Mouse model of congenital polycythemia: Homologous replacement of murine gene by mutant human erythropoietin receptor gene

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Mutations causing truncations of the cytoplasmic domain of the human erythropoietin receptor (EPOR) result in a dominantly inherited disorder—that primary familial congenital polycythemia. This disorder is characterized by increased numbers of erythrocytes (polycythemia) and by in vitro hypersensitivity of erythroid precursors to erythropoietin. The consequences of EPOR truncation in nonerythroid tissues are unknown. We replaced the murine EPOR gene with a wild-type human EPOR gene and a mutant human EPOR gene that we initially identified in a patient with polycythemia. This mutation leads to an EPOR truncated after the first tyrosine residue of the intracellular domain. Mice heterozygous for this mutant allele and a wild-type human EPOR allele mimicked the human disorder. Interestingly, mice that were homozygous for the mutant human allele were severely polycytemic but viable. Our results provide a model for functional studies of EPOR-triggered signaling pathways in erythropoiesis. These animals can now be used to investigate the molecular pathophysiology of this gain-of-function EPOR mutation in erythroid tissue and in those nonerythroid tissues that express EPOR.

Primary familial congenital polycythemia (PFCP), or familial erythrocytosis, is a proliferative disorder of erythroid progenitors (1). Mutations of erythropoietin receptor (EPOR) cause the polycythemia phenotype in some, but not all, PFCP families (2). In families with EPOR mutations, the disease is inherited in an autosomal dominant fashion, suggesting a gain-of-function mutation. We and others (2–4) have described several EPOR mutations associated with the disease phenotype; all of the mutations cause truncations of the intracellular domain of the EPOR protein. These EPOR COOH-terminal truncations leave the EPOR box 1 and 2 (and at least the most proximal tyrosine—Y343) intact, but remove either all or part of the EPOR negative regulatory domain (5). This domain, which includes tyrosine Y429, binds hematopoietic protein tyrosine phosphatase (SHP1), which is responsible for dephosphorylation and inactivation of JAK2 (6). Deletion of the EPOR binding site for SHP1 was shown to prolong JAK2 activity and to cause hypersensitivity to erythropoietin (EPO) in cellular models (6, 7). The role of truncated EPOR in vivo remains to be elucidated; only heterozygous states of truncated EPOR have been reported (2–4). To mimic interactions of an EPOR gain-of-function mutation in a heterozygous patient, we created an animal model (EPOR) locus, and this animal model mimics the human disease. To assess further the obligatory and/or redundant roles in EPOR signaling, we bred the mEPOR heterozygous animals to homozygosity. Here we show that the dominant effect caused by the deletion of the negative regulatory domain of EPOR is phenotypically present in both homozygous and heterozygous animal states, and that a severely truncated EPOR is sufficient to maintain in vivo erythropoiesis.

Materials and Methods

Targeting Vector Construction and Generation of Embryonic Stem (ES) Cell Homologous Recombinants. Homologous sequences flanking the mEPOR gene were derived from a bacterial artificial chromosome clone of isogenic mouse strain 129 DNA (Research Genetics, Huntsville, AL). The human EPOR (hEPOR) gene was subcloned as a DraI fragment from a clone isolated from a human genomic library. The tagging targeting vector contained a phosphoglycerate kinase (PGK) promoter linked to a hypo- xanthine phosphoribosyltransferase (HPRT) minigene (PGK/ HPRT; ref. 8) flanked by a 6.0-kb SalI–NaeI 5’ homology and a 2.0-kb ClaI–XbaI 3’ homology fragments. The PGK/thymidine kinase gene was inserted into the 3’ XbaI site. The exchange targeting vectors contained the wild-type human EPOR (wEPOR) or mEPOR gene flanked by 8.6 kb of mEPOR homology sequences. The vectors were constructed from a 6.0-kb SalI–NaeI 5’ mEPOR fragment, a 4.7-kb ApaI (2673)–BglII (7349) fragment, and a 2.6-kb ClaI–EcoRV mEPOR fragment. Megaprimer mutagenesis was used to link the 5’ and 3’ mouse untranslated region sequences with the hEPOR gene. The primers to link the mEPOR and hEPOR sequences between NaeI and ApaI sites were as follows: M (Start) 5’-GAGATACTGGCTGGAGCGAGAGC-3’ and M/H (Start) 5’-GTCCATGATGCAGCCCTAGC-3’ for the PCR generation of the 5’ megaprimer, which was then used with the H (Start) 3’-CAAGTTTTCGCGCTTACTGTCG-5’ primer to amplify the NaeI–ApaI fragment. The primers used to link the hEPOR and mEPOR sequences between BglII and ClaI sites were: H/M...

