HEMATOPOIESIS and hemostasis involve the continuous production of 6 major types of blood cells (erythrocytes, megakaryocytes, monocytes, granulocytes, B lymphocytes, and T lymphocytes) at tightly controlled rates. This is regulated in part through the differential expression and activation of lineage- and stage-specific hematopoietic growth factor (HGF) receptors of the type 1 superfamily. For example, lymphocyte lineage- and stage-specific hematopoietic growth factor (HGF) receptors are known to assemble with activated HGF receptor complexes. Despite this intriguing complexity, recent investigations in cell lines and retrovirally transduced murine fetal liver cells suggest that most of these factors and signals may be functionally nonessential. To test this hypothesis in erythroid progenitor cells derived from adult tissues, a truncated Epo receptor chimera (EE372) was expressed in transgenic mice using a GATA-1 gene-derived vector, and its capacity to support colony-forming unit-erythroid proliferation and development was analyzed. Expression at physiological levels was confirmed in erythroid progenitor cells expanded ex vivo, and this EE372 chimera was observed to support mitogenesis and red blood cell development at wild-type efficiencies both independently and in synergy with c-Kit. In addition, the activity of this minimal chimera in supporting megakaryocyte development was tested and, remarkably, was observed to approximate that of the endogenous receptor for thrombopoietin. Thus, the box 1 and 2 cytoplasmic subdomains of the Epo receptor, together with a tyrosine 343 site (each retained within EE372), appear to provide all of the signals necessary for the development of committed progenitor cells within both the erythroid and megakaryocytic lineages.

Signals provided by the erythropoietin (Epo) receptor are essential for the development of red blood cells, and at least 15 distinct signaling factors are known to be associated with activated Epo receptor complexes. Despite this intriguing complexity, recent investigations in cell lines and retrovirally transduced murine fetal liver cells suggest that most of these factors and signals may be functionally nonessential. To test this hypothesis in erythroid progenitor cells derived from adult tissues, a truncated Epo receptor chimera (EE372) was expressed in transgenic mice using a GATA-1 gene-derived vector, and its capacity to support colony-forming unit-erythroid proliferation and development was analyzed. Expression at physiological levels was confirmed in erythroid progenitor cells expanded ex vivo, and this EE372 chimera was observed to support mitogenesis and red blood cell development at wild-type efficiencies both independently and in synergy with c-Kit. In addition, the activity of this minimal chimera in supporting megakaryocyte development was tested and, remarkably, was observed to approximate that of the endogenous receptor for thrombopoietin. Thus, the box 1 and 2 cytoplasmic subdomains of the Epo receptor, together with a tyrosine 343 site (each retained within EE372), appear to provide all of the signals necessary for the development of committed progenitor cells within both the erythroid and megakaryocytic lineages.

HEMATOPOIESIS and hemostasis involve the continuous production of 6 major types of blood cells (erythrocytes, megakaryocytes, monocytes, granulocytes, B lymphocytes, and T lymphocytes) at tightly controlled rates. This is regulated in part through the differential expression and activation of lineage- and stage-specific hematopoietic growth factor (HGF) receptors of the type 1 superfamily. For example, lymphocyte lineage- and stage-specific hematopoietic growth factor (HGF) receptors are known to assemble with activated HGF receptor complexes. Despite this intriguing complexity, recent investigations in cell lines and retrovirally transduced murine fetal liver cells suggest that most of these factors and signals may be functionally nonessential. To test this hypothesis in erythroid progenitor cells derived from adult tissues, a truncated Epo receptor chimera (EE372) was expressed in transgenic mice using a GATA-1 gene-derived vector, and its capacity to support colony-forming unit-erythroid proliferation and development was analyzed. Expression at physiological levels was confirmed in erythroid progenitor cells expanded ex vivo, and this EE372 chimera was observed to support mitogenesis and red blood cell development at wild-type efficiencies both independently and in synergy with c-Kit. In addition, the activity of this minimal chimera in supporting megakaryocyte development was tested and, remarkably, was observed to approximate that of the endogenous receptor for thrombopoietin. Thus, the box 1 and 2 cytoplasmic subdomains of the Epo receptor, together with a tyrosine 343 site (each retained within EE372), appear to provide all of the signals necessary for the development of committed progenitor cells within both the erythroid and megakaryocytic lineages.

