Development of primitive and definitive hematopoiesis from non-human primate embryonic stem cells in vitro

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Accepted 8 January 2004

Development 131, 1869-1879
Published by The Company of Biologists 2004
doi:10.1242/dev.01065

Summary

Although information about the development of primitive and definitive hematopoiesis has been elucidated in murine embryos and embryonic stem (ES) cells, there have been few in vitro studies of these processes in primates. In this study, we investigated hematopoietic differentiation from cynomolgus monkey ES cells grown on OP9, a stromal cell line deficient in macrophage colony-stimulating factor. Primitive erythrocytes (EryP) and definitive erythrocytes (EryD) developed sequentially from ES cells in the culture system; this was confirmed by immunostaining and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of embryonic, fetal and adult globin genes. EryP were detected on day 8 without exogenous erythropoietin (EPO), whereas EryD appeared on day 16 and had an indispensable requirement for exogenous EPO. RT-PCR analysis of the cultures revealed a sequential expression of genes associated with primitive and definitive hematopoietic development that was equivalent to that seen during primate ontogeny in vivo. Vascular endothelial growth factor (VEGF) increased, in a dose-dependent manner, not only the number of floating hematopoietic cells, but also the number of adherent hematopoietic cell clusters containing CD34-positive immature progenitors. In colony assays, exogenous VEGF also had a dose-dependent stimulatory effect on the generation of primitive erythroid colonies. More efficient primitive and definitive erythropoiesis was induced by re-plating sorted CD34-positive cells. Thus, this system reproduces early hematopoietic development in vitro and can serve as a model for analyzing the mechanisms of hematopoietic development in primates.

Key words: ES cells, Primate, Primitive hematopoiesis, Definitive hematopoiesis

Introduction

During murine embryogenesis, primitive (embryonic) hematopoiesis is first detected in blood islands of the yolk sac at approximately 7.5 days postcoitum (dpc). It is followed by definitive (fetal and adult) hematopoiesis in aorta-gonad-mesonephros regions, which shifts to the fetal liver, spleen and bone marrow (Dzierzak and Medvinsky, 1995; Xu et al., 2001). Primitive hematopoiesis generates mainly nucleated primitive erythrocytes (EryP), whereas definitive hematopoiesis generates all hematopoietic lineages, including nucleated and enucleated definitive erythrocytes (EryD) and hematopoietic stem cells with a long-term repopulating activity. Pluripotent murine embryonic stem (ES) cells have been used as a powerful tool for studying the mechanisms of hematopoietic development in various culture conditions (Doestchmann et al., 1985; Wiles and Keller, 1991; Keller et al., 1993; Nakano et al., 1996; Nishikawa et al., 1998). Among these conditions, co-culture with OP9 stromal cells, which lack functional macrophage colony-stimulating factor (M-CSF; CSF1 – Mouse Genome Informatics) (Yoshida et al., 1990), can induce primitive and definitive hematopoietic development in murine ES cells in vitro in a pattern similar to that seen in murine ontogeny (Nakano et al., 1996).

Recently, it has been reported that primate hematopoiesis occurs in a manner similar to that of mice, based on immunohistochemical studies of human embryos (Tavian et al., 1996; Tavian et al., 1999). In the erythrocytes of primates, the embryonic (ε and ζ), fetal (γ) and adult (β and α) globin genes are expressed sequentially during development, although small amounts of fetal and adult globin chains are detected even during primitive hematopoiesis (Johnson et al., 2000; Stamatoyannopoulos et al., 2001). Concomitant switches in the α cluster (replacement of the ζ globin gene by the α) and β cluster (replacement of the ε globin gene by the γ, and

replacement of the γ globin gene by the β) occur during development, and coincide with the transition from yolk sac to fetal liver, and finally to bone marrow hematopoiesis.

The hematopoietic development of primates remains to be elucidated, in part because of the ethical restrictions on experiments using their embryos. Old World monkeys, such as the cynomolgus monkey (*Macaca fascicularis*), are widely used for medical research (Hanazono et al., 2000) and have globin gene expression that is similar to that of humans (Johnson et al., 2000). Therefore, their ES cells might be used as a model for elucidating primate hematopoietic development. Recently primate ES cell lines were established (Thomson et al., 1995; Thomson et al., 1996; Thomson et al., 1998; Suemori et al., 2001), and hematopoietic differentiation from primate ES cells was also induced successfully in vitro (Kaufman et al., 2001; Li et al., 2001; Lu et al., 2002; Chadwick et al., 2003). However, compared with the murine system, little work has been done to precisely analyze primitive and definitive hematopoietic development.

To address this problem, we induced hematopoietic differentiation in cynomolgus monkey ES cells by co-culture with OP9 stromal cells. This is the first report to demonstrate that primitive hematopoiesis and its transition to definitive hematopoiesis can be induced from primate ES cells in vitro.

Materials and methods

Maintenance of cynomolgus monkey ES cell line

The ES cell line CMK6 was established from cynomolgus monkey blastocysts produced by in vitro fertilization or intracytoplasmic sperm injection and maintained as previously described (Suemori et al., 2001). Briefly, cells were transferred onto a feeder layer of mitomicin C-inactivated mouse embryonic fibroblasts (MEF) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (Sigma, St Louis, MO), supplemented with 20% KnockOut SR serum replacer (Gibco BRL, Grand Island, NY), 100 μM 2-mercaptoethanol (2ME) (Nakalai Tesque, Kyoto, Japan), 2 mM L-glutamine and 1% nonessential amino acids (Gibco BRL). ES cells were passaged every 3 days to maintain their undifferentiated state, and were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air.

Cytokines and growth factors

Recombinant human granulocyte colony-stimulating factor (G-CSF; CSF2 – Human Gene Nomenclature Database), EPO, interleukin 3 (IL3), stem cell factor (SCF) and thrombopoietin (THPO) were kindly provided by Kirin Brewery (Tokyo, Japan). Recombinant human VEGF, basic fibroblast growth factor (bFGF), and bone morphogenetic protein (BMP) 4 were all purchased from R&D Systems (Minneapolis, Minnesota).

Antibodies

The primary antibodies used in this study were: mouse anti-human CD41 (clone 5B12) and CD45 (clone T16) antibodies (Dako, Kyoto, Japan); mouse anti-human CD34 (clone 563) antibody (Becton-Dickinson, San Jose, CA); KIT antibody (Nichirei, Tokyo, Japan); rabbit anti-human hemoglobin (Hb) polyclonal antibody (Cappel, Aurora, Ohio); and sheep anti-human fetal hemoglobin (HbF) polyclonal antibody (Bethyl, Montgomery, TX). The mouse anti-human embryonic ε-globin (HbEmb) monoclonal antibody, and anti-human fetal liver kinase (FLK) 1 monoclonal antibody were used as described previously (Luo et al., 1999; Sawano et al., 2001). Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-sheep IgG, fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG and alkaline phosphatase (ALP)-conjugated donkey anti-mouse IgG (all purchased from Jackson ImmunoResearch Laboratories, West Grove, PA), and allophycocyanin (APC)-conjugated goat anti-mouse IgG (Becton-Dickinson), were used as secondary antibodies.

In vitro hematopoietic differentiation from ES cells

OP9 stromal cells were kindly provided by Dr Hiroaki Kodama, and were maintained in αMEM (Gibco BRL) supplemented with 20% fetal calf serum (FCS) (EQUITECH-BIO, Kerrville, TX). Trypsin–treated ES cells (4×10^3 cells/well) were transferred onto confluent OP9 stromal cells in αMEM supplemented with 10% FCS and 50 μM 2ME, in the presence or absence of VEGF, bFGF or BMP4. On day 6 of differentiation, the induced cells were harvested with cell dissociation buffer (Invitrogen, Carlsbad, CA). Then the cells were filtered through a 70 μm nylon cell strainer (Falcon, Lincoln Park, NJ), and 1×10^5 cells/well were transferred onto fresh confluent OP9 cells in 6-well plates and cultured in αMEM supplemented with 10% FCS and 50 μM 2ME, in the presence or absence of EPO (10 U/ml). The medium was changed every 2 or 3 days during the induction of differentiation. Adherent hematopoietic cell clusters, which consisted of more than 20 round blast-like cells, were counted using an inverted microscope. The same series of experiments was performed at least three times.

Staining

For cytochemical staining, the floating cells were centrifuged onto glass slides and analyzed by microscopy after May-Giemsa or myeloperoxidase staining.

For immunostaining, floating cells spun onto glass slides were fixed in 4% paraformaldehyde and permeabilized with phosphate buffered saline (PBS) containing 5% skim milk (Becton-Dickinson) and 0.1% Triton X-100 for 30 minutes. The cells were then incubated with primary antibodies overnight, washed three times with PBS containing 5% skim milk, and then incubated with FITC- or Cy3-conjugated secondary antibodies for 30 minutes. Nuclei were labeled with Hoechst 33342 (Molecular Probes, Eugene, Oregon). The cells were then washed three times with PBS and observed by fluorescence microscopy (Olympus, Tokyo, Japan). In the human erythroblastic cell line K562, which is known to express ε, ζ, γ and α globins (Rutherford et al., 1981), all erythroid cells were positive for Hb, HbF and HbEmb. In adult cynomolgus bone marrow, all erythrocytes were positive for Hb and a few were positive for HbF, whereas HbEmb-positive erythrocytes were rarely detected (data not shown). The adherent cells were fixed and incubated with primary and ALP-conjugated secondary antibodies, as described above, and positive cells detected using a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). Endogenous ALP activity was blocked by 2 mM levamisole (Wako, Osaka, Japan).

Methylcellulose colony forming assays

The medium was replaced with a fresh semisolid medium consisting of αMEM, 0.9% methylcellulose, 30% FCS, 10% bovine serum albumin and 50 μM 2ME, and a mixture of human G-CSF 10 ng/ml, EPO 2 U/ml, IL3 20 ng/ml, SCF 100 ng/ml and THPO 10 ng/ml, as previously reported (Sui et al., 1995). All cultures were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Seven days later, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, washed twice with PBS, and processed for May-Giemsa staining, immunostaining and RT-PCR analysis. Colonies (≥50 cells) were counted using an inverted microscope according to the criteria previously reported (Nakahata and Ogawa, 1982; Tajima et al., 1996).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by using TRIzol (Invitrogen) according to the manufacturer's protocol. Each total RNA sample was then reverse-

transcribed using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified in a final volume of 20 μl PCR buffer containing 2.5 mM $MgCl_2$ and 250 μM dNTP, using Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). As cynomolgus monkey-specific sequences were unavailable, we employed those of corresponding human or other Old World monkey genes to design our PCR primers, based on the generally close homology between human and cynomolgus monkey gene sequences. The EPO receptor (EPOR) and α-fetoprotein (AFP)-specific primers have been described previously (Schuldiner et al., 2000; Yokomizo et al., 2002). Samples were initially denatured at 94°C for 5 minutes, followed by amplification rounds consisting of 94°C for 1 minute (denaturing), 57-66°C for 1 minute (annealing) and 72°C for 1 minute (extension), and then a final extension at 94°C for 7 minutes. The oligonucleotide primers were:

ε-globin (450 bp), 5′-TGCATTTTACTGCTGAGGAGA-3′ (sense) and 5′-AAGAGAACTCAGT GGTACTT-3′ (antisense);

γ-globin (524 bp), 5′-AGACGCCATGGGTCATTTCACA-3′ (sense) 5′-GCCTATGGTTGAAAGCTCTGIAT-3′ (antisense);

β-globin (196 bp), 5′-GGGCAGGCTGCTGGTGGTCTAC-3′ (sense) and 5-CGTGCTGGTGTGTGTGCTGG-3′ (antisense);

ζ-globin (223 bp), 5′-TTCCTCAGCCACCCGCAGAC-3′ (sense) and 5′-AGCAGGCAGTGGGACAGGAG-3′ (antisense);

α-globin (152 bp), 5′-TGCACGCGCACAAGCTTCGG-3′ (sense) and 5′-GCACGGTGCTCACAGAAGCCAG-3′ (antisense);

