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Erythropoietin Receptor Signaling Regulates Both Erythropoiesis and Megakaryopoiesis In Vivo

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Abstract

Transgenic expression of a gain-of-function truncated mouse erythropoietin receptor gene (*EpoR*) leads to expansion of the HSC pool in response to human erythropoietin (Epo). We have re-examined this observation using a knock-in mouse model, wherein the mouse *EpoR* gene was replaced in its proper genetic locus by a single copy of either a wild type human or a polycythemia-inducing truncated human *EPOR* gene. Bone marrow cells obtained from knock-in mice were transplanted together with competitor bone marrow cells in a model that allows tracking of erythroid, platelet, and leukocyte contributions by each genotype. Secondary transplants were also performed. Stem/progenitor cells were identified phenotypically and isolated for colony-forming assays to evaluate cytokine responsiveness by cells with the wild type human or truncated human *EPOR* gene.

Augmented Epo signaling increased erythroid repopulation post-transplant as expected, but had no effect on short-term or long-term leukocyte repopulation. However, the wild type human *EPOR* knock-in mouse showed decreases in both erythroid and platelet repopulation compared to marrow cells from the mutant human *EPOR* knock-in mouse or normal B6 animals. These results provide evidence supporting a role for Epo signaling in megakaryopoiesis in vivo and suggest a role for Epo signaling early in hematopoietic development.

Keywords

erythropoietin; hematopoietic stem cells; progenitor cells; erythrocytes; platelets

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AUTHOR CONTRIBUTIONS

Xiaosong Huang: Collection and assembly of data, data analysis and interpretation, manuscript writing.

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Gerald J. Spangrude: Conception and design, financial support, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

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INTRODUCTION

Erythropoietin (Epo) is the major regulator of red blood cell development. It inhibits the apoptosis of late erythroid progenitors and stimulates their proliferation, ultimately expanding red cell mass [1]. In recent years, Epo has been shown to have roles outside of the erythroid lineage [2]. A role for Epo signaling in hematopoietic stem cell (HSC) proliferation has recently been suggested by experiments in which murine HSC carrying a gain-of-function truncated mouse gain-of-function erythropoietin receptor (*EpoR*) transgene were reported to have a competitive advantage over wild-type mouse HSC when exogenous human Epo was provided following marrow transplantation [3,4]. Similar results were reported in a retroviral transduction model [5]. These observations may have relevance to the rare subset of polycythemia patients that go on to develop hematologic malignancies. However, due to the nature of the transgenic and retroviral model systems, in which the transgene is incorporated into the genome without normal regulatory elements, *EpoR* transgenes were possibly abnormally expressed in HSC or early progenitor cells. This misregulated expression could result in functional consequences that would not normally occur under proper regulatory control of the genetic context of endogenous mouse *EpoR*.

We have re-examined the effect of hyperactive Epo signaling on HSC function, utilizing mouse models with wild type human *EPOR* (*wthEPOR*) or mutant human gain-of-function *EPOR* (*mthEPOR*) genes knocked into the mouse *EpoR* locus [6]. This animal model has functional Epo signaling in all tissues that normally express EpoR. Phenotypically, *wthEPOR* mice are anemic with decreased Epo-mediated signal transduction, likely due to decreased affinity of endogenous mouse Epo for the *wthEpoR*, or decreased signaling downstream of the receptor. In contrast, *mthEPOR* mice are polycythemic with increased JAK2 and STAT5 phosphorylation [7]. To evaluate HSC and progenitor cell function in the context of these two knock-in models, we compared the engraftment efficiency and self-renewal of *mthEPOR* or *wthEPOR* marrow cells relative to normal C57BL/6 mouse marrow cells in a competitive marrow transplantation assay. We tracked the relative contribution of donor marrow cells to peripheral blood leukocytes, erythrocytes and platelets over time using flow cytometry and quantitation of hemoglobin isoforms by high pressure liquid chromatography (HPLC). Our results show that hyperresponsive human EpoR stimulation resulted in a pronounced enhancement of erythrocyte differentiation without obvious effects on other hematopoietic lineages, suggesting that primitive hematopoietic stem and progenitor cells are not affected by excess EpoR stimulation. In contrast, competitive transplantation of *wthEPOR* knock-in mouse marrow cells resulted in decreased engraftment of both erythrocyte and platelet lineages as well as a decrease in the frequency of primitive stem and progenitor cells in the bone marrow. These observations suggest an *in vivo* role for Epo signaling in maintenance of the primitive hematopoietic compartment.

