Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9126–9131, August 1996
Medical Sciences

Thrombopoietin rescues *in vitro* erythroid colony formation from mouse embryos lacking the erythropoietin receptor

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ABSTRACT **The interaction of the hormone erythropoietin and its receptor (EpoR) is thought to be required for normal hematopoiesis. To define the role of EpoR in this process, the murine EpoR was disrupted by homologous recombination. Mice lacking the EpoR died *in utero* at embryonic day 11–12.5 with severe anemia. Embryonic erythropoiesis was markedly diminished, while fetal liver hematopoiesis was blocked at the proerythroblast stage. Other cell types known to express EpoR, including megakaryocytes, mast, and neural cells were morphologically normal. Reverse transcription-coupled PCR analysis of RNA from embryonic yolk sac, peripheral blood, and fetal liver demonstrated near normal transcripts levels for EKLF, thrombopoietin (Tpo), c-MPL, GATA-1, GATA-2, and α- and embryonic βH1-globin but none for adult βmaj-globin. While colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) colonies were not present in cultures derived from EpoR−/− liver or yolk sac cells, hemoglobin-containing BFU-E colonies were detected in cultures treated with recombinant Tpo and Kit ligand or with Tpo and interleukin 3 and 11. Rescued BFU-E colonies expressed adult β-globin and c-MPL and appeared morphologically normal. Thus, erythroid progenitors are formed *in vivo* in mice lacking the EpoR, and our studies demonstrate that a signal transmitted through the Tpo receptor c-MPL stimulates proliferation and terminal differentiation of these progenitors *in vitro*.**

The hematopoietic system in vertebrates is capable of generating a diverse array of differentiated cell types, including red blood cells, white blood cells, and platelets, from a few self-renewing stem cells. Interactions between specific cytokines and their receptors transmit signals for proliferation and possibly differentiation within hematopoietic progenitors. The erythropoietin receptor (EpoR) (1, 2) and its ligand erythropoietin (Epo) (3) figure prominently in erythroid proliferation and differentiation (4, 5).

Several lines of evidence suggest that erythroid and megakaryocytic lineages arise from a common cell (6). Both lineages coexpress transcription factors (7, 8), as well as the EpoR and the Tpo receptor (c-MPL) (9–12), which are structurally related members of the cytokine receptor superfamily (13–15). Moreover, the ligands for these receptors exhibit significant structural homology (16, 17). These structural similarities suggest common ancestry and raise the possibility of vestigial overlap in downstream receptor-mediated signaling cascades (18).

Mice lacking either c-MPL (19, 20) or the EpoR (refs. 21 and 22 and see below) have been generated by homologous recombination in embryonic stem (ES) cells. c-MPL−/− mice display a significant, albeit incomplete, reduction in platelet production but apparently normal erythropoiesis (19, 20). By contrast, both Epo and EpoR−/− mice demonstrate a major defect in both embryonic and liver erythropoiesis but normal megakaryopoiesis (refs. 21 and 22 and see below).

Recombinant Epo has been shown to increase human megakaryocytic colony formation (23), as well as murine platelet production (24, 25). On the other hand, thrombopoietin (Tpo) stimulation in syngeneic bone marrow transplant recipients resulted in increased numbers of erythroid blast-forming units (BFU-E), as well as higher erythrocyte nadir counts and accelerated recovery of erythrocytes (17, 26). To address the potential regulation of erythropoiesis by Tpo, we have generated EpoR−/− mice and examined erythroid progenitor proliferation and differentiation in the presence of recombinant Tpo. Our results demonstrate that EpoR−/− erythroid progenitor cells express c-MPL and that exogenous Tpo in combination with either Kit ligand (KL) or interleukin (IL) 3 plus IL-11 rescues BFU-E proliferation and terminal differentiation. These findings indicate that a signal provided by Tpo–c-MPL interaction to cultured erythroid precursor cells can substitute for that normally transmitted through the EpoR and raises the possibility that erythropoiesis might be regulated in part *in vivo* by Tpo.

METHODS

Generation of Targeted Embryonic Stem Cell Clones and EpoR Null Mice. The splice acceptor site of exon II and all of exon III of the EpoR (GenBank accession no. X53081) were excised (*Xho*I to *Bam*HI) and replaced by a neomycin cassette (Fig. 1*A*). Twenty-five micrograms of the targeting construct, which also contained a thymidine kinase cassette for negative selection, was linearalized by *Not*I digestion and electroporated into J1 embryonic stem cells, and recombinants were identified as described (27). Individual clones were selected and injected into C57BL/6 blastocysts, which were transferred into C57BL/6 or BALB/c pseudopregnant foster mothers. Chimeras were backcrossed to C57BL/6 mice and germ-line transmission was detected by Southern blot analysis of tail DNA as described (27). Genotyping was performed both by Southern blot analysis using *Avr*II digestion followed by probing with the 550-bp *Bam*HI–*Avr*II fragment of exon VIII (Fig. 1*B*) or by PCR (data not shown). Staged embryos were removed on embryonic days (E) 10 to E15 (the morning of vaginal plug discovery was taken as E0) and disected free of maternal tissue without disrupting the placenta or visceral yolk sac (27).

