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

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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

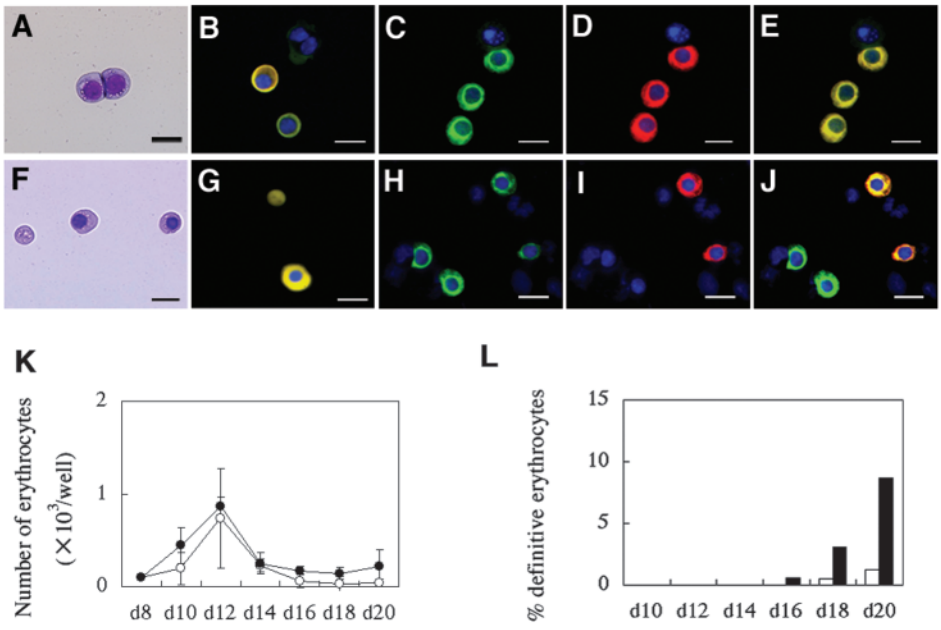

Fig. 1. Development of primitive and definitive erythrocytes. Primate ES cells (4×10^3 cells/well) were cultured onto OP9 stromal cells for 6 days. The induced cells were harvested and re-cultured at a concentration of 1×10^5 cells per well onto fresh OP9 stromal cells, with or without 10 U/ml EPO. The 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.

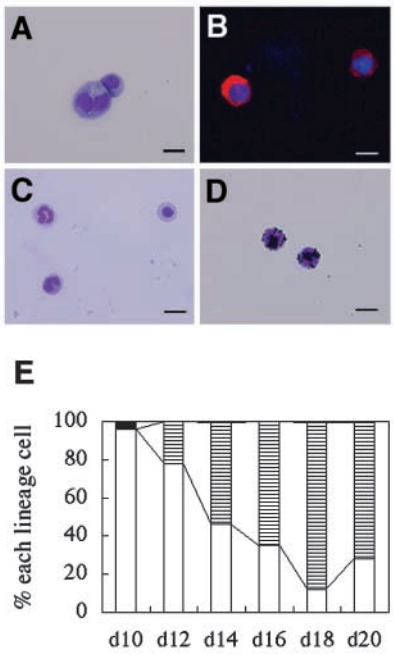

Fig. 2. Development of megakaryocytes and myeloid cells. (A,B) May-Giemsa staining (A, left cell) and CD41 (Cy3) staining (B) of megakaryocytes on day 12. Nuclei were labeled with Hoechst 33342 in B. (C,D) May-Giemsa staining (C) and myeloperoxidase staining (D) of myeloid cells on day 18. Scale bars: 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