EPOR (183 bp), 5′-TGGTATCTGACTCTGGCATCTC-3′ (sense) and 5′-TCCCTGATCATCTGCAGCC-3′ (antisense);

Brachyury (253 bp), 5′-AAGGTGGATCTTCAGGTAGC-3′ (sense) and 5′-CATCTCATTGGTGAGCTCC-3′ (antisense);

FLK1 (239 bp), 5′-AAAACCTTTTGTTGCTTTTGG-3′ (sense) and 5′-GAAATGGGATTGGTAAGGATG-3′ (antisense);

SCL (185 bp), 5′-TCTCGGCAGCGGGTTCTTTG-3′ (sense) and 5′-AAGGCCCCGTTCACAT TCTGC-3′ (antisense);

LMO2 (213 bp), 5′-CTGATGCTTGGCCTTCTCTCC-3′ (sense) and 5′-GGCCCAGTTTGTAGTAGAGGC-3′ (antisense);

MYB (307 bp), 5′-CACGCTGGGCCTGTCATCAAC-3′ (sense) and 5′-GCATGGCTCTTCGTGTTATAGC-3′ (antisense);

GATA-2 (303 bp), 5′-TGGCGCACAACTACATGGAAC-3′ (sense) and 5′-GAGGGGTGCAGTGGCGTCTT-3′ (antisense);

Nestin (718 bp), 5′-GGCAGCGTTGGAACAGAGGTTGG-3′ (sense) and 5′-CTAAACTGGAGTGGTCAGGGCTG-3′ (antisense);

AFP (678 bp), 5′-AGAACCTGTCACAAGCTGTG-3′ (sense) and 5′-GACAGCAAGCTGAGGATGTC-3′ (antisense);

REX1 (489 bp), 5′-CGCGGTGTGGGCCTTATGTG-3′ (sense) and 5′-TCTCAGGGCAGCTCTATTCCTC-3′ (antisense); and

β-actin (280 bp), 5′-GCAGGAGATGGCCACGGCGCC-3′ (sense) and 5′-TCTCCTTCTGCATCCTGTCGGC-3′ (antisense).

The PCR reactions were carried out as follows: ε-globin, 35 cycles; γ-globin, 35 cycles; β-globin, 35 cycles; ζ-globin, 35 cycles; α-globin, 35 cycles; EPOR, 40 cycles; Brachyury, 45 cycles; FLK1, 40 cycles; SCL, 45 cycles; LMO2, 40 cycles; MYB, 40 cycles; GATA2, 35 cycles; Nestin, 35 cycles; AFP, 35 cycles; REX1; 35 cycles; β-actin, 35 cycles. The analysis of globin gene expression in individual erythroid colonies was performed for 40 cycles. PCR products were visualized by 1.5% agarose gel electrophoresis using ethidium bromide staining. cDNA from cynomolgus monkey bone marrow or K562 cells was used as a positive control. For semi-quantitative comparisons, samples were normalized by dilution to give equivalent signals for β-actin. DNA sequencing was performed for genes from which we were unable to obtain adequate PCR products from the positive control.

Flow cytometric analysis and cell sorting

The cells induced from CMK6 were harvested sequentially with cell dissociation buffer (Invitrogen), filtered through a 70 μm nylon cell strainer (Falcon), and incubated in human IgG for 30 minutes to block the non-specific binding of FCγ-receptors. The cells were then incubated with PE-conjugated CD34, or unconjugated FLK1 and KIT antibodies for 30 minutes. Samples staining with unconjugated antibodies were then incubated with APC-conjugated goat anti-mouse antibody (BD PharMingen) for 30 minutes. The cells were then washed and analyzed using a FACScaliber with the CellQuest program (Becton-Dickinson). Forward- and side-scatter plots were used to exclude MEF or OP9 stromal cells, and propidium iodide co-staining was used to exclude non-viable cells. Mouse IgG1 (Dako) was used as an isotype control. On day 10, the cultured cells were harvested and labeled with CD34 antibody as described above. CD34-positive cells were collected using a FACSVantage flow cytometer

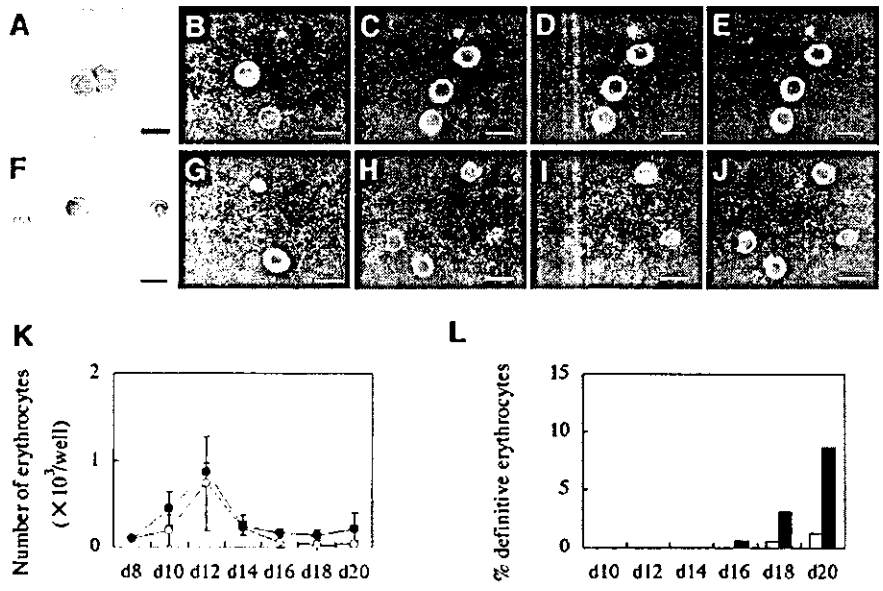

Fig. 1. Development of primitive and definitive erythrocytes. Primate ES cells (4×10^3 cells/well) were cultured onto OP9 stromal cells for 6 days. The induced cells were harvested and re-cultured at a concentration of 1×10^5 cells per well onto fresh OP9 stromal cells, with or without 10 U/ml EPO. The floating cells were harvested every other day and analyzed by May-Giemsa staining and immunostaining against human hemoglobin (Hb), fetal hemoglobin (HbF) and embryonic hemoglobin (HbEmb). (A-E) Day 12; (G-J) day 18. (A,F) May-Giemsa staining of erythrocytes. (B,G) Hb (FITC) and HbF (Cy3) staining of erythrocytes. (C-E,H-J) Hb (FITC) and HbEmb (Cy3) staining of erythrocytes. Merged images are shown in B,E,G and J. Nuclei were labeled with Hoechst 33342 in B-E and G-J. Scale bars: 10 μm. (K) Sequential analysis of the number of erythrocytes, with (black circles) or without (white circles) EPO. (L) Sequential analysis of the proportion of definitive erythrocytes (EryD) among total erythrocytes, with (black columns) or without (white columns) EPO. EryD were defined as Hb- and HbF-positive, HbEmb-negative erythrocytes, whereas primitive erythrocytes were Hb-, HbF- and HbEmb-positive. Data represent the mean±s.d. of triplicate wells. Representative results from one of three independent experiments are shown.

(Becton-Dickinson) and re-plated at a concentration of 2×10^4 cells per well onto fresh OP9 stromal cells in αMEM supplemented with 10% FCS and 50 μM 2ME, in the presence or absence of EPO (10 U/ml). Floating cells were processed for May-Giemsa staining, immunostaining and RT-PCR analysis.

Results

Primitive and definitive hematopoiesis developed sequentially in primate ES cells co-cultured with OP9 stromal cells

Initially, we tried to induce hematopoietic differentiation from ES cells co-cultured on OP9 stromal cells without any additional cytokines or growth factors in the culture medium. Floating hematopoietic cells, which mainly consisted of large nucleated erythrocytes, first appeared on day 8 of the differentiation induction (Fig. 1A). Immunostaining with antibodies against anti-human hemoglobins demonstrated that all of the large erythrocytes were positive for Hb, HbF and HbEmb (Fig. 1B-E), a pattern that corresponds to primitive erythrocytes (EryP). Thereafter, small nucleated or enucleated erythrocytes appeared faintly on day 16 or day 18 (Fig. 1F). Many of the small erythrocytes were positive for Hb and HbF, but negative for HbEmb (Fig. 1G-J), a pattern that corresponds to definitive (fetal) erythrocytes (EryD). These results suggested that EryP and EryD sequentially developed under the culture conditions.

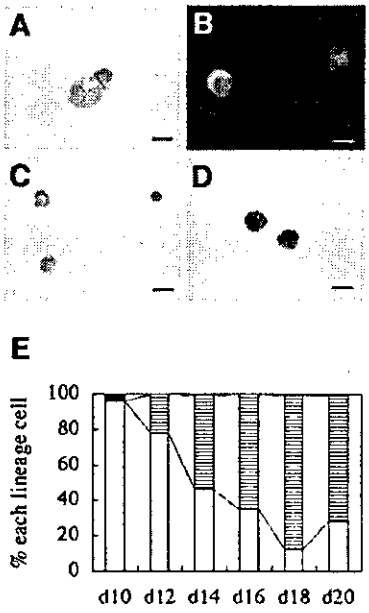

Fig. 2. Development of megakaryocytes and myeloid cells. (A,B) May-Giemsa staining (A, left cell) and CD41 (Cy3) staining (B) of megakaryocytes on day 12. Nuclei were labeled with Hoechst 33342 in B. (C,D) May-Giemsa staining (C) and myeloperoxidase staining (D) of myeloid cells on day 18. Scale bars: 10 μm. (E) Sequential analysis of the percentages of erythrocytes (white columns), myeloid cells (striped columns) and megakaryocytes (black columns) among total hematopoietic cells in the presence of EPO. Each column represents the mean of triplicate wells. Representative results from one of three independent experiments are shown.

Time-course analysis demonstrated that the number of erythrocytes increased to a maximum on day 12 but gradually decreased thereafter (Fig. 1K). The presence of exogenous EPO resulted in more prominent EryD development (Fig. 1L), although it exerted only minor effects on the number of erythrocytes (Fig. 1K).

CD41-positive megakaryocytes appeared on day 8 (Fig. 2A,B), and MPO-positive myeloid lineage cells appeared on day 12 (Fig. 2C,D). Time-course analysis demonstrated that the number of megakaryocytes peaked on day 10 but decreased thereafter, whereas myeloid lineage cells gradually increased until they comprised more than half of the total number of hematopoietic cells on day 14 and thereafter (Fig. 2E).

The sequential expression of embryonic (ε and ζ), fetal (γ) and adult (β and α) globin genes in erythrocytes cultured in the presence of EPO was examined using RT-PCR analysis. As shown in Fig. 3, ε and ζ globin genes were expressed on day 6, and their expression was upregulated to high levels on day 8 and thereafter. The expression of the γ and α globin genes remained at low levels on day 8 but was upregulated on day 12 and thereafter, whereas β globin gene expression remained at low levels on day 12 and was not upregulated until day 18. Expression of EPOR, which was detected in undifferentiated ES cells, declined on day 4 but was upregulated again on day 8. These results confirmed that erythropoiesis begins on day 6 and the transition from primitive to definitive erythropoiesis occurs on day 12 at the mRNA level.

Adherent hematopoietic cell clusters developed from primate ES cells

In the OP9 co-culture system, adherent hematopoietic cell clusters first appeared on day 8, irrespective of the presence of

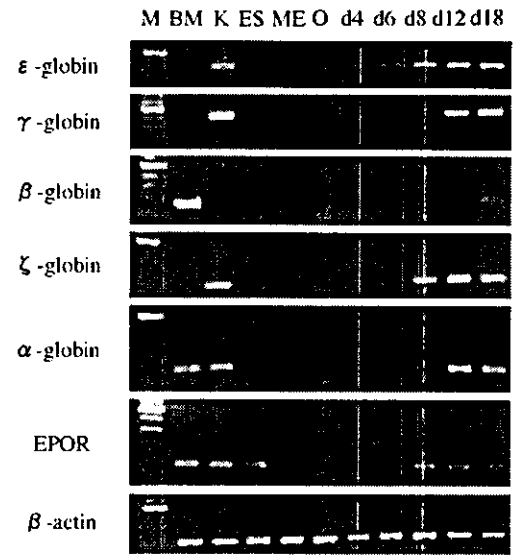

Fig. 3. Sequential RT-PCR analysis of embryonic (ε and ζ), fetal (γ) and adult (β and α) globin expression in erythrocytes during liquid culture differentiation. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; ES, undifferentiated ES cells; ME, mouse embryonic fibroblasts; O, OP9 stromal cells; d, days after the induction of differentiation in the presence of EPO.

