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Generation of Committed Erythroid BFU-E and CFU-E Progenitors Does Not Require Erythropoietin or the Erythropoietin Receptor

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Summary

Erythropoietin (EPO) is the principal growth factor regulating the production of circulating erythrocytes. We introduced null mutations into both *Epo* and the EPO receptor (*EpoR*) gene. Both heterozygotes appeared normal. Homozygous animals exhibited reduced primitive erythropoiesis and died around embryonic day 13, owing to failure of definitive fetal liver erythropoiesis. Both types of mutations exhibited identical phenotypes, indicating that EPO and the EPOR are crucial for definitive erythropoiesis in vivo and that no other ligands or receptors can replace them. Committed erythroid BFU-E and CFU-E progenitors were present in both homozygous fetal livers. Thus, neither EPO nor the EPOR is required for erythroid lineage commitment or for the proliferation and differentiation of BFU-E to CFU-E progenitors. EPO and the EPOR are crucial in vivo for the proliferation and survival of CFU-E progenitors and their irreversible terminal differentiation.

Introduction

Blood cells are formed continuously from a small group of pluripotent stem cells that generate progenitors committed to one or a few hematopoietic lineages. Control of these differentiation pathways is determined, in part, through specific growth factors binding to their cognate receptor(s) (Metcalf, 1989). Erythropoietin, a 34 kDa glycoprotein hormone, is the crucial growth factor that promotes the survival, proliferation, and differentiation of mammalian erythroid progenitor cells (Krantz, 1991). EPO is remarkably specific for erythroid progenitors, owing to the tissue-specific expression of its receptor (EPOR) (D'Andrea et al., 1989).

Within the mammalian embryo, erythropoiesis occurs initially in the yolk sac blood islands (primitive erythropoiesis) and then shifts to the fetal liver, spleen, and eventually to the bone marrow (definitive erythropoiesis). EPO is produced primarily by the fetal liver during midgestation and by the kidney from late gestation to adulthood (Koury et al., 1988a, 1988b, 1991). The source of EPO prior to the fetal liver stage and the function of EPO in primitive erythropoiesis are not clear. Maternal EPO might support fetal erythropoiesis via transfer across the placenta (Koury et al., 1988a); EPOR has been detected in placenta (Anagnostou et al., 1994), where it may function in transepithelial transport of EPO.

Committed erythroid progenitor cells that specifically respond to EPO are detected by the formation of discrete erythroid colonies following in vitro culture in plasma clots or methylcellulose and are termed the colony-forming unit-erythroid (CFU-E) and the burst-forming unit-erythroid (BFU-E) (Gregory and Eaves, 1977; Gregory and Eaves, 1978). As demonstrated by thymidine suicide experiments, the CFU-E is a rapid dividing cell that is highly responsive to low concentrations of EPO and gives rise to erythroblast colonies of 8–49 cells in 7 days (human) or of 8–64 cells in 2 days (mouse). The BFU-E is a more immature cell that divides less frequently. This cell requires EPO as well as other growth factors (Emerson et al., 1985), such as interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF), and stem cell factor (SCF) to develop into grouped clusters of erythroblasts or larger colonies (bursts) of greater than 500 erythroblasts by 15 days (human) or 7–10 days (mouse) in culture (Gregory and Eaves, 1977, 1978). Development from the earliest BFU-E to the latest CFU-E is a continuous process, with an intermediate progenitor often termed the mature BFU-E (Gregory and Eaves, 1977, 1978). The sensitivity of these progenitors to EPO is transient. Beyond the late basophilic erythroblast stage, the level of the EPOR drops, and the cells are no longer dependent on EPO for continued maturation (Koury and Bondurant, 1988). While EPO is thought to be required for proliferation from BFU-E to CFU-E and subsequent proliferation of the CFU-Es, the role of EPO in regulating erythroid differentiation is less defined.

We are interested in several aspects of the in vivo function of EPO and the EPOR. Specifically, we want to know whether maternal EPO can support fetal erythropoiesis; whether EPO plays a similar role in primitive and definitive erythropoiesis; whether EPO is an essential factor in determining the commitment of cells to the erythroid lineage; and whether EPO is crucial for proliferation and differentiation of some or all types of erythroid progenitor cells. To address these questions, a defined in vivo system is needed. We have generated mouse strains carrying null mutations in the *Epo* or *EpoR* genes by gene targeting in embryonic stem cells. We show that heterozygous mice lacking one allele of the *Epo* or *EpoR* genes are viable and fertile and have normal erythrocytes and leukocytes. Homozygous mice, however, die by embryonic days 13–15 (E13–E15) with severe anemia. By culturing fetal livers from both types of homozygous embryos, we showed that BFU-E and CFU-E progenitors were present. Thus, neither EPO nor the EPOR is required for erythroid lineage commitment or for the proliferation and differentiation of BFU-E to CFU-E progenitors. Our results reveal an essential role for EPO in regulating definitive erythropoiesis by controlling processes such as proliferation, survival, and irreversible terminal differentiation of the late progenitors (CFU-E).

Results

Disruption of the Murine *Epo* and *EpoR* Genes

The murine *Epo* gene is encoded by five exons within 2.7 kb of genomic DNA (McDonald et al., 1986; Shoemaker and Mitsock, 1986). The translational start site (ATG) is located in the first exon. We constructed a replacement-type targeting vector, pEpo-M1, that contains 2.5 kb upstream and 5.0 kb downstream flanking sequences of the *Epo* gene, and the PGKneopA cassette for positive selection (Figure 1A). Exons 2–5 were completely deleted so that no EPO protein could be produced from the mutant allele. After electroporation and drug selection, G418-resistant (G418^r) clones from embryonic stem (ES) cells were isolated (see Experimental Procedures). DNAs from individual clones were prepared and digested with EcoRV. After gel separation, filters were hybridized with an external probe from the 3′ flanking region, as indicated in Figure 1A. A 10.0 kb band corresponding to the targeted allele was detected in 10.8% of ES clones isolated (data not shown).