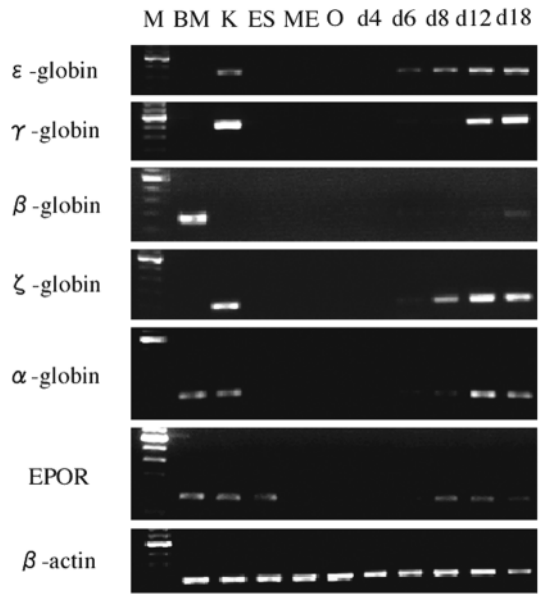

Fig. 3. Sequential RT-PCR analysis of embryonic (ε and ζ), fetal (γ) and adult (β and α) globin expression in erythrocytes during liquid culture differentiation. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; ES, undifferentiated ES cells; ME, mouse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

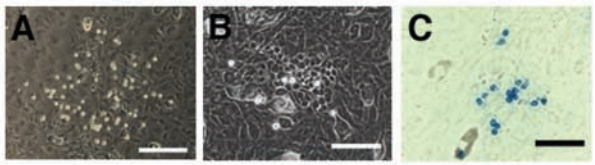

Fig. 4. Development of adherent hematopoietic cell clusters. (A,B) An adherent hematopoietic cell cluster (A) and a cluster with a cobblestone appearance that grew underneath the OP9 stromal cells (B). (C) Alkaline phosphatase staining of adherent hematopoietic cell clusters immunolabeled with human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

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

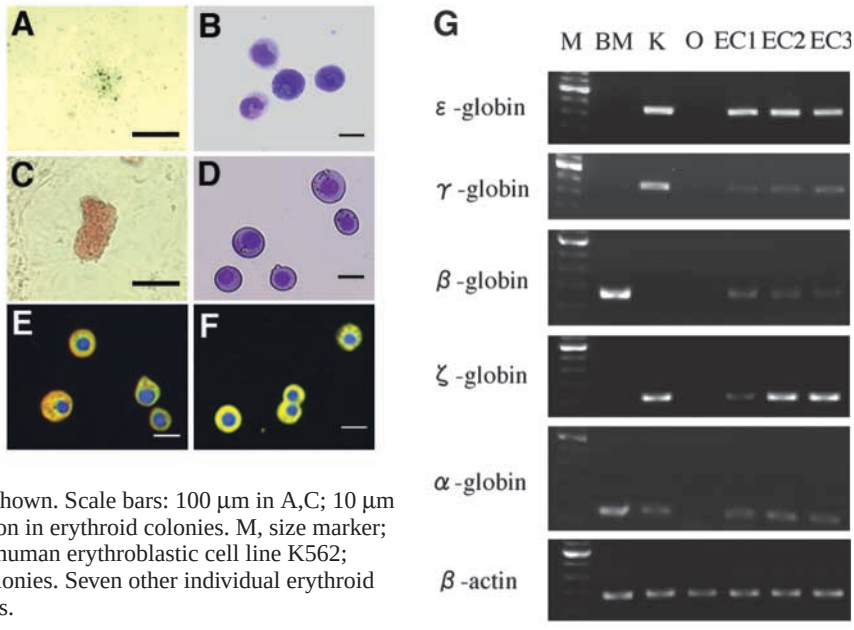

Fig. 5. Development of hematopoietic colonies. Methylcellulose colony-forming assays were performed on day 8. Using adherent cells dissociated with trypsin treatment, only a few GM colonies were observed (A,B). When the medium was replaced with methylcellulose-containing medium, erythroid colonies were 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.