Abbreviations: PFCP, primary familial congenital polycythemia; EPO, erythropoietin; EPOR, erythropoietin receptor; SHP1, protein tyrosine phosphatase; PGK, phosphoglycerate kinase; HPRT, hypoxanthine phosphoribosyltransferase; hEPOR, human EPOR; mEPOR, murine EPOR; mthEPOR, mutant human EPOR; wEPOR, wild-type human EPOR; ES, embryonic stem (cell); RT-PCR, reverse transcription–PCR; BFU-E, burst-forming unit erythroid; rh, recombinant mouse; rh, recombinant human; CFU-E, colony-forming unit erythroid.

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Homologous Recombination in ES Cells and Generation of hEPOR Knock-In Mice. The tagging targeting vector was linearized with BspHI and introduced into the HM-1 (HPRT-deficient) ES cell line. Hypoxanthine/aminopterin/thymidine and gancyclovir selection was used to enrich for homologous recombinants. ES cell clones containing the HPRT-tagged allele (mEPOR-null mutation) were injected into C57BL/6 blastocysts. Chimeric males were backcrossed to C57BL/6 mice, and germ-line transmission of the mutant allele was detected by Southern blot analysis of tail DNA from agouti offspring. Two independent HPRT-tagged clones that transmitted the mEPOR deletion to the germ-line were used for the second round of gene targeting—the wthEPOR or mthEPOR replacement. The exchange-targeting vector was linearized in the BsrBI site and electroporated into HPRT-tagged cell lines. Targeted clones were enriched by simultaneous positive selection for G418 resistance and negative selection (6-thioguanidine) against HPRT. Positive clones with a correctly replaced hEPOR gene were injected into C57BL/6 blastocysts. Resulting chimeric males were mated to C57BL/6 females to obtain offspring heterozygous for wthEPOR or mthEPOR replacement. The exchange-targeting vector was linearized in the BsrBI fragment of wthEPOR exon 8 was replaced by this fragment that contains the HPRT-tagged allele (the mEPOR homology sequences. A gene targeting vector was linearized in the BsrBI site and electroporated into HPRT-tagged cell lines. Targeted clones were enriched by simultaneous positive selection for G418 resistance and negative selection (6-thioguanidine) against HPRT. Positive clones with a correctly replaced hEPOR gene were injected into C57BL/6 blastocysts. Resulting chimeric males were mated to C57BL/6 females to obtain offspring heterozygous for wthEPORneo or mthEPORneo allele.

Expression Analysis and Hematological Analyses. The EPOR transcripts in bone marrow cells were determined with reverse transcription–PCR (RT-PCR) using primers m-hEPOR-F: 5′-GGACGCGCTACCTACGTCG-3′ and m-hEPOR-R: 5′-TCTTGGCGATCTGATCTGC-3′ (to amplify both mEPOR and hEPOR transcripts) or with primers m-hEPOR-F and m-hEPOR-R2: 5′-GCTTACACAACTCCCCGTCAG-3′ (to amplify the wthEPOR and mEPOR transcripts). In the last cycle of amplification, a [γ-32P]ATP-labeled 5′ primer was added to the reaction. Aliquots of PCR products were then digested with EcoO7-III, which specifically recognizes the hEPOR product, or digested with AccI, which specifically recognizes the mEPOR product, and analyzed on an agarose gel. Hematocrits were obtained from 7 μl of tail vein blood by using heparinized microhematocrit capillary tubes (Fisher Scientific). Complete blood count measurements were performed on a CELL-DYN 3500R blood analyzer (Abbott) from larger volumes of blood obtained by exsanguination.