METHODS

Mice

Mice harboring *wthEPOR* or *mthEPOR* knocked into the endogenous mouse *EpoR* locus were generated by gene targeting [6] and then backcrossed to the C57BL/6 background. C57BL/6 mice carrying the hemoglobin- β diffuse isoform allele (B6-Hbb^d) were kindly provided by Dr. David Harrison [8] and were bred in our animal facility. Two strains of green fluorescent protein (GFP) transgenic mice, both of which were derived by microinjection of C57BL/6 oocytes, were utilized in these studies. Initial experiments utilized mice provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan) [9] in which the GFP transgene is driven by a chicken β -actin promoter. Mice carrying both the GFP transgene and the Hbb^d allele were generated in our breeding colony by mating B6-Hbb^d mice with B6-GFP mice and selecting for

cosegregation of GFP and Hbb^d. We refer to these mice as B6-GFP/Hbb^d. A second strain of GFP transgenic mice (C57BL/6-Tg(UBC-GFP)30Scha/J), in which the GFP transgene is driven by a human ubiquitin C promoter [10], was obtained from Jackson Labs (Bar Harbor, ME). We refer to the mice as B6-UBC-GFP. Because GFP expression is observed in the leukocytes, platelets, and erythrocytes of these mice, crossing to the B6-Hbb^d congenic strain was not necessary in order to track tri-lineage engraftment. All animals were maintained in the animal resources center at the University of Utah under protocols approved by the institutional animal care and use committee.

Bone marrow transplantation

Donor marrow cells were obtained from either *wthEPOR* or *methEPOR* knock-in mice or normal C57BL/6 mice. The competitor bone marrow cells were obtained from B6-GFP/Hbb^d or B6-UBC-GFP mice. Equal numbers of donor and competitor marrow cells were mixed (4×10^6 total) and transplanted intravenously into lethally irradiated (¹³⁷Cs, 13Gy delivered in a split dose) recipient mice (B6-GFP/Hbb^d, B6-UBC-GFP, or C57BL/6 were used as recipients in three separate experiments) through the retro-orbital route.

Peripheral blood analysis

Peripheral blood samples were collected from the retro-orbital sinus under isoflurane anesthesia delivered using the E-Z Anesthesia system (Euthanex Corp., Palmer, PA). Blood was collected in heparinized capillary tubes and mixed with acid citrate dextrose at a 10:1 ratio prior to determination of the complete blood count using a Serono System 9010⁺ CP hematology counter (Serono Diagnostics, Allentown, PA). Samples were then mixed with an equal volume of 2% Dextran T500 (Amersham Biosciences, Piscataway, NJ) in phosphate-buffered saline (PBS) and incubated at 37°C for 30 minutes. The upper layer, containing leukocytes, platelets, and residual erythrocytes, was collected for flow cytometry analysis, in which blood cells derived from test and competitor marrow grafts were distinguished by GFP expression. The sedimented erythrocytes were collected and evaluated for relative Hbb^s and Hbb^d ratios using HPLC (see below).

Determination of Hbb variants by HPLC

A cation exchange protocol was developed to separate and quantitate Hbb variant alleles by HPLC [11]. A stock solution of 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 10 mg of DTNB in 250 µl of dimethyl sulfoxide and stored at -20°C. Erythrocytes obtained from heparinized blood were washed three times in dextrose-gelatin-veronal buffer and stored as packed pellets at 4°C for up to 2 weeks prior to analysis. Samples were derivatized by adding 10 µl of packed erythrocytes to 200 µl of 40 mM NaCl, 2 mM DTNB and incubating at room temperature for 30 minutes. Following centrifugation (12,000g for 2 minutes), samples were analyzed using a VARIANT hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA) with a modified β-thalassemia short program (details provided on request).