From the Programs in Genetics and Cell and Developmental Biology, The Pennsylvania State University, University Park, PA, and the Laboratory of Chemical Biology, NIDDK, National Institutes of Health, Bethesda, MD.

Supported by National Institutes of Health Grant No. DK40242 (D.M.W.).

Address reprint requests to Don M. Wojchowski, PhD, 115 Henning Bldg, The Pennsylvania State University, University Park, PA 16802; e-mail: dmw1@psu.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

From www.bloodjournal.org by guest on November 11, 2017. For personal use only.
prediction that this EE372 transgene also should be expressed in megakaryocytic progenitor cells, the ability of this minimal chimera to support megakaryocyte development also has been investigated.

**MATERIALS AND METHODS**

**Transgenic mice.** For expression in mice, a cDNA encoding a minimal hEGF receptor/murine Epo receptor chimera (EE372) was subcloned into a GATA-1 gene vector that contains an upstream activating region, exons 1a and 1b, and the nontranslated region of exon 2. A 17-kb BamI-ClaI restriction fragment containing this linked cDNA construct was injected into pronuclei of fertilized eggs, and eggs were implanted into pseudo-pregnant BDF1 females. Transgene-positive founders were identified initially by polymerase chain reaction (PCR) using primers specific to the hEGF receptor (5'-TCC ATA CAG TGC CAC CCA GAG-3') and murine Epo receptor (5'-AGC AGC CAC AGC TGG AAG TTA-3'). Southern blotting was performed on BglII digests of genomic DNA using a 770-bp XbaI fragment of the murine Epo receptor cDNA. 

**Erythroid progenitor cell preparations and proliferation assays.** Enriched populations of erythroid progenitor cells were prepared from the spleens of mice treated subcutaneously with either thiamphenicol (TAP) or phenylhydrazine (PHZ) and from bone marrow. Briefly, TAP was administered as an implant on day 1 (14 g/kg), mice were phlebotomized on days 2 through 4, TAP was withdrawn on day 6, and splenocytes were prepared on day 9. PHZ was injected subcutaneously on days 1, 2, and 4 (50 mg/kg), and splenocytes were prepared on day 5. Mice were anesthetized with metofane (Schering-Plough, Union, NJ) during all procedures and prior to sacrifice. Disrupted spleen or bone marrow preparations were passed through a 70-µm cell strainer (Fisher, Pittsburgh, PA), collected, exposed for 4 minutes to a freshly diluted acetylcholinesterase, washed, and adjusted to 10^7 cells/mL in 0.5% fetal bovine serum and 0.5% bovine serum albumin and were incubated sequentially for 1 hour at 2°C with the Fc fragment of murine IgG (5 µg/mL; Pierce, Rockford, IL) and for 1.5 hours (in the presence of 0.02% NaN3) with a monoclonal antibody (EGFR.1; Pharmingen, San Diego, CA) to the hEGF receptor extracellular domain (3.3 µg/mL). Cells (2 x 10^6 cells/sample) were then washed, incubated for 30 minutes at 2°C with a phycoerythrin-conjugated antibody specific to murine IgG F(ab')2 (Jackson Research Labs, West Grove, PA), washed, and analyzed by flow cytometry (Coulter XL-MCL system; Coulter, Miami, FL). EE372 receptor density was estimated in a regression analysis by comparing fluorescence values from EE372-positive cells with values obtained for phycoerythrin-molecular equivalent beads (Spherotech, Libertyville, IL). Estimates accounted for a stoichiometry of 1 molecule of phycoerythrin per conjugated secondary antibody and the binding of 2 phycoerythrin antibodies per primary receptor-bound antibody.

