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Introduction of Human Erythropoietin Receptor Complementary DNA by Retrovirus-Mediated Gene Transfer Into Murine Embryonic Stem Cells Enhances Erythropoiesis in Developing Embryoid Bodies

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ABSTRACT

To evaluate the role of the erythropoietin (Epo) receptor (R) in erythropoiesis in more primitive stem cells, we assessed the influence of retrovirus-mediated gene transfer of human (h) *EpoR* complementary DNA (cDNA) into murine embryonic stem (ES) cells on erythroid differentiation of these cells. The h*EpoR* cDNA was efficiently transduced into ES cells, forming h*EpoR* that stably expressed ES (ES-h*EpoR*) cells. Expression of h*EpoR* cDNA was confirmed in ES-h*EpoR* cells by reverse transcriptase–polymerase chain reaction and Northern blot analysis. Colony assays demonstrated that definitive erythroid and primitive erythroid colonies were significantly increased from ES-h*EpoR* cells, when compared with mock virus–transduced ES (ES-Neo) cells, during the time course of differentiation induced by withdrawal of leukemia inhibitory factor, in either the presence or the absence of Epo. Multipotential colony-forming units (CFU-Mix) were also increased in ES-h*EpoR* cells at different stages of differentiation, but no changes were detected for CFU–granulocyte-macrophage colonies (CFU-GM). Time course studies by Northern blot analysis demonstrated elevated levels of expression of β-H_1 and β-Major globin genes in embryoid bodies derived from ES-h*EpoR* cells stimulated with Epo, when compared with similar expression from ES-Neo cells. Expression of the GATA-1 gene was enhanced in ES-h*EpoR* cells, when compared with ES-Neo cells, beginning immediately after initiation of the cultures until 8 days of differentiation. These data indicate that primitive and definitive erythropoiesis in differentiating embryoid bodies can be enhanced by retrovirus-mediated gene transfer of an h*EpoR* gene.

KEY WORDS

Erythropoiesis • Embryonic stem cells • Erythropoietin receptor • Gene transfer

INTRODUCTION

Erythropoietin (Epo) is a principal regulator of erythropoiesis, which supports the survival of erythroid progenitor cells and stimulates their proliferation and differentiation via binding to its cognate cell surface receptor (*EpoR*) [1,2]. *EpoR* is a member of the cytokine receptor superfamily and lacks an intrinsic tyrosine kinase domain. Binding of Epo to the *EpoR* elicits rapid tyrosine phosphorylation events in a series of intracellular signaling molecules. This includes activation of an *EpoR*-associated tyrosine kinase, Janus kinase 2, and signal transduction and activation transcriptional factor 5 [3-12]. The murine (m) and human (h) *EpoR* genes have been isolated and cloned [13-19]. *EpoR* has been reported to be primarily expressed on erythroid progenitors, megakaryocytes, placental cells, fetal liver cells, endothelial cells, and neural cells [20-27]. In erythroid lineages, expression of *EpoR* is very low or absent on immature progenitor cells, such as the burst-forming unit–erythroid (BFU-E), but increases with the maturation of these cells into colony-forming unit–erythroid (CFU-E) and then decreases rapidly with differentiation into mature red blood cells [24,25]. Epo provides both prolifera-

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tion signals to BFU-E and differentiation signals to CFU-E [1,2]. Overexpression of *EpoR* in immature hematopoietic cells enhances BFU-E numbers in response to Epo [27,28].

The Epo–*EpoR* signaling pathway plays an important role in early embryonic erythropoiesis as well. *EpoR* messenger RNA (mRNA) accumulation begins in murine mesoderm cell masses of the developing yolk sac of the neural plate-stage embryo (E7.5) before the development of morphologically recognized erythroblasts, and high levels of *EpoR* mRNA are expressed in yolk sac blood islands at the early somite stage (E8.5) [29]. Epo–*EpoR* signaling is functionally active during the initial proliferation and differentiation of primary yolk sac erythroblasts [29]. Targeted gene disruption of either the Epo or the *EpoR* gene leads to significant decreases in the number of mesoderm yolk sac erythroblasts circulating in the blood stream of E11-12 mutant murine embryos, failure of definitive fetal liver erythropoiesis, severe anemia, and fetal death. However, almost normal numbers of primitive erythroblasts were found in 2 9.5 *EpoR*–null embryos, suggesting that in vivo, *EpoR* contributes to definitive erythropoiesis in yolk sac in mesoderm [30,31].

However, whether Epo–*EpoR* induces an instructive signal for erythroid differentiation in hematopoietic stem cells or simply serves as a survival factor for committed erythroid progenitor cells remains uncertain. Injection of a recombinant retrovirus carrying a constitutively active *EpoR*, or normal *EpoR*, induces erythropoiesis in infected mice, suggesting that *EpoR* induces recruitment of immature erythroid precursors [27,32]. *EpoR* expressed in interleukin (IL)-3–dependent Ba/F3 lymphocytes transmits both proliferation and differentiation signals, and accumulation of globin gene mRNA was found in the transduced cells after induction with Epo, suggesting that *EpoR* plays a role in determining a cell's lineage [33-35]. On the other hand, chimeric receptors constructed by replacing the cytoplasmic tail of constitutively active *EpoR* with diverse cytokine receptors (eg, c-mpl and granulocyte-colony stimulating factor [G-CSF] receptor) support the final erythroid development in vitro and in vivo [36]. Prolactin receptor fully supports differentiation of $EpoR^{-/-}$ erythroid progenitor cells [37]. *EpoR* is also not required for embryonal primitive erythropoiesis [31]. Pluripotential hematopoietic progenitor cells expressing *EpoR* do not differentiate preferentially toward erythropoiesis in transgenic mice [38]. These results suggest that *EpoR*-specific signals are not required for erythroid differentiation.

Previous studies have shown that the profile of hematopoietic progenitor cells from cord blood can be enhanced toward CFU–granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) and BFU-E by retrovirus-mediated gene transfer at the single-cell level [28] and at the single daughter cell level [39]. Based on these studies, we believed that the effect of exogenously expressed h*EpoR* on hematopoiesis needed to be further addressed in more primitive hematopoietic stem cells.

Embryonic stem (ES) cells were derived from the inner cell mass of 3.5-day blastocytes with totipotent differentiation to committed-lineage hematopoietic cells and other cells such as myocardial and endothelial cells [40-44]. In vitro, spontaneous differentiation by withdrawal of leukemia inhibitory factor (LIF) contributes to cystic embryoid bodies (EBs) with endoderm, mesoderm, and ectoderm resembling the 6- to 8-day egg cylinder stage in normal embryonic development. Some EBs develop yolk sac–like blood islands [41,44]. *EpoR* mRNA has been shown to be slightly expressed in undifferentiated ES cells, and its expression can be easily detected immediately after differentiation [44]. Therefore, the aim of this work was to evaluate the effects of exogenously transduced *EpoR* on erythropoiesis in differentiating ES cells by means of retrovirus-mediated gene transfer of an h*EpoR* complementary DNA (cDNA).