The *EpoR* gene is encoded by eight exons with the transmembrane domain encoded by exon 6. The minimum promoter is located 0.5 kb upstream of the transcription initiation site (Youssoufian et al., 1993). To silence the *EpoR* gene completely, we constructed the pEpoR-M1 plasmid, in which a 4.7 kb BamHI–XbaI fragment from the *EpoR* locus, including 1.6 kb from the 5′ flanking region and exons 1–6, was deleted and replaced with PGKneopA for positive selection (Figure 1B). Among 400 G418^r ES clones isolated, homologous recombination occurred at a frequency of 13.5%.

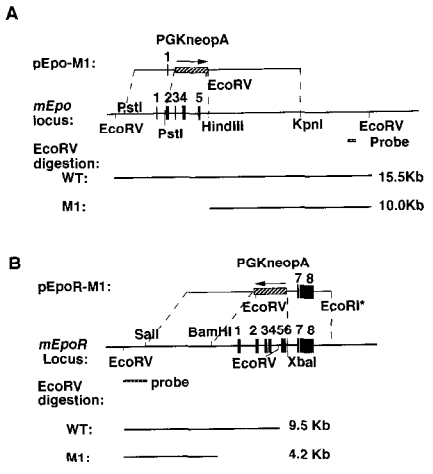

Figure 1. *Epo* and *EpoR* Genomic Maps and Targeting Vectors

(A) A restriction map of the genomic region containing the *Epo* gene is shown in the middle with the exons numbered. The targeting vector pEpo-M1 and the location of the PGKneopA cassette are shown at the top. By EcoRV digestion of genomic DNA, a 15.5 kb or a 10 kb band is detected from the wild-type or targeted allele, respectively, through use of an external probe located at the 3′ end of the gene.

(B) A restriction map of the *EpoR* locus containing eight exons is shown in the middle. At the top, the targeting vector and the location of the PGKneopA cassette are indicated. An external probe derived from the 5′ end of the gene, which hybridizes to a 9.5 kb and a 4.2 kb band, respectively, from the wild-type and targeted allele, is used to screen colonies and to determine the genotypes of the mice.

Epo$^{-/-}$ and *EpoR*$^{-/-}$ Mice Are Embryonic Lethal

Targeted ES clones carrying null mutations in the *Epo* or *EpoR* genes were injected into C57BL/6 or BALB/c blastocysts, and the resulting chimeric mice were backcrossed. Germline transmission of the mutant allele was detected by Southern blot analysis of tail DNA from F1 offspring. Four and two independently isolated ES clones transmitted the mutant *Epo* and *EpoR* alleles, respectively, to the germline.

Heterozygotes carrying deletions in either the *Epo* or *EpoR* genes appeared normal and were viable and fertile. The levels of blood hemoglobin, hematocrit, erythrocytes, and leukocytes and the protein composition of plasma were within normal ranges (data not shown). Heterozygotes derived from independent ES clones were then intercrossed to determine whether mice homozygous for either mutation were viable. No mice homozygous for the *Epo* or *EpoR* null mutation were found at 3 weeks of age; since no postnatal lethality was observed, the homozygous *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ mice were embryonic lethal. Examination of embryos at successive stages of embryonic development revealed that homozygous *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ embryos died between days 13 and 15 of gestation (data not shown). No difference was seen in mice derived from different ES clones or under different genetic backgrounds.

Definitive Erythropoiesis in *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ Fetal Liver Is Completely Impaired

Gross examination of mutant embryos revealed that the most dramatic consequence of the *Epo* and *EpoR* mutations was severe anemia. No significant differences were observed between wild-type and heterozygous littermates (data not shown). Homozygous embryos, however, developed normally until day 13 but appeared very pale; no red coloring characteristic of the normal fetal liver (Figure 2A) could be seen in the *Epo*$^{-/-}$ (Figure 2B) or *EpoR*$^{+/-}$ (data not shown) embryos. Placentas surrounding the homozygous embryos were of normal size but had fewer erythrocytes. Fetal livers were four to five times smaller than those in the normal littermates and were very pale (data not shown), indicating a significant reduction in erythrocyte production.

Histological examination of fetal liver sections obtained from wild-type and homozygous mutants allowed the evaluation and comparison of erythropoietic foci within the liver parenchyma. Erythropoietic islands were numerous in the livers from wild-type (Figure 3A, arrows) or heterozygous (data not shown) animals but were not identifiable in livers from *Epo*$^{-/-}$ embryos (Figure 3B). In the wild-type or heterozygous fetal livers, erythroid cells at all stages of differentiation could be seen. In contrast, the only erythroid cells seen in the homozygous fetal livers were large erythroblasts (see below). All liver sections also showed yolk sac–derived erythrocytes with characteristic abundant eosinophilic cytoplasm and large nuclei (Figures 3A and 3B, arrowheads). Many pycnotic nuclei, characteristic of apoptotic cells, were seen in liver sections from the *Epo*$^{-/-}$ em-

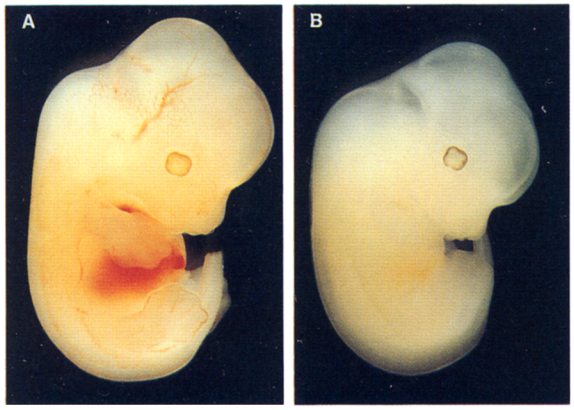

Figure 2. Phenotypic Comparison of E13 Littermates from $Epo^{+/-}$ Parents

The wild-type mouse is shown on the left (A), and the homozygous mutant littermate is at the right (B). The mutant mouse appears to be grossly normal except for the significant reduction of circulating erythrocytes and pale coloring of the liver.