**Assays of erythroid and megakaryocyte colony formation.** In CFUe assays, splenocytes and bone marrow cells were prepared as described above and were cultured at 37°C, 7.5% CO2 for 48 hours in Methocult HCC3242 media (Stem Cell Technologies, Vancouver, British Columbia, Canada) at 3 x 10^5 cells/mL. Cultures contained either Epo (5 U/mL) or hEGF (5 to 10 ng/mL) and, when specified, murine stem cell factor (50 ng/mL; Peprotech, Rocky Hill, NJ). Hemoglobin-positive colonies were stained with a freshly combined solution (1:1) of benzidine (3% in 90% glacial acetic acid) plus 5% H2O2. Megakaryocyte development from marrow progenitor cells was assayed using a serum-free Megacult collagen system (Stem Cell Technologies). Cells were plated at both 1 x 10^5 and 3 x 10^5 cells/mL in the presence of murine IL-3 (50 ng/mL) and either hEGF (10 ng/mL) or Tpo (50 ng/mL). At day 7 of culture, cultures were dried, fixed, and stained for acetylcholinesterase-positive colonies.

**RESULTS**

**Transgenic expression and proliferative activity of the minimal hEGF/Epo receptor chimera EE372 in erythroid progenitor cells.** To test the ability of a minimal membrane-proximal cytoplasmic domain of the Epo receptor to support erythropoiesis in adult tissues, this domain was linked to the extracellular domain of the hEGF receptor and the resulting hEGF-activatable chimera was expressed in transgenic mice. In this

---

**Fig 1.** The hEGF receptor (hEGF R)-murine Epo receptor (mEpo R) chimera, GATA-1 gene-derived transgenic expression vector, and integration of pG1-EE372 in transgenic mice. (A) Diagrammed are the wild-type (wt) murine Epo receptor and the minimal chimeric construct EE372. In EE372, the extracellular domain is that of the human EGF receptor, and the murine Epo receptor cytoplasmic domain is truncated to delete 7 of 8 sites of tyrosine (Y) phosphorylation. Also diagrammed is the GATA-1 gene-derived vector used to express EE372 in transgenic mice (pG1-EE372), uas, upstream activating sequence. pA, polyadenylation signal. (B) Southern blot analyses shown are for representative litters from f3 and f4 generations. Indexed are Bgl II products from the endogenous Epo receptor gene and the pG1-EE372 transgene.
CFU-e and CFU-meg VIA EpoR Jak2/STAT5 DOMAINS

Given the evidence has been generated to suggest that SCF signaling via its receptor tyrosine kinase c-Kit may even depend on lateral evidence has been generated to suggest that SCF signaling via its receptor tyrosine kinase c-Kit may even depend on lateral

The capacity of this minimal EE372 chimera to signal hEGF-induced proliferation in erythroid progenitor cells from adult mice was assayed in primary tests of function. To provide for an enriched population of erythroid cells, mice were treated with either TAP or PHZ under regimens shown in pilot experiments to maximize CFUe production in spleen. TAP in particular was used to generate a developmentally synchronized population of splenic CFUe while limiting the representation of megakaryocytic progenitor cells (see below). In [3H]dT incorporation assays, hEGF proved to support the proliferation of erythroid splenocytes from EE372 mice at rates that paralleled those induced by Epo via the endogenous wild-type Epo receptor (Fig 2). Data shown are for 3 transgenic mice (and nontransgenic control animals) and are representative of results obtained for 6 animals from 3 distinct generations at 5 to 20 weeks of age. These results confirmed the expression of functional EE372 receptors and indicated an essentially wild-type activation of proliferative response pathways. To directly confirm that EE372 receptors were expressed at high frequencies with low variegation (and to discount the possibility that observed proliferative response profiles might be accounted for by EE372 receptor overexpression within subpopulations), the density and distribution of EE372 transgene expression were assayed in erythroid progenitor cells expanded ex vivo. Marrow cells from transgenic and control mice were cultured under conditions recently shown to selectively support erythroid progenitor cell expansion from human CD34+ cells.40 At day 3 of culture, colony-forming assays showed that approximately 50% of expanded cells corresponded to CFUe (data not shown). For cells at this stage, an antibody to the human EGF receptor extracellular domain was used to assay EE372-positive cells (Fig 3). In cells expanded from transgenic mice, 47% of size-gated cells were positive for EE372 expression and mean densities of EE372 expression among gated cells approximated 5,000 receptors per cell (calculated as described in Materials and Methods). Thus, this EE372 transgene is expressed at high frequencies among erythroid progenitor cells at levels within the same order of magnitude as reported for endogenous Epo receptors.44

