°C for

1 minute (denaturing), 60°C to 62°C for 1 minute (annealing), 72°C for 1 minute (extension), and a final extension at 94°C for 7 minutes. Primers for *eNOS*, *SCL*, *GATA-2*, *RUNX1*, and *GAPDH* are described elsewhere.[23,34] Other primers used included α4-integrin (434 bp) (sense, 5′-AGATGG-GATCTCGTCAACCTTC-3′; antisense, 5′-TGGACACCTGTATGCTTC-CTG-3′), *VWF* (472 bp) (sense, 5′-GGGACCTTTCGGATCCTAGTG-3′; antisense, 5′-AGGAGGAATCCACCATCGTC-3′), and mouse β-actin (613 bp) (sense, 5′-ATCCTGACCCTGAAGTACCCCATT-3′; antisense, 5′-CCAAGAAGGAAGGCTGGAAAAGAG-3′). cDNA from adult cynomolgus monkey BM cells, human erythroblastic cells (K562), and human umbilical vein endothelial cells were used as positive controls, and mouse OP9 cells were used as a negative control. For semiquantitative comparison, samples were normalized by dilution to produce equivalent signals for *GAPDH*.

Statistical analysis

Statistical analyses were conducted using the Student *t* test or Fisher exact test. Statistical significance was defined as a *P* value below .05.

Results

Development of primate ESC-derived VE-cadherin$^+$CD45$^-$ endothelial cells

Initially, we investigated when cells positive for hematopoietic or endothelial markers emerged during ESC differentiation. GFP-transfected ESCs were induced to differentiate by coculture with OP9 stromal cells in the presence of exogenous VEGF. Sequential FACS analysis for various surface markers was performed, and the percentage of positive cells among viable GFP$^+$ cells was quantified (Figure 1A). Undifferentiated ESCs expressed low levels of VEGFR-2 but not other hematopoietic or endothelial markers, such as CD31, CD34, VE-cadherin, CD41a, or CD45 (Figure 1B). VEGFR-2 was down-regulated by day 4 of culture but subsequently was re-expressed on a fraction of differentiating cells. CD31$^+$, CD34$^+$, and VE-cadherin$^+$ cells initially appeared on day 6, and CD41a$^+$ and CD45$^+$ cells appeared on day 12. Thus, cells positive for VE-cadherin, an endothelial marker, emerged in the OP9 coculture earlier than those positive for the hematopoietic marker CD41a or CD45.

VE-cadherin belongs to the cadherin family of adhesive transmembrane proteins and is expressed solely in endothelial cells.[35] The protein is additionally detected in all developing vessels from early embryonic stages.[36] To date, VE-cadherin has been used for the isolation of cells committed to endothelial lineage as an endothelial-specific marker.[15,16,26,37] Accordingly, we used anti–VE-cadherin and CD45 mAbs to purify endothelial cells and exclude hematopoietic cells (Figure 1C). Indeed, isolated VE-cadherin$^+$CD45$^-$ cells on day 10 exclusively coexpressed CD31 and CD34 but not CD41a (Figure 1D). Moreover, all VE-cadherin$^+$CD45$^-$ cells on day 10 had Ac-LDL uptake capacity and expressed VEGFR-2 and eNOS but not Hb (Figure 1E-I). On the other hand, only 27.4% ± 7.8% cells expressed VWF (n = 3) (Figure 1J), suggesting that most were functionally immature. Our results indicated that VE-cadherin$^+$CD45$^-$ cells generated on day 10, before the emergence of hematopoietic cells, had several endothelial characteristics.

VE-cadherin$^+$CD45$^-$ population generates hematopoietic and endothelial cells

To determine the hematoangiogenic capacity of isolated VE-cadherin$^+$CD45$^-$ cells, VE-cadherin$^+$CD45$^-$ and VE-cadherin$^-$CD45$^-$ cells were reseeded separately onto fresh, confluent OP9 cells and cultured with hematopoietic cytokine mixture (see "Materials and methods") for hematopoietic differentiation or with VEGF for endothelial differentiation.

In hematopoietic differentiation cultures, adherent hematopoietic clusters initially emerged from the VE-cadherin$^+$CD45$^-$ population at approximately day 10 + 3 (3 days after sorting on day 10) (Figure 2A) and covered large areas of OP9 stromal layers by day 10 + 21 (Figure 2B). In contrast, adherent hematopoietic clusters from the VE-cadherin$^-$CD45$^-$ population were rare and small and disappeared by day 10 + 12 (data not shown). Under hematopoietic differentiation conditions, the numbers of CD45$^+$ and CD45$^+$CD34$^+$ hematopoietic

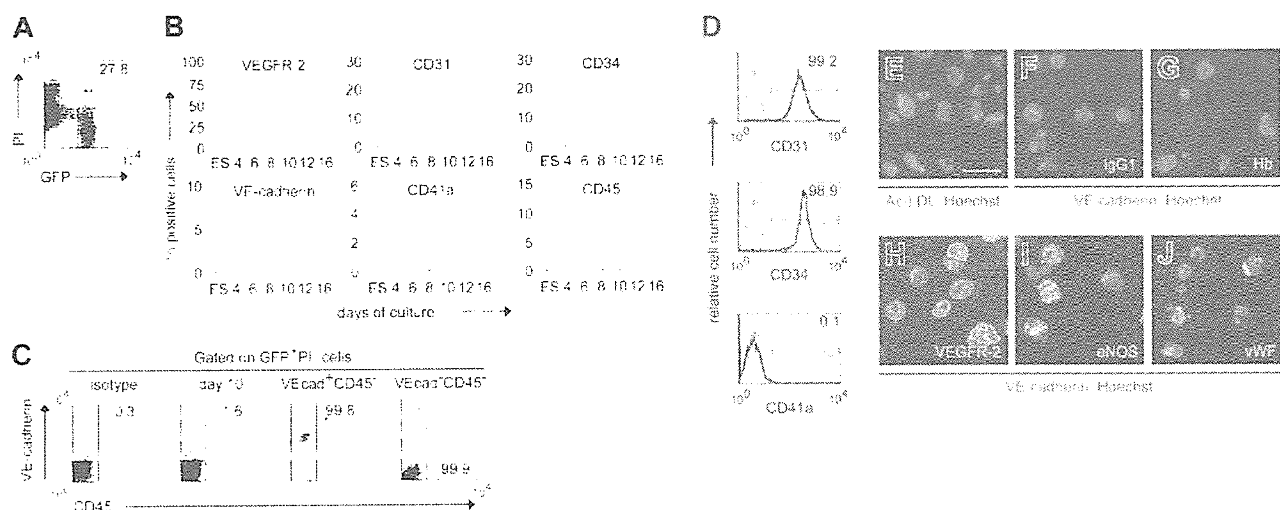


Figure 1. ESC-derived VE-cadherin$^+$CD45$^-$ cells have endothelial properties. Undifferentiated GFP-transfected ESCs (ES) and subsequent differentiating cells were analyzed by FACS. GFP$^+$PI$^-$ cells were gated as ESC-derived viable cells. (A) Percentage of gated cells among the total cells is specified. (B) Sequential analysis of the percentage of cells positive for each antigen among ESC-derived viable cells. Data are presented as mean ± SD of 3 independent experiments. (C) VE-cadherin$^+$CD45$^-$(VEcad$^+$CD45$^-$) or VE-cadherin$^-$CD45$^-$(VEcad$^-$CD45$^-$) cells were sorted on day 10. Representative FACS dot plots and percentages of gated cells are shown. Purities of viable VE-cadherin$^+$CD45$^-$ and VE-cadherin$^-$CD45$^-$ cells are 99.2% ± 0.6% and 99.9% ± 0.1%, respectively, from at least 3 independent experiments. (D) VE-cadherin$^+$ cells on day 10 were analyzed by FACS with various mAbs. Percentages of cells positive for each antigen among VE-cadherin$^+$ cells are shown. Gray line indicates isotype control; black line, VE-cadherin$^+$ cells. (E-J) Untransfected ESC-derived VE-cadherin$^+$CD45$^-$ cells sorted on day 10 were evaluated by the DiI-Ac-LDL incorporation assay (E) or immunostaining with IgG1 (F), anti-Hb (G), VEGFR-2 (H), eNOS (I), or VWF (J) Abs. Hoechst 33342 (E-J) and anti–VE-cadherin mAb (F-J) were used to detect nuclei and endothelial cells, respectively. Original magnification × 200. Scale bar, 50μm.