bryos (Figure 3B, inset, small arrows), but few were visible in fetal livers from wild-type and $Epo^{+/-}$ embryos. Confirming these results, cytospin preparations from livers of normal (Figure 3C) and homozygous (Figure 3D) mice were analyzed by Giemsa staining. Erythropoietic cells at all stages of differentiation, including many fetal liver–derived nonnucleated erythrocytes (N) and few yolk sac–derived nucleated erythrocytes (Y), were seen in the wild-type liver preparation (Figure 3C), while only proerythroblasts (E) and yolk sac–derived nucleated erythrocytes (not shown) were identifiable in the preparation from the $Epo^{-/-}$ homozygotes (Figure 3D). The same results were obtained by analyzing the fetal livers from the $EpoR^{-/-}$ embryos, compared with those from the heterozygous and wild-type embryos (data not shown). These results indicate that EPO and EPOR are essential for controlling production of definitive erythrocytes in fetal liver.

Fetal Livers from $Epo^{-/-}$ Embryos Have BFU-E and CFU-E Progenitors

To determine at which stage of erythropoiesis EPO plays a crucial role, we quantified erythroid progenitors in fetal livers from $Epo^{-/-}$ embryos. Consistent with the anatomical and histological analyses, livers from $Epo^{-/-}$ fetuses had significantly decreased numbers of nucleated cells: an approximately 6-fold and 17-fold reduction at E12.5 and E13.5, respectively, compared with wild-type and heterozygous littermates (Figure 4A).

If the $Epo^{-/-}$ fetal livers do contain CFU-Es and BFU-Es, we should be able to detect benzidine-positive colonies after 2–3 days (for CFU-E) or 7–10 days (for BFU-E) of cultivation when exogenous EPO is supplemented, since these progenitors express the EPOR on their surface. For this purpose, single cell suspensions were prepared and plated in methylcellulose culture supplemented with a cocktail of growth factors including EPO, and benzidine-positive colonies were counted. As shown in Figures 4B and 4C, fetal livers from $Epo^{-/-}$ embryos contained erythroid progenitors, and the relative number of both CFU-E and BFU-E progenitors per 10^5 nucleated cells was significantly increased over that in the wild-type and heterozygous fetal livers (Figures 4B and 4C). These results suggested that the homozygous fetal livers were enriched in progenitor cells. Cytospin preparations of cloned BFU-Es showed only adult-type definitive erythroid cells (data not shown). The numbers of CFU-granulocyte/macrophage and CFU-megakaryocyte in cultures from homozygous $Epo^{-/-}$ fetal livers were normal (data not shown), suggesting that the function of EPO in vivo is restricted to the erythroid lineage. Interestingly, in the control fetal livers, progenitor cells undergo rapid proliferation and differentiation to generate more mature forms of erythroid cells (see Figure 3C), and the number of nucleated cells per fetal liver increased 4-fold from E12.5 to E13.5 (Figure 4A). On the other hand, the relative number of progenitors decreases with embryonic development: a 3-fold and a 2-fold reduction in the numbers of CFU-E and BFU-E per 10^5 nucleated cells, respectively, were observed (Figures 4B and 4C). In contrast, no significant increase in the number of nucleated cells was observed in livers from $Epo^{-/-}$ embryos, suggesting that, in the absence of EPO, fetal liver cells either become growth-arrested or undergo apoptosis (Figure 4A). Interestingly, the number of BFU-E progenitors in homozygous fetal livers stayed constant from E12.5 to E13.5, while the number of CFU-Es decreased almost 2-fold (Figures 4B and 4C), indicating that in the absence of EPO some of the CFU-Es underwent apoptosis, as suggested by the histologic analysis in Figure 3B. No differences were seen in either the size or the degree of hemoglobinization among erythroid colonies derived from the $Epo^{-/-}$ homozygotes or their wild-type or heterozygous littermates (data not shown).

Maternal EPO Cannot Support Fetal Erythropoiesis

It has been suggested that maternal EPO could cross the placental barrier and support fetal erythropoiesis (Koury et al., 1988a). In particular, in the $EPO^{-/-}$ embryos the amount might be sufficient to allow generation of BFU-Es and CFU-Es in the fetal liver yet insufficient to support CFU-E differentiation. However, higher concentrations of EPO (3 U/ml) are required for in vitro differentiation of the earlier BFU-Es than for the later CFU-Es (0.03 U/ml) (Gregory and Eaves, 1978). The facts that $Epo^{-/-}$ embryos died at E13–E15 gestation stages, right after the switch from primitive to definitive erythropoiesis, and that no differences were seen between $Epo^{-/-}$ and $EpoR^{-/-}$ mice, also suggested that maternal sources of EPO cannot substantially affect the definitive erythropoiesis in either $Epo^{-/-}$ or wild-type embryos.

To confirm that maternal EPO did not affect fetal erythropoiesis, $Epo^{+/-}$ heterozygous females, 0–7 days after detection of the vaginal plug, were injected daily with 3000 U/kg recombinant murine EPO (rmEPO). An approximately 20% increase in hematocrit was detected in these females after 8 days of injection (data not shown). However, no $Epo^{-/-}$ homozygotes were found in 83 embryos