The minimal chimera EE372 promotes red blood cell development independently and in synergy with c-Kit. Given the uniform expression of mitogenically competent chimeric receptors in EE372 mice described above, investigations next assessed the ability of the above EE372 receptor form to support red blood cell production. Specifically, the ability of hEGF to support the differentiation of CFUe within marrow and in splenocytes from TAP or PHZ treated EE372 mice was tested.

For erythroid progenitor cells from each source and among all EE372 transgenic mice assayed, hEGF promoted red cell colony formation at efficiencies essentially equivalent to levels promoted by Epo at equimolar concentrations (ie, via endogenous Epo receptors; Table 1). Colony morphology (typically 16-cell hemoglobinized colonies) was indistinguishable from CFUe differentiated from control or transgenic mice in the presence of Epo (data not shown), and hEGF failed to support any detectable differentiation or outgrowth of CFUe from nontransgenic animals.

The development of CFUe at high efficiencies is known to depend on coexposure to stem cell factor (SCF),27 and some evidence has been generated to suggest that SCF signaling via its receptor tyrosine kinase c-Kit may even depend on lateral signaling through Epo receptor complexes.45 Based on these
considerations, whether the truncated chimera EE372 might act in synergy with SCF/c-Kit to support CFUe development also was tested. In control experiments, red cell formation due to Epo was enhanced markedly in the presence of SCF (Fig 4). Interestingly, this effect also was exerted at full efficiency for erythroid splenocytes from EE372 transgenic mice after their exposure to hEGF plus SCF (Fig 4). Thus, the membrane-proximal cytoplasmic subdomain of the Epo receptor efficiently

Table 1. Wild-Type Activity of EE372 in Supporting CFUe Formation From Adult Erythroid Progenitor Cells

<table>
<thead>
<tr>
<th>Mouse</th>
<th>CFUe Formation, % of Epo-Induced Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Epo, No EGF</td>
</tr>
<tr>
<td>Nontransgenic no. 1</td>
<td>0</td>
</tr>
<tr>
<td>Nontransgenic no. 2</td>
<td>0</td>
</tr>
<tr>
<td>EE372 no. 45</td>
<td>0</td>
</tr>
<tr>
<td>EE372 no. 161</td>
<td>0</td>
</tr>
<tr>
<td>EE372 no. 178</td>
<td>0</td>
</tr>
<tr>
<td>EE372 no. 194*</td>
<td>0</td>
</tr>
<tr>
<td>EE372 no. 181†</td>
<td>10</td>
</tr>
</tbody>
</table>

The ability of splenic erythroid progenitor cells from EE372 and nontransgenic control mice to form hemoglobinized colonies was assayed in the presence of either Epo (5 U/mL), hEGF (20 ng/mL), or no cytokines. Benzidine-positive colonies (CFUe) were scored at 48 hours of culture. Values are the means of duplicate samples expressed as the percentage of colonies induced in the Epo treatment groups. The mean values (± standard error) of Epo-induced colonies for mice no. 1, 2, 45, 161, 178, 194, and 181 are 10.5 ± 3.5, 34 ± 3.0, 13 ± 2, 19.5 ± 0.5, 28 ± 6.0, 8.7 ± 1.7, and 25 ± 13.7, respectively (n = 2 for all samples except no. 194, where n = 3).

*Bone marrow preparation of erythroid progenitor cells.
†hEGF included at 4 ng/mL.