Fig. 4. Development of adherent hematopoietic cell clusters. (A,B) An adherent hematopoietic cell cluster (A) and a cluster with a cobblestone appearance that grew underneath the OP9 stromal cells (B). (C) Alkaline phosphatase staining of adherent hematopoietic cell clusters immunolabeled with human CD34 antibody. Scale bars: 100 μm.

exogenous EPO (Fig. 4A), and contained some typical cobblestone-like cells, which are known to be characteristic of immature hematopoietic progenitors (Fig. 4B). The adherent fraction has been reported to contain more progenitors than the floating fraction in the OP9 co-culture system (Suwabe et al., 1998). The number of clusters increased to a maximum on day 10, but rapidly decreased thereafter. Immunostaining demonstrated that more than half of the cells in the clusters were positive for CD34 (Fig. 4C).

Primitive erythroid colonies were generated from primate ES cells in methylcellulose colony-forming assays

To evaluate the generation of various progenitors in this system, we first performed methylcellulose colony-forming assays in the presence of a mixture of cytokines, including G-CSF, EPO, IL3, SCF and THPO, under stromal-free conditions using dissociated adherent cells with trypsin treatment. However, only a few granulocyte and macrophage colonies were observed (Fig. 5A,B), and erythroid colonies were never generated at any time during differentiation induction. To improve the system, the medium in the 6-well plates was sequentially replaced with a methylcellulose-containing medium that included a mixture of cytokines. Erythroid colonies were generated from the cultures replaced on day 7 of induction, with a peak on day 8 or day 9 (Fig. 5C,D). The number of erythroid colonies gradually decreased, and no colony formation was observed from day 12 and thereafter. The colonies were first identified 5 days after the addition of methylcellulose-containing medium. Immunostaining demonstrated that all the erythrocytes in the colonies were positive for Hb, HbF and HbEmb (Fig. 5E,F), corresponding to EryP. All 10 of the representative erythroid colonies examined expressed not only embryonic (ε and ζ), but also fetal (γ) or adult (β and α) globin genes (Fig. 5G). These results suggest that the erythroid colonies were derived from primitive hematopoiesis.

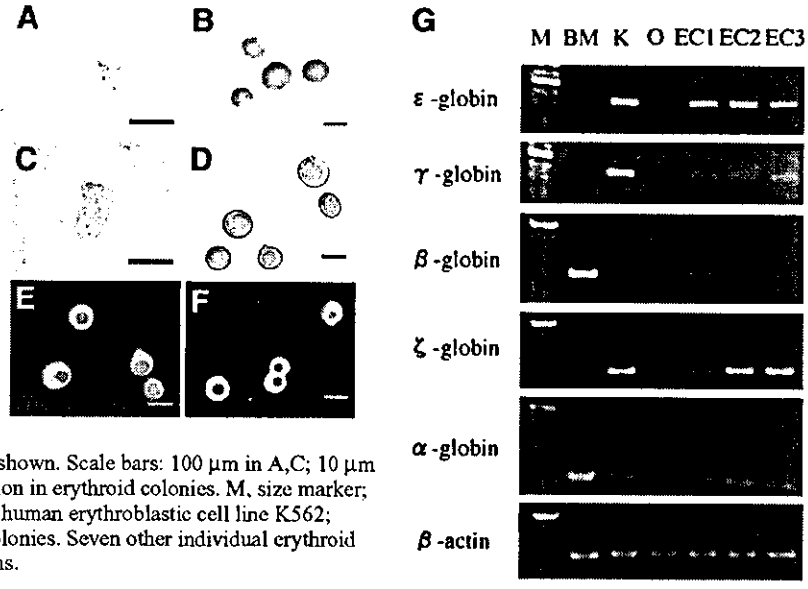

Fig. 5. Development of hematopoietic colonies. Methylcellulose colony-forming assays were performed on day 8. Using adherent cells dissociated with trypsin treatment, only a few GM colonies were observed (A,B). When the medium was replaced with methylcellulose-containing medium, erythroid colonies were generated on the OP9 stromal cell layer (C-F). (A,C) Morphology of a GM colony (A) and a primitive erythroid colony (C). (B,D) May-Giemsa staining of a GM colony (B) and a primitive erythroid colony (D). (E,F) Human hemoglobin (Hb; FITC) and fetal hemoglobin (Cy3; E), and Hb (FITC) and embryonic hemoglobin (Cy3; F) staining of erythrocytes in a primitive erythroid colony. Nuclei were labeled with Hoechst 33342. Merged images are shown. Scale bars: 100 μm in A,C; 10 μm in B,D-F. (G) RT-PCR analysis of globin gene expression in erythroid colonies. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; O, OP9 stromal cells; EC1-EC3, primitive erythroid colonies. Seven other individual erythroid colonies showed similar globin gene expression patterns.

Flow cytometric and RT-PCR analysis of hematopoietic development in the OP9 co-culture system

To investigate whether the temporal expression pattern of genes involved in hematopoietic development is also reproduced in the OP9 co-culture system, we first examined the expression of KIT, FLK1 and CD34, which are surface markers expressed by early hematopoietic cells. As shown in Fig. 6, flow cytometric analysis of undifferentiated ES cells revealed that almost all cells expressed KIT and FLK1, although CD34-positive cells were not detected. The expression of KIT and FLK1 declined on day 4, but was upregulated on day 6. CD34-positive cells were first observed on day 6, and increased thereafter.

We also examined the expression of genes associated with hematopoietic development by RT-PCR (Fig. 7). Brachyury, an early mesodermal marker, was expressed on day 4 but its expression declined on day 6. The expression of FLK1, LMO2, MYB and GATA2, which were detected in undifferentiated ES cells, declined on day 4, but was upregulated on day 6, just before hematopoietic development. SCL expression was first detected on day 6 and remained constant thereafter. By contrast, the marker genes that are indicative of ectoderm (Nestin), endoderm (AFP), and undifferentiated ES cells (REX1) were expressed during differentiation induction. Altogether, these results suggest that the hematopoietic development that is induced in the co-culture system parallels

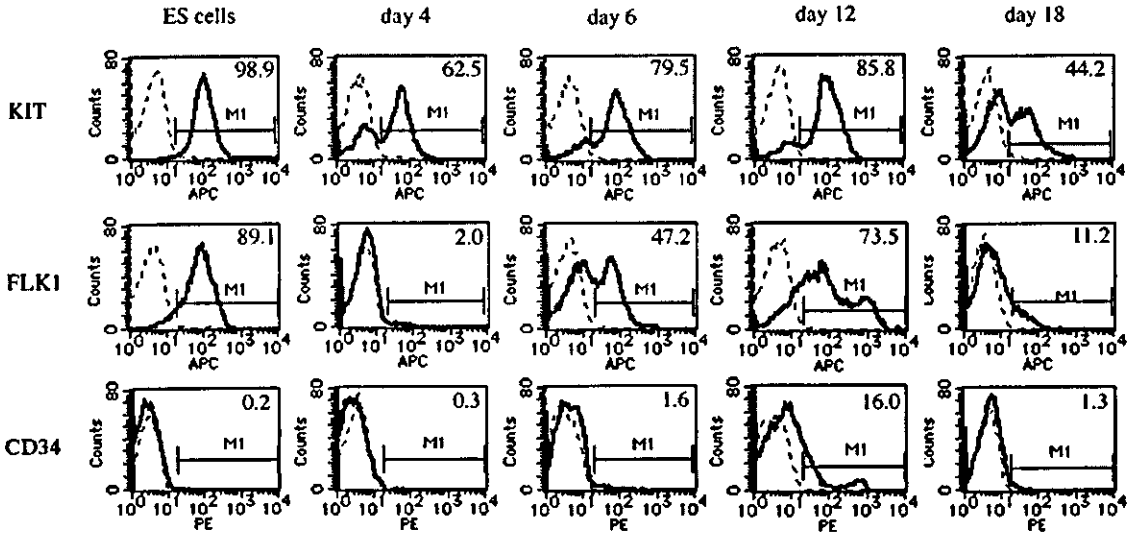

Fig. 6. Sequential flow cytometric analysis of KIT, FLK1 and CD34 during differentiation induction. Undifferentiated ES cells, or the cultures on day 4, 6, 12 and 18, were stained with antibodies specific for KIT, FLK1 or CD34, or with control IgG1. Plots show the isotype control IgG1 staining profile (dashed lines) versus the specific antibody staining profiles (solid lines). Representative results from one of three independent experiments are shown.

that found in the embryo, and that the development of other lineages also occurred concomitantly.

VEGF enhances primitive and definitive hematopoiesis in a dose-dependent manner in the OP9 co-culture system and methylcellulose assay

Although the transition from primitive to definitive hematopoiesis was induced, definitive hematopoiesis was less efficient in our culture system, irrespective of exogenous EPO. As VEGF, bFGF, and BMP4 have been shown to promote primitive and definitive hematopoietic development in murine ES cells (Johansson and Wiles, 1995; Faloon et al., 2000; Nakayama et al., 2000), we tested these growth factors in our culture system.

Exogenous VEGF increased the number of erythrocytes in a dose-dependent manner until day 14, in the presence or absence of EPO (Fig. 8A). These erythrocytes consisted exclusively of EryP. EryD were rarely observed in the presence of VEGF alone (data not shown), whereas exogenous VEGF plus EPO enhanced EryD production more prominently with time (Fig. 8B).

The total number of hematopoietic cells was increased by exogenous VEGF irrespective of the presence of EPO (Fig. 8C). VEGF did not affect the percentage of each lineage population (Fig. 8D), and the increase in myeloid cells, major components of definitive hematopoiesis, contributed to the increase of the total number of hematopoietic cells after day 14. Furthermore, exogenous VEGF enhanced the increase in both adherent cell clusters and primitive erythroid colonies in a dose-dependent manner (Fig. 8E,F), suggesting that VEGF affects hematopoiesis at the progenitor level. Exogenous bFGF did not alter the process of hematopoiesis, or the number of adherent clusters and erythroid colonies. By contrast, exogenous BMP4 exerted an adverse effect on hematopoiesis (Fig. 8E-H).

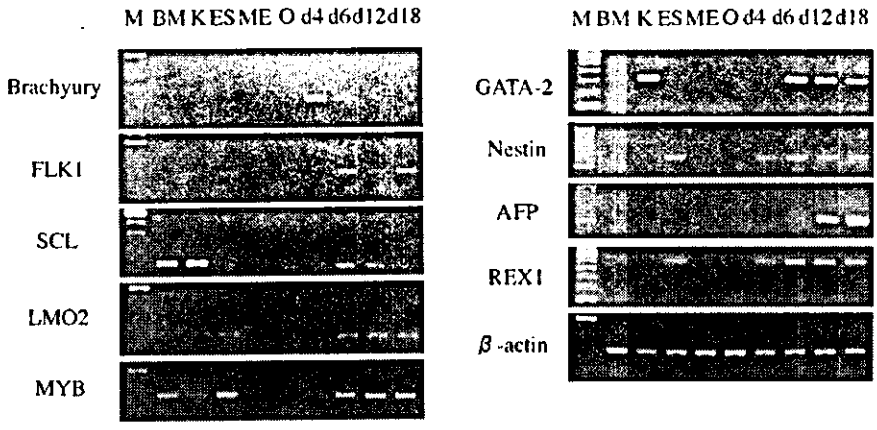

Fig. 7. Sequential RT-PCR analysis of genes associated with hematopoietic development in cultures during liquid culture differentiation. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; ES, undifferentiated ES cells; ME, mouse embryonic fibroblasts; O, OP9 stromal cells; d, days after the induction of differentiation in the presence of EPO.