MATERIALS AND METHODS

Cells

The ES-D3 cell, a murine 129/Sv blastocyst-derived ES cell line, and murine embryonic fibroblast cells used as feeder layers [45] were gifts from Dr. Jay A. Tischfield, Rutgers University (Piscataway, NJ). Undifferentiated ES-D3 cells were maintained in ES culture medium (Dubecco's modified Eagle's medium [DMEM], high glucose; Gibco BRL, Grand Island, NY) supplemented with 15% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratory, Logan, UT), 2 mmol/L glutamine, 0.1 mmol/L β-mercaptoethanol (Sigma, St. Louis, MO), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mmol/L nonessential amino acids (Gibco BRL) on gelatin-coated culture dishes in the presence of 1000 U/mL recombinant murine LIF (Gibco BRL). To prepare cells for gene transfer, frozen ES cells were thawed and cultured on mitomycin C (Sigma)–treated embryonic fibroblast feeder layers in the presence of LIF. To prepare feeder layers, confluent, healthy mouse embryonic fibroblasts were split onto gelatin-coated dishes containing ES medium and allowed to grow to confluency. The medium was then replaced with fresh DMEM with 10% FCS and 1 μg/mL mitomycin C. ES cells were incubated at 37°C in 5% CO_2 for 2 hours. The dishes were washed with phosphate-buffered saline (PBS) 3 times and resuspended in fresh ES medium. The mitomycin C–treated cells were used within 5 days. After gene transfer and selection in G418 (Gibco BRL) (0.75 mg/mL), polyclonal ES cells were maintained in ES medium with LIF and incubated at 37°C in 5% CO_2. The cells were passaged every 2 to 3 days by trypsinization in 0.25% trypsin (Gibco BRL) in PBS containing 1% FCS, washed, and resuspended in fresh ES medium at 5×10^5 cells/mL.

Retrovirus-Mediated Gene Transduction

The retroviral vector encoding h*EpoR* and the neomycin phosphotransferase (Neo) gene pLh*EpoR*SN was constructed from an *Eco*RI/*Xho*I fragment containing the h*EpoR* coding sequence inserted into the cloning site of pLXSN, as previously described [28]. Before gene transduction, rapidly growing and healthy single ES cells were replated into fresh ES medium until the cells had grown to 80% confluency. The medium was changed for fresh ES medium containing half the amount of retroviral supernant with polybrene (8 μg/mL) (Aldrich Chemical, Milwaukee, WI). G418 was added 40 hours later at a concentration of 0.75 mg/mL to aid in selecting G418-resistant cells. The stable h*EpoR* cDNA–transduced cells, known as ES-h*EpoR* cells, that contain the h*EpoR* coding sequence and the Neo gene cDNA mock virus–transduced ES (ES-Neo) cells were obtained after 2 weeks of selection in ES medium containing

G418. Expression of Neo gene and h*EpoR* gene in these cell pools was confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) and Northern blot analysis.

In Vitro Differentiation of ES Cells

To initiate differentiation, ES cells were dissociated by trypsinization, and differentiation was performed in suspension culture in 60-mm bacterial petri dishes in the same ES medium mentioned above, in the absence of LIF and in the presence of 1 U/mL recombinant human Epo. Single ES cells were replated at 2×10^5 cells/mL in a final volume of 5 mL medium to form EBs. EBs were maintained in a humidified condition at 37°C and 5% CO_2. Medium was changed every 2 days by allowing EBs to settle down in a tube, replacing the medium, and gently pipetting EBs into fresh petri dishes. At various times of differentiation, the EBs were harvested by centrifugation and used for colony assay and extraction of total RNA.

Colony Assay

For colony assay, EB-derived cells were harvested by dissociating them in 0.25% collagenase medium at 37°C for 1 hour and passing the cells slowly through a 23-gauge syringe needle. Cells were washed in PBS containing 1% FCS, and live cells were counted by trypan blue exclusion. Single undifferentiated ES cells and EB-derived cells, harvested at days 6, 10, and 14 of differentiation, were cultured at a concentration of 10^4 cells/mL in 1% methylcellulose, 30% FCS, 2 mmol/L glutamine, 0.1 mmol/L β-mercaptoethanol, and 0.1 mmol/L hemin (Eastman Kodak, Rochester, NY) in 35-mm diameter culture dishes for colony formation as previously described [28,39].

Recombinant murine preparations of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and Steel factor (SLF) were purchased from R&D Systems (Minneapolis, MN), and used at concentrations of 200 U/mL, 200 U/mL, and 50 ng/mL, respectively. Recombinant human Epo was purchased from Amgen (Thousand Oaks, CA) and used at a concentration of 1 U/mL. Cells were cultured at 37°C, 5% CO_2, and 5% O_2. Colony-forming unit–granulocyte macrophage (CFU-GM), multipotential CFU (CFU-Mix), and definitive erythroid colonies (Ery-D) were scored after 8 days of culture, and primitive erythroid colonies (Ery-P) were scored after 4 days of culture [44,46]. Individual Ery-P and Ery-D colonies were distinguished by the size of the cells within the colonies and by the fact that Ery-P colonies were a more brilliant red color than Ery-D colonies. Cells in Ery-P colonies are large and nucleated, whereas cells in Ery-D colonies are smaller and enucleated [47].

To evaluate the formation of blood island–containing EBs, ES cells were plated in 1% methylcellulose culture medium as mentioned above, in either the presence or the absence of growth factors. Cells were allowed to differentiate for 7 to 14 days; EBs were counted; and those EBs containing hemoglobinized cells were scored as erythroid cell positive [44]. Significant differences between groups were determined by the Student *t* test.

RT-PCR Analysis

Total RNA was isolated from undifferentiated ES, ES-Neo, ES-h*EpoR* cells (day 0), and from EB-derived cells at days 2, 4, 6, 8, 10, and 14 of differentiation by Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany) as suggested by the manufacturer. Total RNA was treated with recombinant human deoxyribonuclease I (DNase I) for 15 minutes at room temperature and was inactivated by heating at 95°C for 10 minutes before the addition of reverse transcriptase (RT) to eliminate any contamination of remaining genomic DNA. One microgram of total RNA lysate in 20 μL RT buffer (RT buffer: 25 mmol/L Tris-HCl [pH 8.3], 37.5 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 10 mmol/L deoxyribonucleoside triphosphate mixture, 0.5 mg/L Oligo $[dT]_{15}$ primer], 26 units RNasin, and 200 units murine Moloney leukemia virus reverse transcriptase [Promega, Madison, WI]) was used for RT. After cDNA synthesis, 1 μL of the cDNA solution synthesized from 50 ng total RNA was used for PCR amplification. This was performed in 50 μL of reaction mixture containing 1 × PCR buffer, 60 μmol/L of dNTP, 2 units Taq polymerase (Boehringer Mannheim, Mannheim, Germany), and 0.5 μmol/L of each specific primer. PCR was performed by a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). For Neo gene expression, the sequence of primers were 5′ CAA GAT GGA TTG CAC GCA GGT TCT CCG 3′ as sense strand and 5′ CCA GAG TCC CGC TCA GAA GAA CTC GTC 3′ as antisense strand. The PCR condition was denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and polymerization at 72°C for 2 minutes for 35 cycles. For expression of transduced h*EpoR* gene, the sequence of primers was as follows: 5′ CCC CTA CCC ACC CCA CCT AA 3′ of h*EpoR* cDNA as sense strand and 5′ ACC TGC GTG CAA TCC ATC TTG 3′ corresponding to the Neo gene of the pLXSN as antisense strand as previously described [28,39]. The PCR condition was denaturation at 95°C for 25 seconds, annealing at 65°C for 30 seconds, and polymerization at 72°C for 2 minutes for 35 cycles. The primers and annealing temperature of amplification for globin genes and transcription factor genes are shown in the Table [41,49]. Ten microliters amplified product were electrophoresed, transferred to membrane, and hybridized with a [^{32}P]dCTP (Amersham, Arlington Heights, IL)-labeled *Xho*I-*Eco*RI digested pLXSN Neo gene fragment and a *Hind*III-*Eco*RI digested h*EpoR* gene fragment from the pLh*EpoR*SN plasmid as a probe. Hybridization was performed overnight at 42°C, and filters were washed with 0.1× SSC in 0.1% SDS at 55°C for 60 minutes, dried, and exposed to x-rays at –70°C.