Analysis of Peripheral Blood, Yolk Sac, and Liver Cells. The embryos were rinsed in PBS to remove maternal red cells and exsanginated via the umbilical and vitelline vessels in 1 ml of

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Abbreviations: EpoR, erythropoietin receptor; Epo, erythropoietin; Tpo, thrombopoietin; IL, interleukin; KL, Kit ligand; E, embryonic day(s); RT–PCR, reverse transcription-coupled PCR; CFU-E, erythroid colony-forming unit(s); BFU-E, erythroid blast-forming unit(s).

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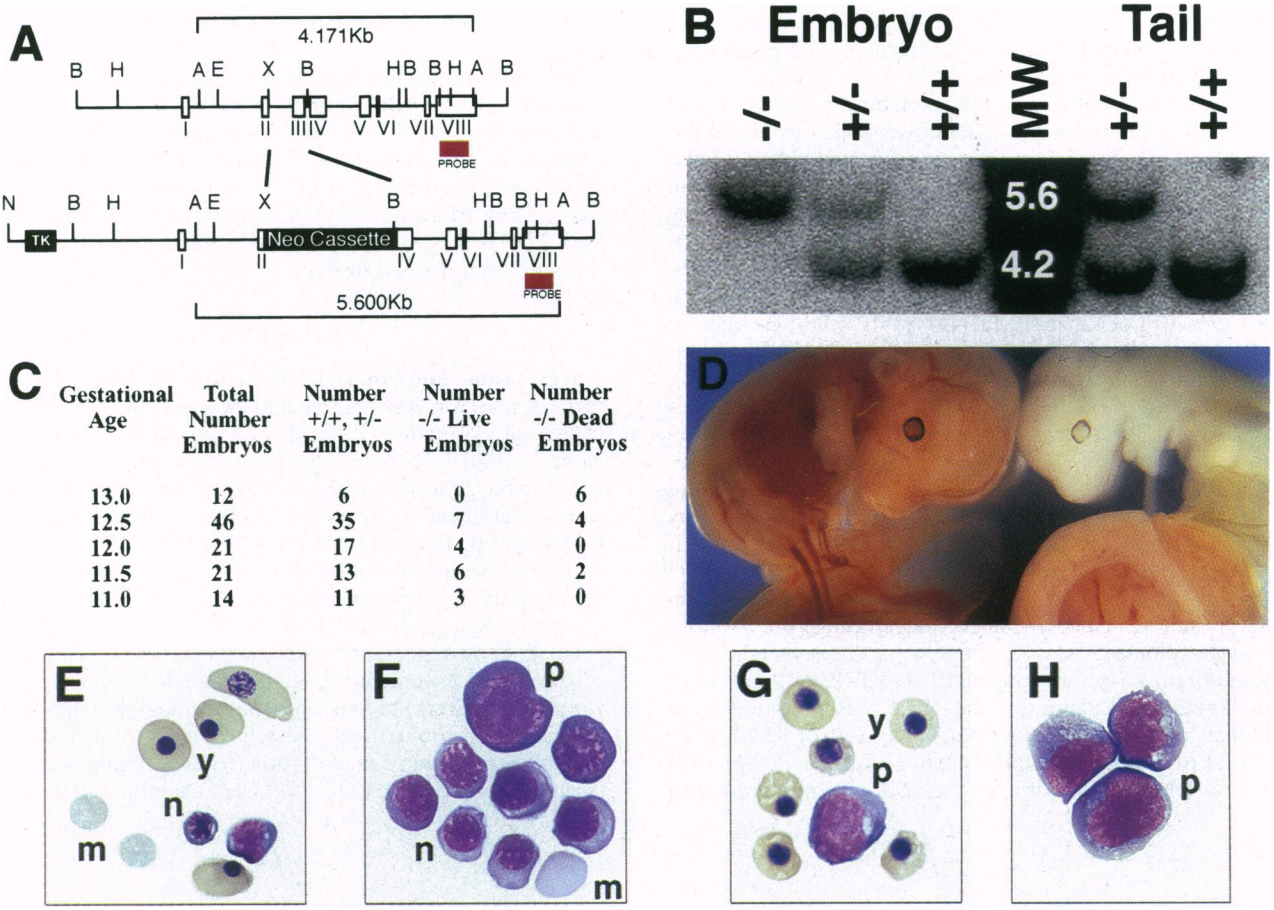

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Gestational Age	Total Number Embryos	Number +/+, +/- Embryos	Number -/- Live Embryos	Number -/- Dead Embryos
13.0	12	6	0	6
12.5	46	35	7	4
12.0	21	17	4	0
11.5	21	13	6	2
11.0	14	11	3	0