More efficient primitive and definitive hematopoiesis is induced by re-plating sorted CD34-positive cells

As previously shown, other lineages developed concomitantly in our culture system. Consequently, we purified the CD34-positive cells in the cultures and seeded them onto fresh OP9 stromal cells on day 10 (Fig. 9A-

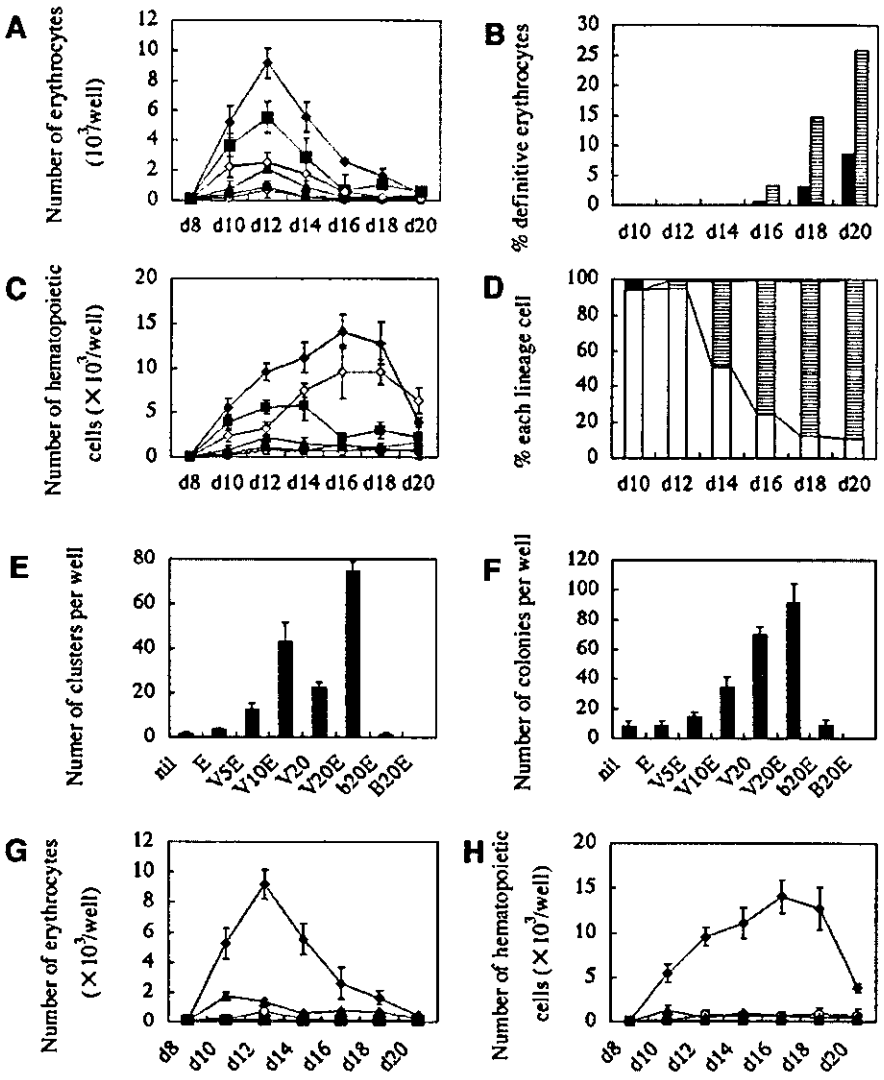

Fig. 8. Effects of EPO and various growth factors on primitive and definitive hematopoiesis. Primate ES cells were cultured for 6 days with VEGF, bFGF or BMP4. The induced cells were harvested and re-cultured with (black circles) or without (white circles) 10 U/ml EPO. The floating cells were analyzed as described in Fig. 2. Adherent clusters (≥20 cells) were counted on day 10. Primitive erythroid colonies (≥50 cells) were counted 7 days after replacing the medium with methylcellulose-containing medium on day 8. (A,C) Effects of EPO and various concentrations of VEGF (0 ng/ml, circles; 5 ng/ml, triangles; 10 ng/ml, squares; 20 ng/ml, diamonds) on the number of erythrocytes (A) and total hematopoietic cells (C). (B) Sequential analysis of the proportion of definitive erythrocytes (EryD) among total erythrocytes in the presence of EPO alone (black columns), or EPO plus VEGF (20 ng/ml; striped columns). (D) Sequential analysis of the percentages of erythrocytes (white columns), myeloid cells (striped columns) and megakaryocytes (black columns) among total hematopoietic cells in the presence of EPO plus VEGF (20 ng/ml). (E,F) Effects of EPO and growth factors on the number of adherent hematopoietic clusters (E) and primitive erythroid colonies (F). E, EPO; V, VEGF (5 to 20 ng/ml); b, bFGF (20 ng/ml); B, BMP4 (20 ng/ml). (G,H) Effects of bFGF (20 ng/ml, triangles), BMP4 (20 ng/ml, squares) and VEGF (20 ng/ml, diamonds) on the number of erythrocytes (G) and total hematopoietic cells (H). Data represent the mean±s.d. of triplicate wells. Representative results from one of three independent experiments are shown.

C). In the presence of EPO, approximately on day 25 (i.e. 15 days after the cell sorting), adherent hematopoietic cell clusters grew larger and had a cobblestone appearance (Fig. 9D). The number of floating cells, most of which were erythrocytes, increased with a peak on day 28 (Fig. 9E,H,I). Nearly 40% of these erythrocytes were definitive, and many were enucleated (Fig. 9E-G,J,K). In the absence of EPO, hematopoietic development after cell sorting was barely observed. These results indicate that CD34-positive cells in cultures contained progenitors of both primitive and definitive hematopoiesis.

Discussion

In this study, we have demonstrated that nonhuman primate ES cells are a suitable tool for dissecting the molecular and cellular mechanisms of primitive and definitive hematopoietic development. The capacity of murine ES cells to differentiate into hematopoietic cells has been investigated intensively in many culture systems (Doestchmann et al., 1985; Wiles and Keller, 1991; Keller et al., 1993; Nakano et al., 1996; Nishikawa et al., 1998); in particular, the transition from primitive to definitive hematopoiesis can be induced in murine ES cells by co-culture on OP9 stromal cells in vitro (Nakano et al., 1996). This culture system can reproduce hematopoietic development from murine ES cells with a pattern similar to that observed in developing mouse embryos, and has been established as a powerful experimental tool for elucidating the regulation of hematopoietic development and differentiation (Suwabe et al., 1998; Era et al., 2000; Kitajima et al., 2002).

The hematopoietic development of primates, however, is different from that of mice. For example, expression of the γ gene during the fetal period is an event that occurs only among primates (TomHon et al., 1997). Therefore, in vitro and in vivo studies of primate hematopoietic development should be performed using primate-derived materials. In recent studies in primate ES cells, Kaufman et al. and Li et al. demonstrated that the definitive hematopoiesis, but not the primitive hematopoiesis, of in vivo differentiation is recapitulated (Kaufman et al., 2001; Li et al., 2001). Lu et al. revealed that

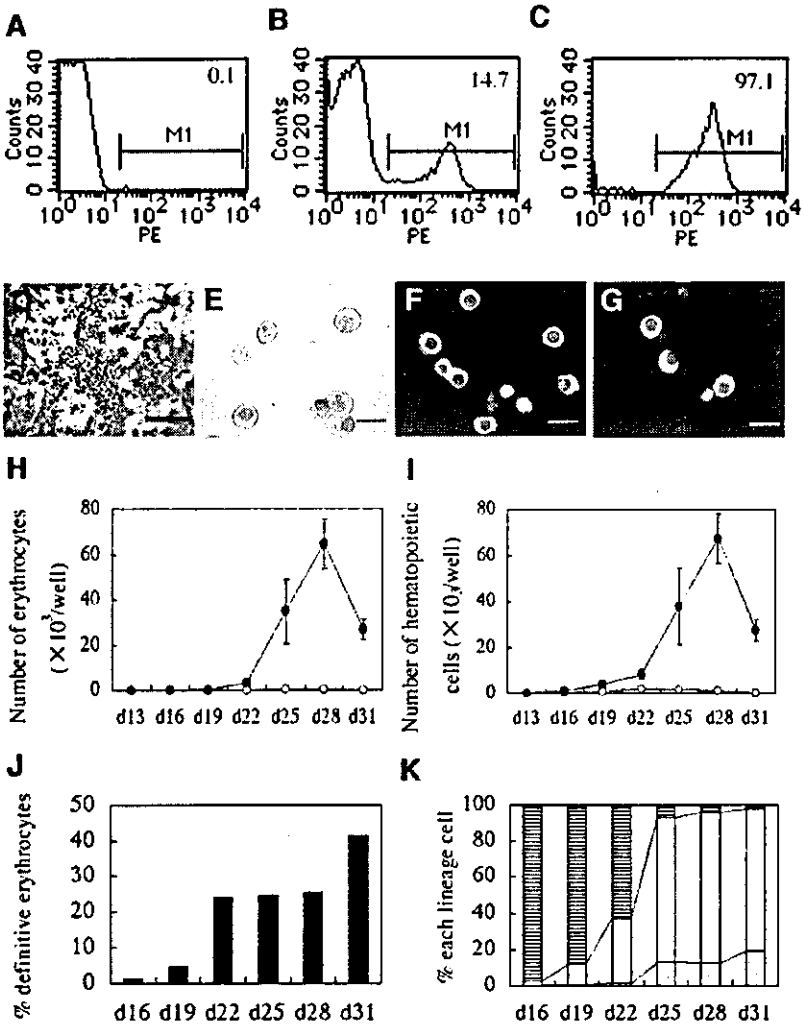

Fig. 9. More efficient primitive and definitive hematopoiesis is induced by re-plating sorted CD34-positive cells. (A-C) Flow cytometric analysis and cell sorting of cultures on day 10 with human CD34 antibody. A sorting gate in B was defined according to the intensity of staining with an isotype control antibody (A). Reanalysis of the sorted cells confirmed the purity as 95 to 98% (C). (D) Large adherent hematopoietic cell cluster with a cobblestone appearance on day 25. Scale bars: 100 μm. (E-G) May-Giemsa staining (E), human hemoglobin (Hb; FITC) and embryonic hemoglobin (Cy3; F), and Hb (FITC) and fetal hemoglobin (Cy3; G) staining of day 25 erythrocytes grown in the presence of EPO. Nuclei were labeled with Hoechst 33342. Merged images are shown. Scale bars: 10 μm. (H,I) Sequential analysis of the number of erythrocytes (H) and total hematopoietic cells (I), with (black circles) or without (white circles) 10 U/ml EPO. (J) Sequential analysis of the proportion of definitive erythrocytes among total erythrocytes. (K) Sequential analysis of the percentages of enucleated erythrocytes (stippled columns), nucleated erythrocytes (white columns) and myeloid cells (striped columns) in the presence of EPO. Data represent the mean±s.d. of triplicate wells. Representative results from one of three independent experiments are shown.

the coexistence of primitive and definitive hematopoiesis is recapitulated at the mRNA level (Lu et al., 2002). In this study, we have demonstrated for the first time the transition from primitive to definitive hematopoietic development in primate ES cells at both the transcriptional and the translational level in vitro. Immunostaining using human hemoglobin antibodies demonstrates that embryonic and definitive (fetal) erythrocytes appear on day 8 and day 16, respectively. Sequential RT-PCR analysis of globin genes demonstrates upregulation of primitive (ε and ζ) globin gene expression on day 8 and of definitive (γ and α) globin genes on day 12, which indicates that the erythropoietic transition can be recapitulated in ES cells at the mRNA level. Therefore, our in vitro system is superior in precisely reflecting the ontogeny of hematopoietic cells in vivo, and should be a useful tool to define the mechanisms of primate hematopoiesis. The generation of adherent hematopoietic cell clusters containing CD34-positive cells onto the OP9 cell layer indicates that this induction system also recapitulates hematopoietic development at the progenitor level from primate ES cells, as has been observed in murine ES cells (Nakano et al., 1996; Suwabe et al., 1998; Era et al., 2000; Kitajima et al., 2002).