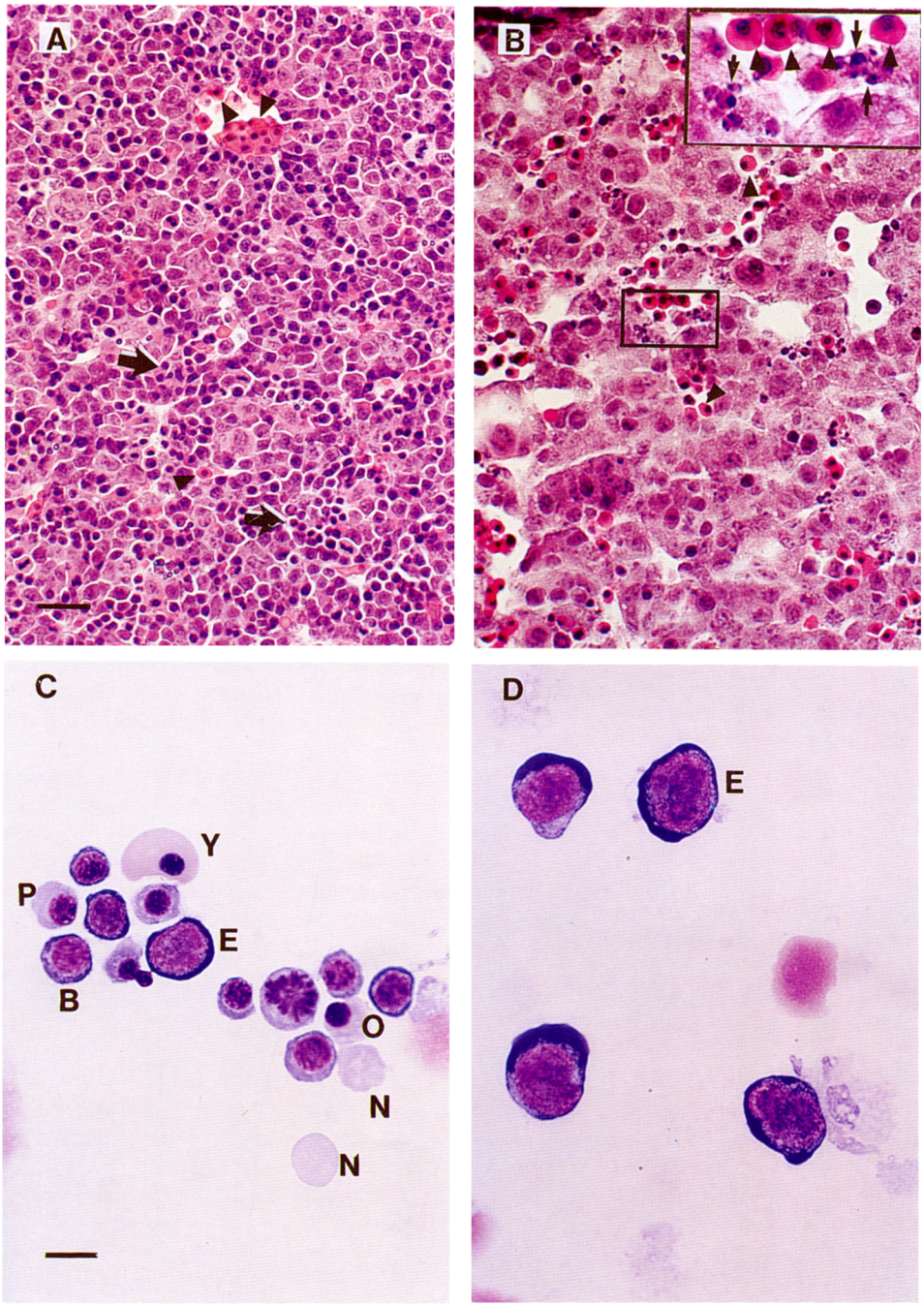

Figure 3. Liver Section and Liver Cell Cytospin

Preparations from wild-type and *Epo*$^{-/-}$ mutant fetuses.

(A) Hematoxylin- and eosin-stained section of liver from a E13 wild-type fetus. The liver contains numerous islands of erythropoietic cells (arrows) and some yolk sac–derived nucleated erythrocytes (arrow heads). Scale bar, 25 μm.

(B) Liver preparation from an E13 *Epo*$^{-/-}$ mutant fetus; hematoxylin and eosin staining. Liver cells are large. Nucleated yolk sac–derived erythrocytes (arrow heads) are prominent in the absence of significant definitive erythropoiesis. Apoptotic figures are seen (see inset, small arrows). Same magnification as in (A).

(C) Liver cytospin preparation from a wild-type E13 fetus; Giemsa staining. Numerous erythropoietic cells in all stages of differentiation are seen, including proerythroblasts (E), basophilic erythroblasts (B), polychromatic erythroblasts (P), orthochromatic erythroblasts (O), and nonnucleated erythrocytes (N). Yolk sac–derived nucleated erythrocytes were also seen (Y). Scale bar, 10 μm.

(D) Liver cytospin preparation from a E13 *Epo*$^{-/-}$ mutant fetus; Giemsa staining. Proerythroblasts (E) are prominent with noticeable absence of cells at later stages of differentiation; yolk sac–derived nucleated erythrocytes are occasionally seen (data not shown). Same magnification as in (C).

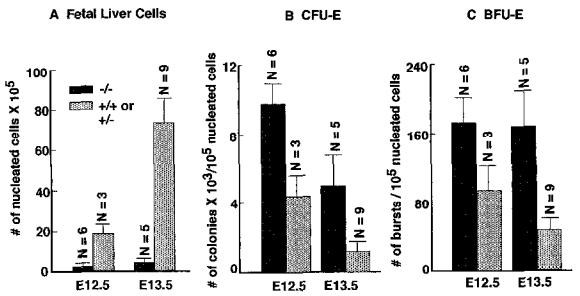

Figure 4. Erythroid Progenitor Cells in Normal and *Epo*$^{-/-}$ Mutant Fetal Livers

(A) Total number of nucleated cells per fetal liver.
(B) Number of CFU-E detected after 2 days of cultivation in α-methylcellulose supplemented with a cocktail of growth factors.
(C) Number of BFU-E observed after 7–10 days of cultivation in α-methylcellulose medium supplemented with a cocktail of growth factors.
CFU-E and BFU-E numbers are expressed per 10^5 nucleated fetal liver cells. The error bars represent one standard deviation of the mean. The numbers at the top of each error bar indicate the number of fetuses analyzed.