FIG. 1. (*A*) Targeted disruption of the mouse EpoR gene. The EpoR locus and partial restriction map is shown (*Upper*) with the targeting construct (*Lower*). Tk, PGK-thymidine kinase cassette. Restriction enzymes are as follows: B, *Bam*HI; H, *Hin*dIII; A, *Avr*II; E, *Eco*RI; X, *Xho*I; N, *Not*I. (*B*) Southern blot analysis of tail and embryo genomic DNA demonstrates the 4.2-kb wild-type and 5.6-kb mutant alleles. (*C*) Viability and genotypic analysis of embryos during gestation (days). (*D*) Phenotypic comparison of E13 wild-type (*Left*) and EpoR homozygous mutant (*Right*). (*E* and *F*) Wild-type day 12.5 peripheral blood (E) or fetal liver (F) May–Grunwald–Geimsa-stained cytospins. Mature primitive yolk sac-derived erythroid cells (y) as well as fetal liver-derived definitive erythroid cells, including proerythroblasts (p), normoblasts (n), and mature enucleated red cells (m) are present. (*G* and *H*) EpoR−/− peripheral blood (G) or fetal liver (H) May–Grunwald–Geimsa-stained cytospins. Mature primitive yolk sac-derived erythroid cells (y) as well as liver-derived definitive proerythroblasts (p) but no mature definitive liver-derived erythroid cells are present.

PBS by surgical removal of the placenta. Single-cell suspensions of fetal liver were prepared by gentle passage through a 26-gauge needle, and single-cell suspensions from yolk sacs were prepared by incubation for 1 h in 0.1% collagenase in 20% fetal calf serum/PBS at 37°C followed by passage through 25- and 26-gauge needles. Cell number and viability were determined by trypan blue exclusion. Peripheral blood, yolk sac, and liver cytocentrifuge preparations were made by centrifugation at 400 rpm in a Shandon Cytospin 3 rotor for 4 min and stained in May–Grunwald–Geimsa (27).

Preparation of RNA, DNA, and cDNA from Cells and Tissue. Total RNA was prepared from tissues using a commercial kit (RNAzol B, Tel-Test, Friendswood, TX). Genomic DNA was prepared from tails and carcasses by standard methods. cDNA was prepared using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) by the manufactures recommendations.

Primer Oligonucleotides for PCR. PCRs were performed using *Taq* DNA polymerase (Boehringer Mannheim) according to the manufacture's instructions. Reactions were performed with equal percentages of total RNA (5%) obtained from homozygous mutant or wild-type littermate embryos. Each reaction mixture contained Mg^{2+} at a final concentration of 2 mM, with 0.2 μM oligonucleotide primers, all four dNTPs (each at 250 μM), and 0.1 μCi [α-^{32}P]dCTP (3000 Ci/mM; 1 Ci = 37 GBq; Amersham). Reactions were controlled by including amplification primers for β-actin or HPRT. Reactions were incubated in a Perkin–Elmer 9600 thermal cycler at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. Aliquots were removed after a variable number of cycles and electropheresed through a 6% polyacrylamide gel. Image analysis was performed using a PhosphoImager and IMAGEQUANT software (Molecular Dynamics). All analyses were performed on samples within the linear range of amplification. The following primer sets were used: EKLF, 5′-TCGCCGGAGACGCAGGCT-3′ and 5′-CCCAGTCCTTGTGCAGGA-3′; c-MPL, 5′-CCATCGATCTAGAGCTGCGCCCCCGAGC-3′ and 5′-CGGAATTCGTAGGAATGTATAGGTCTGC-3′; TPO, 5′-TCTGTCCAGCCCCGTAGGTC-3′ and 5′-GTTCCATCCACAGTCCGTG-3′ (28); EpoR, 5′-GGGATGGACTTCAACTACAG-3′ and 5′-TGATGCGGTGATAGCGAGGAGA-3′; GATA2, 5′-CGGAATTCGACACACCACCCGATACCCACCTAT-3′ and 5′-CGGAATTCGCCTACGCCATGGCAGTCACCATGCT-3′ (29); GATA1, 5′-CATTGGCCCCTTGTGAGGCCAGAGA-3′ and 5′-ACCTGATGGAGCTTGAAATAGAGGC-3′ (30); α-globin, 5′-CTCTCTGGGGAAGACAAAAGCAAC-3′ and 5′-GGTGGCTAGCCAAGGTCACCAGCA-3′ (29); βmajor-globin, 5′-CTGACAGATGCTCTCTTGGG-3′ and 5′-CACAACCCCAGAAACAGACA-3′ (29); βH1-globin, 5′-AGTCCCCATGGAGTCAAAGA-3′ and 5′-CTCAAGGAGACCTTTGCTCA-3′ (29);

β-actin, 5′-GTGACGAGGCCCAGAGCAAGAG-3′ and 5′-AGGGGCCGGACTCATCGTACTC-3′ (29).