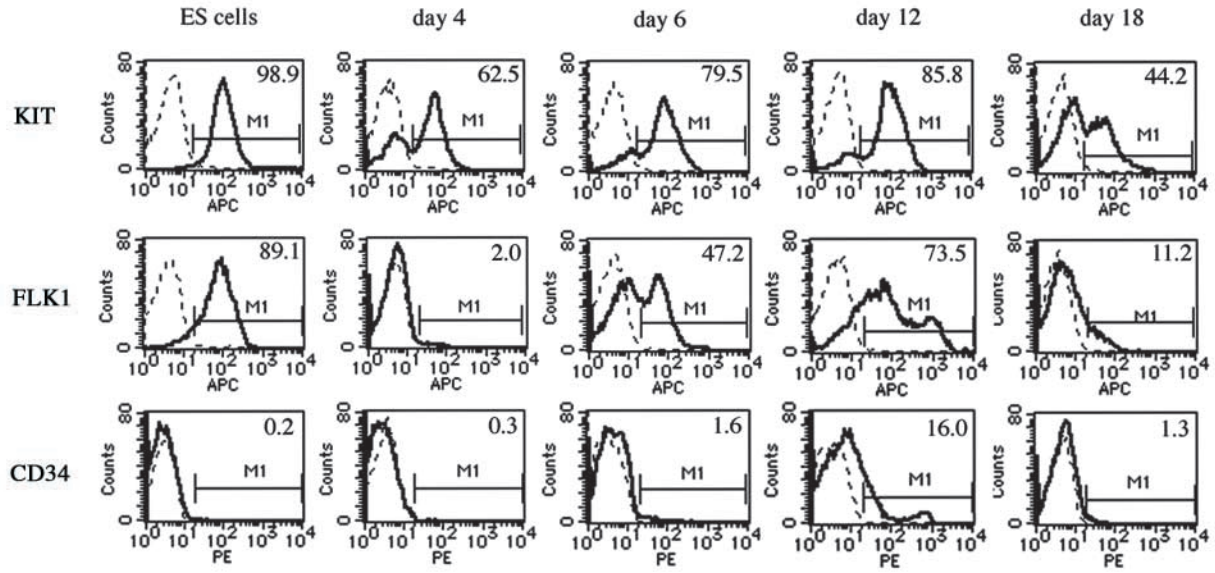

Fig. 6. Sequential flow cytometric analysis of KIT, FLK1 and CD34 during differentiation induction. Undifferentiated ES cells, or the cultures on day 4, 6, 12 and 18, were stained with antibodies specific for KIT, FLK1 or CD34, or with control IgG1. Plots show the isotype control IgG1 staining profile (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).

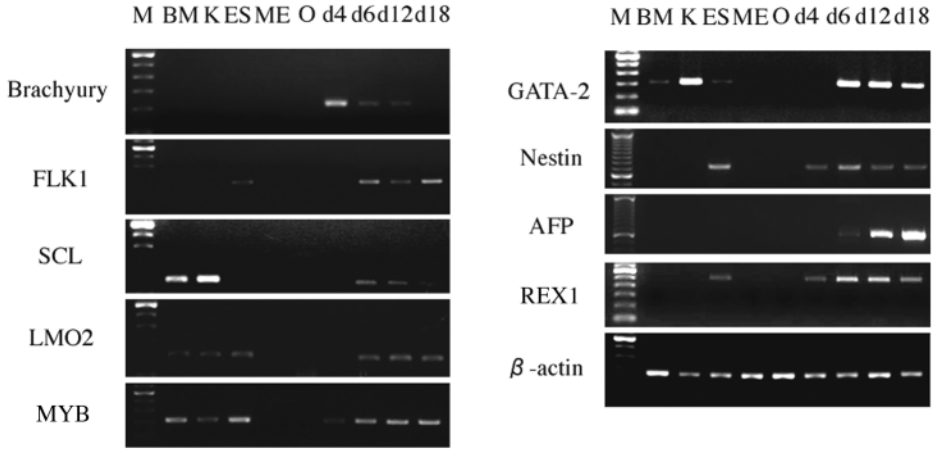

Fig. 7. Sequential RT-PCR analysis of genes associated with hematopoietic development in cultures during liquid culture differentiation. M, size marker; BM, adult cynomolgus monkey bone marrow cells; K, human erythroblastic cell line K562; ES, undifferentiated ES cells; ME, mouse embryonic fibroblasts;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