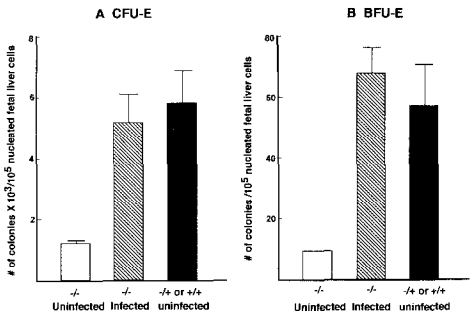

Figure 5. Erythroid Progenitor Cells in E12.5 Normal and *EpoR*$^{-/-}$ Mutant Fetal Livers

The cells from E12.5 *EpoR*$^{-/-}$ livers were incubated with or without SFFVEpoR for 2–3 hr on ice. Heterozygous and wild-type fetal liver cells were also incubated under the same conditions but without virus. After infection, the cells were washed and then plated in methylcellulose; benzidine-positive colonies were counted after 2 days (CFU-E, [A]) and 7–10 days (BFU-E, [B]) of cultivation. All values are expressed per 10^5 nucleated fetal liver cells. The error bars represent one standard deviation of the mean.

examined after E15 of gestation, and no improvement in erythropoiesis was observed in the homozygous embryos at E12–E13 gestation stages (data not shown). These results strongly suggest that maternal EPO cannot support fetal erythropoiesis and that the development of BFU-E and CFU-E progenitors in the *Epo*$^{-/-}$ fetal liver is not due to maternal EPO.

EpoR$^{-/-}$ Fetal Livers Also Have BFU-E and CFU-E Progenitors

Definitive evidence that neither EPO nor the EPOR is required for the generation of BFU-E and CFU-E progenitors came from an analysis of homozygous *EpoR*$^{-/-}$ embryos. Direct detection of CFU-Es and BFU-Es in fetal livers from the *Epo*$^{-/-}$ embryos should not be possible by culture in methylcellulose, since EPO, acting through the EPOR, is essential for the production of both types of colonies. Indeed, when fetal liver cells from *EpoR*$^{-/-}$ embryos were cultured with EPO, no CFU-Es or BFU-Es could be seen (data not shown). Low levels of CFU-Es and BFU-Es, however, were detected when cultures were supplemented with a cocktail of growth factors including EPO (Figure 5, uninfected). Therefore, we infected fetal liver cells from E12.5 *EpoR*$^{-/-}$ embryos with a recombinant retrovirus expressing the wild-type *EpoR* (Pharr et al., 1993), and the cells were subsequently placed in methylcellulose culture without added growth factors or were supplemented with a cocktail of growth factors including EPO. In the absence of added growth factors, no CFU-Es and BFU-Es were detected after retroviral infection (data not shown). In the presence of the cocktail of growth factors, the number of erythroid progenitors per 10^5 nucleated cells was about the same as that in normal fetal livers (Figure 5). Since *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ embryos died at the same developmental stage and their fetal livers contained similar numbers of nucleated cells (data not shown), we assume that the total number of progenitor cells in *EpoR*$^{-/-}$ fetal livers is very similar to that in *Epo*$^{-/-}$ fetal livers. On this basis, we estimate that the infection efficiencies for BFU-E and CFU-E progenitor cells were 40% and 53%, respectively. Thus, the generation of BFU-E and CFU-E progenitors in mouse fetal liver proceeds independently of the production and expression of *Epo* or *EpoR*, and the essential function of EPO and EPOR in vivo is to enable the CFU-E progenitors to survive and to trigger their proliferation and then irreversible terminal differentiation.

Primitive Erythropoiesis in the *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ Yolk Sac Is Partially Impaired

In early embryogenesis (E7–E11), the yolk sac is the first site of erythropoiesis. By E12, however, the major site of erythropoiesis has shifted from the yolk sac to the liver. The fact that *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ mutants developed normally and survived to E13 suggested that primitive erythropoiesis at the yolk sac stage might be normal. However, both *Epo*$^{-/-}$ (data not shown) and *EpoR*$^{-/-}$ embryos at E10–E11 of gestation were very pale (Figure 6A, right), allowing unequivocal identification of homozygous mutants. Blood vessels in the yolk sac of *EpoR*$^{-/-}$ embryos had many fewer erythrocytes (Figure 6A, right) than did the normal embryos (Figure 6A, left). Peripheral blood was collected from phenotypically normal and homozygous *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ embryos at E11–E12 of gestation, and total blood cells were counted. There was a 5- to 10-fold reduction in the number of blood cells in either *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ mutants as compared with that in wild-type or heterozygous littermates (data not shown). Importantly, some primitive erythrocytes were present in the yolk sac surrounding the *EpoR*$^{-/-}$ (Figure 6C) and *Epo*$^{-/-}$ (data not shown) embryos, though many fewer than in normal embryos (Figure 6B). Thus, a low level of EPO and EPOR-independent erythropoiesis in the yolk sac allows homozygous *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ embryos to develop and survive from E7 to E13 of gestation.