Our results from the colony assays also demonstrate for the first time that primitive erythroid colonies are generated with the aid of stromal cells, but that definitive colonies do not emerge in the presence or absence of stromal cells. By contrast, a recent study has shown that definitive erythroid colonies are generated from primate ES cells under stromal-free conditions, but that primitive colonies are not (Kaufman et al., 2001). These differences may be partially due to differences in the culture conditions, the colony assays, and/or the ES cells and stromal cells that were used for the induction of differentiation. Notably, our individual primitive erythroid colonies express not only embryonic but also fetal and adult globin genes, which is consistent with the results obtained by plating human embryonic or fetal cells (Peschle et al., 1984; Stamatoyannopoulos et al., 1987). Fetal and adult hemoglobin synthesis, and factors regulating their synthesis, have been intensively analyzed in human cord blood, and in neonatal and adult erythroid colonies (Stamatoyannopoulos et al., 2001). However, precise analysis of hemoglobin synthesis in primitive erythroid colonies has not been performed. Thus, our culture system will also serve as a powerful tool for elucidating the regulatory mechanisms of primitive hematopoiesis.

Co-culture on OP9 stromal cells alone induces hematopoietic development less efficiently in primate ES cells than in murine ES cells. VEGF, bFGF and BMP4 have been reported to promote primitive or definitive hematopoietic development in previous studies in murine ES cells (Johansson and Wiles, 1995; Faloon et al., 2000; Nakayama et al., 2000). Therefore, we quantified the stimulatory effects of these

growth factors on both types of hematopoiesis. Unexpectedly, exogenous BMP4 fails to induce hematopoietic differentiation in our culture system. There are two possible explanations for this discrepancy. One is that human BMP4 does not work on the cynomolgus ES cell line we used. However, considering that human BMP4 functions in both murine and primate ES cells (Johansson and Wiles, 1995; Nakayama et al., 2000; Li et al., 2001; Chadwick et al., 2003) the possibility seems unlikely. Another possibility is that BMP4 causes the OP9 stromal cells to differentiate and thereby impairs their interaction with ES cells. Supporting this notion is the fact that we observed that exogenous BMP4 resulted in an increase of adipocytes on the OP9 cell layer (data not shown). This observation is also consistent with a previous report that showed that BMP4 induces the differentiation of mesenchymal progenitors into distinct various mesenchymal cell lineages including adipocytes (Ahrens et al., 1993).

Of course, there is a common requirement for cytokines or growth factors during hematopoietic differentiation from both primate and murine ES cells. We demonstrated that in primate erythropoiesis, exogenous EPO is required for EryD development, whereas EryP develop independently of EPO, despite substantial expression of EPO receptor. This result is consistent with reports showing that murine primitive and definitive erythrocytes have different requirements for EPO (Wu et al., 1995; Lin et al., 1996). As previously reported in murine ES cells (Heberlein et al., 1992; Keller et al., 1993), the EPOR is expressed in undifferentiated primate ES cells. However, it is unlikely that erythrocytes are contained in undifferentiated ES cells, because no globin gene expression is detected before the induction of differentiation. Further studies will be required to analyze the function of EPOR expressed in undifferentiated ES cells.

Among growth factors examined in this study, VEGF, a ligand for FLK1, was the only one to stimulate both primitive and definitive hematopoiesis. FLK1 is required for the development of primitive and definitive hematopoietic cells, as well as endothelial cells, in the murine embryo (Shalaby et al., 1995; Shalaby et al., 1997). Recent studies on the differentiation of murine ES cells in vitro also indicate that primitive and definitive hematopoietic and endothelial cell lineages can be generated from FLK1-positive cells (Choi et al., 1998; Nishikawa et al., 1998; Faloon et al., 2000). In our study, the expression of FLK1 was upregulated on day 6, before hematopoietic development. This result is consistent with the recent report on vascular progenitor cell differentiation from cynomolgus monkey ES cells onto OP9 stromal cells (Sone et al., 2003). Furthermore, we observed that exogenous VEGF also enhances the development of vascular endothelial cadherin-positive endothelial colonies under the same culture conditions (K.U., T.H. and T.N., unpublished). Taken together, these results strongly suggest that primitive and definitive hematopoietic, as well as endothelial, lineage progenitors are derived from FLK1-positive cells in culture. Further studies, by single cell culture of FLK1-positive cells to differentiate into both lineage cells, will be needed to confirm this possibility.

We also examined indispensable genes associated with hematopoietic development. GATA2 has been reported to be necessary for the proliferation and survival of both primitive and definitive hematopoietic progenitors (Tsai et al., 1994). Its expression in our system supports the proposed role it plays in the generation of hematopoietic progenitors. The expression of Brachyury, an early mesodermal marker (Herrmann et al., 1994), was upregulated on day 4, and was followed by the upregulation of SCL, MYB and LMO2 expression on day 6, before hematopoietic development. SCL (Robb et al., 1996; Porcher et al., 1996) and LMO2 (Warren et al., 1994; Yamada et al., 1998) are required for both primitive and definitive hematopoietic development, whereas MYB is essential for the development of definitive hematopoiesis only (Mucenski et al., 1991). SCL is also crucial for the development of hemangioblasts (Faloon et al., 2000; Chung et al., 2002). These results suggest that a similar profile of genes is involved in hematopoiesis in culture as is involved in early hematopoiesis in vivo. These observations will also facilitate the genetic manipulations of ES cells that may shed light on the unresolved molecular mechanisms behind hematopoietic development.

As sequential RT-PCR analysis of Nestin, AFP and REX1 indicated that other lineage cells and undifferentiated ES cells also grow during the differentiation induction process, we purified the CD34-positive cells in the cultures and seeded them onto fresh OP9 stromal cells. Analyses after cell sorting indicated that enhanced definitive hematopoiesis was generated on day 25 and thereafter, although primitive hematopoiesis was still produced. These results indicate that both hematopoietic processes originate from the sorted CD34-positive population. Further experiments to quantitatively analyze definitive hematopoiesis will be performed using this improved assay.

In conclusion, the sequential development of primitive and definitive hematopoiesis can be induced from primate ES cells by co-culture with OP9 stromal cells. This induction system will provide new approaches for elucidating the mechanisms regulating primate hematopoietic development and differentiation during embryogenesis.

We would like to thank Tanabe Seiyaku Co. Ltd. (Osaka, Japan), for help in primate ES cell preparation, and Ken-ichi Suzuki (Yamanouchi Seiyaku) for providing cynomolgus monkey bone marrow cells. We also thank Drs T. Yasumi, R. Nishikomori and M. Ogawa for critical reading of the manuscript. This study was supported by grants from the Scientific Research on Priority Areas, the Creative Science Research, the Japan Society for the Promotion of Science, and the Ministry of Education, Culture, Sports, Science and Technology.

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Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation

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ABSTRACT **We evaluated the expression of the FLK1, one of the lateral mesoderm early markers where cardiogenesis occurs, to characterize and isolate cardiac stem/progenitor cells from ES cells. Dissociated cells from embryoid bodies (EBs) on day 3, 4, or 5 were collected into two subpopulations with or without FLK1 expression and coculture on OP9 stromal cells was continued to examine whether contracting colonies came out or not. FLK1$^+$ cells from EBs at days 3 and 4 formed spontaneous contracting colonies more efficiently than FLK1$^-$ cells on the same days, but not at day 5. Most contracting cardiac colonies derived from FLK1$^+$ cells mainly on day 4 were detected on endothelial cells along with hematopoietic cells. Further characterization of cells with these capabilities into three lineages revealed the FLK1$^+$ CD31$^-$VE-cadherin$^-$ phenotype. Our findings indicate that FLK1$^+$ cells, especially FLK1$^+$ CD31$^-$VE-cadherin$^-$ cells, could act as cardiohemangioblasts to form cardiac cells as well as endothelial cells and hematopoietic cells.—Iida, M., Heike, T., Yoshimoto, M., Baba, S., Doi, H., Nakahat, T. Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. *FASEB J.* 19, 371–378 (2005)**

Key Words: embryoid bodies · ES cells · cardiomyocyte differentiation · blastocysts

CURRENT THERAPEUTIC MODALITIES to treat end-stage cardiac failure include pharmacological therapy and mechanical left ventricular assist devices, but their applications are limited because they are inadequate. Cardiac transplantation is the ultimate treatment of choice for end-stage cardiac disease but is hampered by the limited availability of donor organs, complications of immunosuppressive therapy, and long-term failure of grafted organs (1–5). Thus, the development of alternative therapeutic strategies to overcome intractable cardiac disease remains an important therapeutic goal.

Embryonic stem (ES) cells are continuously growing stem cell lines of embryonic origin first isolated from the inner cell mass of mouse blastocysts. These unique cells are characterized by their capacity to proliferate in undifferentiated state for a prolonged period in culture and by their ability to differentiate into every tissue type in the body. It is anticipated that ES cells might become feasible sources to repair or regenerate ischemic or damaged tissues, including the heart. Investigation of murine ES cells has reviewed important insights into the early steps of development in the mammalian heart, including patterns of gene expression, myofibrillogenesis, ion channel development and function, receptor development, and calcium handling (6). ES cells can differentiate into various derivatives of three primary germ layers, including cardiomyocytes as well as neuronal cells and hematopieitic cells simultaneously in vitro, leading to the difficulty of isolating tissue-specific cells. This difficulty in preparing pure populations of cardiac lineage has hampered dissection of the mechanism underlying cardiac lineage differentiation. Therefore, development of the privileged characterization system into cardiac lineage is indispensable for further investigation and clinical application.

During differentiation into hematopoietic or endothelial cells from ES cells in vitro, characterization of stem/progenitor cells into these lineages was done using flow cytometer based on cell surface molecules (7–9). FLK1, a receptor for vascular endothelial growth factor, is a marker for lateral plate mesoderm and the earliest differentiation marker for endothelial cells and blood cells (10). Nishikawa et al. have elucidated that FLK1 could be induced during differentiation of ES cells using an EB system or 2-dimensional culture on plates coated with type IV collagen and characterized hemagioblasts, whose derivatives could form blood cells and vessels (9, 11). In a cardiac development system, cell sorting approaches have been done based on the expression of transcription factors. Generally, expression of transcription factor genes precedes that of structural genes. Several transcription factors are expressed in the heart primordium, where they play a pivotal role during heart development by controlling

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doi: 10.1096/fj.04-1998com

the expression of many cardiac muscle-specific genes (12). Among these, Nkx2.5 is known to be expressed most strongly in cardiomyocytes of the heart tube during early cardiac development (13). Hidaka et al. generated an ES cell line in which a GFP reporter gene had been knocked into one of the Nkx2.5 loci and isolated the cells, which differentiated into cardiomyocytes during in vitro culture (14). However, Nkx2.5 expression cannot be evaluated by flow cytometer based on its expression on cell surface; therefore, this methodology encounters difficulties when applying the characterization of cardiac stem/progenitor cells from the ES cells line without a genetical marker insertion under Nkx2.5 gene promoter before use.

The heart originates from the splanchnic mesoderm. During development, two primordia of epithelial cells and precardiac mesoderm are observed at paired regions of anterior lateral mesoderm that eventually fuse in the midline of the body, leading to formation of a tubular heart. Cells from the lateral mesodermal region are known to express a FLK1 gene differentially with the potential to differentiate into hematopoietic and endothelial cells (15, 16). In this paper, we evaluated the differentiation potential of cells with FLK1 expression derived from EB cells and elucidated that these cells could differentiate into cardiomyocytes as well as hematopoietic or endothelial cells.