Phenotypic analysis of HSC populations

Marrow cells from *wthEPOR* and *methEPOR* knock-in mouse strains were incubated in a cocktail of antibodies to CD2, CD3, CD5, CD8, Mac-1, Gr-1, TER119, CD45R, and CD19. Lineage depletion was carried out by 2 successive incubations in sheep anti-rat Ig-coupled magnetic beads (Dyna, Oslo, Norway). Lineage-negative (Lin^{neg}) cells were incubated with 0.2 µM rhodamine-123 in Hank's balanced salt solution (HBSS) containing 5% newborn calf serum (HBSS) at 37°C for 20 minutes. After washing once, the cells were resuspended in fresh HBSS and incubated at 37°C for another 20 minutes to allow for efflux of the dye. After washing, the cells were stained with phycoerythrin (PE) conjugated anti-Sca-1 and

allophycocyanin (APC) conjugated anti-c-kit antibodies. The cells were then washed and resuspended in HBSS containing propidium iodide (PI) for exclusion of dead cells and analyzed using a modified two-laser FACScan flow cytometer (BD Biosciences, San Jose, CA; modified by Cytex Development, Fremont, CA). In experiments evaluating GFP expression of the bone marrow compartment of transplant recipients, the rhodamine-123 staining was omitted because of the conflict with GFP emission. In these cases, the hematopoietic stem and progenitor cell compartment was evaluated as the c-kit⁺, lineage-negative, Sca-1⁺ (KLS) population and the percentage of donor-derived cells was measured based on GFP expression.

In vitro CFC assay

Lin^{neg} cells prepared as described above were stained with PE-conjugated anti-Sca-1, APC-conjugated anti-c-kit, and biotinylated anti-Flt3. The biotinylated antibody was detected using streptavidin-FITC, and dead cells were excluded based on PI staining as described above. The c-kit⁺ Lin^{neg} Sca-1⁺ Flt3^{neg} (KLSF) cell population was isolated by aseptic cell sorting using a FACS Vantage or FACS Aria instrument (BD Biosciences, San Jose, CA) and plated in alpha-modified Eagle's medium (αMEM)-based methylcellulose containing 10% fetal calf serum, 10% deionized bovine serum albumen, antibiotics, glutamine, and 2-mercaptoethanol. Colony growth and differentiation were stimulated with recombinant cytokines in combination as indicated, including stem cell factor (SCF; 100 ng/ml), G-CSF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (20 ng/ml), a peptide thrombopoietin (Tpo) mimetic (5 nM), and erythropoietin (4 U/ml) (cytokines obtained from PeproTech, Rocky Hill, NJ; the Tpo mimetic was synthesized by the University of Utah core peptide synthesis facility as described [12]). After seven days of culture, quadruplicate plates were stained using benzidine hydrochloride and scored for total colonies and for colonies containing hemoglobinized erythroid lineage cells. Results are reported as mean number of colonies per 100 cells plated, and error bars represent standard error values. Statistical analysis utilized a one-tailed, unpaired T-test with equal variance.

RESULTS

Tri-lineage engraftment of *wthEPOR* and *mthEPOR* knock-in progenitors in recipient bone marrow

To evaluate the effects of augmented or decreased Epo signaling on the engraftment potential of HSC and progenitor cells, we performed competitive repopulation assays by co-transplanting 2×10^6 C57BL/6, *wthEPOR*, or *mthEPOR* marrow cells (donor) together with 2×10^6 GFP transgenic marrow cells (competitor) into lethally irradiated recipient mice. Using GFP and hemoglobin-β isoforms as markers, we tracked the relative contributions of donor and competitor marrow cells into leukocytes, platelets and erythrocytes in the peripheral blood of recipient mice up to five months post-transplantation [11].