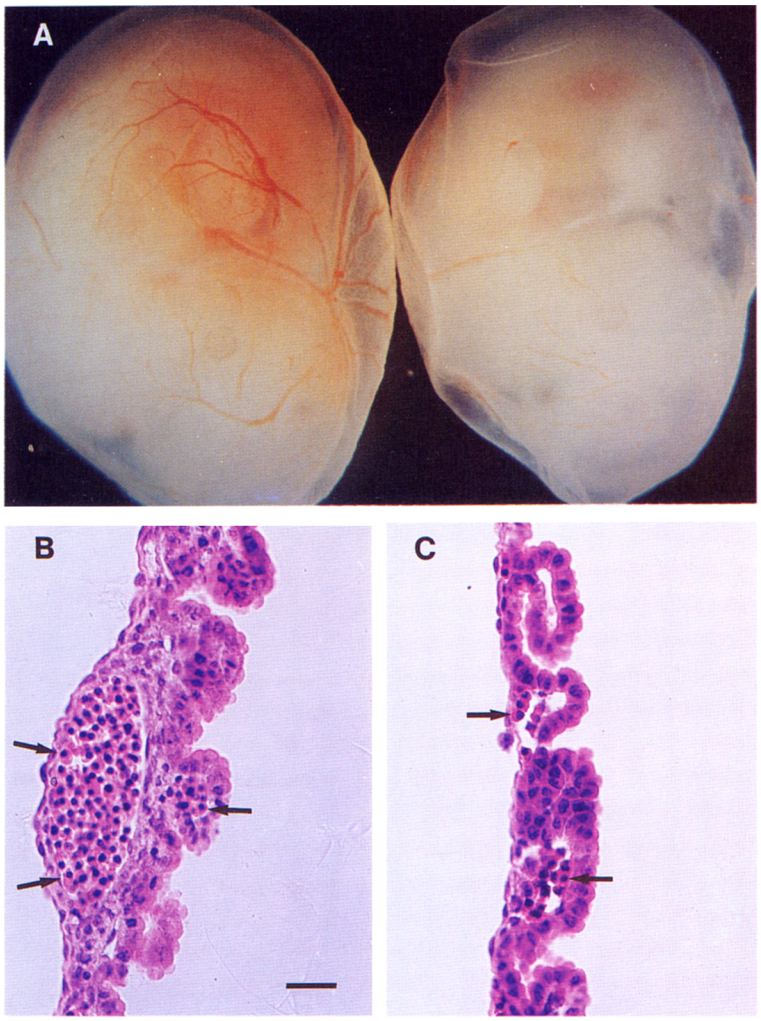

Figure 6. Whole Embryos and Sections of the Yolk Sac from Wild-Type and *EpoR*$^{-/-}$ Mice

(A) Phenotypic comparison of E11 wild-type (left) and *EpoR*$^{-/-}$ (right) embryos. The embryos are within the yolk sac; the mutant yolk sac appears very pale with significant reduction in circulating erythrocytes.

(B) Yolk sac section from an E11 wild-type embryo; hematoxylin and eosin staining. Yolk sac–derived nucleated erythrocytes fill the vessels (arrows). Scale bar, 25 μm.

(C) Yolk sac section from a E11 *EpoR*$^{-/-}$ mutant embryo; hematoxylin and eosin staining. Most of the vessels are empty. Yolk sac–derived erythrocytes are seen occasionally (arrows). Same magnification as in (B).

Discussion

In this study, we generated mouse strains in which either the endogenous *Epo* or the *EpoR* gene was inactivated via homologous recombination in ES cells. Our results reveal a pivotal role for EPO and EPOR in erythropoiesis. Yolk sac hematopoiesis is significantly reduced and leads to severe anemia. Definitive erythropoiesis, which begins in the fetal liver and thereafter in the spleen and bone marrow of adults (Figure 7), is completely impaired, resulting in embryonic lethality by E13–E15. The deficiency in erythropoiesis is not at the stem cell or progenitor cell level, since both BFU-E and CFU-E progenitors are present in the fetal livers derived from both *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ mice. Our data indicate that the major function of EPO is to trigger proliferation and then irreversible terminal differentiation of the committed late erythroid CFU-E progenitors. EPO may also act to prevent apoptosis of CFU-Es.

EPO Does Not Cross the Placenta into the Fetus

Whether a maternal source of EPO can support fetal erythropoiesis is controversial. In sheep and monkeys, administration of EPO to pregnant females causes significant increases in the level of circulating maternal EPO and in maternal erythropoiesis (Zanjani et al., 1993). However, there was no increase in the level of fetal plasma EPO or in fetal reticulocyte production unless EPO was directly injected into the fetus, suggesting that the fetal erythrocyte production is not affected by maternal levels of EPO (Zanjani et al., 1993). One study using mice demonstrated the transfer of maternally administrated [^{125}I]EPO into the fetus (Koury et al., 1988a). Although the small size of the mouse fetus and the short gestation period in mice did not permit direct assessment of the physiological significance of transplacental transfer of EPO, this finding raises the possibility that EPO from a maternal source may be involved in the regulation of erythropoiesis in the fetus.

This question was directly addressed here in our study of *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ mice. If significant amounts of maternal EPO do cross the placenta into the fetus, we would expect *Epo*$^{-/-}$ mice to survive until birth. If maternal EPO cannot support fetal erythropoiesis, then *Epo*$^{-/-}$ mice, like *EpoR*$^{-/-}$ mice, should die during embryogenesis. No *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ homozygous mice survived to birth, and both *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ mice died at the same period of gestation with severe anemia. Administration of high doses of

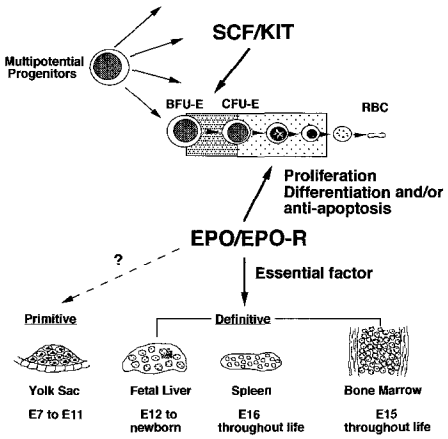

Figure 7. Growth Factors Involved in Regulating Murine Erythropoiesis In Vivo

SCF and its receptor KIT are important for the proliferation and differentiation of committed erythroid progenitors from the BFU-E to the CFU-E stage. EPO and EPOR are crucial for the survival of the late CFU-E progenitors, their irreversible differentiation, or both. EPO and EPOR are essential for definitive erythropoiesis in the fetal liver and have a partial role in controlling primitive erythropoiesis in the yolk sac.

rmEPO to pregnant $Epo^{+/-}$ females significantly increased the hematocrit of the mothers but had no effect on the erythropoiesis in the livers of $Epo^{-/-}$ fetuses or on the survival of the homozygous embryos. Thus, our results provide definitive physiological and functional evidence that, in mice, maternal EPO cannot cross the placenta and regulate fetal erythropoiesis.