In Vitro Hematopoietic Colony Assays. For burst-forming unit erythropoietic (BFU-E) assays, the bone marrow cells were obtained from 3-month-old adult mice. MethoCult medium M3236 (which contains 1% BSA, 10 μg/ml bovine insulin, and 200 μg/ml iron-saturated transferrin) with the addition of 15% (vol/vol) FBS (StemCell Technologies, Vancouver), 10 ng/ml recombinant mouse (rm) IL-3, 10 ng/ml recombinant human (rh) IL-6, 50 ng/ml rm stem cell factor (all StemCell Technologies, Vancouver) and appropriate doses of rm EPO (a kind gift from E. Goldwasser, University of Chicago) were used. In parallel experiments, rh EPO (Amgen Biologicals) dose–responses were tested. Cultures were scored at day 6 of incubation. For colony-forming unit erythrocytic (CFU-E) assays, MethoCult medium M3236 with addition of 30% (vol/vol) FBS and appropriate doses of rm or rh EPO were used. Cultures were scored within 48–72 h of incubation. Serum-free CFU-E assay was carried out in M3236 media and appropriate doses of rh EPO.

Results and Discussion

Double-Replacement Gene Targeting in ES Cells to Replace an mEPOR Gene with its Human Homologue. The site-specific replacement of the mouse gene by its human counterpart was accomplished by double-replacement gene targeting (tag and exchange) in mouse ES cells (9). In the first step, the mEPOR gene was deleted in ES cells via homologous recombination, and the locus was tagged with a positive selectable marker—PGK/HPRT minigene (Fig. 1a). The correctly targeted ES cell lines were tested in vivo for their totipotency. Two cell lines that transmitted the knockout allele to the germ-line were used for the exchange step—a second gene-targeting event. The exchange vectors contained either the wthEPOR or mthEPOR gene (from the translational start site up to the stop codon) flanked by 5′ and 3′ mEPOR homology sequences. A loxp-flanked PGK/neo expression cassette (floxed-neo) was introduced into intron VI of hEPOR gene in a reverse orientation relative to EPOR transscript (Fig. 1b; ← indicates the orientation). Simultaneous negative selection (6-thioguanine) against HPRT and positive selection for G418 resistance was used to select homologous recombinants that lost the HPRT minigene through replacement by the hEPOR gene (Fig. 1b).

Cre-Mediated Reactivation of Hypomorphic Allele: Mice Survive Solely on hEPOR. Insertion of the neo cassette into the intron VI of the hEPOR gene created a hypomorphic allele. Heterozygous mEPOR/hEPORfloxed-neo animals were indistinguishable from their wild-type littermates; however hEPORfloxed-neo homozygotes have not been observed and may die in utero (10, 11). Expression analysis of the hEPORfloxed-neo allele demonstrated that the gene was silenced (data not shown). The hypomorphic allele was converted into a fully expressed allele by mating the hEPORfloxed-neo mice to cytomegalovirus/cre transgenic animals (Fig. 1c). The levels of mEPOR and hEPOR transcripts in mEPOR/hEPOR heterozygous animals were approximately equal (Fig. 2A), suggesting proper regulation of the hEPOR gene with flanking mouse cis-acting elements.

To determine whether a mouse can survive solely on hEPOR, the F1 heterozygous mice were first assessed for deletion of the neo marker gene (Fig. 1c) and then bred to homozygosity. Examination of wthEPOR/hEPORfloxed-neo animals revealed significantly lower hematocrits than the wild-type mouse littermates (Fig. 3a). The weight of spleens from wthEPOR/hEPOR animals was 80–140 mg. and the weight of spleens of control animals was 100–180 mg. Despite the known compatibility of human and mouse EPO with their respective receptors (12), the in vivo efficiency of hEPOR signaling by mouse EPO may be lower than that of the human EPO ligand, suggesting subtle mouse/human species differences of EPO/EPOR interactions. These interactions may not be detectable in vitro studies in which high nonphysiological EPO concentrations are used. The reduced efficiency of EPO signaling may be caused by a decreased binding affinity between hEPOR and mEPO or a different affinity between the cytoplasmic domain of hEPOR (signal-transducing domain) and mouse signal transduction factors such as JAK2 kinase and STAT5.

