ERYTHROPOIETIN MODULATION OF PODOCALYXIN, AND A PROPOSED ERYTHROBLAST NICHE

Running title: Epo regulation of adhesion/migration factors

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ABSTRACT

Epo's erythropoietic capacity is ascribed largely to its anti-apoptotic actions. In part via gene profiling of bone marrow erythroblasts, Epo is now shown to selectively down-modulate the adhesion/migration factors chemokine receptor-4 (Cxcr4) and integrin alpha-4 (Itga4), and to up-modulate growth differentiation factor-3 (Gdf3), oncostatin-M (Onco-M) and podocalyxin like-1 (PODXL). For PODXL, Epo dose-dependent expression of this CD34-related sialomucin was discovered in Kit<sup>pos</sup>CD71<sup>high</sup> pro-erythroblasts, and was sustained at subsequent Kit<sup>neg</sup>CD71<sup>high</sup> and Ter119<sup>pos</sup> stages. In vivo, Epo markedly induced PODXL expression in these progenitors and in marrow-resident reticulocytes. This further was associated with a rapid release of PODXL<sup>pos</sup> reticulocytes to blood. As studied in erythroblasts expressing minimal Epo receptor (EpoR) alleles, efficient PODXL induction proved to depend upon an EpoR-PY343 Stat5 binding site. Moreover, in mice expressing an EpoR-HM F343 allele, compromised Epo-induced PODXL expression correlated with abnormal anucleated red cells representation in marrow. By modulating this select set of cell surface adhesion molecules and chemokines, Epo is proposed to mobilize erythroblasts from a hypothesized stromal niche, and possibly promote reticulocyte egress to blood.
INTRODUCTION

To maintain tissue oxygenation, red cell formation from progenitors in bone marrow, spleen and fetal liver is strictly regulated. In early progenitors, erythroid lineage commitment is directed by a unique set of DNA binding and transcription factors (e.g., GATA-1, EKLF-1 and FOG-1). Subsequent pro-erythroblast expansion is likewise sharply controlled, in part, by the glycoprotein hormone erythropoietin (Epo). Epo is expressed in adult kidney via hypoxia-inducible transcription factor pathways. Epo's subsequent interactions with its single transmembrane receptor (EpoR) are then thought to selectively support erythroblast survival. Redundant EpoR-activated survival pathways, in fact, have been described that depend upon PI3 kinase and AKT-dependent regulation of Foxo3a and (m)TOR, as well as EpoR/Jak2/Stat5-dependent induction of Pim1 kinase and the Bcl2 orthologue Bcl-xl. These response pathways likely contribute in important ways to Epo's clinical utility as an anti-anemia agent, and apparent cytoprotective actions on injured cardiac, endothelial, neuronal and renal cells.

Less attention has been paid to other possible key Epo action modes. Recently, our laboratory described a core EpoR/Jak2 signaling axis that supports steady-state erythropoiesis, but interestingly fails to support accelerated red cell production during anemia. Efficient stress erythropoiesis is rescued, however, upon the selective restoration of EpoR/PY343/Stat5 signaling. These findings prompted a search for new Epo-(and Stat5-) response genes that might promote stress erythropoiesis. This initially involved the first-time profiling of such genes in purified bone marrow-derived primary erythroblasts. Outcomes reveal Epo regulation of several chemokines and adhesion/migration factors including growth differentiation factor-3 (Gdf3), oncostatin-M (Onco-M), chemokine receptor-4 (Cxcr4), integrin alpha-4 (Itga4) and the CD34-related sialomucin, podocalyxin like-1 (PODXL).
Among this novel set of Epo-modulated factors, Onco-M acts via its JAK- and Stat- coupled hetero-dimeric receptor \(^1\) and can affect cell growth, differentiation and/or migration in tissue and context specific fashions \(^1\). Gdf3 acts as a TGF-beta receptor family antagonist, and is best characterized by its effects on embryonic dorsal axis formation \(^1\). Cxcr4 and Itga4, respectively, are the seven trans-membrane receptors for the Cxc chemokine SDF-1 \(^\text{22}\), and an integrin alpha-4 subunit that (as associated with beta-1 integrin) mediates binding to vascular cell adhesion molecule 1 (VCAM-1), fibronectin, and paxillin \(^\text{16,23,24}\).