As described in the methods section, two strains of GFP transgenic mice were utilized for these studies. Our initial experiments utilized B6-GFP/Hbb^d mice, in which leukocytes and platelets but not erythrocytes express the GFP transgene. In these animals we followed erythrocyte engraftment based on analysis of hemoglobin alleles using HPLC as previously described [11]. We subsequently obtained the UBC-GFP transgenic strain, in which all blood cells are GFP⁺, and performed two transplant experiments using these mice as a source of bone marrow competitor cells. Regardless of the strain of GFP transgenic mice used, we observed a leukocytosis in transplant recipients that increased over time. This effect was most pronounced when UBC-GFP mice served as the source of competitor bone marrow cells as well as the irradiated transplant recipients, and leukocyte counts ranged from 20 to 30×10^3 cells/μL by 20 weeks post-transplantation compared to 5 to 10×10^3 cells/μL in unmanipulated UBC-GFP mice. The majority of the leukocytosis was derived from GFP-negative lymphocytes, which by several months post-transplant represented about 90% of the peripheral blood leukocytes

regardless of the genotype of the bone marrow donor strain (C57BL/6, *wthEPOR*, or *mthEPOR*). Apart from the pronounced leukocytosis, no other abnormalities were observed in the animals. Spleen and thymus cellularity and immunophenotype were essentially normal, and no obvious signs of autoimmunity were observed. Since erythrocyte and platelet counts were normal in all experimental groups (with the exception of the recipients of polycythemic *mthEPOR* marrow cells), we focused on the analysis of these parameters in this study.

The data in Figure 1 show the relative contributions of the donor marrow (shaded bars) and competitor marrow cells (open bars) relative to the total cell counts for the erythroid and platelet lineages over time. Recipients of a 50:50 mixture of C57BL/6 and GFP transgenic marrow cells showed approximately 50% chimerism in both the erythroid and platelet lineages over time. In contrast, marrow cells obtained from *mthEPOR* knock-in donors provided a higher contribution to engraftment of the erythroid lineage and subsequent productive erythropoiesis compared to competitor marrow cells over the time course of the experiment, and recipients of *mthEPOR* cells exhibited a polycythemic phenotype. Thus, the total erythrocyte count from recipients of *mthEPOR* knock-in donors at 17 weeks post-transplantation was $11.4 \pm 1.6 \times 10^6$ cells/ μ L (mean $\pm$ SD) compared to a count of $8.0 \pm 0.3 \times 10^6$ and 8.9 ± 0.5 cells/ μ L in recipients of *wthEPOR* and C57BL/6 bone marrow, respectively. An average of 88% of the erythrocytes in recipients of a 50:50 mix of bone marrow from *mthEPOR* and GFP transgenic animals was derived from the *mthEPOR* marrow. Consistent with the anemic phenotype of *wthEPOR* mice, marrow cells from these animals competed poorly with competitor marrow cells in erythroid reconstitution, such that *wthEPOR* red cells represented less than 2% of the total red cell count 17 weeks after transplantation. This result is consistent with the observation that *wthEPOR* is not optimally stimulated by mouse Epo in vivo, as previously described [6, 7]. Thus, competitive transplantation of *wthEPOR* and *mthEPOR* bone marrow cells recapitulates the phenotypes of the donor mouse strains.

Analysis of platelet reconstitution revealed that *wthEPOR* knock-in bone marrow showed a progressively reduced contribution to platelets compared to the competitor graft, while normal C57BL/6 and *mthEPOR* knock-in marrow maintained consistent levels of platelets relative to the competitor marrow cells (Figure 1, lower panel). At 17 weeks post-transplant, recipients of *wthEPOR* knock-in marrow had about one half the number of donor-derived platelets relative to C57BL/6 recipients ($p = 0.001$) or *mthEPOR* recipients ($p = 0.003$). The decrease in relative contribution of *wthEPOR* knock-in mice marrow cells to the platelet count was seen throughout the time course of our study and regardless of the GFP competitor strain in three separate experiments. Since the contribution of competitor marrow to megakaryopoiesis compensated for the decrease in donor-derived platelet counts in recipients of *wthEPOR* marrow, the total platelet counts did not differ between the three groups. These observations indicate that the defect in megakaryopoiesis was intrinsic to the hypomorphic *wthEPOR* knock-in mice marrow cells, and implicates a role for Epo signaling in the regulation of platelet production, consistent with previous in vitro studies [13,14].