Heterozygous mthEPOR Animals Are Polycythemic and Mimic the Human Disease. To establish that the mthEPOR causes polycythemia, we bred the heterozygous mEPOR/mthEPORfloxed-neo mice to cre transgenic mice or to the wthEPORfloxed-neo/cre homozygous mice. After neo deletion through cre recombination, mthEPOR gene expression was reactivated (Fig. 2A), and the mthEPOR heterozygous animals developed polycythemia within 3–6 weeks after birth (Fig. 3a). The mice had increased...
hematocrit and elevated hemoglobin concentration but did not exhibit any red cell morphological abnormalities. The C5964G mutation leads to an EPOR truncated after the first tyrosine residue of intracellular domain. This membrane-proximal domain of EPOR is associated with JAK2 (13) which, through phosphorylation of EPOR tyrosines, generates docking sites for the transcription activator STAT5 (14). JAK2

\[ \text{STAT5} \]

plays a nonredundant, essential role in EPO

\[ \text{EPOR} \]

mediated regulation of erythropoiesis (15, 16). The mechanism by which the truncation leads to hyperactive EPOR is hypothesized to be through the absence of the negative regulatory domain of EPOR (5–7). At least three negative regulators of erythropoiesis bind to this EPOR domain: CIS (17), CIS3

\[ \text{SOCS3} \]

and SHP1 (6). By binding to phosphorylated tyrosines, CIS prevents binding of other SH-2-containing proteins to the cytoplasmic domain of EPOR (17). CIS3/SOCS3 binds to both JAK2 and EPOR Y401, and suppresses EPO-dependent JAK2/STAT5 signaling (18). The deletion of the Y401 binding site leads to reduced CIS inhibitory effect on EPO/EPOR-induced STAT5 activation (18). SHP1 (hematopoietic cell phosphatase) is recruited by EPOR Y429 and, after it attaches to the cytoplasmic EPOR domain, it dephosphorylates JAK2 (6). The inactivation of SHP1 binding site was shown to lead to prolonged phosphorylation of JAK2/STAT5 (6), and the same mechanism (i.e., prolonged activation of JAK2/STAT5) applies to signaling mediated by the truncated EPOR (20).

**Homozygous mthEPOR Mice Are Severely Polycythemic but Viable.** The phenotype of mice homozygous for mthEPOR could not be predicted because the deleted cytoplasmic EPOR sequence contains both positive and negative regulators of EPO signaling. To assess the homozygous state of the mthEPOR, we created homozygous mice by crossing the mthEPOR heterozygous animals. Mice of all expected genotypes were born in Mendelian ratios, indicating that homozygosity for the mthEPOR is compatible with life and that the most proximal cytoplasmic tyrosine of the severely truncated mthEPOR is permissive for maintaining erythroid differentiation and maturation. Analyses of peripheral blood of adult mice (in microhematocrit capillaries) demonstrated elevated hematocrits often >60% (mean 58%), the most severe polycythemia of all disease-causing genotypes (Fig. 3a). The polycythemia became apparent first at 3 to 6 weeks after birth. Three homozygous mthEPOR and five age-matched control wild-type mEPOR animals were available for complete blood counts and red cell indices measurements. The erythrocytes bearing mthEPOR were increased in number, exhibited significant anisocytosis, and trended toward microcytosis. Abnormalities compatible with expanded red cell mass and iron

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**Fig. 1.** Double replacement gene targeting in the mEPOR locus. (a) Tagging of the mEPOR locus (depicted in red) by PGK/HPRT minigene (indicated by a green box). The targeting vector contains 7.5 kb of total homology that permitted deletion of the mEPOR gene between NaeI and Clai restriction sites (at positions 1315 and 6226). A PGK/thymidine kinase expression cassette was introduced into the XbaI site of the vector. The eight exons are shown as rectangles; 5’ and 3’ untranslated regions are stippled. (b) Knock-in of the hEPOR gene into the mEPOR locus. The exchange targeting vector contained a wtEPOR or mthEPOR gene (depicted in blue) flanked by mEPOR homology sequences. The PGK/neo cassette (a yellow oval) flanked by loxP sites was introduced into the HpaI site of intron 6. (c) Removal of the neo cassette through cre-mediated recombination. The lox-flanked PGK/neor-selectable marker was deleted in vivo by crosses with cytomegalovirus/Cre-expressing mice. Both “tag and exchange” recombination events were detected by Southern blotting with an EcoRV-SalI (5’ probe) and a XbaI–EcoRV (3’ probe) mouse-genomic fragments. The recognized restriction fragments of recombinant (three independent clones) and wild-type alleles are indicated. Restriction sites were RV, EcoRV; Xho, Xhol; H, HindIII; and E, Eco47II.