***In Vitro* Progenitor Colony Assay.** Progenitor cell assays were performed in 0.9% methylcellulose, Dulbecco's modified Eagle's medium, 20% fetal calf serum, and recombinant growth factors. About 7.5% of total yolk sac or fetal liver cells was plated per 35-mm Petri dish. For erythroid colony-forming unit (CFU-E) analysis, 5×10^5 cells from wild-type or 2×10^4 cells from mutants were cultured for 2 days in Epo (2 units/ml) or partially purified recombinant Tpo (500 ng/ml; a gift from Amgen). For CFU-mast analysis, 1×10^5 cells from wild-type or 0.5×10^4 cells from mutants were cultured for 7–10 days in Kit ligand (KL) (50 ng/ml). For BFU-E analysis, 0.5–1.0 $\times 10^5$ wild-type cells or 0.25–0.5 $\times 10^4$ mutant cells were cultured for 7 days in one of four growth factor combinations: KL (50 ng/ml)/Epo (2 units/ml), KL/Tpo (500 ng/ml), and IL-3 (10 ng/ml)/IL-11 (25 ng/ml) plus either Epo or Tpo. Megakaryocyte (CFU-meg), granulocyte–macrophage (CFU-GM), granulocyte (CFU-G), and macrophage (CFU-M) colony-forming units were scored in duplicate plates containing IL-3, IL-11, and Tpo. In an independent assay for CFU-GM, 5×10^4 cells were cultured for 7 days in granulocyte–macrophage colony-stimulating factor (GM-CSF) (5 ng/ml). Lineage identity was confirmed by microscopic examination of May–Grunwald–Geimsa-stained cytocentrifuge preparations from single colonies.

Reverse Transcription-Coupled PCR (RT–PCR) Analysis of Single Erythroid Colonies. Total RNA was prepared from individual "red" colonies generated in KL/Epo or KL/Tpo, by lysis in 20 μl of water containing 0.8% Nonidet P-40, 5 mM DTT, and RNAsin (0.5 unit/ml). cDNA was prepared with Moloney murine leukemia virus-derived reverse transcriptase and semi-quantitative RT–PCR was performed as described above.

Immunofluoresence Staining of Fetal Liver with GATA-1 Monoclonal Antibodies. Immunofluoresence for GATA-1 was performed on fetal liver cell suspensions from day E12.5 embryos as described (M. J. Weiss, personal communication) using a rat monoclonal antibody to murine GATA-1 (NG1, Santa Cruz Biotechnology). Cell nuclei were counterstained with 0.001% 4′6-diamidino-2-phenylindole (DAPI, HCl) for 5 min and then washed in PBS.

RESULTS

Generation and Analysis of EpoR−/− Mice. An EpoR targeting vector was constructed by replacing the splice acceptor site of exon II and all of exon III with a neomycin cassette (Fig. 1*A*). After electroporation and drug selection, G418- and gancyclovir-resistant J1 embryonic stem clones were isolated and genotyped by Southern blot analysis using a 0.55-kb probe derived from exon VIII (Fig. 1*B*). Six (of 138) embryonic stem cell clones with the targeted disruption of the EpoR were injected into blastocysts. One of the resulting chimeras transmitted the EpoR-targeted mutation through the germ line. In agreement with published data (21, 22), heterozygous mice were viable, fertile, and phenotypically normal for all parameters studied, including hemoglobin, hematocrit, mean red cell count, mean red cell volume (MCV), and mean red cell hemoglobin concentration (MCHC) (data not shown). Staged litters (from E10 to E15) were examined to determine