Secondary transplantation of 10^7 bone marrow cells obtained from the primary transplant recipients recapitulated the results seen in the primary transplant experiment (Figure 1). This result demonstrates that the differences in the engraftment potential for platelet and erythroid lineages between *wthEPOR* and *mthEPOR* bone marrow cells are largely due to the activity of progenitor cells, since the presence of a stem cell effect in either mouse strain would predict a more pronounced effect upon secondary transplantation.

***wthEPOR* knock-in mice have a lower proportion of hematopoietic progenitor cells compared to *mthEPOR* knock-in or wild-type mice**

To investigate whether there are effects of Epo signaling on the frequency of hematopoietic progenitor cells, we determined the proportion of these cells in marrow cells from *wthEPOR*

and *mthEPOR* knock-in mice. In *wthEPOR* knock-in mice, the Rh123^{low} subset of Lin^{neg}c-kit⁺Sca1⁺ (KLS-R) cells represented $0.011 \pm 0.003\%$ of marrow cells while in *mthEPOR* knock-in mice these cells represented $0.023 \pm 0.006\%$ of marrow cells (mean $\pm$ SEM, $n = 8$, $p = 0.025$). In normal C57BL/6 mice, these cells represented $0.031 \pm 0.003\%$ of marrow cells (mean $\pm$ SEM, $n = 4$), which is not significantly different from *mthEPOR* knock-in mice. We conclude from the combined results of phenotypic and functional analysis that the replacement of the endogenous mouse *EpoR* gene with the polycythemic *mthEPOR* gene does not alter the characteristics of the HSC compartment, in contrast to the effect on erythropoiesis resulting in polycythemia. The reduction of the frequency of KLS-R cells in the marrow of *wthEPOR* knock-in mice may reflect a reduced frequency of early multipotent progenitor cells that are not resolved from primitive HSC by our immunophenotyping methods.

To further evaluate the effects of the *wthEPOR* in hematopoiesis, we evaluated the bone marrow of the competitive transplant studies shown in Figure 1. Bone marrow cells obtained from these animals were evaluated for the frequency of KLS cells derived from either the GFP + competitor graft or the GFP-donor-derived graft. The results are shown in Figure 2. Recipients of equal numbers of C57BL/6 and UBC-GFP bone marrow cells showed equal representation (75%) of C57BL/6 donor-derived KLS cells and erythroid lineage cells in the bone marrow 18 weeks after the transplantation. In contrast, less than 50% of the KLS cells in recipients of *wthEPOR* bone marrow were derived from the knock-in cells ($p < 0.001$), and erythrocytes were predominantly (>95%) derived from GFP+ competitor bone marrow cells. The frequency of donor-derived KLS cells in recipients of *mthEPOR* bone marrow cells approximated that seen in recipients of C57BL/6 bone marrow, while the erythroid compartment was predominantly derived from the donor cells in these animals. These results are consistent with the decreased frequency of KLS-R cells in the bone marrow of unmanipulated *wthEPOR* mice, suggesting that the decreased signaling through the human receptor results in a decrease in the frequency of hematopoietic progenitor cells.

HSC of both *wthEPOR* and *mthEPOR* knock-in mice exhibit synergistic cytokine responsiveness

One hallmark characteristic of primitive HSC and progenitor cells is their synergistic responsiveness to cytokine stimulation, particularly with cytokine combinations that include SCF. Synergy between SCF and Epo is particularly relevant to erythropoiesis [15], and the impact of hyperactive *EPOR* signaling in the context of synergy with other cytokines has not been investigated at the HSC level. To evaluate the effect of augmented erythropoietin signaling on cytokine responsiveness, progenitor cell frequency, and differentiation activity of primitive stem and progenitor cells obtained from the two knock-in mouse strains, we isolated c-kit⁺ Lin^{neg} Sca-1⁺ Flt3^{neg} (KLSF) marrow cells from each strain and cultured these cells in the presence of various combinations of cytokines. Colonies were evaluated after 7 days of culture, and colonies that included hemoglobinized cells were distinguished based on benzidine staining.