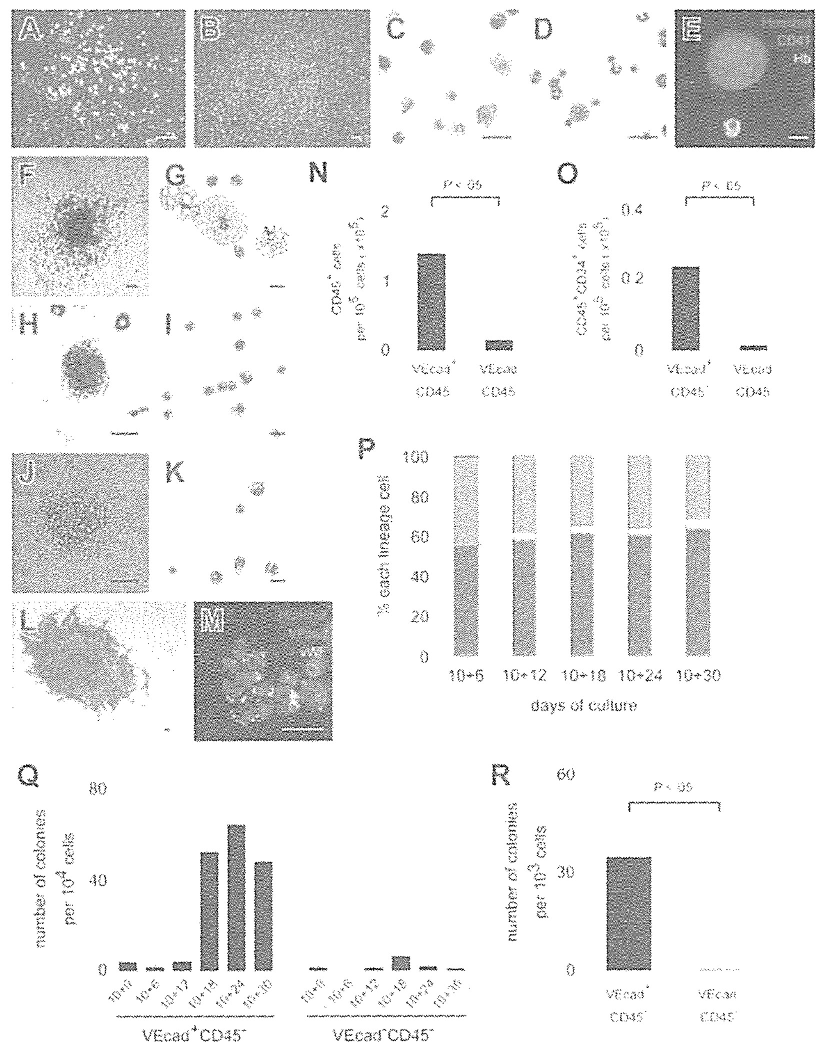


Figure 2. ESC-derived VE-cadherin$^+$CD45$^-$ cells generate hematopoietic and endothelial cells/colonies. Light micrographs of adherent hematopoietic clusters on days 10 + 5 (A) and 10 + 23 (B). (C-D) May-Giemsa staining of floating hematopoietic cells on days 10 + 6 (C) and 10 + 30 (D). (E) Immunostaining of floating hematopoietic cells on day 10 + 30 with anti–CD41 (red) and Hb (green) Abs. (F-K) Light micrographs and May-Giemsa staining of a GM (F-G), erythroid (H-I), or mixed colony (J-K) are depicted. (L) Immunostaining of an endothelial colony with anti–VE-cadherin mAb (blue). (M) Immunostaining of VE-cadherin$^+$CD45$^-$ cell-derived cells after 7-day culture with anti–VE-cadherin (red) and VWF (green) Abs. (E, M) Nuclei were labeled with Hoechst 33342. (N, O) Numbers of CD45$^+$ (N) or CD45$^+$CD34$^+$ cells (O) derived from 1×10^5 VE-cadherin$^+$CD45$^-$ (VEcad$^+$CD45$^-$) or VE-cadherin$^-$CD45$^-$ (VEcad$^-$CD45$^-$) cells. (P) Sequential analysis of the percentages of nucleated erythrocytes (red), enucleated erythrocytes (yellow), myeloid lineage cells (green), and megakaryocytes (blue) among floating cells. Each bar represents the mean of 3 independent experiments. (Q) Sequential analysis of the numbers of hematopoietic colonies per 1×10^4 VEcad$^+$CD45$^-$ or VEcad$^-$CD45$^-$ cell-derived cells. (R) Numbers of endothelial colonies per 1×10^3 VEcad$^+$CD45$^-$ or VEcad$^-$CD45$^-$ cells. (A-P) Data obtained from VEcad$^+$CD45$^-$ cells are shown. Data are presented as mean ± SD of 3 independent experiments in N-O and Q-R. Each experiment was performed in triplicate in P-R. Original magnification × 40 (B, F, L), × 100 (A, H, J), and × 200 (C-E, G, I, K, M). Scale bars, 20 μm (C-E, G, I, K), 50 μm (M), and 100 μm (A-B, F, H, J, L).

cells generated from the VE-cadherin$^+$CD45$^-$ population were 9.5- and 16.1-fold higher, respectively, than from the VE-cadherin$^-$CD45$^-$ population at day 10 + 7 ($P < .05$) (Figure 2N-O). It should be noted that VE-cadherin$^-$CD45$^-$ cells generated a small but significant minority of hematopoietic cells at day 10 + 7.

Floating hematopoietic cells emerged from the VE-cadherin$^+$CD45$^-$ population at approximately day 10 + 3. Their number was low until day 10 + 12 but increased exponentially afterward. Cells consisted exclusively of mature hematopoietic cells, such as erythrocytes, myeloid lineage cells, and megakaryocytes, as revealed by May-Giemsa staining and immunostaining (Figure 2C-E). Floating erythrocytes on day 10 + 6 were apparently larger than those on day 10 + 30 (Figure 2C-D). The percentage of erythrocytes among floating cells was approximately 60% to 70% throughout the experiments, which increased to some degree with time (Figure 2P). Enucleated erythrocytes accounted for less than 1% of total erythrocytes on day 10 + 6 and 6% to 10% from day 10 + 12 onward. Again, floating cells from the VE-cadherin$^-$CD45$^-$ population were rarely observed.