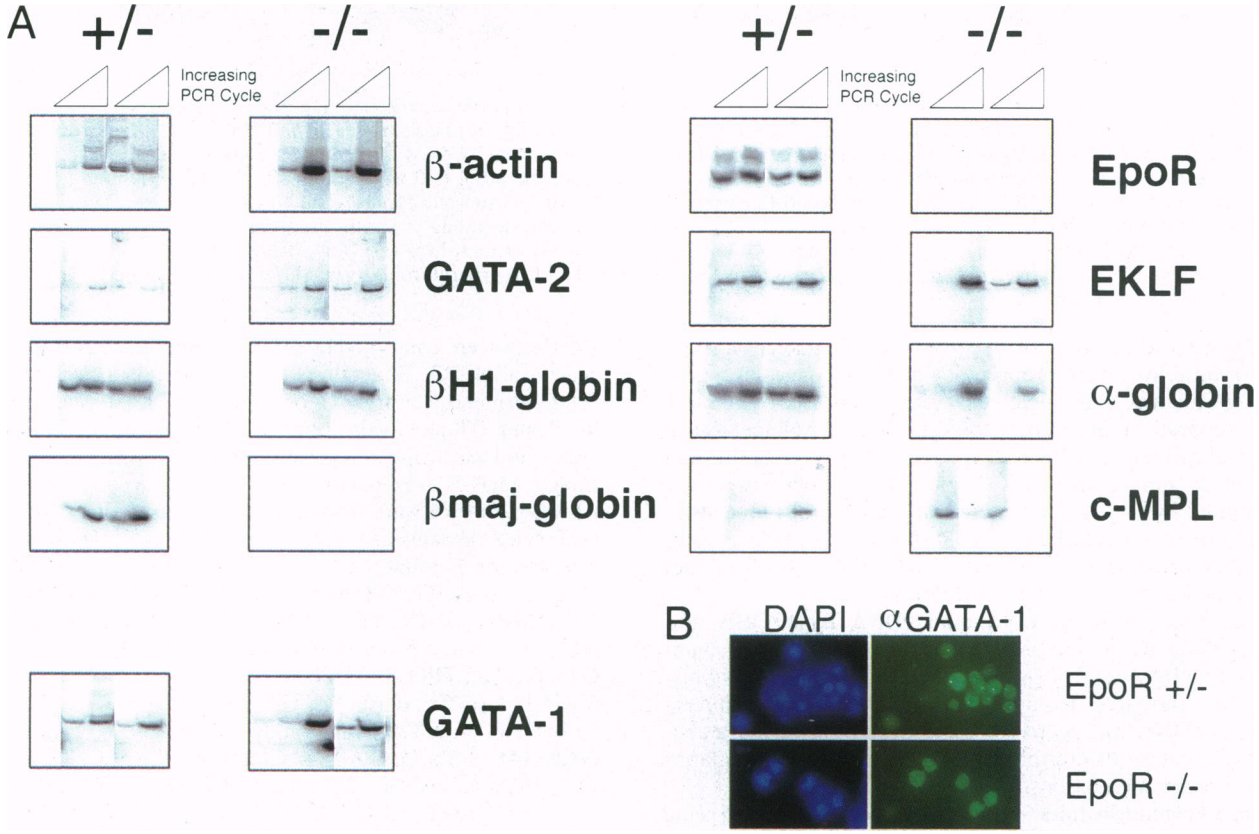

FIG. 2. (*A*) RT–PCR analysis of globins, cytokine receptors, erythroid transcription factors, and other gene products. Semi-quantitative RT–PCR was performed on E12 peripheral blood RNA from two wild-type and mutant samples using 5% of the total RNA sample. Aliquots were removed every two cycles over the linear range of amplification (determined for each primer set). Wild-type cell preparations contain approximately 20-fold greater numbers of cells compared with mutants and cell cycle was adjusted accordingly. (*B*) GATA-1 immunofluorecence of fetal liver cells. Cells stained by DAPI (*Left*) or rat anti-mouse GATA-1 (*Right*). Wild-type cells (*Upper*) stain with approximately equal intensity to EpoR−/− cells (*Lower*) although areas of focal concentration of GATA-1 were absent in the later. Controls incubated with anti-rat fluorescein isothiocyanate alone were devoid of staining (data not shown).

Table 1. Fetal liver *in vitro* progenitor assay

Colony type	Growth factor(s)	No. of colonies EpoR−/−	No. of colonies EpoR+/− or +/+
CFU-E	Epo	0 ± 0	7270 ± 3293
	Tpo	0 ± 0	230 ± 86
BFU-E	KL	0 ± 0	12 ± 18
	KL/Epo	0 ± 0	199 ± 74
	KL/Tpo	20 ± 10	107 ± 79
	IL-3/IL-11/Tpo	21 ± 12	74 ± 41
Ery/Meg	KL/Tpo	19 ± 8	60 ± 37
Ery/Mix	IL-3/IL-11/Tpo	38 ± 11	108 ± 53
Mast cell	KL	15 ± 7	117 ± 61
Mega	IL-3/IL-11/Tpo	6 ± 4	21 ± 14
GM	IL-3/IL-11/Tpo	48 ± 34	205 ± 69
Macrophage	IL-3/IL-11/Tpo	94 ± 30	245 ± 79

Equivalent percentages (7.5%) of total liver cells were plated in methylcellulose (EpoR−/− livers contain approximatly 20-fold fewer cells). Colonies were scored from duplicate plates and are expressed ± SD. CFU-E, erythroid colony-forming unit; Ery, erythrocyte; Meg or Mega, megakaryocyte; GM, granulocyte–macrophage.

the timing and cause of death of EpoR null embryos. The gestational age, genotype, and viability of embryos is presented in Fig. 1*C*.

Some heterogeneity was evident in the EpoR−/− phenotype early in development. Prior to the onset of fetal liver erythropoiesis, E10–E11.5 EpoR−/− embryos were often viable but invariably pale. Histologic analysis of the yolk sacs revealed that blood islands were reduced in number and size (data not shown and refs. 21 and 22) but not absent. EpoR−/− embryos exsanguinated into PBS by disruption of the uterine and vitelline vessels demonstrated 10- to 20-fold fewer embryonic red cells on average than normal littermates (data not shown). However, the cells that were present had a normal appearance after histochemical staining (y in Fig. 1 *E* and *G*) suggesting that a proportion of EpoR−/− embryonic erythroid precursors were able to complete a normal program of proliferation and differentiation. EpoR−/− embryos died of anemia between E11.5 and E13, in agreement with recent reports (21, 22). The fetal livers of EpoR−/− embryos were smaller in size and paler compared with littermates (Fig. 1*D*). Hematoxylin/eosin-stained histological sections of EpoR−/− fetal liver revealed hypocellularity and nuclear fragmentation consistent with apoptosis, as described (refs. 21 and 22 and data not shown). May–Grunwald–Geimsa-stained fetal liver and peripheral blood cytocentrifuge preparations revealed normal appearing proerythroblasts, but no hemoglobinized normoblasts or enucleated red cells (Fig. 1 *E–H*). No gross morphological abnormalities were identified in the brain, which is reported to express EpoR (31). Thus, some primitive hematopoiesis occurs in the absence of the EpoR, but definitive hematopoiesis requires the receptor.