METHODS

Cell lines

EB5 ES cell line and its derivative, the GACT4 ES cell line, produced by stable transfection of EGFP gene driven by ubiquitous CAG promoter were kindly provided by Dr. Ogawa. The self-renewal activity and differentiation potential could not be distinguished between these lines. The continuous expression of GFP gene during differentiation was confirmed by flow cytometry (data not shown). The cells were maintained on gelatin-coated tissue culture dishes in GMEM containing 10% knockout-SR (Gibco, Grand Island, NY, USA), 1%FCS, 5×10^{-5} M^2 mercaptoethanol (2ME), nonessential amino acids, 5000 units/mL leukemia inhibitory factor, Puromycin, and Brastcidine. The OP9 stromal cell line, kindly provided by Dr. Kodama, was cultured as described previously (17, 18).

Differentiation of ES cells

ES cells were differentiated as described previously (15). Briefly, single-cell dispersions of undifferentiated ES cells were suspended in α-MEM supplemented with 10% FCS and 5×10^{-5} M 2ME at a concentration of 2×10^4 cells on bacteriological grade 100 mm Petri dishes. On days 3–5 after differentiation, formed EBs were subjected to additional experiments.

FACS analysis and in vitro cultures of sorted cell populations

ES cells were harvested with a cell dissociation buffer (Gibco), followed by dissociation. Dissociated single cells were stained with phycoerythrin (PE) -conjugated anti-FLK1 antibody (PharMingen, San Diego, CA, USA) and PECAM-1 (anti-CD31) (PharMingen). Monoclonal antibodies AVAS12 (anti-FLK1) (8) and VECD (anti-VE-cadherin) (19) were purified from hybridoma cultured supernatants on a protein G-Sepharose column (Pharmacia, Piscataway, NJ, USA) and labeled with allophycocyanin. These antibodies had not influenced the differentiation potential of ES cells (7–9, 11). Sorted FLK1 positive and negative cells were cocultured with OP9 stromal cells at a cell density of 2×10^3/well in 24-well plate culture dishes. FACS sorting systems in this study did not damage ES cell differentiation as in previous reports (9, 11). These experiments were repeated 10 times and quantified the differentiation capacity.

Immunohistochemical staining

For immunological staining of the contracting cardiac colonies derived from $FLK1^+$ and $FLK1^-$ cells on OP9 stromal cells, cultured cells were fixed in situ by 4% paraformaldehyde in phosphate1-buffered saline (for 10 PBS) min at 4°C. After washing with PBS, 2% skim milk in PBS was incubated as a blocking solution for 1 h at room temperature. The fixed dishes were incubated with MF20, monoclonal antibody against myosin heavy chain (Hybridoma bank) and anti-CD45 antibody (Becton Dickinson, Franklin Lakes, NJ, USA) overnight at 4°C, followed by incubation with alkaline phosphatase (ALP)-conjugated antimouse IgG (H^+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. After each step, the cultured cells were washed three times with PBS containing 0.05% Tween 20 (Wako Chemical, Kyoto, Japan). Cells were visualized using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate (Roche Molecular Biochemicals, Basel, Switzerland). Endogenous ALP activity was blocked by 2 mmol/L levamisole (Sigma, St. Louis, MO, USA) before incubation with MF20. Anti-CD45 antibody was peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories Inc.) and visualized using DAB Substrate Kit (Vector Laboratories, Inc., Burlingame, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from $FLK1^+$ and $FLK1^-$ cells dissociated before and after coculture with OP9 cells by acid-phenol extraction using Trizol Reagent (Gibco). Reverse transcription was performed with 1 μg of total RNA treated with RNase-free DNaseI (Gibco) as a template and oligo (dT) as a primer. The cDNA was amplified by PCR using the following primers: brachyury-specific primers 5′-TGCTGCCTGTGAGTCATAAC-3′ and 5′-TCCAGGTGCTATATATTGCC-3′, which should give a 947 bp product; goosecoid-specific primers 5′-GCACCATCTTCACCGATGAG-3′ and 5′-AGGAGGATCGCTTCTGTCGT-3′, which should give a 179 bp product; Nkx-2.5-specific primers 5′-GTGGGTCTCAATGCCTATG-3′ and 5′-CTCTTTCCCTACCAGGCTC-3′, which should give a 233 bp product. PCR conditions for brachyury and goosecoid were performed by denaturing the DNA at 94°C for 5 min, followed by 30 cycles of amplification: 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and a final extension step at 72°C for 7 min. For Nkx2.5, PCR reactions were performed by denaturing the DNA at 94°C for 5 min, followed by 30 cycles of amplification: 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and a final extension step at 72°C for 7 min. To normalize the amount of template, 18S rRNA was used as an internal

control according to the manufacturer's instruction (QuantumRNA Internal Standards Kit, Ambion, Austin, TX, USA). Amplified fragments were separated on a 2.0% agarose gel and visualized with ethidium bromide staining.

RESULTS

Differential progression into contracting cardiomyocytes between FLK1$^+$ and FLK1$^-$ cells from EBs

ES cells do not express FLK1 gene in an undifferentiated state. However, differentiation in EB culture induces various kinds of differentiated markers, including FLK1. We first evaluated the temporal switch of FLK1 gene expression during EB culture on days 3–5 sequentially. As demonstrated in Fig. 1*A*, FLK1$^+$ cells could be detected during this culture although barely detected in undifferentiated state at day 0. Day 4 was when the highest percentage of FLK1$^+$ cells could be detected. FLK1$^+$ cells or FLK1$^-$ cells generated from EBs on days 3–5 were sorted, respectively, followed by coculture on OP9 stromal cells (Fig. 1*B*). Expression of brachyury gene could be detected in FLK1$^+$ cells but not in FLK1$^-$ cells (data not shown). FLK1$^+$ cells and FLK1$^-$ cells generated from EBs on day 3 differentiated into contracting cardiac colonies efficiently after a further 8-day culture on OP9 cells (day 3+7), though differentiation from FLK1$^+$ cells preceded by 1 day that of FLK1$^-$ cells. Mouse fibroblast cell line 3T3 could not support this differentiation at all (data not shown). Similarly, both populations from EBs on day 4 differentiated into contracting cardiac colonies; differentiation from FLK1$^+$ cells preceded by 1 day that of FLK1$^-$ cells. In contrast, FLK1$^+$ cells and FLK1$^-$ cells from EBs on day 5 failed to form contracting cardiac colonies. This experiment was repeated 10 times with the same tendency, describing a representative result. These results imply that both populations of FLK1$^+$ and FLK1$^-$ cells on day 3 or 4 had the capability of differentiation into contracting cardiac cells, with a higher tendency in FLK1$^+$ cells.

Cardiac muscle-specific gene expression during differentiation into cardiomyocyte

We examined the expression of cardiac muscle-specific or mesoderm-specific gene during differentiation. RT-PCR analysis was performed to determine the expression of cardiac muscle-specific gene Nkx2.5 (20, 21) and expression of early mesoderm markers goosecoid and brachyury (22, 23) (Fig. 1*B*). It was confirmed that OP9 cells did not express early mesoderm markers (goosecoid and brachyury) (data not shown). Cardiac muscle-specific Nkx2.5 gene was already detected in FLK1$^+$ cells from dissociated EBs on days 3–5, with the highest expression on day 4. In FLK1$^-$ cells, Nkx2.5 gene expression could be faintly or barely confirmed. In contrast, the highest expression of the early mesoderm markers goosecoid and brachyury was confirmed in both populations from EBs at day 3, but decreased gradually on days 4 and 5. The results imply that each population of cells from EBs on days 3–5 has different properties as to developmental stage, leading to the distinct differentiation capability.

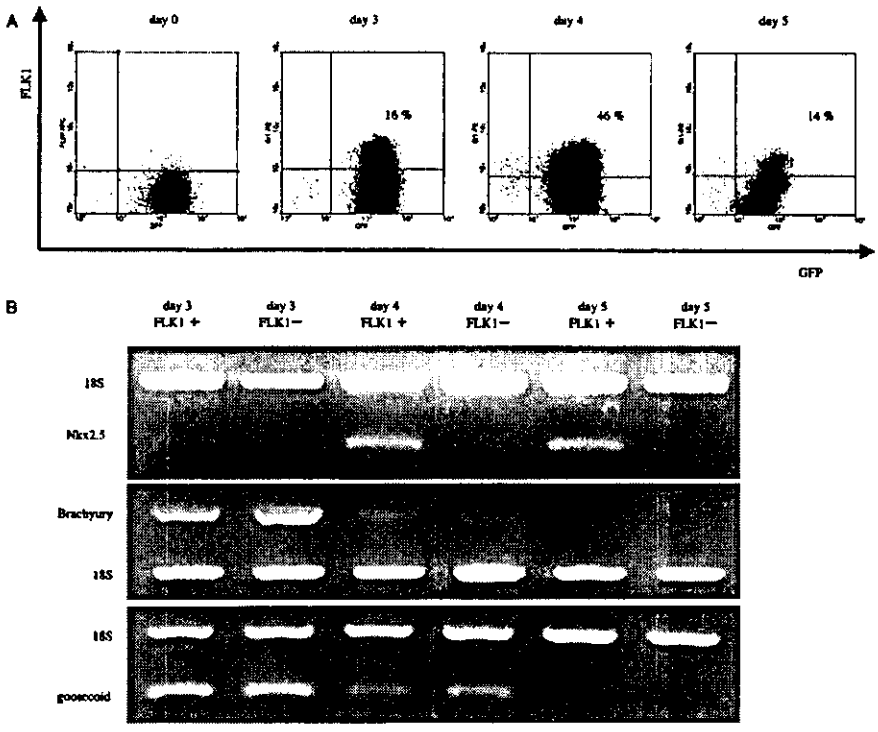

Figure 1. *A)* ES cells were differentiated as described (17). On days 3–5 after differentiation, EBs formed were stained with phycoerythrin (PE)-conjugated anti-FLK1 antibody. Stained cells were sorted using a FACS Vantage cell sorter. Sorted FLK1 positive and negative cells were cocultured with OP9 stromal cells at a cell density of 2×10^5/well in 24-well plate culture dishes. Contracting cardiac colonies after 7-day culture on OP9 cells (day 3+7, day 4+7, and day 5+7) differentiated from FLK1$^+$ cells and FLK1$^-$ cells generated from EBs on days 3–5. *B)* Total RNA was isolated from FLK1$^+$ cells and FLK1$^-$ cells were generated from EBs on day 3, 4, and 5. By acid-phenol extraction using Trizol Reagent (Gibco), reverse transcription was performed with 1 μg of total RNA treated with RNase-free DNaseI (Gibco) as a template and oligo (dT) as a primer. PCR amplification was performed by RT-PCR analysis for cardiac muscle-specific gene and early mesoderm markers after sorting. Amplified fragments were separated on a 2.0% agarose gel and visualized with ethidium bromide staining.

Simultaneous generation of endothelial cells and hematopoietic cells during Flk1$^+$ cell differentiation into cardiomyocytes

FLK1$^+$ cells or FLK1$^-$ cells generated from EBs on days 3 and 4 differentiated into contracting cardiac colonies after coculture on OP9 cells. During differentiation of FLK1$^+$ cells from EBs at day 4, endothelial sheet formations were observed along with contracting cardiac colonies for the most part, some of which were accompanied with blood cells simultaneously (**Fig. 2A-b, e, *B***). These observations were not identified in FLK1$^-$ cells from day 4. On day 3, a part of contracting colonies derived from FLK1$^+$ population was accompanied with endothelial cells and hematopoietic cells but not from the FLK1$^-$ population (Fig. 2*A*-a, d). FLK1$^+$ cells and FLK1$^-$ cells from EBs on day 5 did not differentiate into contracting cardiac colonies, although FLK1$^+$ cells preserved the capability to differentiate into cobble stone-like hematopoetic clusters and endothelial sheets, which were not identified in FLK1$^-$ cultures (Fig. 2*A*-c, f). These observations imply that FLK1$^+$ cells or FLK1$^-$ cells had different differentiation potential depending on the harvesting date during EB culture, and only FLK1$^+$ cells exhibited the phenotype of cardiohemangioblasts.