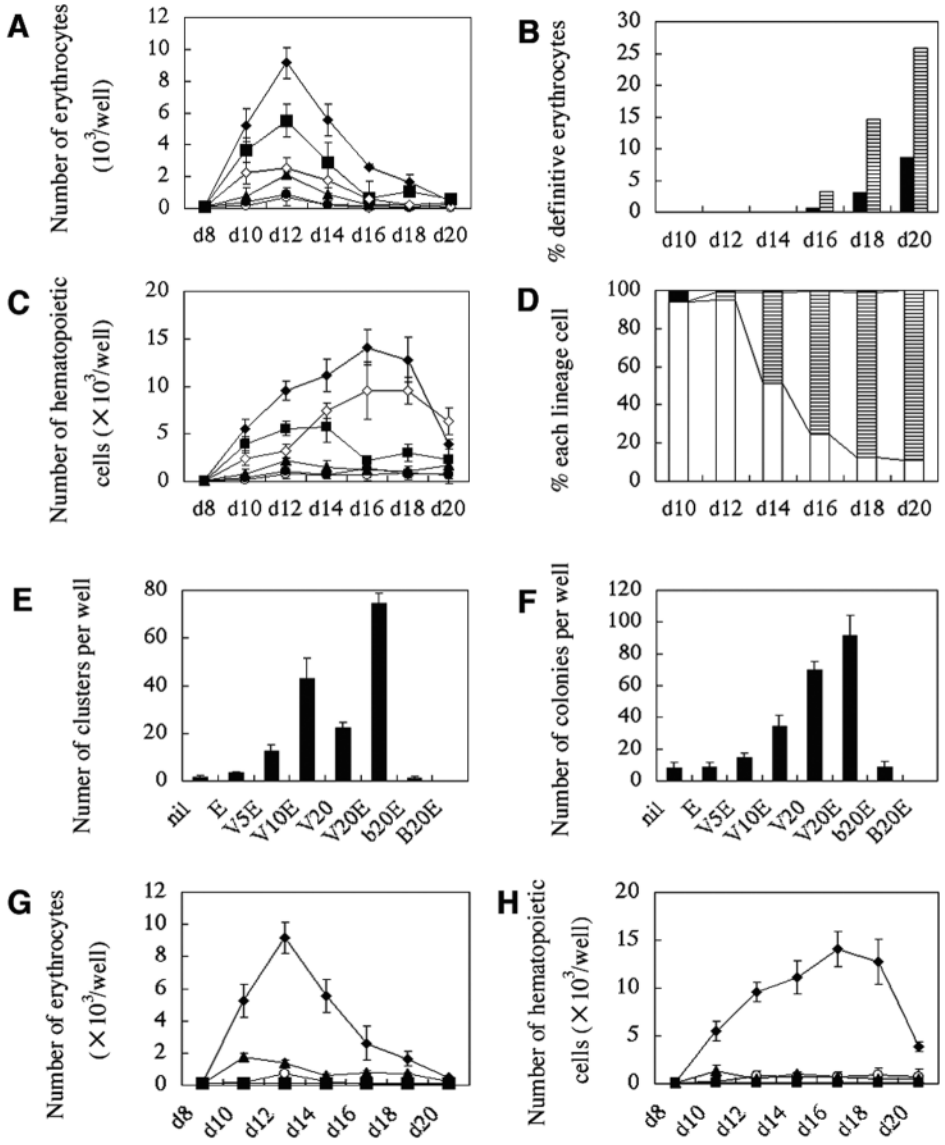

Fig. 8. Effects of EPO and various growth factors on primitive and definitive hematopoiesis. Primate ES cells were cultured for 6 days with VEGF, bFGF or BMP4. The induced cells were harvested and re-cultured with (black circles) or without (white circles) 10 U/ml EPO. The floating cells were analyzed 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