Northern Blot Analysis

A total of 15 μg of RNA extracted from parental ES, ES-Neo, or ES-h*EpoR* cells was electrophoresed on 1% agarose-formaldehyde gel, blotted to Genescreen Plus nylon membrance, and hybridized with [^{32}P]dCTP-labeled h*EpoR* gene fragment as a probe. For expression of murine *EpoR*, β-*H*$_1$, β-Major and GATA-1 genes, the probes were generated by RT-PCR amplification. PCR products were cloned into pGEM-T vector (Promega). The inserted fragments were confirmed by direct sequencing. Probe labeling was performed by using an oligolabeling kit (Pharmacia Biotech, Piscataway, NJ). Prehybridization was performed at 68°C in Hyperexpress hybridization solution (Clontech, Palo Alto, CA) with 100 μg/mL denatured salmon-testes DNA (Sigma) for 1 hour, and hybridization was performed under the same

Oligonucleotide Primers Used for Gene Expression Analysis by reverse transcriptase–polymerase chain reaction (RT-PCR) [41,49]

Gene	Size (bp)*	T(C°)†	5′ Primer	3′ Primer
GATA-1	581	55	5′-ATGCCTGTAATCCCAGCACT-3′	5′-TCATGGTGGTAGCTGGTAGC-3′
c-*myb*	681	55	5′-TTCAAGGCCAGCATTCTTGC-3′	5′-CCTCTAGGAGCTCATTTGTG-3′
scl	396	55	5′-TATGAGATGGAGATTTCTGAT G-3′	5′-GCTCCTCTGTGTAACTGTC-3′
NF-E2	391	62	5′-GAGCCCTGGCCATGAAGATTCC-3′	5′-CACCATCAGCAGCCTGTTGCAG-3′
EKLF	359	55	5′-TCGCCGGAGACGCAGGCT-3′	5′-CCCAGTCCTTGTGCAGGA-3′
EpoR	452	55	5′-GGACACCTACTTGGTATTGG-3′	5′-GACGTTGTAGGCTGGAGTCC-3′
β-H_1	256	55	5′-ATGCCCCATGGAGTCAAAGA-3′	5′CTCAAGGAGACCTTTGCTCA-3′
β-Major	578	55	5′-CTGACAGATGCTCTCTTGGG-3′	5′-CACAACCCCAGAAACAGACA-3′

*Predicted size of RT-PCR products.
†Annealing temperature.

conditions as prehybridization for 1 hour without salmon-testes DNA. After hybridization, the filter was washed with 2× SSC in 0.05% SDS and 0.1× SSC in 0.1% SDS at room temperature for 60 minutes, dried, and exposed to x-rays at –70°C overnight. Visualized bands were quantitated using Sigma Gel Software and Scanner (Jandel Scientific, San Rafael, CA).

RESULTS

Expression of Human *EpoR* and Endogenous Murine *EpoR* Gene in ES-h*EpoR* Cells

Parental ES cells were transduced with retroviral pLh*EpoR*SN or mock control pLXSN and selected with G418. After 2 weeks of selection, the cells integrating and expressing transduced Neo, a selectable marker gene, survived, expanded, and were designated as ES-h*EpoR* (for those cells integrating transduced Neo) and ES-Neo (for those cells expressing transduced Neo). G418 was added every 2 weeks for all the experiments, and each individual experiment was performed using freshly thawed frozen cells. The predicted products of the transduced h*EpoR* gene in ES-*EpoR* cells and the Neo gene in both the ES-h*EpoR* and ES-Neo cells were detected by RT-PCR analysis (Figure 1A). Northern blot analysis was applied to further confirm expression of the transduced h*EpoR* gene, and a specific 1.6-kilobase (kb) band transcripted from pLh*EpoR*SN was detected using hybridization with ^{32}P-labeled h*EpoR* gene fragment in ES-*EpoR* cells, but not in parental ES or ES-Neo cells (Figure 1B). Expression of the transduced h*EpoR* gene was constantly detected by Northern blot analysis in ES-h*EpoR* cells, but not ES-Neo cells, undergoing differentiation, although a slight decrease was observed during differentiation (Figure 2). In contrast, the expression of murine *EpoR* was detected at a very low level in undifferentiated ES-Neo cells and was greatly enhanced at day 4 to 14 of differentiation. Transduction of h*EpoR* did not change the expression pattern of endogenous murine *EpoR* (Figure 2). No difference was detected for the expression of murine *EpoR* between ES-Neo and parental ES cells (data not shown). The expression pattern of murine *EpoR* in parental ES cells is consistent with that in other reports [41,44,48].

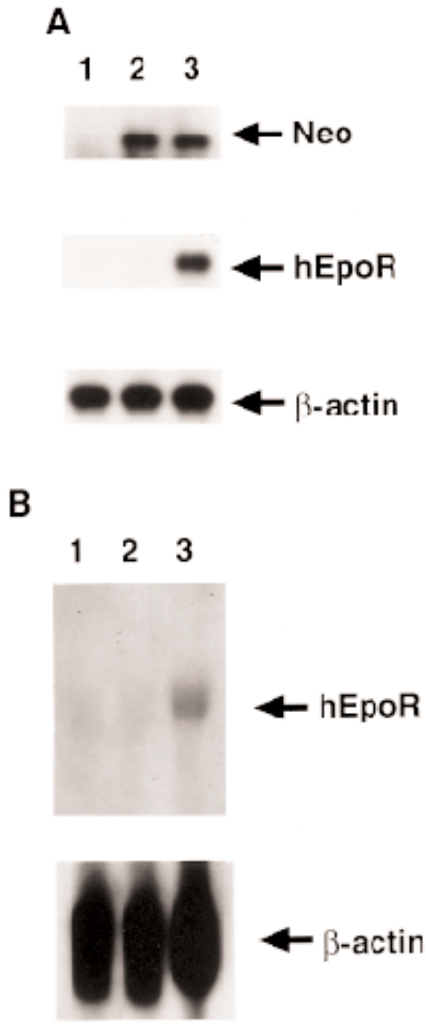

Figure 1. Detection of the expression of the transduced h*EpoR* gene and the Neo gene in undifferentiated embryonic stem (ES) cells. A. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. The amplified products were subjected to Southern blotting using ^{32}P-labeled h*EpoR* and Neo gene fragments as probes. B. Northern blot analysis. Fifteen micrograms of total RNA was electrophoresed on a 1% agarose gel, transferred to Genescreen Plus nylon membrane, and hybridized with a ^{32}P-labeled h*EpoR* cDNA fragment. β-actin was used as messenger RNA control. 1 indicates parental ES cells; 2, ES-Neo cells; 3, ES-h*EpoR* cells.