The ability of splenic erythroid progenitor cells from EE372 and nontransgenic control mice to form hemoglobinized colonies was assayed in the presence of either Epo (5 U/mL), hEGF (20 ng/mL), or no cytokines. Benzidine-positive colonies (CFUe) were scored at 48 hours of culture. Values are the means (± standard error) of 3 assays and are representative of 2 independent experiments.

Fig 3. Expression of EE372 receptors on adult erythroid progenitor cells expanded ex vivo. (A) Using conditions developed by Panzenbock et al, erythroid progenitor cells were expanded from the marrow of transgenic (EE372) and control mice (wt, wild-type). EE372 expression was assayed using an antibody specific to the hEGF receptor extracellular domain. PE, phycoerythrin fluorescence intensity. FALS, forward angle light scatter. (B) Example estimate of EE372 receptor densities. Phycoerythrin molecular equivalent microbeads were used to generate a calibration profile (3,800, 12,000, 34,000, 124,000, and 300,000, reading left to right). This profile (and regression analyses) then were used to estimate EE372 receptor densities on marrow cells expanded ex vivo (see inset).

Fig 4. EE372 mediates hEGF-dependent CFUe development and synergizes with c-Kit. The ability of erythroid splenocytes from TAP-treated EE372 mice to form hemoglobinized colonies was assayed in the absence of cytokines or the presence of either Epo (5 U/mL), hEGF (20 ng/mL), SCF (50 ng/mL), Epo plus SCF, or hEGF plus SCF. Benzidine-positive colonies (CFUe) were scored at 48 hours of culture. Values are the means (± standard error) of 3 assays and are representative of 2 independent experiments.
mediates the synergistic effects of SCF/c-kit on red blood cell
development from adult erythroid progenitor cells ex vivo.

**Megakaryocyte development is supported efficiently via the**
**minimal Epo receptor chimera EE372.** Structurally, the single
transmembrane receptor for thrombopoietin (Mpl) is related
closely to the Epo receptor, and each is known to signal via Jak2
and to activate STAT5. In addition, substantial overlap exists
between lineage-restricted transcription factors known to
dictate commitment to megakaryocytic and erythroid lineages.35,46
This includes GATA-1, and the GATA-1 gene-derived vector
used in these studies has been shown to direct expression in
megakaryocytes.35 Based on these considerations and on the
prediction that EE372 mice should also express this receptor
chimera in megakaryocytic cells, the ability of the EE372
receptor to support the development of megakaryocytic colonies
(colony-forming unit-megakaryocyte [CFU-meg]) was
assayed. Remarkably, colony-forming assays in serum-free
medium showed that CFU-meg development was supported
by this minimal receptor form in response to hEGF at
levels approximating that supported by Tpo (Fig 5A). Also, the
morphology of acetylcholinesterase-positive megakaryocytic
colonies was essentially indistinguishable from those of CFU-
meg colonies cultured in the presence of Tpo (Fig 5B). Thus, the
box 1 and 2 domains of the Epo receptor together with tyrosine
343 appear to provide all signals necessary to support both
erthroid and megakaryocytic development.

**DISCUSSION**

Novel features of the present investigation that merit discus-
sion include the following: (1) the nature of signals provided by
the receptor form EE372 that support erythropoiesis and megakary-
ocyctic cell development from adult hematopoietic tissues and (2)
advantages of this unique transgenic model for investigations of
receptor-derived signals that promote CFUe and CFU-meg
survival, proliferation, and terminal differentiation. With re-
gards first to erythropoiesis, previous studies of the activities of
carboxyl terminal-truncated and (P)Y-mutated Epo receptor
forms in cell lines and fetal liver have provided compelling
evidence that the minimal cytoplasmic subdomain contained in
EE372 efficiently supports proliferation and differentiation
signaling in these models. However, whether this recep-
tor subdomain and derived signals might also support red blood
cell development from CFUe in adult tissues has not been
studied to date. This is an important issue, because fetal and
adult erythropoiesis are known to differ in several notable ways.
Direct evidence that Epo receptor activation mechanisms in
fetal liver differ from those in adult tissues recently has been
provided based on the ability of the gp55 protein of the
anemia-inducing strain of Friend virus to promote Epo receptor-
dependent erythropoiesis from fetal liver but not from mar-
row. In addition, progenitor cells in fetal liver are known to
reconstitute hematopoiesis in irradiated mice at efficiencies
higher than progenitor cells from marrow, and Epo produced
by fetal liver per se may target stromal cells and affect their
activity in supporting progenitor cell development. Despite
these differences, the present studies clearly show that, when expressed at levels comparable
with the endogenous Epo receptor, the minimal Epo receptor
cytoplasmic subdomain containing only the box 1 and box 2
domains and 1 Y343 site exhibits wild-type activity in promot-
ing proliferation and hemoglobinization of erythroid progenitor
cells derived from adult spleen and marrow.