Gene Expression in Peripheral Blood and Fetal Liver Hematopoietic Cells. Since the number of erythroid cells in EpoR−/− mice was limiting, gene expression was evaluated by semi-quantitative RT–PCR and compared with normal littermate controls. Representative results using E12 peripheral blood as a source of both embryonic and definitive red cell RNA are presented in Fig. 2. As expected, no EpoR PCR product was observed from RNA samples of mutant mice using different primer pairs (Fig. 2 and data not shown). α- and βH1-globin RNAs were abundant and approximately equal in samples from EpoR−/− mice and littermate controls, as was anticipated based on the presence of well-hemoglobinized embryonic red cells in smears of peripheral blood (Fig. 1*G*). By contrast, βmaj-globin RNA expression was absent in EpoR−/− embryos. The expression of transcripts encoding

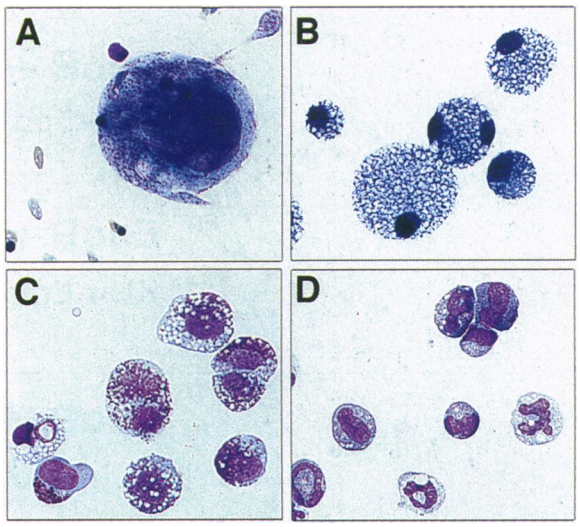

FIG. 3. Normal megakaryocytes, mast cells, macrophages, and granulocytes derived from EpoR−/− colonies. (*A*) Megakaryocyte derived from an EpoR−/− erythroid–megakaryocyte colony grown in IL-3, IL-11, and Tpo. (*B*) Mast cells derived from an EpoR−/− colony grown in KL. (*C*) Macrophages derived from an EpoR−/− colony grown in IL-3, IL-11, and Tpo. (*D*) Granulocytes derived from an EpoR−/− colony grown in IL-3, IL-11, and Tpo. (*A*, ×200; *B–D*, ×600.)

erythroid transcription factors, including EKLF, GATA-1, and GATA-2, was not appreciably different between mutant and wild-type littermates. To verify that GATA-1 was expressed in EpoR−/− cells, fetal liver cytospin preparations were stained with a GATA-1 specific monoclonal antibody. As anticipated from the RT–PCR analysis (Fig. 2*A*) and RNase protection (data not shown), expression of GATA-1 was detected in EpoR−/− cells, although focal accumulations were decreased compared with wild-type cells (32). Finally, c-MPL expression was present in both mutant and littermate control samples (Fig. 2*A*), thereby demonstrating that its expression does not require EpoR signaling.

***In Vitro* Progenitor Colony Assays.** Mast cells and megakaryocytes (33) express the EpoR, where as granulocytes and macrophages do not. To study the effects of the null mutation on these cell lineages, progenitor colony assays were performed. Nonerythroid progenitors were present in normal numbers when corrected for total number of fetal liver cells plated (Table 1). Moreover, May–Grunwald–Geimsa-stained cells derived from these colonies appeared normal (Fig. 3). These results suggest that the EpoR is not required for the development of these lineages.

Tpo Rescues Erythroid Differentiation of BFU-E. We characterized further the proliferative and differentiative capacity of EpoR−/− erythroid progenitors. No CFU-E or BFU-E colonies were derived from EpoR−/− fetal livers or yolk sacs in the presence of recombinant KL, Epo, Tpo, or KL/Epo (Table 1). Surprisingly, BFU-E colonies were detected in cultures from EpoR−/− embryos grown in the presence of either KL/Tpo or IL-3/IL-11/Tpo (Fig. 4*A* and Table 1). Cells from these colonies contained mature enucleated erythrocytes (m) and late normoblasts (n), which were indistinguishable from wild-type BFU-E grown in KL and Epo (Fig. 4*B*). Furthermore, colonies rescued in the presence of recombinant KL/Tpo expressed GATA-1 and wild-type levels of βmaj-globin RNA (Fig. 4*C*). EpoR−/− BFU-E express c-MPL RNA at similar (Figs. 2*A* and 4*C*), albeit low, levels compared with wild-type BFU-E (Fig. 4). Thus, Tpo signaling through the c-MPL receptor rescues the block to proliferation and differentiation in EpoR−/− erythroid precursors.