Increases in Formation of Blood Islands in EBs, but Not in Plating Efficiencies of EBs, From ES-h*EpoR* Cells

In vitro differentiation of ES cells by withdrawal of LIF can generate EBs in either suspension culture or methylcel-

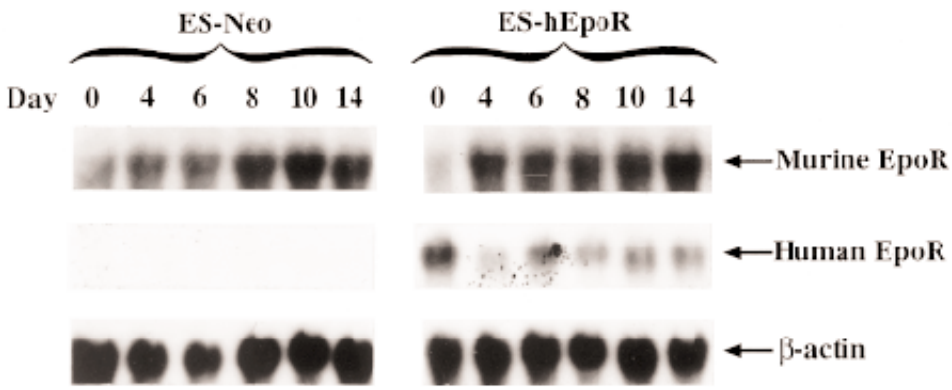

Figure 2. Detection by Northern blot analysis of expression of the transduced h*EpoR* gene and the endogenous murine *EpoR* gene in ES-Neo and ES-h*EpoR* cells during differentiation. Results from 1 of 3 representative experiments are shown. Fifteen micrograms of total RNA extracted from undifferentiated embryonic stem (ES) cells (day 0) and embryoid bodies (EBs) harvested at days 4, 6, 8, 10, and 14 of differentiation in the presence of 1 U/mL Epo were subjected to electrophoresis on 1% agarose-formaldehyde gel, blotting to Genescreen Plus nylon membrane, and hybridization with [^{32}P]dCTP-labeled h*EpoR* (1.8 kb) or murine *EpoR* (0.5 kb) gene fragment as a probe. The expression of β-actin gene was used as messenger RNA (mRNA) control. Note that because different-length probes were used to detect mRNA expression of the human and murine *EpoR*, and blot signals are related to hybridization efficiencies, which may vary between these probes, it is difficult to directly compare the relative expression of the human *EpoR* to that of the murine *EpoR*.

lulose culture [44,49]. When ES cells were seeded at a concentration of 10^4 cells in 1 mL methylcellulose culture medium without hematopoietic growth factors, a total of 300 to 350 EBs were generated by day 7 of differentiation. The addition of GM-CSF, IL-3, and SLF with or without Epo did not significantly influence plating efficiencies. Expression of the h*EpoR* gene in ES cells neither changed the plating efficiencies nor modified the independence of EBs to growth factors (Figure 3A).

Some EBs contained visible blood islands after 7 to 10 days of cultures. This early stage of erythropoiesis appeared as a relatively small red area within the center of the EBs; hemoglobinization increased swiftly during the ensuing days of differentiation, with bright red color occurring at about day 14 of differentiation (Figure 4A). Some EBs ruptured, releasing red erythroid colonies that remained at the periphery of the EBs (Figure 4B).

In this study, we observed that 33.7% and 31.2% of EBs from parental ES and ES-Neo cells, respectively, contained visible blood islands at day 14 of differentiation after culture in the presence of GM-CSF, IL-3, SLF, and Epo (Figure 3B). Expression of the h*EpoR* gene in ES cells contributed to the significantly enhanced formation of blood island–containing EBs (53.8%) at day 14 of differentiation (see Fig-

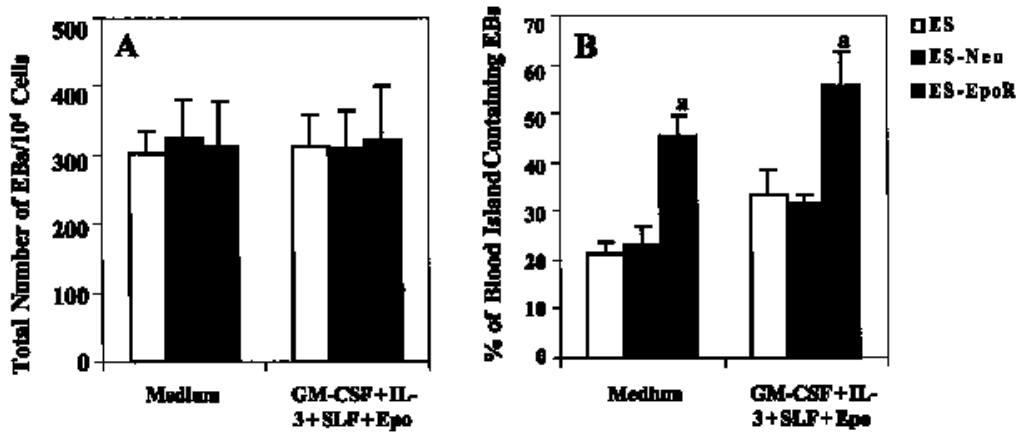

Figure 3. Development of embryoid bodies (EBs) and blood islands within EBs during differentiation of parental embryonic stem (ES), ES-Neo, and ES-h*EpoR* cells. A. The total number of EBs generated from 10^4 cells in 1 mL methylcellulose culture medium with or without hematopoietic growth factors, as indicated, at day 7 of differentiation. B. The percentage of blood island–containing EBs on day 14 of differentiation in the absence and presence of Steel factor (SLF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (Epo). Results from 3 separate experiments are expressed as mean ± SD. a indicates significant ($P < .05$) differences from parental ES and ES-Neo cells.

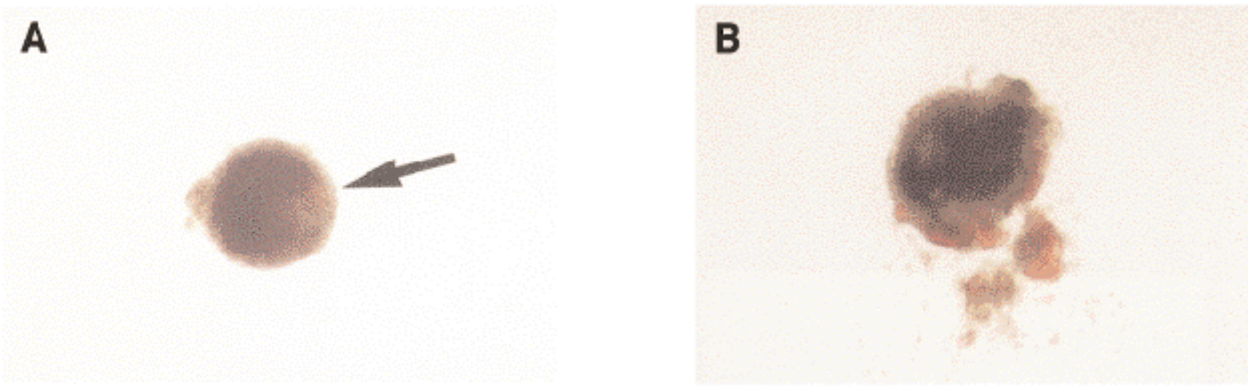

Figure 4. Representative blood island–containing embryoid bodies (EBs) on day 14 of differentiation of embryonic stem (ES) cells. A. The blood island within the center of the EB from ES-Neo cells is indicated by the arrow. B. Ruptured EB derived from an ES-h*EpoR* cell releasing large erythroid burst colonies in the periphery of the EB. (Original magnification, ×40)