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deficiency, likely secondary to augmented erythropoiesis, were as follows (data shown as mean and SD): for mthEPOR mice, red blood cell count (RBC, million/mm³) 11.63 and 1.19; hemoglobin concentration (Hb, g/dl) 16.7 and 1.73; hematocrit (Hct) 51.4 and 3.72; mean corpuscular volume (MCV, fl) 44.2 and 1.81; red cell distribution width (RDW, %) 19.07 and 0.32. For wt mEPOR mice, RBC 8.85 and 1.08; Hb 13.3 and 1.56; Hct 41.8 and 3.20; MCV 47.5 and 2.52; and RDW 16.04 and 1.22. Differences in RBC, Hct, and RDW achieved statistical significance with P = 0.039, 0.024, and 0.032, respectively. The spleens of mthEPOR homozygous were enlarged to the weight of 200–350 mg.

**EPO Hypersensitivity of mthEPOR Erythroid Progenitors in Vitro.** To establish whether the polycythemic phenotype of the mthEPOR animals was directly related to the sensitivity of erythroid progenitors to EPO, we tested early, more primitive erythroid progenitor (BFU-E) and late erythroid progenitor (CFU-E) responses to various EPO doses in serum-containing cultures (Fig. 3a and b). As illustrated in Fig. 3c, the polychromatophilic phenotype was accompanied with the highest _in vitro_ hypersensitivity of the CFU-Es to EPO; these _in vitro_ differences were paralleled by the differences in hematocrits (Fig. 3a). The _in vitro_ hypersensitivity of MethEPOR homozygous BFU-Es was comparable to that of the mthEPOR heterozygous erythroid progenitors (Fig. 3b). The CFU-Es are responsive mainly to EPO, whereas the earlier erythroid progenitors are regulated by other cytokines as well. Thus the CFU-E analysis in serum-containing cultures may better reflect _in vivo_ erythropoiesis. The hypersensitivity of erythroid progenitors bearing mthEPOR was demonstrable with both rm EPO and rh EPO (not shown). To test whether other (non-EPO) factors in FBS contribute to the observed hypersensitivity of CFU-Es, mthEPOR homozygous late erythroid progenitors were assayed in serum-free conditions (containing BSA, insulin, and transferrin supplement). In this condition, the animal homozygous for mthEPOR had a number of EPO-independent CFU-Es comparable to that of its littermate with mEPOR genotype, and only partially maintained EPO hypersensitivity in the lowest doses of rh EPO (data not shown). These results are in agreement with earlier studies (21) showing that either serum or, in serum-free conditions, insulin-like growth factor-I is required to maintain the EPO hypersensitivity of cells expressing truncated EPOR.

**Functional Consequences of Truncated EPOR in Hematopoiesis.** To determine whether the mthEPOR leads to expansion of erythroid pools, we compared the absolute numbers of BFU-E and CFU-E progenitors in homozygous mutants and controls. Fig. 3d demonstrates increases in both early and late mutant erythroid progenitors compared with wtEPOR animals. This fact, in conjunction with CFU-E hypersensitivity, splenomegaly (mouse spleen is an erythropoietic organ composed largely of late erythroid progenitors), and normal leukocyte and platelet counts of mthEPOR/mthEPOR animals indicated that the late erythroid progenitors, CFU-Es, are the main targets of up-regulated EPO signaling. The contribution of negative regulators of EPO signaling in the observed phenotypes can be judged from the _in vitro_ behavior of erythroid progenitors derived from mice null for these factors. The SOCS3-deficient hematopoietic progenitors had an increased proliferative capacity and formed larger colonies then normal controls (19), a phenomenon not observed in mthEPOR cells. In contrast, the SHP1-deficient (motheaten viable) mice had EPO-hypersensitive CFU-Es, forming colonies in serum-containing cultures in the absence of exogenous EPO (22). These data, together with the absence of a polycythemia phenotype in mthEPOR newborns (SOCS3 is a negative regulator of fetal liver erythropoiesis; ref. 19) suggest that SHP1 (or rather, the deletion of its docking site) plays the principal role in polycythemia phenotype.