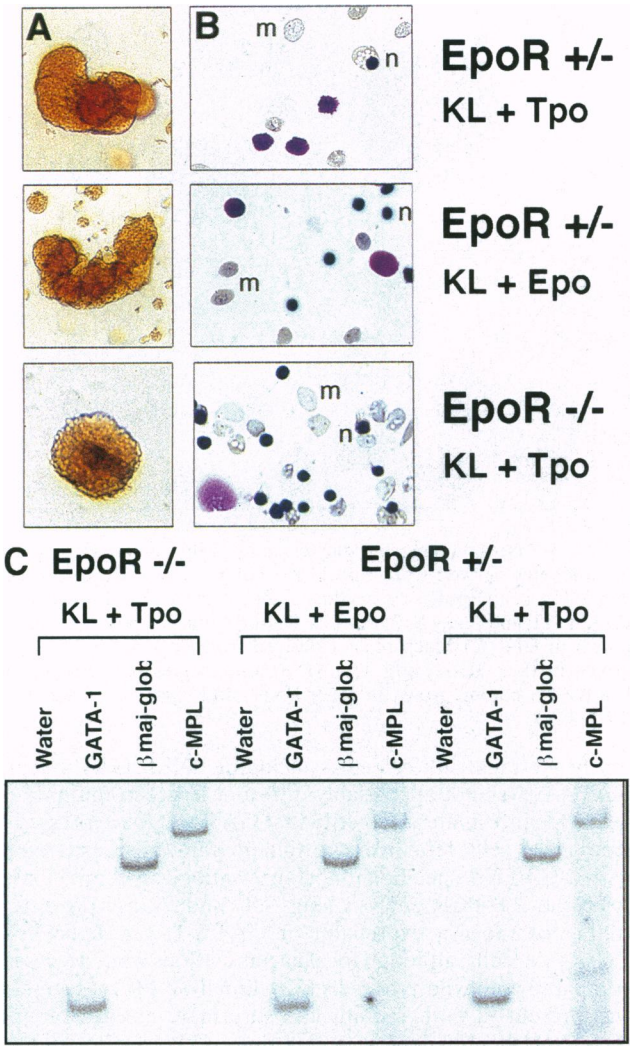

FIG. 4. Tpo rescues erythroid differentiation of EpoR−/− BFU-E. (*A*) Photographs of hemoglobinized E12.5 red BFU-E in 7-day-old methylcellulose cultures of fetal liver cell suspensions. EpoR ± cells were cultured in either KL and Tpo (*Top*) or KL and Epo (*Middle*) and EpoR−/− cells were cultured in KL and Tpo (*Bottom*). (×100.) No red colonies were generated from EpoR−/− cells grown in KL and Epo (see Table 1). (*B*) May–Grunwald–Geimsa-stained cytocentrifuge preparations of the colonies depicted in *A*, demonstrating well hemoglobinized late normoblasts (n) or mature enucleated erythrocytes (m). (×600.) (*C*) RT–PCR analysis of pools of eight BFU-E of the morphology depicted in *A*. EpoR−/− BFU-E grown in KL and Tpo express c-MPL and equivalent levels of GATA-1 and βmaj-globin compared with EpoR ± BFU-E grown in KL and Epo.

DISCUSSION

To study the role of the erythropoietin receptor in the development of the hematopoietic system, EpoR−/− mice were generated by homologous recombination. Our results confirm two recent reports (21, 22) that the EpoR is essential for the proliferation and differentiation of definitive erythroid cells in the fetal liver. Proerythroblasts were present, but more differentiated erythroid cells were absent in EpoR−/− fetal livers, possibly because of programmed cell death (ref. 22, results herein).

Expression of the EpoR is not required for erythroid lineage commitment since BFU-E are present in normal (or slightly increased) numbers in the fetal livers of EpoR−/− mutant embryos. Although these BFU-E could not be detected under routine culture conditions in Epo ± KL or IL-3 and IL-11 because of a failure to respond to Epo, their presence was unmasked by culture in recombinant Tpo and KL or Tpo with IL-3 and IL-11. These rescued colonies appeared morphologically normal and expressed βmaj-globin. Thus, defective erythropoiesis can be corrected *in vitro* by the addition of Tpo. Similar results were obtained by culturing EpoR−/− cells with a retroviral vector expressing a constitutively active EpoR (21), confirming progenitor formation occurs independently of EpoR−/− *in vivo*.

No CFU-E or BFU-E were detected when EpoR−/− fetal liver or yolk sac progenitor cells were cultured in recombinant KL or IL-3 and IL-11 either alone or in the presence of Epo. This observation contrasts with recent reports describing a significant number of BFU-E derived from EpoR−/− fetal liver samples cultured in pokeweed mitogen-stimulated spleen conditioned medium (SCM) (21, 22). In light of our results with recombinant Tpo, it seems likely that the mixture of nonrecombinant factors present in SCM includes either sufficient amounts of Tpo or other cytokines that are also capable of stimulating these cells. The rescue of definitive erythroid colonies has important implications for developmental regulation of erythropoiesis and megakaryopoiesis, as well as for the specificity of hematopoietic cytokine receptor signaling.