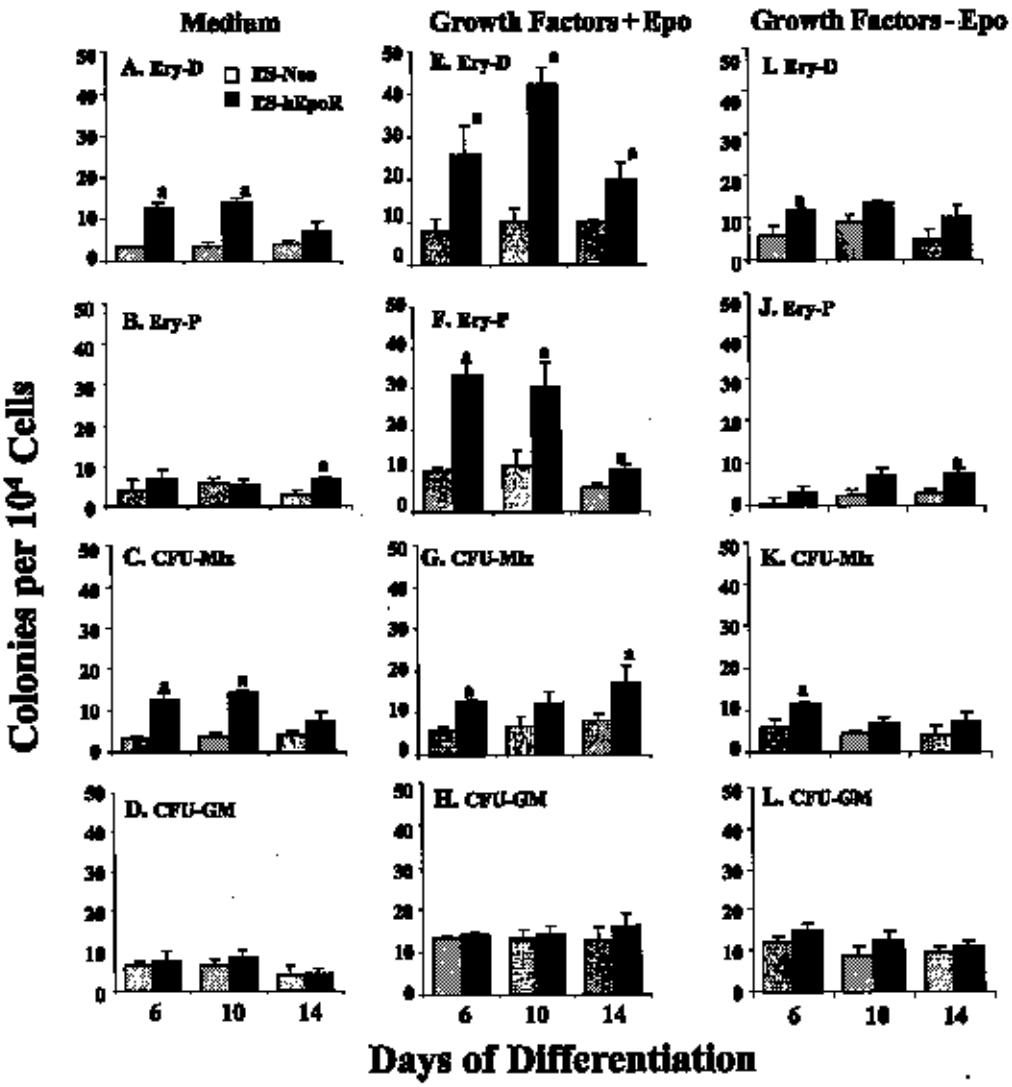

Figure 5. Time course of development of hematopoietic colonies from ES-Neo and ES-h*EpoR* cells. Cells were plated at 10^4/mL in methylcellulose culture in the absence (A-D) or presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and Steel factor (SLF) with (E-H) and without (I-L) Epo. Results from 5 separate experiments are expressed as mean ± SD. a indicates significant ($P < .05$) differences from parental ES-Neo cells. Ery-D indicates definitive erythroid colony; Ery-P, primitive erythroid colony; CFU-Mix, multipotential colony-forming unit; CFU-GM, colony-forming unit–granulocyte/macrophage.

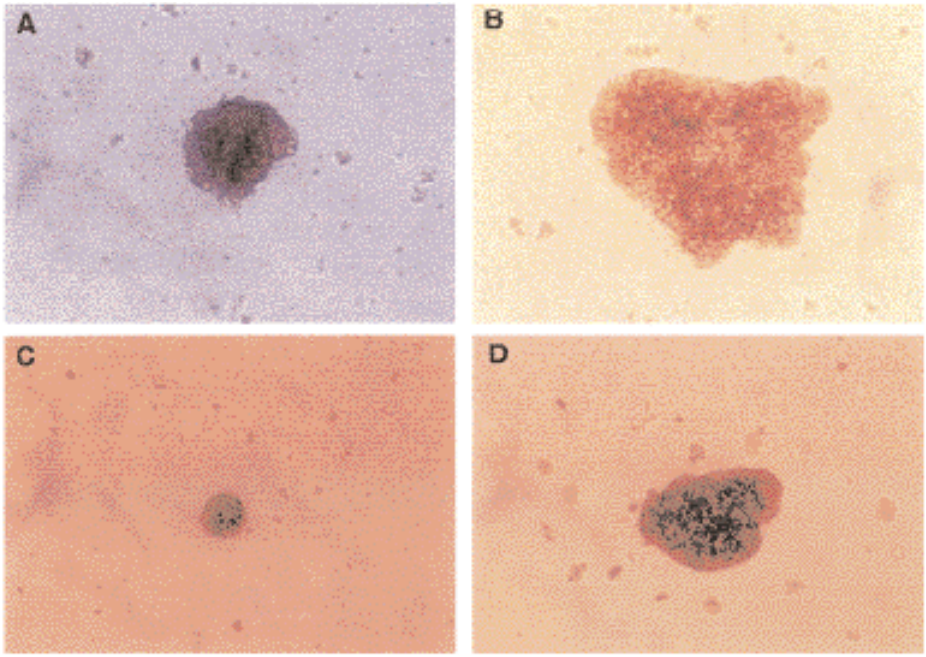

Figure 6. Representative definitive erythroid colonies (Ery-D) from ES-Neo cells (A) and ES-h*EpoR* cells (B), and primitive erythroid colonies (Ery-P) from ES-Neo cells (C) and ES-h*EpoR* cells (D). (Original magnification, ×80)

ures 3B and 4B) when compared with parental ES and ES-Neo cells grown in the presence of these same cytokines. This enhancement of formation of blood island–containing EBs was also observed from ES-h*EpoR* cells in the absence of cytokines (Figure 3B).