We also performed a sequential standard methylcellulose colony assay to evaluate the clonogenic potential of VE-cadherin$^+$CD45$^-$-derived or VE-cadherin$^-$CD45$^-$-derived cells. As shown in Figure 2Q, few colonies were generated from VE-cadherin$^+$CD45$^-$ cells (denoting day 10 + 0) and coculture of VE-cadherin$^+$CD45$^-$ cells on the OP9 layer for another 12 days (day 10 + 12), consisting exclusively of granulocyte-macrophage (GM) colonies. After 18 days of coculture (day 10 + 18), other colonies, including GM (Figure 2F-G), erythroid (Figure 2H-I), and mixed (Figure 2J-K), were detected. During the experiment, few colonies were generated from the coculture of VE-cadherin$^-$CD45$^-$ cells on the OP9 layer.

In endothelial differentiation cultures, sheetlike or cordlike VE-cadherin$^+$ endothelial colonies were generated after 7 days almost exclusively from the VE-cadherin$^+$CD45$^-$ population ($P < .05$) (Figure 2L, R). In 7-day culture, all endothelial cells had Ac-LDL uptake capacity and expressed VE-cadherin, VWF, VEGFR-2, CD31, CD34, and eNOS (Figure 2M and data not shown). Our results indicated that VE-cadherin$^+$CD45$^-$ cells isolated on day 10 composed a population of early endothelial cells with hemogenic properties that could differentiate into mature endothelial cells.

VE-cadherin$^+$CD45$^-$ population contains single cells with hematopoietic and endothelial capacities

We performed a single-cell deposition assay to analyze whether the VE-cadherin$^+$CD45$^-$ population contained common progenitors for hematopoietic and endothelial lineages. Individual wells of a 96-well plate were subjected to fluorescence microscopy 24 hours after cell deposition, and wells that contained more than one GFP$^+$ cell (ESC-derived cells) were excluded from subsequent analyses (5 of 2885 wells). Consistent with previous reports,[23] when a mixture of anti–CD45, CD41, and γ-globin mAbs was used, all

the round cells belonging to the hematopoietic lineage were stained positively.

Of the 2880 wells analyzed, 269 (8.6%) demonstrated clonal outgrowth consisting of endothelial progeny only (7.4%; 213 wells) (Figure S1A, available on the *Blood* website; see the Supplemental Figures link at the top of the online article), hematopoietic progeny only (0.17%; 5 wells) (Figure S1B), and both endothelial and hematopoietic progeny (1.1%; 31 wells) (Figure S1C). Thus, our results clearly demonstrated that the VE-cadherin$^+$CD45$^-$ population contained common progenitors for hematopoietic and endothelial lineages.

VE-cadherin$^+$CD45$^-$ cells generated primitive and definitive erythrocytes sequentially

To determine whether the erythrocytes derived from the VE-cadherin$^+$CD45$^-$ population were primitive or definitive, we analyzed the expression patterns of embryonic (ζ and ϵ), fetal (α and γ), and adult (β) globins in floating erythrocytes by sequentially immunostaining for various globin chains (Figure 3). Until day 10 + 6, all floating erythrocytes expressed ϵ- and ζ-globins, whereas β-globin expression was hardly detected (less than 1%). The percentage of floating erythrocytes positive for β-globin increased gradually from day 10 + 12, and almost all erythrocytes were positive by day 10 + 30. Meanwhile, expression of ϵ- and ζ-globins declined gradually to approximately 90% and 80% by day 10 + 30, respectively. All floating erythrocytes expressed α- and γ-globins throughout the experimental period. Others and we have found β-globin the most specific type of globin genes for the identification of definitive erythrocytes.[4,25,29,30,34] Results here showed that β-globin, a specific marker for definitive erythrocytes,

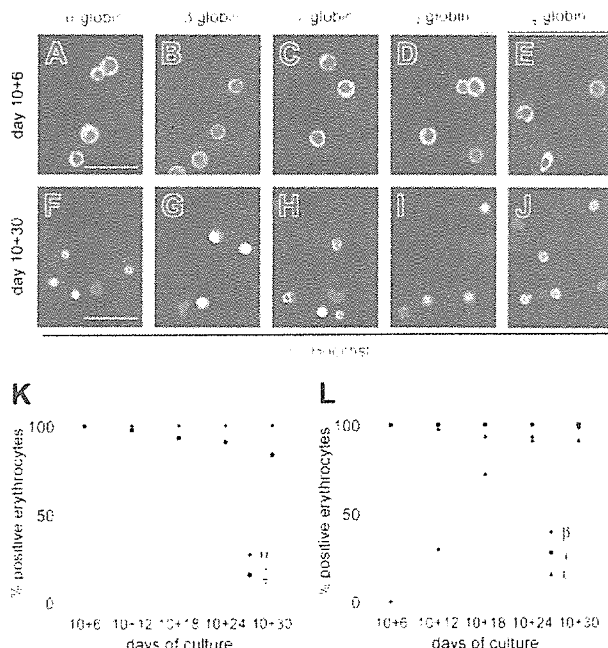


Figure 3. ESC-derived VE-cadherin$^+$CD45$^-$ cells generate primitive and definitive erythrocytes sequentially. Immunostaining of floating erythrocytes on days 10 + 6 (A-E) and 10 + 30 (F-J). Cy3 detection of erythrocytes stained with anti–α-, anti–β-, anti–ϵ-, anti–γ-, or anti–ζ-globin mAbs (red) and FITC detection with anti-Hb polyclonal Ab (green). Anti-Hb Ab, which reacts with embryonic, fetal, and adult erythrocytes, was used to detect all erythrocytes. Nuclei were labeled with Hoechst 33342. Merged images are shown. Original magnification × 200. Scale bars, 50 μm. (K-L) Sequential analysis of the proportion of erythrocytes positive for anti–α- or anti–ζ-globin mAbs (K) and anti–β-, anti–γ-, or anti–ϵ-globin mAbs (L) among the total erythrocytes. Data are presented as mean ± SD of 3 independent experiments.

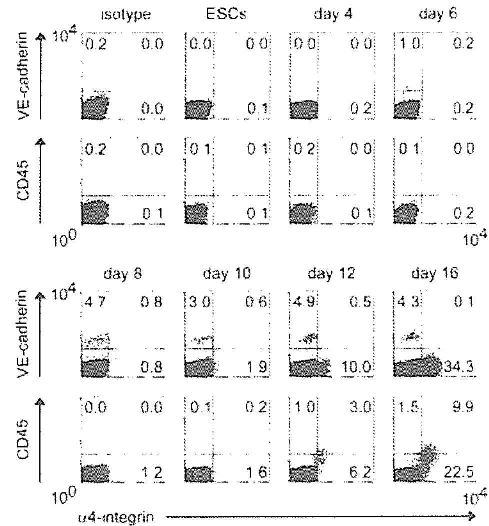


Figure 4. Temporal emergence of VE-cadherin$^+\alpha$4-integrin$^+$ cells during ESC differentiation. As described for Figure 1, undifferentiated GFP-transfected ESCs and subsequent differentiating cells were analyzed by FACS. Percentages of cells in each quadrant among the total viable GFP$^+$ cells are indicated. Representative results from 1 of 3 independent experiments are shown.

is up-regulated gradually in the OP9 coculture and that VE-cadherin$^+$CD45$^-$ cells generate primitive and definitive erythrocytes sequentially.