As shown in Figure 3, both the number of total colonies as well as the number of colonies containing erythroid cells were similar between *wthEPOR* and *mthEPOR* knock-in mouse KLSF cells stimulated with SCF as a single cytokine. This result demonstrates that the hyperactive *EPOR* by itself is not sufficient to synergize with SCF. Interestingly, stimulation with IL-6 consistently resulted in more colonies in *wthEPOR* cultures compared to *mthEPOR* cultures, while stimulation with Epo alone resulted in colonies only in *mthEPOR* cultures. Synergy between SCF and IL-6 was similar between the two genotypes, while the combination of SCF with Epo promoted only additive responses from both *wthEPOR* and *mthEPOR* KLSF cells. The combination of SCF, IL-3, IL-6, Flt3 ligand, G-CSF and Tpo, with or without human Epo, resulted in 30 to 50% cloning efficiency, demonstrating that most KLSF

cells in both knock-in strains require multiple cytokines to achieve optimal proliferation. Under these conditions, neither maximal colony formation nor the frequency of colonies containing hemoglobinized cells differed between the two knock-in strains or wild type C57BL/6 mice in replicate experiments (data not shown). We conclude that when present in the mouse genome under appropriate regulatory control, *methEPOR* does not contribute additional growth or differentiation signals in primitive stem and progenitor cells compared to *wthEPOR* or the cells with endogenous *EpoR* gene.

DISCUSSION

Epo stimulates growth, prevents apoptosis, and promotes differentiation of erythroid progenitors. However, Epo and EpoR expression are not restricted to erythrocytes and erythroid progenitor cells. The function of EpoR in HSC is controversial. Transgenic mice expressing gain-of-function *EpoR* under the control of the β -actin promoter showed an enhanced HSC self-renewal capability when exogenous human Epo was provided [4]. In the present study, we evaluated HSC and progenitor cell function in a mouse model in which a hyperactive form of the human *EPOR* (*methEPOR*) or the wild type human *EPOR* (*wthEPOR*) were placed under the control of the endogenous mouse *EpoR* genetic regulatory elements. The *methEPOR* knock-in mouse model had increased Epo signaling and increased red blood cell mass and erythroid progenitor cell number, while the *wthEPOR* knock-in mouse model was hypomorphic and had decreased Epo signaling and an anemic phenotype [6]. Using these mouse models, we performed a competitive repopulation assay to assess the effect of enhanced or decreased Epo signaling on the functions of HSC and progenitor cells.

Consistent with the phenotypes of the knock-in mouse models, *methEPOR* mouse marrow in competition with normal mouse marrow had augmented repopulation of erythrocytes in recipient mice, while the *wthEPOR* mouse marrow had a severe defect in erythrocyte repopulation. These results coincide with the polycythemia and anemia phenotypes of these two mouse models, respectively. Interestingly, we observed no significant differences of leukocyte repopulation between *methEPOR*, *wthEPOR*, and C57BL/6 marrow cells up to 5 months post-transplantation in primary transplantation studies and 16 weeks after secondary transplantation. Furthermore, *methEPOR* knock-in mice had an equivalent number of phenotypically-defined HSC relative to wild-type mice, and this together with the transplant data suggests that hyperactive EpoR signaling does not influence the number of hematopoietic progenitor cells in the bone marrow.