Increased Erythroid Progenitors in ES-h*EpoR* Cells

To investigate the possible effects of the human *EpoR* gene on the proliferation and differentiation of erythroid precursors in EBs, EB-derived cells, at different times of differentiation in suspension culture, were replated in methylcellulose cultures for colony formation. Differentiating ES cells generated CFU-Mix, Ery-P, Ery-D, and GM-CFU. Results from 5 experiments showed that in the absence of cytokines, transduction of h*EpoR* gene in ES cells, slightly but significantly increased colony numbers from Ery-D and Ery-P (Figure 5A and 5B). In the presence of GM-CSF, IL-3, SLF, and Epo, Ery-D colonies were further enhanced by 3.3-, 4.2-, and 2.0-fold at days 6, 10, and 14 of differentiation from ES-h*EpoR* cells, respectively, when compared with differentiation from ES-Neo cells (Figure 5E). Similar results were detected in Ery-P colonies from ES cells. In the presence of the same growth factors, a 3.3- and 2.7-fold increase in Ery-P occurred at days 6 and 10 of differentiation from ES-h*EpoR* cells, respectively, when compared with differentiation from ES-Neo cells (Figure 5F). GM-CSF, IL-3 and SLF, in the absence of Epo, did not enhance formation of Ery-D (Figure 5I) or Ery-P (Figure 5J) colonies from ES-h*EpoR* cells beyond that of cells cultured in medium control without growth factors. Furthermore, no differences in formation of colonies were noted between parental ES and ES-Neo cells (data not shown). The erythroid colonies formed from ES-hEopR cells were larger and more hemoglobinized than those from ES-Neo cells (Figure 6). These results suggest that the human *EpoR* gene contributed to the enhanced growth of both primitive and definitive erythroid progenitors in EBs.

Transduction of the h*EpoR* gene also contributed to significant increases in CFU-Mix colonies from days 6 to 14 of differentiation in the absence of cytokines. This increase for CFU-mix was slightly but significantly enhanced on day 14 of differentiation by the addition of Epo to GM-CSF, IL-3, and SLF, but not in the absence of Epo (Figure 5C, 5G, and 5K), suggesting that the h*EpoR* gene might contribute to augmentation of multipotential precursors in the differentiation of ES cells. In contrast, no differences could be detected for CFU-GM colonies in ES-h*EpoR* cells and ES-Neo cells during differentiation with or without growth factors (Figure 5D, 5H, and 5L).

Expression of Globin Genes in Differentiating EBs

To further evaluate the differentiation of erythroid precursors to mature red cells in ES-*EpoR* cells, the expression of fetal and adult globin genes were examined by Northern blot analysis. The expression of the β-H_1 globin gene was first detected at a very low level on day 6 in ES-Neo cells in the presence of Epo. The expression was greatly increased by day 8 of differentiation, with maximal expression occurring on day 10 of differentiation. Elevated expression of the β-H_1 globin gene was noted in ES-h*EpoR* cells, when compared with ES-Neo cells, beginning on day 8 of differentiation (Figure 7A). Relative expression of the β-H_1 globin gene, compared with control expression of the β-actin gene, showed 3- to 5-fold enhancement during days 8 to 14 of differentiation in ES-h*EpoR* cells when compared with similar expression in mock virus–transduced ES cells (Figure 7B). Elevated expression of β-Major globin gene was also detected at days 8, 10,

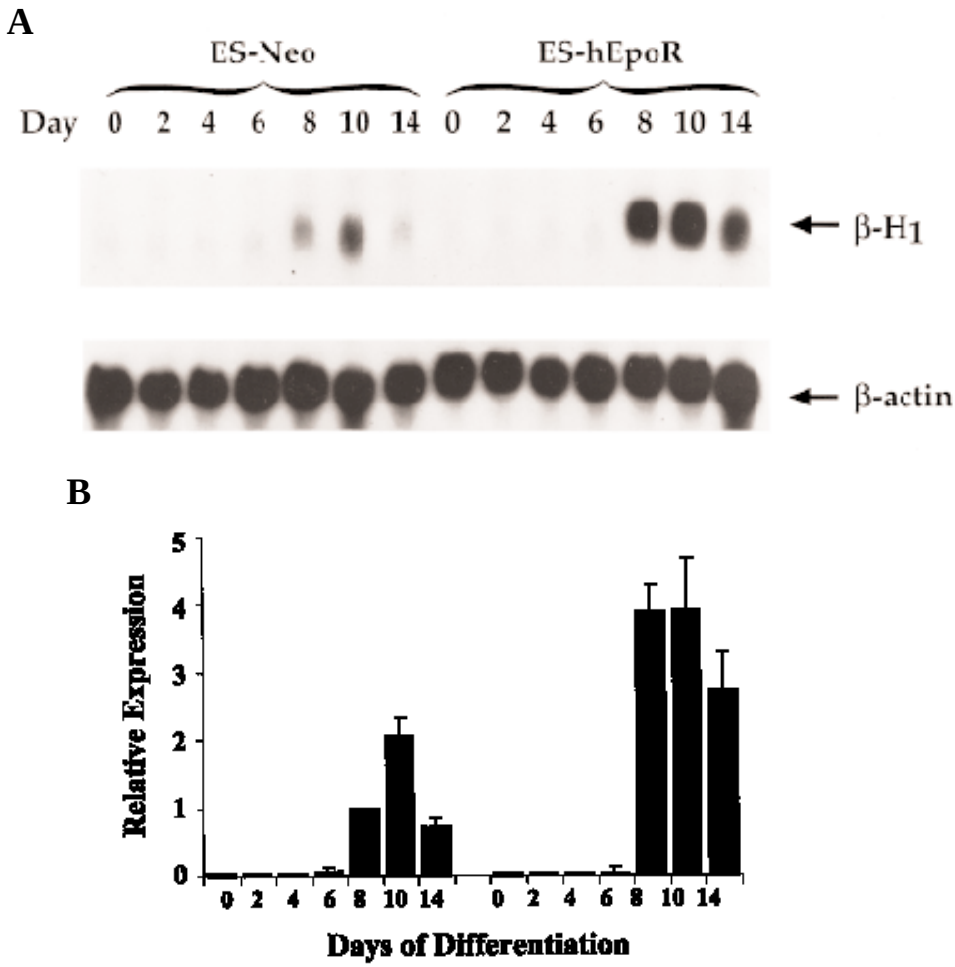

Figure 7. A. Detection by Northern blot analysis of expression of β-H_1 globin genes in ES-Neo and ES-h*EpoR* cells during differentiation; results from 1 of 4 representative experiments are shown. Fifteen micrograms of total RNA extracted from undifferentiated embryonic stem (ES) cells (day 0) and embryoid bodies (EBs) harvested at days 2, 4, 6, 8, 10, and 14 of differentiation after culture in the presence of 1 U/mL erythropoietin (Epo) were subjected to electrophoresis on 1% agarose-formaldehyde gel, blotting to Genescreen Plus nylon membrane, and hybridization with [^{32}P]dCTP-labeled β-H_1 globin gene fragment as a probe. B. Relative expression was calculated by comparison with expression of the β-actin gene. Results from 4 separate experiments are expressed as mean + SD.

and especially 14 of differentiation in ES-h*EpoR* cells when compared with ES-Neo cells (Figure 8).

Expression of Transcription Factor Genes in EBs

To address possible transcriptional regulation of these globin genes, expression of several transcriptional factor genes—GATA-1, EKLF, NF-E2, c-*myb*, and *scl*, which are believed to play important roles in erythropoiesis—was analyzed by RT-PCR (data not shown); results for GATA-1 were confirmed by Northern blot analysis (Figure 9A). The GATA-1 gene was detected at a low level of transcription in ES-Neo cells on days 0 to 4 of differentiation; expression was increased after 6 days of differentiation to a maximal level on days 10 to 14 of differentiation. Expression of the GATA-1 gene in h*EpoR*-transduced ES cells was enhanced 1.5 to 3 times when compared with similar expression in ES-Neo cells during days 0 to 8 of differentiation (Figure 9B). This increased expression of the GATA-1 gene might contribute to earlier and increased expression of globin genes and to the switch of globin genes from fetal to adult. In contrast, no differences in the expression level and pattern of other transcription factor genes (eg, EKLF, NF-E2, c-*myb*, and *scl*) were noted between ES-h*EpoR* and ES-Neo cells during the entire time course of differentiation as assessed by RT-PCR analysis (data not shown).