Conversion of FLK1$^-$ cells into FLK1$^+$ cells during coculture on OP9 cells

Although FLK1$^-$ cells from EBs on day 4 did not express the Nkx2.5 gene, they could differentiate into contracting cardiac muscle cells with a 1-day delay compared with FLK1$^+$ cells. Nkx2.5 gene is a crucial transcription factor for heart development, so Nkx2.5 gene expression was evaluated during the differentiation into contracting cardiac cells of these FLK1$^-$ cells sequentially (**Fig. 3**). As shown in Fig. 3, expression of the Nkx2.5 gene was confirmed in a 1-day delayed manner in FLK1$^-$ cells vs. FLK1$^+$ cells, which corresponded to the delay of phenotypical change. To investigate the role of FLK1 in this system further, we examined the phenotypical conversion of FLK1$^-$ cells into FLK1$^+$ cells during coculture on OP9 cells using ES cells with the GFP gene driven under CAG promoter. After 1-day coculture of FLK1$^-$ cells from EBs at day 4, cells were harvested according to GFP expression and resorted according to the FLK1 expression into two populations with or without FLK1 expression. Surprisingly, nearly 0.1% of FLK1$^-$ cells changed their phenotype into FLK1$^+$ cells. These two populations continued to be cultured on OP9 cells as described above (**Fig. 4*A***). Resorted FLK1$^+$ cells formed contracting colonies more efficiently than resorted FLK1$^-$ cells (Fig. 4*B*). In addition, contracting colonies derived from resorted

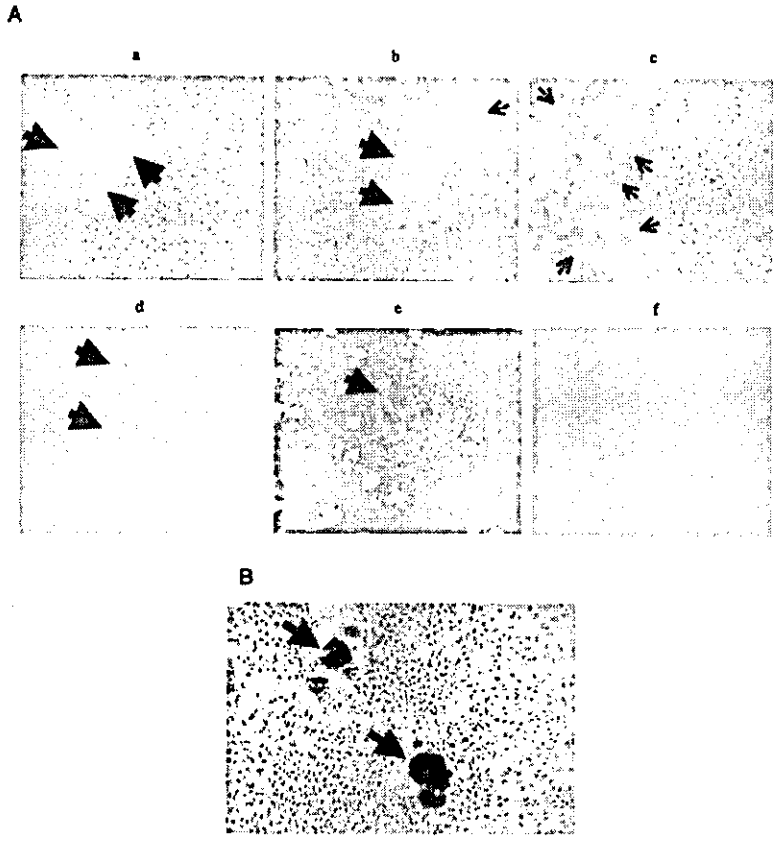

Figure 2. *A)* Differentiation of FLK1$^+$ cells from EBs on days 3 (a), 4 (b), and 5 (c) at day 7 after sorting,.respectively; day 3+7, day 4+7, and day 5+7 and FLK1$^-$ cells from EBs on day 3 (d), 4 (e), and 5 (f). FLK1$^+$ and FLK1$^-$ cells from EBs on days 3 and 4 were differentiated into contracting cardiac colonies (arrows) (a, b, d, e). Asterisks indicate endothelial cells under contracting cardiac colony-derived FLK1$^+$ cells. *B)* Expression of sarcomic myosin on day 4+7. Contracting cardiac colonies (arrows) derived from FLK1$^+$ cells from EBs on day 4 were stained with antibodies to sarcomic myosin (MF20) on OP9 cells.

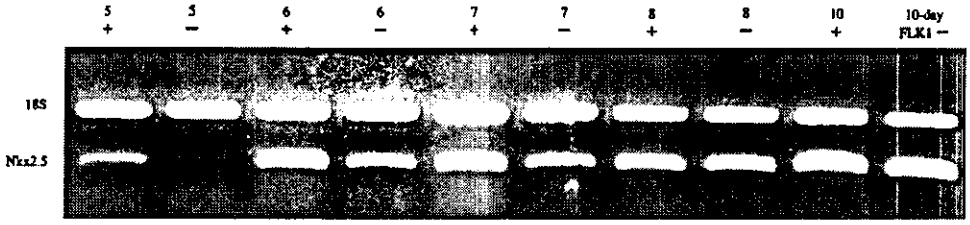

Figure 3. RT-PCR analysis for cardiac muscle-specific gene on sorted FLK1$^+$ and FLK1$^-$ cells generated from EBs on day 4. Total RNA was isolated from FLK1$^+$ and FLK1$^-$ cells dissociated after coculture with OP9 cells by acid-phenol extraction using Trizol Reagent (Gibco). Reverse transcription was performed with 1 μg of total RNA treated with RNase-free DNaseI (Gibco) as a template and with oligo (dT) as a primer, then PCR amplification was performed

FLK1$^+$ cells began to be detected 1 or 2 days earlier than FLK1$^-$ cells. These results suggest that FLK1$^-$ cells might differentiate into contracting cardiac cells after converting into FLK1$^+$ cells predominantly.

Further characterization of cardiac stem/progenitor cells in FLK1$^+$ cells

We further classified FLK1$^+$ cells from EBs on day 3, 4, or 5 according to the expression of endothelial markers

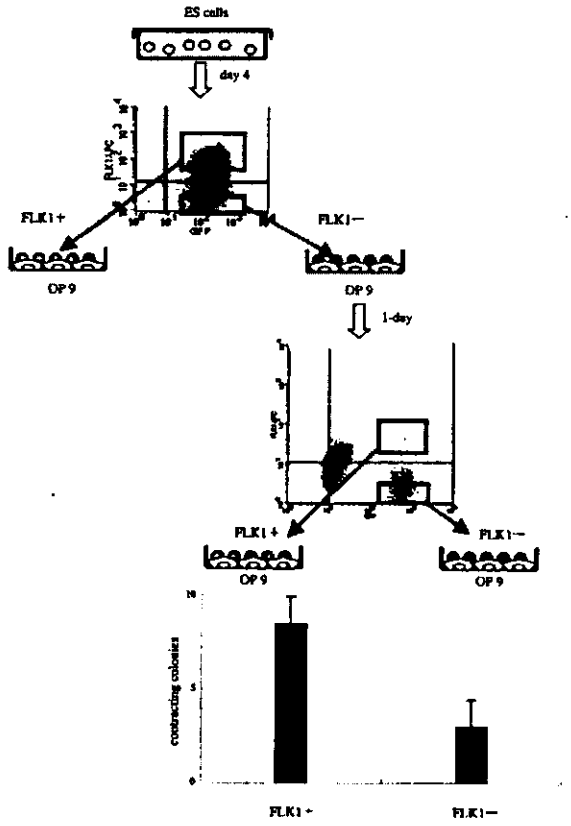

Figure 4. *A)* Schema of our FACS sorting system for cardiac differentiation. We sorted FLK1$^+$ cells and FLK1$^-$ cells from EBs on day 4, recultured 2000 cells/well of 24-well OP9 stromal cell-coated plates, resorted FLK1$^-$ cells on day 4+1, and recultured them on OP9 stromal cells in the same way. *B)* Resorted FLK1$^+$ cells and FLK1$^-$ cells were differentiated into contracting cardiac colonies.

CD31 and VE-cadherin and sorted them into the following four populations: cells expressing both FLK1 and CD31 (F$^+$31$^+$), cells expressing FLK1 without expression of CD31 (F$^+$31$^-$), cells expressing both FLK1 and VE-cadherin (F$^+$V$^+$), and cells expressing FLK1 without expression of VE-cadherin (F$^+$V$^-$), respectively (**Fig. 5*A***). As shown in Fig. 5*B*, F$^+$31$^-$ or F$^+$V$^-$ cells from EBs on days 3 and 4 differentiated predominantly into contracting cardiac cells. Another experiment of single-cell culture with F$^+$31$^-$V$^-$ characteristics demonstrated the differentiation into three lineages of cardiac cells as well as endothelial and hematopoietic cells, confirmed by immunohistochemical analysis (**Fig. 6*A–C***). These results support the notion that cardiac progenitor cells can be identified as F$^+$31$^-$V$^-$ cells.

DISCUSSION

In this paper, we have characterized the cardiac stem/progenitor cells from ES cells using the FACS sorting systems. Cardiac cell differentiation from ES cells could be easily induced in culture in vitro, and many extensive studies have been done from the viewpoint of a functional or physiological standpoint up to now (24). However, this conventional differentiation system comprises various kinds of differentiated tissues other than cardiac tissue; therefore, precise evaluation of cardiomyocyte differentiation has been prevented. In this study, we evaluated the expression of the FLK1 gene, an early marker of lateral mesoderm where cardiogenesis occurs (25), to characterize and isolate cardiac stem/progenitor cells from ES cell. We demonstrated that FLK1, especially FLK1$^+$CD31$^-$VE-cadherin$^-$, was a feasible marker for detecting cardiac stem/progenitor cells.

Several transcription factors are expressed in the heart primordium, where they play a pivotal role during heart development by controlling the expression of many cardiac muscle-specific genes (12). Among these, Nkx2.5 is known to be expressed most strongly in cardiomyocytes of the heart tube during early cardiac development (13) and is recognized as an early marker for cardiac cell differentiation (20, 21). In our experiments, Nkx2.5 gene expression was always detected before that of contracting characteristics, implying that

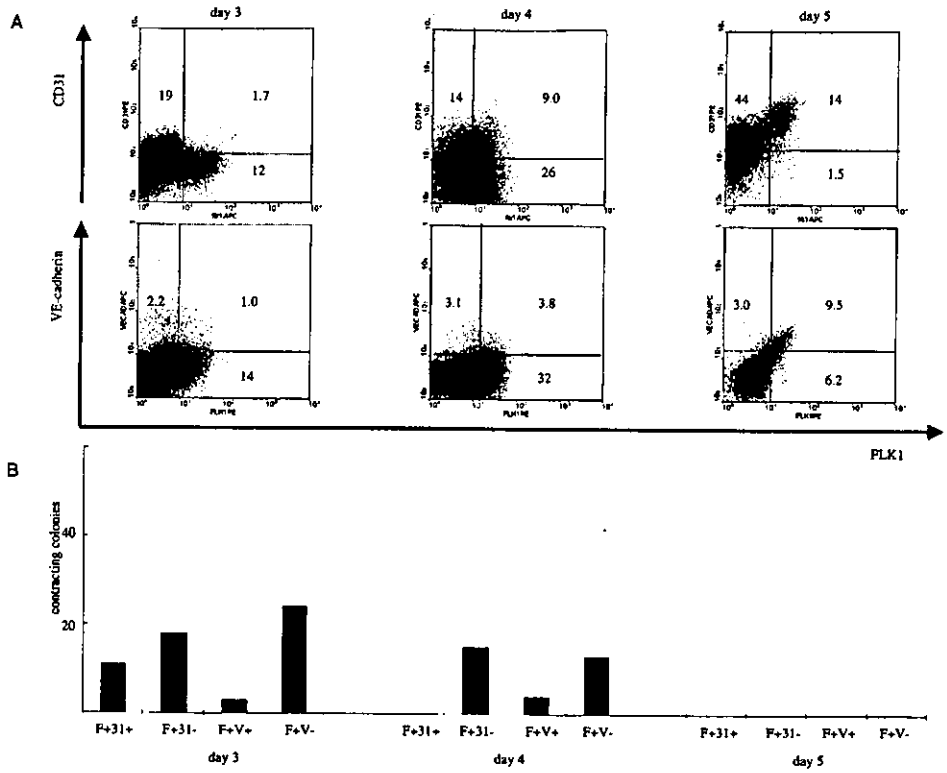

Figure 5. *A)* Dissociated single cells were stained with PE-conjugated anti-FLK1 antibody (PharMingen) and PECAM-1 (anti-CD31) (PharMingen). FACS analyses of FLK1, CD31, and VE-cadherin expression during EB development. *B)* EBs on days 3–5 were sorted for FLK1$^+$CD31$^+$, FLK1$^+$CD31$^-$, FLK1$^+$VE-cadherin$^+$, and FLK1$^+$VE-cadherin$^-$ cells. The contracting colonies were counted on days 3+7, 4+7, and 5+7.