**General Conclusions.** The autosomal dominant inheritance of EPO mutations associated with PFCP is underscored by the fact that PFCP has been observed only in patients heterozygous for the mthEPOR; no homozygous patients with gain-of-function EPO alleles have been described (1–4). The unexpected outcome of our studies is the observation that the _in vivo_ phenotypes of heterozygous and homozygous mthEPOR mice are comparable: both genotypes are viable and exhibit polycythemia. It has been suggested that EPO tyrosine residues other then Y343 may be required to support proliferation and differentiation of erythroid progenitors (23). The PFCP-associated truncations of EPO affect the docking sites for not only the negative but also the positive regulatory elements of signal transduction (reviewed in ref. 24). This fact makes the functional consequence of homodimers (i.e., homozygous states for PFCP-causing mutations) unpredictable. Its gain-of-function nature was called into question by _in vitro_ experiments using transfected murine stem cell models (21). The polyclathrophic phenotype of homozygous mthEPOR mice provides conclusive evidence that signals mediated by distal EPO tyrosines are not mandatory for erythroid proliferation and terminal differentiation. Undoubtedly, EPO is an essential hematopoietic cytokine required for the differentiation and proliferation of erythroid progenitors into red blood cells (10, 11). Of the several signaling pathways

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**Fig. 2.** Expression of the EPOR in the mEPOR locus. mRNA was isolated from bone marrow cells and used as a template for RT-PCR. (A) Relative quantities of mouse and human EPOR transcripts. RT-PCR primers were complementary to both mouse and human EPOR sequences and generated PCR products of the same size; however, only the human EPOR fragment contained an Eco47-III recognition site. For further experimental details, see Materials and Methods. (B) Relative quantities of wthEPOR and mthEPOR mRNAs in wthEPOR/mthEPOR heterozygotes were determined with a Molecular Dynamics PhosphoImager. RT-PCR products were digested with AccI, which specifically recognizes the mthEPOR product. The genotypes of mice and undigested (−) and digested (+) samples are indicated above the blots.
activated by the EPO/EPOR interaction, JAK2/STAT5 seems to be the most critical. Recently, knock-out studies (16, 25) suggested that the JAK2/STAT5 pathway triggers the main antiapoptotic signals mediated by EPOR. Moreover, transgenic studies (reviewed in ref. 26) revealed that the most proximal EPOR cytoplasmic tyrosine Y343, an EPOR-JAK2 interaction site, is capable of maintaining this pathway. The polycythemic phenotype of this animal model supports this conclusion. Other signaling pathways promoting proliferation, differentiation, and prevention of apoptosis are associated with more distal EPOR tyrosines (24). These pathways are more indirect and trigger signals through cascades of signaling molecules before the final acceptor of the signal—transcription factor—is reached. Such formation of signaling networks provides opportunities for crosstalk and leads to redundancy in receptor-mediated signaling (27). Our data suggest that their functional role in erythropoiesis remains to be fully elucidated.

An Animal Model to Study EPO/EPOR Signaling in Nonerythroid Tissues. EPO/EPOR have been shown to function in brain cells (28), endothelial cells (29), heart structures (30), and the uterus (31). The functional disturbances of EPO/EPOR in nonerythroid tissue had been suggested, but had not been unequivocally confirmed or elucidated in detail. To study gain-of-function of EPOR in nonerythroid tissues, the hEPORlox-neo hypomorphic allele can be reactivated by crossing transgenic mice that express cre under the control of tissue-specific promotors. This cre-induced reactivation of knocked-in mthEPOR should facilitate detailed studies of EPO/EPOR signaling pathways and establish its functional importance. Although PFCP is a rare disorder, several individuals we studied have developed otherwise unexplained hypertension, strokes, and coronary disease, suggesting that PFCP and augmented EPO signal transduction may be an independent risk factor for development of hypertension and cardiovascular disease (32). Our preliminary data (not shown) indicate that by the age of 3 months, homozygosity for the truncated EPOR leads to significant elevation of mean arterial blood pressure. These data need to be validated by controlled longitudinal studies; if they are confirmed, this observation extends the usefulness of the animal model to nonerythroid disease states.

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