DISCUSSION

Hematopoietic growth factor receptors play important roles in normal hematopoiesis by binding their cognate ligands and transducing intracellular signals responsible for cell proliferation and terminal differentiation. Lineage-specific cytokine receptors, such as *EpoR*, G-CSFR, M-CSFR and c-*mpl*, are intimately involved in the output of mature cells from lineage-specific committed progenitors, and these receptors are expressed during the commitment of stem cells to their progenitors. However, the role of lineage-specific cytokine receptors in proliferation and differentia-

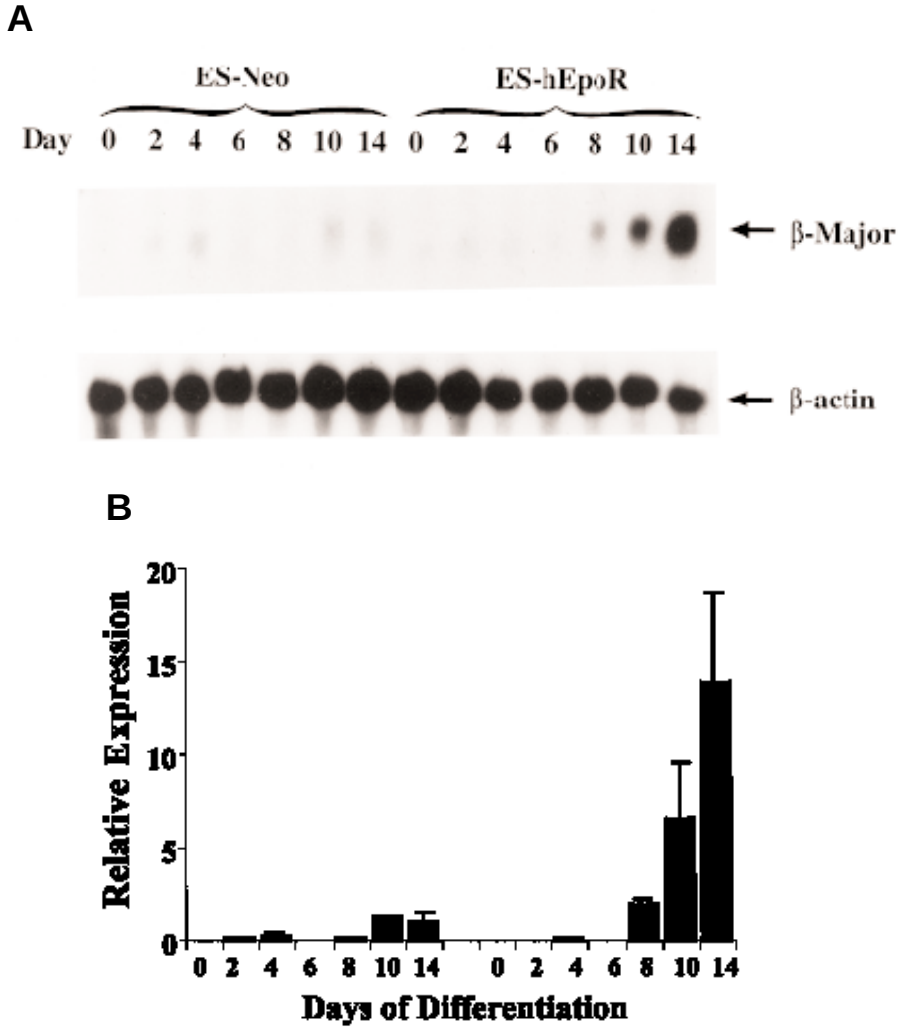

Figure 8. A. Detection by Northern blot analysis of expression of the β-Major globin gene in ES-Neo and ES-h*EpoR* cells during differentiation; results from 1 of 3 representative experiments are shown. Fifteen micrograms of total RNA extracted from undifferentiated embryonic stem (ES) cells (day 0) and embryoid bodies (EBs) harvested at days 2, 4, 6, 8, 10, and 14 of differentiation after culture in the presence of 1 U/mL erythropoietin (Epo) were subjected to electrophoresis on 1% agarose-formaldehyde gel, blotting to Genescreen Plus nylon membrance, and hybridization with [^{32}P]dCTP-labeled β-Major globin gene fragment as a probe. B. Relative expression was calculated by comparison with expression of the β-actin gene. Results from 3 separate experiments are expressed as mean + SD.

tion of embryonic stem cells remains unclear. The establishment of ES cell lines has provided a model system with which to study early hematopoietic development. This system has been used widely to correlate expression of cytokines, their receptors, globins, and a series of hematopoietic cell surface markers throughout hematopoietic development in vitro [40-43,49].

In the present study, significantly increased numbers of erythroid progenitors were noted within EBs generated from h*EpoR* cDNA–transduced and stably expressed ES cells when compared with EBs from mock virus–transduced ES-Neo cells. Increased numbers of erythroid colonies, including Ery-D and Ery-P, were derived from EBs of ES-h*EpoR* cells. Production of CFU-mix colonies, but not CFU-GM colonies, from ES-h*EpoR* cells was also slightly increased. These results, together with the enhanced formation of blood islands within developing EBs after the introduction of h*EpoR* cDNA into ES cells, demonstrate that introduction of the h*EpoR* gene contributes to production of fetal and adult erythroid progenitor cells in developing EBs, and that the enhancement might not be entirely owing to the effects of exogenously added Epo. Several possibilities exist to explain the exogenous Epo-independent erythropoiesis in the development of blood islands and erythroid colonies in *EpoR* cDNA–transduced ES cells in the absence of Epo. First, totipotential ES cells are able to differentiate into all tissue cells, including those cells producing endogenous Epo, which in turn could trigger *EpoR* signals in developing EBs [40,41]. Second, transduced *EpoR* might result in a relatively higher level of expression of *EpoR* under the control of a strong promoter and could induce spontaneous activation or formation of ligand-independent *EpoR* dimerization that might be sufficient to elicit a differentiation signal. Third, it is conceivable that the function of the *EpoR* may be modu-