Hematopoietic progenitors exclusively reside in the α4-integrin$^+$ subpopulation of VE-cadherin$^+$CD45$^-$ cells

In vivo and in vitro experiments in mice show that α4-integrin is a marker of the earliest precursor of hematopoietic cell lineage from endothelial progenitors.[26] To determine whether this is applicable to primates, we sequentially traced the expression patterns of VE-cadherin, CD45, and α4-integrin in differentiating ESCs cocultured with OP9 cells. As shown in Figure 4, VE-cadherin$^+\alpha$4-integrin$^+$ cells first appeared on day 6, peaked at approximately days 8 to 10, and almost disappeared by day 16. In contrast to the previously reported time-course of mouse ESC differentiation,[26] VE-cadherin$^+$ and α4-integrin$^+$ cells simultaneously developed from monkey ESCs. CD45$^+$ cells appeared from day 12 onward, and most coexpressed α4-integrin.

Next, we isolated VE-cadherin$^+\alpha$4-integrin$^-$, VE-cadherin$^+\alpha$4-integrin$^+$, and VE-cadherin$^-\alpha$4-integrin$^+$ cells on day 10 after excluding dead cells and CD45$^+$ cells to evaluate their hematopoietic and endothelial capacity (Figure 5A). Isolated cells were reseeded onto fresh confluent OP9 layers and were cultured with hematopoietic cytokine mixture. Among the VE-cadherin$^+$CD45$^-$ population, only the α4-integrin$^+$ subpopulation gave rise to adherent hematopoietic clusters and floating hematopoietic cells (Figure 5C, F, H). Adherent clusters grew, and the number of floating cells increased throughout the experimental period (Figure 5D, G-H). VE-cadherin$^+\alpha$4-integrin$^+$ cells generated 5- to 10-fold more floating hematopoietic cells than total VE-cadherin$^+$ cells. VE-cadherin$^-\alpha$4-integrin$^+$ cells yielded more adherent clusters and floating cells than VE-cadherin$^+\alpha$4-integrin$^+$ cells until day 10 + 6 (Figure 5B, E). Interestingly, however, the adherent clusters from VE-cadherin$^-\alpha$4-integrin$^+$ cells disappeared by day 10 + 12, and the number of floating cells declined drastically (Figure 5H). Again, the sizes of floating erythrocytes on day 10 + 6 derived from VE-cadherin$^+\alpha$4-integrin$^+$ and VE-cadherin$^-\alpha$4-integrin$^+$ cells were larger than those on day 10 + 30 from VE-cadherin$^+\alpha$4-integrin$^+$ cells (Figure 5E-G).

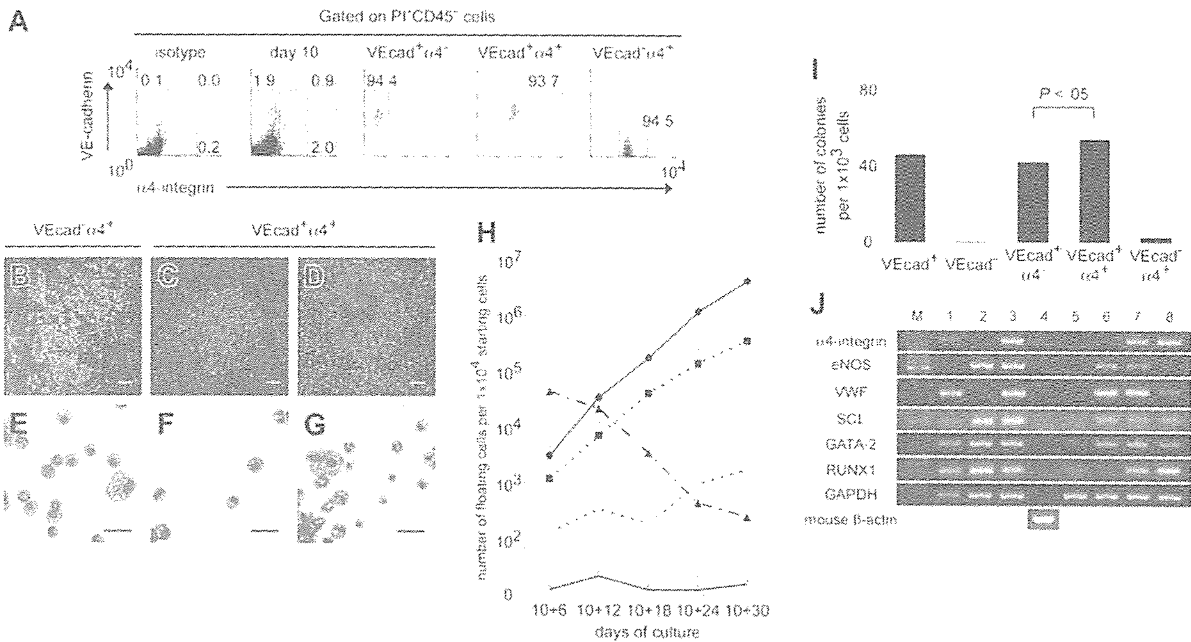


Figure 5. Hematopoietic progenitors exclusively reside in the α4-integrin⁺ subpopulation among VE-cadherin⁺CD45⁻cells. (A) VE-cadherin⁺α4-integrin⁻ (VEcad⁺α4⁻), VE-cadherin⁺α4-integrin⁺ (VEcad⁺α4⁺), or VE-cadherin⁻α4-integrin⁺ (VEcad⁻α4⁺) cells were sorted on day 10 after the exclusion of PI⁺ and CD45⁺ cells. Representative FACS dot plots and percentages of gated cells are shown. Purities of viable VEcad⁺α4⁻, VEcad⁺α4⁺, and VEcad⁻α4⁺ cells are 95.0% ± 3.0%, 94.1% ± 0.5%, and 96.9% ± 2.1%, respectively, from at least 3 independent experiments. (B-D) Light micrographs of adherent hematopoietic clusters on day 10 + 6 from VEcad⁻α4⁺ cells (B) and days 10 + 6 (C) and 10 + 18 (D) from VEcad⁺α4⁺ cells. (E-G) May-Giemsa staining of floating hematopoietic cells on day 10 + 6 from VEcad⁻α4⁺ cells (E) and days 10 + 6 (F) and 10 + 30 (G) from VEcad⁺α4⁺ cells. Original magnification × 40 (B-D) and × 200 (E-G). Scale bars, 100 μm (B-D) and 20 μm (E-G). (H) Sequential analysis of the numbers of floating viable cells per cultured 1 × 10⁴ VE-cadherin⁺ (■), VE-cadherin⁻ (□), VEcad⁺α4⁻ (○), VEcad⁺α4⁺ (●), or VEcad⁻α4⁺ (▲) cells. (I) Numbers of endothelial colonies per 1 × 10³ cells in each subpopulation. (H-I) Data are presented as mean ± SD of 3 independent experiments. Each experiment was performed in triplicate. (J) RT-PCR analysis of genes associated with hematopoietic or endothelial development. Each lane contained cDNA from the following cells: adult cynomolgus monkey BM cells (lane 1), K562 erythroblastic cells (lane 2), human umbilical vein endothelial cells (lane 3), OP9 stromal cells (lane 4), total GFP⁺ ESC-derived cells (lane 5), VE-cadherin⁺α4-integrin⁻ cells (lane 6), VE-cadherin⁺α4-integrin⁺ cells (lane 7), and VE-cadherin⁻α4-integrin⁺ cells (lane 8) sorted on day 10. Representative results from 1 of 3 independent experiments are shown.