Unexpectedly, we observed impaired production of platelets derived from *wthEPOR* mouse marrow compared to recipients of *methEPOR* or normal mouse marrow. This decreased megakaryopoiesis was sustained over time and was also present after secondary transplantation (Figure 1). However, platelet numbers (the sum of donor plus competitor contributions) were normal in both primary and secondary transplant recipients, demonstrating that the defect in megakaryopoiesis was intrinsic to the *wthEPOR* marrow cells. The decrease in the percentage of platelets derived from *wthEPOR* marrow cells was significant, as donor-derived platelet numbers were decreased by 50% or greater relative to control or *methEPOR* cells at 21 weeks post-primary transplant and at 16 weeks post-secondary transplant. This observation is consistent with previous studies suggesting that Epo/EpoR signaling might play a role in megakaryopoiesis [16].

Tpo, the major cytokine influencing platelet production, is clearly the primary regulator of megakaryopoiesis [17]. However, targeted deletion of Tpo or of its receptor c-mpl fails to completely ablate thrombopoiesis [18], suggesting that additional cytokines function to regulate platelet production in vivo. A number of cytokines, including IL-3, IL-6, IL11, and leukemia inhibitory factor, have been shown to promote megakaryocyte growth, but compound

knockout studies have failed to provide evidence supporting the role of these cytokines in the residual production of platelets in the absence of c-mpl signaling [19,20]. Due to the embryonic lethal phenotype of *Epo* or *EpoR* knockouts, similar experiments combining either of these mutations with targeted deletion of *tpo* or *mpl* would require embryo aggregation in an experimental setting that would allow distinction of platelet reconstitution, as we have utilized in our studies. To our knowledge, such studies have not been reported.

The extensive homology between c-mpl and EpoR [21], as well as the common downstream signaling pathways activated by these receptors [22], make EpoR an attractive candidate as a regulator of platelet function in vivo. Epo will act with Tpo in a synergistic manner to promote megakaryocyte colony growth in vitro [13,14] and with IL-6 to promote proplatelet process formation [23]. In this context, Epo/EpoR signaling could act on a common progenitor for the erythrocyte and megakaryocyte lineages, or on committed progenitors of the megakaryocyte lineage in order to exert its role in megakaryopoiesis. However, in light of the lack of a positive effect of the *mtH**EPOR* on platelet reconstitution in our experiments (Figure 1 and Figure 2), our data are consistent with the conclusion that Tpo provides the dominant regulatory effect on platelet numbers in vivo, and that Epo signaling may be responsible for the residual erythropoiesis seen in the absence of Tpo signaling.

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Figure 1. Competitive repopulation of *wthEPOR* or *mthEPOR* knock-in mouse bone marrow with normal mouse bone marrow

Peripheral blood analysis of erythrocyte and platelet cell numbers from each genotype of mouse bone marrow at various times post-transplantation are shown. This experiment utilized UBC-GFP mice as a source of competitor bone marrow cells and as irradiated recipients, and is representative of three experiments. Secondary transplantation was performed by isolating bone marrow from primary recipients 20 weeks after the initial transplant, and transferring 10^7 bone marrow cells into each irradiated recipient. Error bars indicate standard error of the mean.

Figure 2. Analysis of bone marrow cells of primary transplant recipients

Primary transplant recipients were sacrificed at 18 weeks post-transplant and bone marrow cells were isolated. The frequency of donor-derived (GFP-negative) erythrocytes and bone marrow progenitor (KLS) cells was determined by flow cytometry. Each group represents 4 mice, processed individually to obtain statistical analysis. Error bars indicate standard error of the mean.

Figure 3. Synergistic cytokine responsiveness of KLSF cells isolated from *wthEPOR* or *mthEPOR* knock-in mouse marrow

The KLSF stem/progenitor cell population was isolated from the marrow of *wthEPOR* or *mthEPOR* knock-in mouse donor animals as described in Methods. Cell cultures were established in methylcellulose medium containing the indicated cytokines. The plates were stained with benzidine hydrochloride after 7 days of culture and scored for total colonies and for colonies containing hemoglobinized cells 100 cells plated. Data reflects the means of triplicate or quadruplicate plates, and error bars represent standard errors. S36GTF indicates the combination of SCF, IL-3, IL-6, G-CSF, Tpo, and Flt3 ligand.