The Roles of EPO and the EPOR in Primitive and Definitive Erythropoiesis

In the developing mouse embryos, the yolk sac is the original site of hematopoiesis, with the first stem cell colony-forming units (CFU-S) appearing at approximately E7 of gestation (Moore and Metcalf, 1970). By E12, the fetal liver becomes the major hematopoietic center. Fetal liver erythropoiesis and yolk sac erythropoiesis are distinct in several ways. In the blood islands of the yolk sac, erythrocytes mature as a cohort in a somewhat synchronous fashion, which is quite different from the asynchronous differentiation that occurs in the fetal liver (Tavassoli and Yoffey, 1983). Yolk sac erythropoiesis normally generates only large nucleated erythrocytes that synthesize embryonic hemoglobin. Fetal liver erythropoiesis, in contrast, produces nonnucleated erythrocytes that express adult hemoglobin. Some experiments have demonstrated that the fetal liver and yolk sac erythropoiesis differ in their responsiveness to EPO (Cole and Paul, 1966).

In $Epo^{-/-}$ and $EpoR^{-/-}$ embryos, definitive erythropoiesis was completely blocked, suggesting that EPO plays a critical role in regulating the erythrocyte production in the fetal liver. Primitive erythropoiesis in the yolk sac was, however, only partially impaired, with a 5- to 10-fold reduction in erythrocyte production. A small number of primitive erythrocytes are produced in the complete absence of either EPO or the EPOR, indicating that some of the erythroid progenitor cells in the yolk sac are able to proliferate and differentiate independent of either EPO or EPOR. Thus, the low level of CFU-E and BFU-E colonies detected in the $EpoR^{-/-}$ fetal liver prior to retroviral infection (Figure 5, uninfected) could be due to this small group of yolk sac–derived erythroid progenitors that migrated to the fetal liver after the blood circulation started at E9 of gestation. These results are not likely to be due to the leakiness of the knock-out mutations, since functional proteins are not likely to be generated from the mutant alleles (Figure 1). On the other hand, our results raise the very interesting possibility that two distinct populations of erythroid progenitors are present in the murine yolk sac, one being EPO- and EPOR-dependent and the other EPO- and EPOR-independent.

EPO and EPOR Are Not Required for the Formation of Definitive Erythroid Progenitor Cells

The control of erythropoiesis by EPO has been intensively studied both in vivo and in cell culture. Experimental manipulation of EPO levels in animals, either through the induction of anemia that stimulates the endogenous EPO production and increases circulating levels of erythrocytes, or through direct administration of EPO, resulted in a significant change in the number of CFU-Es, but only small and variable effects in the number of BFU-Es in the bone marrow (Hara and Ogawa, 1977; Peschle et al., 1979). These studies suggest that EPO induces the formation of CFU-Es from BFU-E progenitors. Administration of large amounts of EPO in humans led to a significant increase in the number of BFU-Es in the bone marrow and in the percentage of the BFU-Es active in DNA synthesis (Dessypris et al., 1988). Importantly, we do not know whether these effects were due to a direct action of EPO to stimulate the development of BFU-Es and CFU-Es from earlier progenitors or, alternatively, to support their survival. In addition, the effects of EPO may be indirect, possibly through the stimulation of production of other hematopoietic growth factors or through nonspecific stimulation of accessory or stromal cells.

The formation of BFU-Es in cell culture is affected by a number of growth factors besides EPO, including SCF, IL-3, and GM-CSF (Emerson et al., 1985). We do not know how these factors function cooperatively with EPO in vivo in controlling proliferation and differentiation of erythroid progenitors. The level of EPO responsiveness correlates well with the amount of EPOR expressed on the surface of the progenitor cells. The human early BFU-Es have little or no EPOR on their surface and are not responsive to EPO. After 48–72 hr of growth in the presence of IL-3, GM-CSF, or SCF, mature (also called late) BFU-Es develop; they express low levels of *EpoR* and are weakly responsive to EPO (Sawada et al., 1988, 1990). After another 4–5 days in culture, these cells give rise to a number of CFU-Es that are highly responsive to EPO (Gregory and Eaves, 1977) and express approximately 1000 EPORs on their surface (Sawada et al., 1988). On the basis of these experiments, EPO was thought to function with other growth factors, such as IL-3, GM-CSF, and SCF, in control-

ling the proliferation and differentiation of BFU-E progenitors to CFU-Es. EPO is clearly the crucial growth factor for the subsequent proliferation and differentiation of CFU-Es.

Molecular genetic analysis of mice with a null mutation in the gene encoding GM-CSF has indicated that GM-CSF is not crucial for erythropoiesis, or that other factors can compensate for its function (Dranoff et al., 1994). SCF, however, is crucial for the development of BFU-E progenitor cells to the CFU-E stage (Figure 7), since mice lacking SCF (*Sl* mutants) or its receptor KIT (*W* mutants) exhibit a significant reduction of CFU-E progenitors in their fetal liver and suffer from severe anemia (Nocka et al., 1989). Since the survival and proliferation of the CFU-E progenitors depends absolutely on EPO, these results suggest that the committed erythroid progenitors cannot proliferate or mature further unless both the KIT and the EPOR signal transduction pathways are functional. While KIT binds several intracellular signal transduction proteins, such as PI3K and PLCγ1, we do not know how it contributes to proliferation and differentiation of the erythroid cells.