To clarify the difference in hematopoietic kinetics between VE-cadherin⁺α4-integrin⁺ and VE-cadherin⁻α4-integrin⁺ populations, we examined whether floating erythrocytes derived from each population were primitive or definitive. As in the VE-cadherin⁺CD45⁻ population, all floating erythrocytes derived from both populations expressed α-, ϵ-, γ-, and ζ-globins but were devoid of β-globin on day 10 + 6 (Figure S2A-E, K-O), indicative of primitive erythrocytes. Moreover, almost all erythrocytes from the VE-cadherin⁺α4-integrin⁺ population were positive for β-globin by day 10 + 30, whereas the expression of ϵ- and ζ-globins declined gradually (Figure S2F-J), characteristic of definitive erythrocytes.

In endothelial differentiation cultures with VEGF, VE-cadherin⁺α4-integrin⁺ cells generated significantly more endothelial colonies than VE-cadherin⁺α4-integrin⁻ cells after 7-day culture ($P < .05$) (Figure 5I). VE-cadherin⁻α4-integrin⁺ cells barely generated endothelial colonies.

To verify the hematopoietic and endothelial capacities of these 3 populations, gene expression profiles were investigated with the use of RT-PCR (Figure 5J). The presence of α4-integrin was confirmed specifically in the VE-cadherin⁺α4-integrin⁺ and VE-cadherin⁻α4-integrin⁺ populations. *eNOS* and *VWF*, representative endothelial proteins, were expressed in VE-cadherin⁺α4-integrin⁻ and VE-cadherin⁺α4-integrin⁺ populations, whereas *VWF* was expressed weakly in the VE-cadherin⁻α4-integrin⁺ population. *SCL* and *GATA-2*, transcriptional factors associated with hematopoietic and endothelial development,[38,39] were expressed in all 3 populations. Notably, *RUNX1*, a transcriptional factor associated with definitive hematopoiesis,[40,41] was expressed in the VE-cadherin⁺α4-integrin⁺ and VE-cadherin⁻α4-integrin⁺ populations but not in VE-cadherin⁺α4-integrin⁻ cells.

Finally, we performed a single-cell deposition assay of VE-cadherin⁺α4-integrin⁺ cells. We differentiated GFP-transfected ESCs, and single VE-cadherin⁺α4-integrin⁺ cells that were exclusively negative for CD45 were assayed on day 10. Of the 958 wells analyzed (2 of 960 wells were omitted because they contained more than 1 GFP⁺ cell), 106 (11.1%) demonstrated clonal outgrowth consisting of endothelial progeny only (6.3%; 60 wells), hematopoietic progeny only (0.52%; 5 wells), and both endothelial and hematopoietic progeny (4.3%; 41 wells). Hence, common progenitors for hematopoietic and endothelial lineages in the VE-cadherin⁺CD45⁻ population were 4.0-fold more enriched in the α4-integrin⁺ subpopulation ($P < .001$).

Thus, these results suggested that among VE-cadherin⁺CD45⁻ cells, only the α4-integrin⁺ subpopulation participated in primitive and definitive hematopoiesis, whereas α4-integrin⁺ and α4-integrin⁻ subpopulations were involved in endothelial lineage development. Our results also showed that VE-cadherin⁻CD45⁻α4-integrin⁺ and VE-cadherin⁺CD45⁻α4-integrin⁺ cells were primary sources for primitive and definitive hematopoiesis, respectively.

Colonies consisting of primitive and definitive erythrocytes are generated from VE-cadherin⁺α4-integrin⁺ cells

As shown, erythroid colonies were not generated from VE-cadherin⁺CD45⁻ cells by day 10 + 18 with the standard methylcellulose assay (Figure 2Q). Others and we[24,42] have reported the successful development of colonies consisting of primitive erythrocytes on OP9 stromal layers. Colony-forming assays were performed on OP9 layers. Colonies consisting of

primitive erythrocytes were generated after 7-day coculture on OP9 cells from the VE-cadherin+, VE-cadherin+α4-integrin+, and VE-cadherin−α4-integrin+ populations but not the VE-cadherin+α4-integrin− population (Figure 6A-D, I). All erythrocytes in individual colonies from these populations were positive for ζ-globin but devoid of β-globin, indicative of primitive erythrocytes. The primitive erythroid clonogenic progenitors in the VE-cadherin+ population were 8.7-fold more enriched in the α4-integrin+ subpopulation (Figure 6I). On the other hand, the VE-cadherin−α4-integrin+ population yielded a significantly higher number of colonies consisting of primitive erythrocytes and GM than the VE-cadherin+α4-integrin+ population (each $P < .05$), analogous to the patterns for floating hematopoietic cells in both populations (Figure 5H). We used the standard methylcellulose assay to generate colonies consisting of definitive erythrocytes after day 10 + 18 from the VE-cadherin+ and VE-cadherin+α4-integrin+ populations but not the VE-cadherin+α4-integrin− and VE-cadherin−α4-integrin+ populations (Figure 6E-H, J). All erythrocytes in individual colonies were positive for β-globin, and some were devoid of ζ-globin, characteristic of definitive erythrocytes.

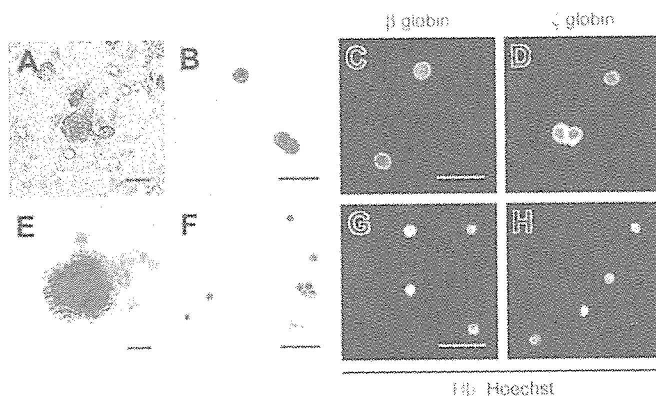
Thus, the VE-cadherin+α4-integrin+ population displayed primitive and definitive erythroid clonogenic activity. Our data showed that hemogenic endothelial cells are not only the sole progenitor population for definitive hematopoiesis, they are deeply involved in primitive hematopoiesis.

Discussion

Despite several similarities, a number of differences were observed between mouse and primate hematopoietic development, such as the pattern of globin switching during the shift of hematopoietic sites. To clarify the pathogenesis and treatment of hematologic disorders in humans, it was important to investigate hematopoietic development using primate materials. In the near future, it may be necessary to apply human ESC-derived products to nonhuman primates as preclinical models for cell transplantation, ahead of their use in clinical settings. However, relatively little is known about hematopoiesis during primate embryogenesis compared with mouse embryogenesis, partly because of poor availability and ethical limitations of primate embryos. Thus, primate ESCs are more promising for studies on primate embryogenesis. In addition, coculture with the OP9 stromal cells has been used successfully for hematopoietic differentiation of mouse and primate ESCs.[23,24,31,42,43] In this report, we used the primate ESC and OP9 coculture system and demonstrated for the first time that α4-integrin+ hemogenic endothelial cells are deeply involved in primitive and definitive hematopoiesis in primates.