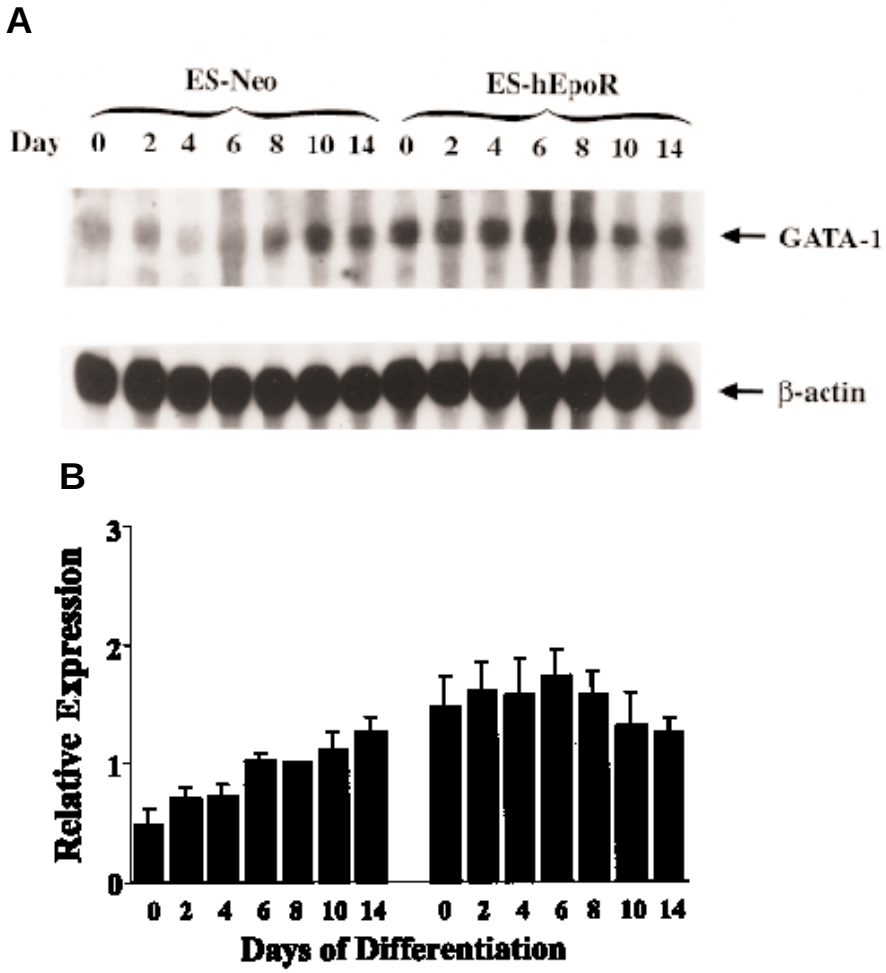

Figure 9. A. Detection by Northern blot analysis of expression of the GATA-1 gene in ES-Neo and ES-h*EpoR* cells during differentiation; results from 1 of 4 representative experiments are shown. Fifteen micrograms of total RNA extracted from undifferentiated embryonic stem (ES) cells (day 0) and embryoid bodies (EBs) harvested at days 2, 4, 6, 8, 10, and 14 of differentiation after culture in the presence of 1 U/mL erythropoietin (Epo) were subjected to electrophoresis on 1% agarose-formaldehyde gel, blotting to Genescreen Plus nylon membrane, and hybridization with [^{32}P]dCTP-labeled GATA-1 gene fragment as a probe. B. Relative expression was calculated by comparison with expression of the β-actin gene. Results from 4 separate experiments are expressed as mean + SD.

lated by other growth factors present in the culture environment or in developing EBs, such as SLF, that can phosphorylate the *EpoR* [50]. Also, the *EpoR* may be activated by trace amounts of bovine factors present in the medium. It has been reported that the lower the concentration of Epo in the culture, the higher the degree of differentiation in *EpoR*-transduced Ba/F3 cells [51].

Enhanced output of erythroid cells was suggested by globin gene expression studies. Although the expression pattern was not significantly changed by introduction of the h*EpoR* gene into ES cells, the relative expression level was elevated in ES-h*EpoR* cells when compared with ES-Neo cells. In both ES-Neo and ES-h*EpoR* cells, expression of fetal globin genes, such as β-H_1 globin, was elevated on days 8 to 14 of differentiation but decreased on day 14 of differentiation. Elevated expression of adult β-Major globin gene was observed on days 8 to 14 of differentiation, but was most apparent at later stages (days 10 and 14) when compared with ES-Neo cells. These results are consistent with globin gene switching from embryonic to fetal/adult globin genes [52] and indicate that both primitive and definitive erythroid progenitors could be enhanced by transduction of the h*EpoR* gene into ES cells. Globin gene switching from fetal to adult type was enhanced in ES-h*EpoR* cells as well.

Of particular interest, one of the transcription factor genes, GATA-1, displayed enhanced expression during differentiation of ES-h*EpoR* cells when compared with mock virus–transduced ES cells. GATA-1 plays a crucial role in eythropoiesis because it binds to a motif (A/T)GATA(A/G) that is found in the promoters and enhancers of nearly all erythroid cell–specific genes studied thus far, including the globin genes and their respective locus control regions [53-56]. Targeted disruption of the GATA-1 gene in mice leads to failure in production of mature red blood cells and apoptosis of committed erythroid precusors [57]. Introduction of a normal GATA-1 gene into GATA-1$^-$ mouse ES cells

restores the developmental potential of primitive and definitive erythroid cells [58]. GATA-1 and Epo cooperate to promote survival of erythroid cells [59]. GATA-1 also appears to be central to the regulation of transcription of the *EpoR* gene [53]. It has been noted that the activation of GATA-1 could be enhanced by Epo–*EpoR* signaling in several cell lines, such as Ba/F3 and J2E [54,60]. In our study, GATA-1 is expressed at a low level in undifferentiated mock virus–transduced ES cells but is increased after 6 days of differentiation. In contrast, elevated expression of this gene was seen at days 0 through 8 of differentiation in ES-*EpoR* cells as compared with ES-Neo cells. It has been reported that Epo–*EpoR* signaling transactivates GATA-1 gene expression after stimulation of cells with Epo. This concurs with the observation that progressive GATA-1 gene expression appears only in later erythroid differentiation stages. A low level of GATA-1 expression occurring by itself before Epo stimulation is not enough to transactivate erythroid-specific gene expression, although activation of the *EpoR* gene by the GATA-1 gene may be possible at this differentiation stage [54]. Activation of the *EpoR* gene, combined with expression of GATA-1, might be able to transactivate erythroid-specific genes at this differentiated stage [61]. Therefore, we believe that enhancement of globin gene expression by transduction of the h*EpoR* gene in ES cells might be mediated, at least partly, by the transactivation of GATA-1, which in turn activates globin gene expression at later differentiation stages in these cells. We failed to detect differences in the expression levels and patterns of other transcription factors, such as NF-E2, c-*myb*, *scl*, and EKLF, in ES-h*EpoR* cells when compared with parental ES or ES-Neo cells, suggesting that these genes might not play an essential role in the observations we have made.

It is conceivable that the enhanced erythropiesis in developing EBs derived from h*EpoR*-transduced ES cells might be a result of signals tranduced by h*EpoR* to support proliferation and differentiation of erythroid progenitors derived from these cells. However, we cannot exclude the possible role of h*EpoR* in transducing specific signals for differentiation of ES cells to erythroid lineage. Although evidence indicates that the Epo–*EpoR* signal is not specifically required for erythroid differentiation [39], our data partially support the role of transduced h*EpoR* in the production of erythroid precursors in differentiating ES cells. Some studies have indicated that the cytoplasmic region of *EpoR* contains a differentiation domain that is unique to the *EpoR* and different from the cytoplasmic region of other hematopoietic growth factor receptors [36]. Human *EpoR* transgenic mice have been established using a genomic fragment containing the h*EpoR* gene, and they exhibit correct developmental hematopoietic and neural expression [62,63]. By using an ES cell model in our study, expression of the h*EpoR* gene was constantly detected during in vitro differentiation. Future studies creating h*EpoR* transgenic mice using this ES cell model may further address the role of *EpoR* in primitive hematopoietic stem cells.

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