the commitment to cardiac differentiation could be concluded by the acquisition of Nkx2.5 gene expression. Cells with Nkx2.5 gene expression were recognized cardiac stem/progenitor cells. Our PCR experiments suggested that close correlation between expression of the Nkx2.5 gene and that of FLK1 was confirmed, implying that FLK1 expression might become a feasible marker for detecting cardiac stem/progenitor cells. This interpretation could also be supported by the observation that FLK1$^-$ cells differentiated into contracting muscle cells 1 or 2 days later

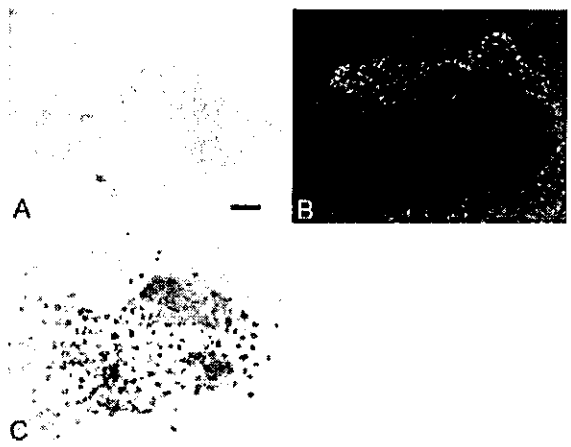

Figure 6. *A)* FLK1$^+$CD31$^-$VE-cadherin$^-$ single cells were cocultured on 96-well OP9 stromal cells-coated plates. *B)* Expression of the endothelial cells were characterized using acetylated low density lipoprotein (DiI-Ac-LDL) (Molecular Probes). *C)* Single-cell culture stained with antibodies to sarcomic myosin (blue) and CD45 (PharMingen) (brown).

than FLK1$^+$ cells, synchronous with Nkx2.5 gene expression. In contrast, FLK1$^+$ cells from EBs on day 5 could not differentiate into contracting cardiac muscle cells, implying FLK1 itself is not the sole decisive marker for cardiac stem/progenitor cells and that cells with FLK1 expression are composed of heterogeneous populations from the viewpoint of development. This discrepancy might be explained by the difference in expression patterns of the brachyury or goosecoid gene. FLK1$^+$ cells from EBs on day 3 or 4 expressed brachyury, or goosecoid gene, but not on day 5, reflecting the immaturity of FLK1$^+$ cells from EBs on day 3 or 4. These fluctuated expressions of Nkx2.5, brachyury, and goosecoid genes during these 3 days suggested that FLK1$^+$ cells might be different qualitatively during this period. Taken together, FLK1$^+$ cells from EBs on day 3 or 4 might be regarded as cardiac stem/progenitor cells. It suggested that FLK1$^+$ cells had quite different characters during differentiation on days 3, 4, and 5; however, until now FLK1 expression was attained only during mesodermal differentiation. Furthermore, these FLK1 characters showed that endothelial and hematopoietic cells had not enhanced development of the cardiomyocyte.

Using the in vitro ES differentiation model system, G. Keller et al. had shown that ES cells differentiated for 2.5–3.5 days contained a unique cell population, the blast colony-forming cell (BL-CFC) (26, 27). BL-CFCs form blast colonies in response to vascular endothelial growth factor (VEGF), a ligand for the receptor tyrosine kinase FLK1 (28), suggesting that these cells express FLK1 gene. These observations are consistent with the interpretation that a fraction of the FLK1$^+$

cells would represent the hemangioblasts (29). Our findings demonstrated that contracting cardiac colonies derived from FLK1$^+$ cells at day 4 appeared on endothelial sheets, sometimes with blood cells, supporting the idea that FLK1$^+$ cells on day 4 contained three populations: cardiac progenitor cells, endothelial progenitor cells, and hematopoietic progenitor cells. We analyzed, sorted, and recultured FLK1$^+$ cells on day 3, 4, or 5 under further classification using VE-cadherin or CD31. Our studies demonstrated that more FLK1$^+$CD31$^-$ or FLK1$^+$ VE-cadherin$^-$ cells on day 4 differentiated into contracting colonies than FLK1$^+$CD31$^+$ cells and FLK1$^+$VE-cadherin$^+$ cells, which predominantly differentiated into endothelial cells, hematopoitic cells. However, these observations could not clarify whether only one kind of single cardiohemangioblasts exists with differential capability into three lineages or whether this population is still heterogeneous, with differential potential only into one or two lineages. Additional experiments are needed to resolve this question.

In our experiments, FLK1$^-$ cells from EBs on day 3 or 4 could differentiate into contracting cardiac cells. This contradicted our idea that FLK1 could be a good marker to isolate cardiac stem/progenitor cells from ES cells. However, precise examination of FLK1 expression on FLK1$^-$ cells 1 day after plating on OP9 cells revealed that conversion of the FLK1 gene in nearly one-third of FLK1$^-$ cells of cells could be observed from negative to positive. Replating of FLK1$^+$ cells or FLK1$^-$ cells on OP9 cells (derived from FLK1$^-$ cells at day 4) revealed that FLK1$^+$ cells differentiated into contracting cardiac muscle cells predominantly. On the other hand, when we plated sorted cells on fibroblast cells, gelatin-coated plates, and collagen type IV-coated plates, the contracting cardiac cells either did not show or were only a few. These results imply that FLK1$^-$ cells with the ability to differentiate into cardiac cells change their phenotype from negative to positive before differentiating into contracting cardiac cells predominantly, leaving the possibility that FLK1$^-$ cells could still differentiate into cardiac cells directly.

In conclusion, the findings in this study demonstrate that FLK1 plays a significant role not only in hematopoietic and endothelial differentiation, but also in cardiac differentiation, and that cardiohemangioblasts populations contribute somewhat to the formation of contracting cardiac muscle cells. The development of isolation and culture conditions described here in which ES cells depend on cardiohemangioblasts to initiate cardiac differentiation will greatly facilitate further analysis of cardiac stem/progenitor cell specification and cell therapy. FJ

This study was supported by a Health and Labor Science Research Grant, Research on Human Genome, Tissue Engineering Food Biotechnology, Ministry of Health, Labor and Welfare, a Grant-in Aid for Science Research on Priority Areas 122150-67, and a Grant-in-Aid for Creative Scientific Research 13GS-0009.

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Received for publication May 3, 2004.
Accepted for publication November 1, 2004.

Differential Requirement for the CD40-CD154 Costimulatory Pathway during Th Cell Priming by CD8α^+ and CD8α^- Murine Dendritic Cell Subsets[1]

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Dendritic cells (DCs) regulate the development of distinct Th populations and thereby provoke appropriate immune responses to various kinds of Ags. In the present work, we investigated the role CD40-CD154 interactions play during the process of Th cell priming by CD8α^+ and CD8α^- murine DC subsets, which have been reported to differently regulate the Th response. Adoptive transfer of Ag-pulsed CD8α^+ DCs induced a Th1 response and the production of IgG2a Abs, whereas transfer of CD8α^- DCs induced Th2 cells and IgE Abs in vivo. Induction of distinct Th populations by each DC subset was also confirmed in vitro. Although interruption of CD80/CD86-CD28 interactions inhibited Th cell priming by both DC subsets, disruption of CD40-CD154 interactions only inhibited the induction of the Th1 response by CD8α^+ DCs in vivo. CD40-CD154 interactions were not required for the proliferation of Ag-specific naive Th cells stimulated by either DC subset, but were indispensable in the production of IL-12 from CD8α^+ DCs and their induction of Th1 cells in vitro. Taken together, in our immunization model of Ag-pulsed DC transfer, CD40-CD154 interactions play an important role in the development of CD8α^+ DC-driven Th1 responses but not CD8α^- DC-driven Th2 responses to protein Ags. ***The Journal of Immunology,*** **2004, 172: 4826–4833.**

Dendritic cells (DCs)[3] are specialized APCs that activate naive T lymphocytes and initiate adaptive immunity (1, 2). In response to Ag presentation by DCs, naive Th cells differentiate into one of several functional subsets that differ in their cytokine secretion patterns and effector functions. Th1 cells secrete IFN-γ and promote cellular immunity by activating CTLs, NK cells, and macrophages. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, which induce Ig class switching to IgE and promote eosinophil-predominant inflammation. There are likely to be other Th subsets, such as Th0 and T regulatory cells, which produce various combinations of cytokines. The balance between these Th subsets determines the nature, strength, and duration of immune responses (3, 4).

The current understanding is that a series of defined signals must be delivered to naive Th cells to initiate effective immune responses. Along with the presentation of antigenic peptides to TCRs, appropriate costimulation must be provided for the activation and proliferation of naive Th cells. Furthermore, a large body of evidence suggests that DCs provide additional signals that instruct the functional differentiation of Th cells. These Th cell differentiation-regulating signals involve many factors, most of which are related to DCs (5, 6).

Two distinct DC subsets in the murine spleen have been characterized with regard to the expression of the CD8 α-chain (7, 8). In vitro studies suggest that CD8α^+ DCs induce a limited T cell response and may play a role in the regulation of immune responses, whereas CD8α^- DCs may be more stimulatory (9–11). In vivo studies have shown that injection of CD8α^+ DCs triggers the development of Th1 cells whereas injection of CD8α^- DCs induces a Th2-type response to soluble Ags (12, 13). It was later shown that neither CD8α^+ nor CD8α^- DCs can induce optimal T cell responses in their immature state and that their maturation is a prerequisite for both subsets to become potent activators of naive T cells (14). Moreover, recent studies revealed that CD8α^+ DCs are responsible for maintaining peripheral tolerance under steady-state conditions (15, 16) but administration of an agonistic anti-CD40 Ab induces them to provoke a CTL response to tissue-associated Ags (16, 17). These studies clearly illustrate that the functional properties of each DC subset are not immutably fixed and that factors such as the maturation state of the DCs and the costimulatory molecules that they express contribute to the regulation of T cell priming.

Of the multiple costimulatory pathways that have been identified, CD40-CD154 interaction is thought to play a pivotal role in the process of T cell priming by DCs (18–20). CD154 is a member of the TNF family and is expressed primarily on activated Th cells. CD40, the receptor for CD154, is expressed on APCs such as B cells, macrophages, and DCs. CD40 ligation induces the maturation and activation of DCs (21) and promotes their secretion of cytokines such as IL-12 (22, 23), which is essential for the development of the Th1 response (24–27). In addition, exposure of DCs

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Received for publication February 18, 2003. Accepted for publication February 9, 2004.

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[1] This work was supported by Grant-in-Aid for Creative Scientific Research 13GS0009 and by the Ministry of Education, Science, Technology, Sports and Culture of Japan Grant 12215067, as well as by the Program for Research on Pharmaceutical and Medical Safety and the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research in Japan.

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[3] Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; hu-IgG, human IgG; ham-IgG, hamster IgG.

0022-1767/04/$02.00