Sequential development of primitive and definitive hematopoiesis from ESC-derived endothelial cells

We showed that VE-cadherin+CD45− endothelial cells derived from nonhuman primate ESCs generate primitive and definitive erythrocytes. To date, several studies demonstrate hematopoietic differentiation of human and nonhuman primate ESCs.[23,24,34,43-46] Previous in vivo and in vitro experiments in humans indicate that at least a proportion of hematopoietic cells originate in vascular endothelial cells.[12,45] However, whether primitive hematopoiesis and definitive hematopoiesis originate in hemogenic endothelium in primates remains to be elucidated. Data obtained in mice are controversial. Numerous investigators report that only multilineage definitive, but not primitive, hematopoietic progenitors arise from



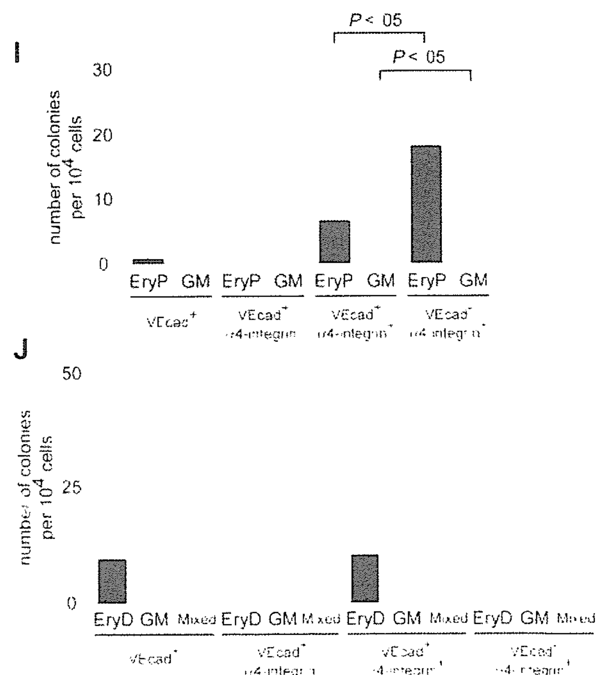


Figure 6. α4-Integrin+ subpopulation among VE-cadherin+CD45− cells generates hematopoietic colonies composed of primitive and definitive erythrocytes. (A-H) Light micrographs and May-Giemsa staining of colonies consisting of primitive (A-B) and definitive (E-F) erythrocytes from VE-cadherin+α4-integrin+ cells are depicted. Immunostaining data from colonies consisting of primitive (C-D) and definitive (G-H) erythrocytes from VE-cadherin+α4-integrin+ cells are also presented. Cy3 detection of erythrocytes stained with anti–β-globin (C, G) or anti–ζ-globin (D, H) mAbs (red) and FITC detection with anti-Hb polyclonal Ab (green). Nuclei were labeled with Hoechst 33342. Merged images are shown. Original magnification × 100 (A, E) and × 200 (B-D, F-H). Scale bars, 50 μm. (I-J) Numbers of hematopoietic colonies per 1 × 10⁴ cells sorted on day 10 (I) or after 30-day coculture on OP9 layers (J) in each subpopulation. VEcad+ denotes the VE-cadherin+CD45− population. EryP and EryD represent colonies consisting of primitive and definitive erythrocytes, respectively. Data are presented as mean ± SD of 3 (I) or 2 (J) independent experiments. Each experiment was performed in duplicate (I) or triplicate (J).

endothelial cells,[47] whereas others show that VE-cadherin$^+$ endothelial cells derived from mouse ESCs generate primitive and definitive hematopoietic cells.[48] Here, we demonstrate that primitive and definitive hematopoietic cells are, at least in part, generated from a subset of endothelial cells in primates. Because primitive hematopoiesis occurs only in the yolk sac, we hypothesized that ESC-derived VE-cadherin$^+$CD45$^-$ endothelial cells are equivalent to those in yolk sac blood islands and possibly in the AGM region in vivo.

VE-cadherin is a specific endothelial lineage marker,[15,16,26,37] whereas CD45 is widely accepted as a specific hematopoietic lineage marker except in erythroid and megakaryocytic lineage cells. Based on reports that VE-cadherin$^+$CD45$^+$ intermediate cells exist in mouse embryos,[49,50] we isolated VE-cadherin$^+$CD45$^-$ cells as definitive endothelial, but not hematopoietic, cells. In addition, our immunochemistry and FACS analyses demonstrated that VE-cadherin$^+$CD45$^-$ cells on day 10 of culture coexpress other endothelial markers, such as CD31, CD34, VEGFR-2, and eNOS, and take up Ac-LDL but that they lack mature endothelial properties, including VWF expression (Figure 1D-J). These results are consistent with the established multiparameter criteria for defining endothelial cells.[45,51] Furthermore, VE-cadherin$^+$CD45$^-$ cells are devoid of hematopoietic specific marker expression, such as hemoglobin, CD45, and CD41a. Thus, the VE-cadherin$^+$CD45$^-$ cells in this study are confirmed as endothelial, albeit immature, cells.

Studies show that β-globin is the most specific type of globin gene for the identification of definitive erythrocytes during human embryogenesis and primate ESC differentiation.[4,25,29,30,34] In our experiments, VE-cadherin$^+$CD45$^-$ cells initially produced larger, nucleated erythrocytes almost with no β-globin expression and later generated smaller, partly enucleated, erythrocytes expressing β-globin (Figures 2C-D, P, 3). This result is morphologically supported by the finding that human ESC-derived erythroblasts devoid of β-globin expression are megaloblastic and similar to primitive erythroid cells found in 4- to 5-week-old human embryos.[44] On the other hand, our results showed that the high proportion of β-globin$^+$ cells on day 10 + 30 also expressed embryonic globins (ϵ and ζ). This is consistent with previous reports that the embryonic globins and β-globin are expressed in early definitive hematopoietic cells.[29,30] Hence, VE-cadherin$^+$CD45$^-$ endothelial cells isolated on day 10 generated primitive and definitive erythrocytes sequentially.

Clonal analysis disclosed that 1.1% of the single VE-cadherin$^+$CD45$^-$ cells yielded endothelial and hematopoietic cells (Figure S1). Our results are in agreement with previous data,[45] and the characteristics of endothelial cells isolated by both groups are similar. Given that VE-cadherin$^-$CD45$^-$ cells almost never generated endothelial colonies, even under endothelial culture conditions (Figure 2R), we suggest that bipotential cells among the VE-cadherin$^+$CD45$^-$ population are not the contaminating cells during cell sorting.

α4-Integrin is a marker of the hemogenic endothelial cells in primates

The differences between hemogenic and nonhemogenic endothelial cells and how a subset of endothelial cells acquires hemogenic capacity during early embryogenesis in primates remain unclear. Here, we used α4-integrin as a candidate marker of hemogenic endothelial cells. To our knowledge, there are no reports on the expression or function of α4-integrin during early primate embryogenesis. Developmentally, in mice, α4-integrin is expressed on yolk sac blood islands and all hematopoietic cells in the fetal liver.[52,53] It is essential for the maintenance of efficient development of multilineage progenitors in the fetal liver[54] and is a marker of the earliest precursor of the hematopoietic cell lineage from endothelial progenitors in vivo and in vitro.[26] We show that the α4-integrin$^+$, not the α4-integrin$^-$ subpopulation among ESC-derived endothelial cells, yields hematopoietic cells. Except for the generation of primitive hematopoiesis, this is consistent with previous findings in mice.[26] In our study, α4-integrin$^+$ hemogenic endothelial cells generated primitive and definitive hematopoietic cells, as confirmed by immunostaining of erythroid cells/colonies (Figures 6, S2), though it remains to be clarified whether primitive and definitive hematopoiesis have common precursors.