EpoR is expressed on nonerythroid hematopoietic cells, such as mast cells and megakaryocytes. Nonetheless, their development proceeds normally in EpoR−/− mice. Similarly, the brain, an additional site of EpoR expression, appeared grossly and histologically normal. Thus, the EpoR is either functionally redundant in these cell types or does not play a critical role in the development of these tissues.

The absence of βmaj-globin mRNA in fetal liver samples suggests either that the cells arrest (and/or undergo apoptosis) prior to the onset of β-globin expression or that the EpoR is necessary for β-globin expression. Cells derived from GATA-1 null ES cells, which are also arrested at the proerythroblast stage, express βmaj-globin (29). Therefore, signaling mediated by EpoR may play a more direct role in βmaj-globin expression, perhaps by altering the activity of one or more critical transcription factors. In this regard, RNA encoding EKLF, a transcription factor necessary for proper β-globin expression (27), is expressed normally in the absence of the EpoR (Fig. 2*A*). Moreover, by gel-shift analysis of nuclear extracts of EpoR−/− erythroid cells, we have detected EKLF protein (data not shown). It is not currently possible, however, to be certain that EKLF protein functions normally in EpoR−/− cells. The development of EpoR−/− precursors into mature erythroid cells in response to Tpo suggests that signaling through c-MPL is able to activate cytoplasmic and nuclear proteins in common with those activated by EpoR.

Our studies are consistent with an overlapping function of the EpoR and c-MPL. The moderate degree of residual embryonic erythropoiesis in EpoR−/− embryos may reflect stimulation of embryonic precursors by relatively high concentrations of Tpo in blood islands (as detected by RT–PCR, data not shown). Alternatively, other growth factors such as BMP-4, activin, fibroblast growth factors, and KL may partially compensate for the loss of EpoR in embryonic cells. Breeding to generate EpoR−/− c-MPL−/− embryos will distinguish these two alternatives. In addition, it is possible that the triggering of c-MPL or EpoR on cells of the megakaryocyte lineage may result in partial redundancy with respect to platelet production. Although many cytokines have some activity on megakaryocytes, some of the residual megakaryocyte and platelet production in c-MPL−/− mice may have resulted from Epo–EpoR-generated signaling on megakaryocyte progenitors.

One model to account for the rescue of erythroid cells by Tpo relies on the coexpression of both of these cytokine receptors, possibly by a common erythroid–megakaryocyte progenitor. We speculate that these progenitors may be sig-

naled by Epo and/or Tpo via the EpoR and c-MPL receptors, respectively. This model predicts the following. (*i*) Tpo should rescue EpoR−/− erythroid cells *in vitro*, as shown here. (*ii*) Tpo present in the developing EpoR−/− embryo may be sufficient for limited erythroid proliferation and differentiation and thus may account for the primative erythrocytes present, as well as the definitive progenitors detected by Tpo rescue. (*iii*) Epo may stimulate megakaryocytic progenitors or some platelet production in c-MPL−/− mice (19, 20). (*iv*) *In vivo* and *in vitro* administration of Epo or Tpo would be expected to increase platelet or erythroid counts, respectively, which has also been reported (26, 34, 35).

The coexpression of c-MPL (Fig. 4*C*) and EpoR on erythroid progenitors, as well as megakaryocytes (33), suggests a common ancestry, consistent with previous data demonstrating expression of an overlapping cohort of transcription factors (7, 8) in the two lineages. Based on the homologies between these receptors, ligands, and signaling cascades, erythroid and megakayocytic lineages may have arisen during evolution from a common primordial cell. In this cell, one ligand with structural similarity to Epo and Tpo signaled a receptor to stimulate proliferation and differentiation. With the requirement of distinct cellular function, such as oxygen delivery and hemostasis, two cell populations were derived. The signaling apparatus from the primordial cell was also likely maintained on both lineages. The cytoplasmic domains of EpoR and c-MPL are related; EpoR contains eight tyrosine residues and c-MPL contains five (10). Furthermore, a similar subset of SH2 domain containing cellular proteins, including Jak2, Stat5, Shc, SHPTP-1 (HCP, motheaten), and SHPTP-2 (Syp) are phosphorylated upon stimulation with either ligand (36, 37). The presence of Epo and Tpo receptors on both the erythroid and megakaryocytic lineages suggests a coordinated response to periods of significant blood loss. Such periods of blood loss (e.g., from trauma) trigger hemostasis and a coordinated stimulation of erythroid and megakaryocytic populations by Epo and Tpo might provide for a rapid and efficient response for the organism. Thus, the rescue of EpoR−/− cells by Tpo indicates a commonality between the Epo and Tpo pathways and suggests that multiple ligand–receptor interactions can activate similar signaling pathways in hematopoietic development.

We thank R. Shivdasani, K. Kotkow, C. Browne, J. Scorer, and M. Rosenblat for technical assistance; B. Paw and D. Ransom for review of this manuscript; C. Brugera for the Technicon analysis of adult blood, Amgen for the Tpo; R. Hughes for isolation of the EpoR genomic clones. M.W.K. is a Clinician-Scientist supported through the MRC of Canada. A.C.P. is a traveling scholar of the Lucille P. Markey Foundation.

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