Recently, we demonstrated (Wu et al., 1995) that SCF could replace EPO in supporting the growth and survival of HCD57 cells, an EPO-dependent erythroid cell line. Interestingly, SCF supported the proliferation of 32D cells expressing KIT only if they also expressed *EpoR*. In HCD57 cells, KIT and the EPOR were complexed together, and SCF rapidly induced tyrosine phosphorylation of the EPOR. These results suggested that KIT may activate the EPO/EPOR signal transduction pathway via tyrosine phosphorylation of the EPOR and, in turn, the activated EPOR could induce the further proliferation and maturation of committed erythroid progenitor cells. Since fetal livers from *EPOR*$^{-/-}$ embryos contain CFU-Es and can be rescued by expression of recombinant *EpoR*, we suggest that interaction of the KIT and EPOR at or around the CFU-E stage may be particularly important for triggering subsequent cell proliferation, differentiation, or both.

In summary, we showed that in the absence of EPO or EPOR, BFU-E and CFU-E progenitors developed normally in vivo but failed to undergo terminal differentiation to form mature erythrocytes. These results suggest that neither EPO nor the EPOR is required for erythroid lineage commitment or for the proliferation and differentiation of BFU-E to CFU-E progenitors. EPO and the EPOR are crucial in vivo for proliferation of CFU-E progenitors and for their survival and irreversible terminal differentiation into erythrocytes (Figure 7). Our results also indicate that there are no other ligands or receptors that can replace EPO and the EPOR in controlling definitive erythropoiesis. However, low levels of EPO- and EPOR-independent erythropoiesis do occur in primitive erythropoiesis at the yolk sac stage, suggesting that other mechanisms may play a critical role.

Experimental Procedures

Construction of Targeting Vectors

Genomic DNA clones corresponding to the *Epo* or *EpoR* locus were isolated from a 129(J1) genomic library (Wu et al., 1994).

pEpo-M1

The targeting vector pEpo-M1 was constructed by first inserting a 2.5 kb PstI–PstI fragment from the 5′ end of the *Epo* gene into a Bluescript vector, forming pEpo-M1-5′. Then a 1.8 kb EcoRI–HindIII fragment from the plasmid pGEM7(KJ1)R (Rudnicki et al., 1992) containing PGKneopA sequences was inserted into the EcoRI and HindIII sites of the pEPO-M1-5′ vector, forming pEpo-M1-neo. Finally, a 5.0 kb HindIII–KpnI fragment from the 3′ region of the *Epo* gene was inserted into the HindIII and KpnI sites of the pEpo-M1-neo vector (Figure 1A).

pEpoR-M1

The targeting vector pEpoR-M1 was constructed by first replacing a BglII–BamHI fragment in the plasmid pGEM7(KJ1)R (Rudnicki et al., 1992) with a 4 kb SalI–BamHI fragment derived from the 5′ end of the *EpoR* locus, yielding pEpoR-M1-5′, and then inserting the 2.7 kb XbaI–EcoRI* (site in the polylinker) fragment derived from the 3′ end of the *EpoR* gene into the XbaI and EcoRI sites of the plasmid pEpoR-M1-5′ (Figure 1B).

Electroporation, Selection of ES Clones, and Southern Blot Analysis

J1 ES cells were cultured essentially as described (Li et al., 1992). To introduce the targeting vector into the endogenous gene, 25 μg of pEpo-M1 or pEpoR-M1 plasmid was linearized at the NotI or SalI site, respectively, and electroporated into 1×10^7 J1 ES cells in a volume of 0.8 ml at 400 V and 25 μF by use of a Bio-Rad Gene Pulser. After 24 hr of culture, the medium was supplemented with 400 μg/ml G418 (GIBCO BRL), and 400 ES clones from each electroporation were isolated after 7–10 days of selection. Individual clones were expanded, and genomic DNAs were prepared as described (Laird et al., 1991). DNAs were digested with EcoRV and resolved on a 0.7% agarose gel. After transferring, filters were hybridized with ^{32}P-labeled probes, as indicated in Figure 1.

Generation of Germline Chimeras

ES clones containing the null mutations were injected into BALB/c or C57BL/6 embryos. Chimeric mice were backcrossed to BALB/c or C57BL/6 mice, and germline transmission of the mutant allele was detected by Southern blot analysis of tail DNA from F1 offspring with agouti coat color.

Histological Analysis of Embryos

Embryos were dissected free of uterine muscle and decidua, and the placenta and yolk sac were saved for genotyping. For histology, the embryos were placed in 10% buffered formalin for 24–48 hr and then in successive ethanol and xylene baths and finally embedded in Paraplast Plus (Oxford) with an Autotechnicon mono embedder (Technicon). Embryos were sectioned by using a Reichert–Jung microtome and stained with hematoxylin and eosin.

Progenitor Cell Assays

Individual fetal livers were dissected free in Iscove's modified Dulbecco's medium (IMDM), disaggregated into single cell suspensions, passed through a Cell Strainer (70 μm, Falcon), and then washed three times in IMDM medium. Cells were diluted 1:20 in 2% acetic acid to lyse nonnucleated mature erythrocytes, and then the remaining cells were counted. Cells from each fetal liver were plated in triplicate in α-methylcellulose without growth factor or supplemented with EPO (3 U/ml) or a cocktail of growth factors (3 U/ml EPO and 1% pokeweed mitogen–stimulated murine spleen cell conditioned medium) (StemCell Technologies, Incorporated). Colony formation was monitored at appropriate times (2–3 days for CFU-E and 7–10 days for BFU-E), and benzidine-positive colonies were counted. For retroviral infection, fetal liver cells were resuspended in medium containing a recombinant spleen focus-forming virus expressing *EpoR* (SFFVEpoR) (Pharr et al., 1993), 4 μg/ml Polybrene and kept on ice for 2–3 hr. Following infection, cells were washed once and plated in α-methylcellulose as described above.

Treatment of Mice with rmEPO

Pregnant *Epo*$^{+/-}$ females, 6–8 weeks old, were injected subcutaneously every day for a total of 8 days with 3000 U/kg rmEPO (in phosphate-buffered saline containing 0.025% bovine serum albumin, provided by Amgen, Incorporated). Blood for hematocrit determinations was

collected directly into hematocrit tubes (Baxter) from the retroorbital sinus under anesthesia. Baseline hematocrit determinations were performed prior to the first injection.

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