In human embryos, yolk sac blood islands are observed from gestational days 16 to 24, and intraaortic hematopoietic cell clusters are observed in the AGM region from days 27 to 40.[55,56] The frequency of hemogenic endothelial cells in vivo reflects the actual blood-forming activity of these hematopoietic tissues as a function of developmental age.[12] Interestingly, α4-integrin$^+$ endothelial cells were detected for a limited period of time in this study (Figure 4), but primitive and definitive hematopoietic cells emerged sequentially, suggesting that precursors of primitive and definitive hematopoiesis arise simultaneously but that the definitive precursors required a maturation phase (on OP9) before they differentiated into hematopoietic cells. This may recapitulate the hemogenic activity of endothelial cells in and temporal lag of hematopoiesis between yolk sac blood islands and the AGM region in vivo.

The transcriptional factor *Runx1* is required for definitive hematopoiesis.[40,41] *Runx1* is expressed in endothelial cells where definitive hematopoietic cells emerge, specifically the yolk sac, vitelline and umbilical arteries, and ventral wall of the dorsal aorta in the AGM region but not in endothelial cells elsewhere in mouse embryos.[57] In addition, *Runx1* is reportedly required for the formation of intraaortic hematopoietic clusters and the emergence of hematopoietic stem cells.[57-59] Our RT-PCR data show that *RUNX1* is expressed in the hemogenic α4-integrin$^+$ subpopulation, but not the nonhemogenic α4-integrin$^-$ subpopulation, among ESC-derived endothelial cells (Figure 5J). *RUNX1* is additionally expressed in the VE-cadherin$^-$α4-integrin$^+$ population generating mainly primitive erythrocytes, consistent with the finding that primitive erythrocytes in the yolk sac express *Runx1* 8 days after coitus in mice.[57]

Based on the results from this study, we propose a model of primitive/definitive hematopoietic and endothelial lineage development from primate ESCs (Figure 7). We suggest that VE-cadherin$^-$CD45$^-$α4-integrin$^+$ and VE-cadherin$^+$CD45$^-$α4-integrin$^+$ cells contain a subset with hemogenic capacity and that these are primary sources for primitive and definitive hematopoiesis, respectively. Moreover, we hypothesize that in primates,

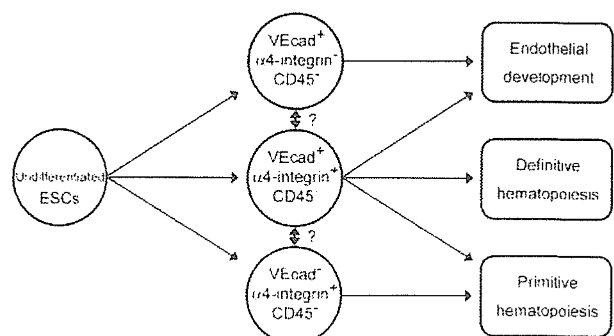


Figure 7. Schematic representation of the differentiation pathway from ESCs into endothelial or primitive and definitive hematopoietic cell lineages on coculture with OP9 cells.

hemogenic endothelial cells are located in the yolk sac and in the AGM region, generate primitive and definitive hematopoietic cells, and are marked by the temporal expression of α4-integrin. The function of α4-integrin during hematopoietic development remains to be elucidated.

In conclusion, we have successfully induced the differentiation of nonhuman primate ESCs into hemogenic endothelial cells, which in turn gave rise to primitive and definitive hematopoietic cells with the OP9 coculture system. Hemogenic activity exclusively resides in the α4-integrin$^+$ subpopulation among endothelial cells. Our culture system should provide an alternative, powerful tool for understanding early hematopoietic development during primate embryogenesis, such as the processes triggering transition from endothelial cells to hematopoietic cells in the yolk sac and the AGM region or the initial emergence of hematopoietic stem cells.

Acknowledgments

This work was supported by grants from Science Research on Priority Areas; Creative Science Research; the Japan Society for the Promotion of Science; the Ministry of Education, Culture, Sports, Science and Technology; and Tanabe Seiyaku (Osaka, Japan) for help with primate ESC preparation.

Authorship

Contribution: G.S. performed all the experiments and wrote the manuscript. K.U. contributed to the study design, data analysis, and writing of the manuscript. T.H. contributed to the study design, data analysis, and writing of the manuscript. M.A. contributed to the study design, writing of the manuscript, and interpretation of the results. A.N. contributed to the study design and developed a method for colony-forming assays. F.M. supervised the flow cytometric analysis. H.S. supervised the culture of primate embryonic stem cells. H.Y.L. contributed vital antibodies. D.H.K.C. contributed vital antibodies and revised the manuscript. R.T. supervised the culture of primate embryonic stem cells. M.S. contributed vital antibodies. N.N. contributed to the study design and supervised the culture of primate embryonic stem cells. T.N. supervised the study and contributed to the study design and to the writing and revision of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Tatsutoshi Nakahata, Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan; e-mail: tnakaha@kuhp.kyoto-u.ac.jp.

References

1. Palis J, Yoder MC. Yolk-sac hematopoiesis: the first blood cells of mouse and man. Exp Hematol. 2001;29:927-936.
2. Palis J, Robertson S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development. 1999;126:5073-5084.
3. Stamatoyannopoulos G, Constantoulakis P, Brice M, Kurachi S, Papayannopoulou T. Coexpression of embryonic, fetal, and adult globins in erythroid cells of human embryos: relevance to the cell-lineage models of globin switching. Dev Biol. 1987;123:191-197.
4. Peschle C, Mavilio F, Care A, et al. Haemoglobin switching in human embryos: asynchrony of zeta → alpha and epsilon → gamma-globin switches in primitive and definite erythropoietic lineage. Nature. 1985;313:235-238.
5. Sabin F. Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during the second day of incubation. Contributions to Embryology. 1920;9:213-262.
6. Murray P. The development in vitro of the blood of the early chick embryo. Proc R Soc London. 1932;11:497-521.
7. Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell. 1997;89:981-990.
8. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376:62-66.
9. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. Development. 1998;125:725-732.
10. Sugiyama D, Ogawa M, Hirose I, Jaffredo T, Arai K, Tsuji K. Erythropoiesis from acetyl LDL incorporating endothelial cells at the preliver stage. Blood. 2003;101:4733-4738.
11. de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity. 2002;16:673-683.
12. Oberlin E, Tavian M, Blazsek I, Peault B. Blood-forming potential of vascular endothelium in the human embryo. Development. 2002;129:4147-4157.
13. Jaffredo T, Gautier R, Brajeul V, Dieterlen-Lievre F. Tracing the progeny of the aortic hemangioblast in the avian embryo. Dev Biol. 2000;224:204-214.
14. Jaffredo T, Gautier R, Eichmann A, Dieterlen-Lievre F. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development. 1998;125:4575-4583.
15. Nishikawa SI, Nishikawa S, Kawamoto H, et al. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity. 1998;8:761-769.
16. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development. 1998;125:1747-1757.
17. Nishikawa SI. A complex linkage in the developmental pathway of endothelial and hematopoietic cells. Curr Opin Cell Biol. 2001;13:673-678.
18. Smith RA, Glomski CA. "Hemogenic endothelium" of the embryonic aorta: does it exist? Dev Comp Immunol. 1982;6:359-368.
19. Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn. 2001;222:273-279.
20. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.
21. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. Biol Reprod. 1996;55:254-259.
22. Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A. 1995;92:7844-7848.
23. Umeda K, Heike T, Yoshimoto M, et al. Identification and characterization of hemoangiogenic progenitors during cynomolgus monkey embryonic stem cell differentiation. Stem Cells. 2006;24:1348-1358.
24. Umeda K, Heike T, Yoshimoto M, et al. Development of primitive and definitive hematopoiesis from nonhuman primate embryonic stem cells in vitro. Development. 2004;131:1869-1879.
25. Umeda K, Heike T, Nakata-Hizume M, et al. Sequential analysis of α- and β-globin gene expression during erythropoietic differentiation from primate embryonic stem cells. Stem Cells. Prepublished on August 3, 2006, as DOI 10.1634/stemcells-2006-0199.
26. Ogawa M, Kizumoto M, Nishikawa S, Fujimoto T, Kodama H, Nishikawa SI. Expression of α4-integrin defines the earliest precursor of hematopoietic cell lineage diverged from endothelial cells. Blood. 1999;93:1168-1177.
27. Furuya M, Yasuchika K, Mizutani K, Yoshimura Y, Nakatsuji N, Suemori H. Electroporation of cynomolgus monkey embryonic stem cells. Genesis. 2003;37:180-187.
28. Sawano A, Iwai S, Sakurai Y, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood. 2001;97:785-791.
29. Luo HY, Liang XL, Frye C, et al. Embryonic hemoglobins are expressed in definitive cells. Blood. 1999;94:359-361.
30. Chui DH, Mentzer WC, Patterson M, et al. Human embryonic zeta-globin chains in fetal and newborn blood. Blood. 1989;74:1409-1414.
31. Suwabe N, Takahashi S, Nakano T, Yamamoto M. GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. Blood. 1998;92:4108-4118.
32. Tajima S, Tsuji K, Ebihara Y, et al. Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human CD34+ cells. J Exp Med. 1996;184:1357-1364.
33. Nakahata T, Ogawa M. Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. J Clin Invest. 1982;70:1324-1328.
34. Zambidis ET, Peault B, Park TS, Bunz F, Civin CI.

Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. Blood. 2005;106:860-870.
35. Lampugnani MG, Resnati M, Raiteri M, et al. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol. 1992;118: 1511-1522.
36. Breier G, Breviario F, Caveda L, et al. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. Blood. 1996;87:630-641.
37. Fraser ST, Ogawa M, Yu RT, Nishikawa S, Yoder MC, Nishikawa S. Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. Exp Hematol. 2002;30: 1070-1078.
38. Kallianpur AR, Jordan JE, Brandt SJ. The *SCL/TAL-1* gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. Blood. 1994;83:1200-1208.
39. Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature. 1994;371:221-226.
40. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A. 1996;93: 3444-3449.
41. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84:321-330.
42. Nakano T, Kodama H, Honjo T. In vitro development of primitive and definitive erythrocytes from different precursors. Science. 1996;272:722-724.
43. Vodyanik MA, Bork JA, Thomson JA, Slukvin, II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood. 2005;105:617-626.
44. Qiu C, Hanson E, Olivier E, et al. Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development. Exp Hematol. 2005;33:1450-1458.
45. Wang L, Li L, Shojaei F, et al. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity. 2004;21: 31-41.
46. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 2001;98:10716-10721.
47. Fujimoto T, Ogawa M, Minegishi N, et al. Stepwise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. Genes Cells. 2001;6:1113-1127.
48. Zhang WJ, Park C, Arentson E, Choi K. Modulation of hematopoietic and endothelial cell differentiation from mouse embryonic stem cells by different culture conditions. Blood. 2005;105:111-114.
49. Taoudi S, Morrison AM, Inoue H, Gribi R, Ure J, Medvinsky A. Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver. Development. 2005;132:4179-4191.
50. Fraser ST, Ogawa M, Yokomizo T, Ito Y, Nishikawa S, Nishikawa S. Putative intermediate precursor between hematogenic endothelial cells and blood cells in the developing embryo. Dev Growth Differ. 2003;45:63-75.
51. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med. 2003;9:702-712.
52. Hamamura K, Matsuda H, Takeuchi Y, Habu S, Yagita H, Okumura K. A critical role of VLA-4 in erythropoiesis in vivo. Blood. 1996;87:2513-2517.
53. Sheppard AM, Onken MD, Rosen GD, Noakes PG, Dean DC. Expanding roles for alpha 4 integrin and its ligands in development. Cell Adhes Commun. 1994;2:27-43.
54. Arroyo AG, Yang JT, Rayburn H, Hynes RO. α4 Integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. Immunity. 1999;11:555-566.
55. Tavian M, Peault B. Embryonic development of the human hematopoietic system. Int J Dev Biol. 2005;49:243-250.
56. Tavian M, Hallais MF, Peault B. Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. Development. 1999; 126:793-803.
57. North T, Gu TL, Stacy T, et al. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development. 1999;126:2563-2575.
58. North TE, de Bruijn MF, Stacy T, et al. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity. 2002;16:661-672.
59. Yokomizo T, Ogawa M, Osato M, et al. Requirement of Runx1/AML1/PEBP2αB for the generation of haematopoietic cells from endothelial cells. Genes Cells. 2001;6:13-23.

概論

幹細胞を用いた再生医療の進展

中畑龍俊

再生医療という言葉がマスコミにしばしば登場し，医学関係者ばかりでなく広く社会の関心を集めている．従来の医療は，臓器障害をできるだけ早期に発見し，その原因の除去および生体防御反応の修飾により，障害を受けた臓器の自然回復を待つものであった．しかしながら，臓器障害も一定の限度を超えると不可逆的となり，臓器の機能回復は困難となる．このような患者さんに対して障害を受けた細胞，組織，さらには臓器を体内で再生し（*in vivo*法），あるいは体外で目的とする細胞，組織，臓器を人為的に再生して移植する（*ex vivo*法）ことにより治療しようというのが再生医療である．このための基礎研究が盛んに行われ，その成果が続々と臨床の場に持ち込まれようとしている．

再生医療の基盤となる細胞は幹細胞であり，この細胞のもつ自己複製能は，再生医療が成立するための理論的根拠を与えている．すなわち，幹細胞を用いた再生医療を一旦患者さんに施すと，幹細胞は分裂し，自分と同じ能力をもった細胞を失うことなく作りながら，一方では求められる機能をもった成熟細胞を生み出し続けることにより治療が成立すると考えられるからである．このような幹細胞を用いた再生医療が続々と開始されている．第3章ではその中から特に研究が進んでいる分野を取り上げ，第一線で活躍されている先生方に臨床応用の現状を概説していただいた．第4章では幹細胞を用いた臨床応用に伴う倫理的な側面や将来の産業化に向けた開発の現状などについて述べていただいた．

1．臨床応用される幹細胞

第1部で詳述されているように，幹細胞は受精卵を培養して樹立された胚性幹細胞（embryonic stem cell：ES細胞）とわれわれの体の中に存在する体性幹細胞に分けることができる．幹細胞には階層性（hierarchy）があり，より上位の未分化な幹細胞は自己複製能が高く，非常

[キーワード]
再生医療，胚性幹細胞，胎性幹細胞，階層性（hiearachy），モデル動物，臨床研究
MAPCs：multipotent adult progenitor cells

Progress of regenerative medicine using stem cells
Tatsutoshi Nakahata：Department of Pediatrics, Kyoto Univesity, School of Medicine（京都大学大学院医学研究科発生発達医学講座発達小児科学）