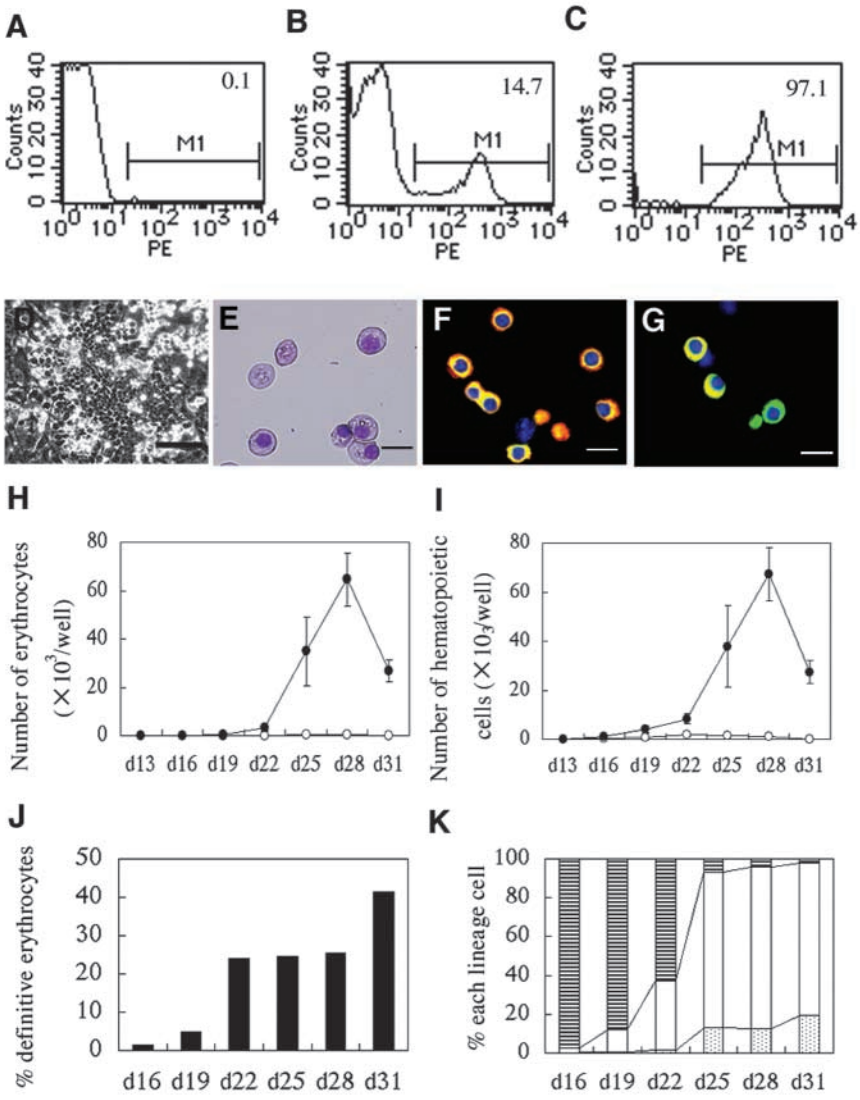

Fig. 9. More efficient primitive and definitive hematopoiesis is induced by re-plating sorted CD34-positive cells. (A-C) Flow cytometric analysis and cell sorting of cultures on day 10 with human CD34 antibody. A sorting gate in B was defined according to the intensity of staining with an isotype control 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.

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