The observed ability of the minimal Epo receptor chimera
EE372 to also support megakaryopoiesis raises interesting
questions concerning the nature of key signals that this and the
endogenous receptors for Tpo and Epo transduce. In each,
membrane proximal box 1 and 2 cytoplasmic domains are
conserved and signaling depends critically on Jak2. However,
in the Tpo receptor, tyrosine phosphorylation is limited to
only 2 carboxyl terminal sites (ie, Y599, Y604 and Y626, Y631 in the murine and human receptors, respectively). In murine Mpl, Y599 and Y604 each appear to contribute to the activation of STAT5a/b and possibly STAT3. The Epo receptor and EE372 also selectively activates STAT5, and this signal transducer and activator of transcription therefore comprises 1 shared effector. Moreover, in the Tpo receptor, Epo receptor, and EE372, the selective uncoupling of STAT5 activation has been demonstrated to blunt proliferative and/or differentiation signaling in at least certain cell line models. However, in mice nullizygous for STAT5a and b, erythropoiesis and megakaryopoiesis are essentially unperturbed. Thus, this latter result either is the consequence of compensatory mechanisms or no essential roles exist for STAT5a or b in these lineages. In the Tpo receptor system, Shc recently has emerged as an apparently important transducer and is recruited to PY599 of murine Mpl. Mutation of this site inactivates differentiation signaling in megakaryocytic F36P cells as well as in myeloid WEHI3B-D, and 32D cells. Beyond this, Shc is known to be linked to Grb2/mSos/Ras/Ras and SHIP signaling and to induced polyploidy and gpIIb/IIIa expression in F36P cells is inhibited by a dominant-negative variant of c-H-Ras (S17N) and is stimulated by a dominant-active form (G12V). Thus, the intensity of Mpl and Shc-mediated activation of Ras has been suggested to regulate megakaryocyte development. In EE372, candidate PY sites for Shc recruitment are deleted. However, He et al previously have described an Epo receptor and Jak2-dependent pathway to Shc activation that efficiently proceeds in the absence of receptor tyrosine phosphorylation. Thus, Shc is proposed to comprise an attractive candidate effector of EE372, Epo, and Tpo receptor-mediated erythropoiesis and megakaryopoiesis. In addition, it is considered likely that a number of as yet undiscovered targets likely lie downstream of Jak2 per se, and it is proposed that certain of these also play important roles in Epo and Tpo response pathways. The observed ability of the minimal Epo receptor domain to support megakaryocyte formation in the absence of Tpo is inconsistent with an instructive minimal Epo receptor domain to support megakaryocyte formation in at least certain cell line models. However, in mice nullizygous for STAT5a and STAT3 the tyrosine phosphorylation of CBL and association with CRK adaptor proteins. Blood 89:1033, 1997

ACKNOWLEDGMENT

The authors thank Dr S.H. Orkin (Children's Hospital, Boston, MA) for generously providing pA2GATA as a transgene expression vector, Dr T.L. Blankenship-Paris for expert veterinary advice, and Dr E. Kunze for directing flow cytometric analyses.

REFERENCES


A Minimal Cytoplasmic Subdomain of the Erythropoietin Receptor Mediates Erythroid and Megakaryocytic Cell Development

Chris P. Miller, Zi Y. Liu, Constance T. Noguchi and Don M. Wojchowski