PODXL is a sulphated sialomucin that is expressed at high levels by renal podocytes, and supports glomerular diaphragm slit formation via anti-adhesive and/or charge repulsion effects \(^\text{18,25}\). However, PODXL also is a marker for developing hemangioblasts and hematopoietic stem cells \(^\text{26}\), and can apparently exert anti-adhesive effects in cell migratory contexts \(^\text{18}\). Within the erythroid lineage, the presently discovered marked Epo- induction of PODXL (and the above chemokines and adhesion/migration factors) in erythroblasts is therefore proposed to promote transit from a hypothesized early stage niche, as well as reticulocyte egress from marrow, especially during anemia.

In bone marrow, niches for stem cells have been characterized at sinusoidal endothelia which affect self-renewal vs. differentiation \(^\text{27}\). Similarly, B-cell progenitor fates have been shown to depend on niche- associated interactions with sinusoidal reticular, and CXCL12\(^\text{high}\) stromal cells \(^\text{28}\). Erythroid islands also clearly exist, are broadly distributed in marrow, and are comprised of approximately ten erythroid cells plus a central resident macrophage \(^\text{29}\). Island formation depends in part upon ICAM4 and alpha-V integrin interactions, and appears to affect primarily late-stage erythroblast maturation \(^\text{29,30}\). By comparison, the presently proposed erythroid niche includes early stage erythroblasts, predicted stromal components, and several previously undescribed Epo target genes. Overall, findings suggest that Epo functions as more than a simple survival factor, and may dynamically modify the erythroblast cell surface and its microenvironment.
MATERIALS AND METHODS

Mice: Mice expressing EpoR-HM and EpoR-H alleles (and congenic controls) as described \(^{12,31}\), were used in IACUC-approved procedures at age 8-12 weeks. Hematocrits and reticulocytes were assayed by microcentrifugation, and flow cytometry (ReticCount-Reagent\textsuperscript{TM}, BD Biosciences) \(^{12}\). Epoietin-alpha was administered intraperitoneally at 1 and 24 hours at the doses indicated.

Primary erythroblast preparations: Marrow was flushed from femurs and tibiae in Iscove's modified Dulbecco's medium (IMDM, Invitrogen #12440-053) containing 2% FBS, passed through a 40 µm strainer, washed and resuspended in 1 mL of phosphate buffered saline (PBS) (Invitrogen #14190-144). Following a two-minute exposure to 9 mL of buffered 0.8% ammonium chloride (Stem Cell Technologies), 10 x PBS (1.1 mL) was added and cells were collected through 50% FBS in PBS and washed in IMDM. Ex vivo culture was at 8x10\(^5\) cells/mL in StemPro-34 (Invitrogen) supplemented with 2.5 U/mL Epo, 100 ng/mL mSCF, 1 uM dexamethasone, 1 uM beta-estradiol, 75 µg/mL h-transferrin, (Sigma #T0665), 0.5% BSA (Stem Cell Technologies, #9300), 0.1 mM 2- mercaptoethanol and 1.5 mM L-glutamine (i.e., “SP34-EX” medium) \(^{12}\). At day-3 of expansion, CD71\textsuperscript{pos}Ter119\textsuperscript{neg} erythroblasts were isolated by two rounds of Lin\textsuperscript{pos} cell depletion (Stem Cell Technologies) [cat#: 17066 as biotinylated antibodies to CD5 (Ly-1), CD45R/B220, CDllb (Mac1), Ter119, Ly6G (Gr1)]. Kit\textsuperscript{pos}CD71\textsuperscript{high} erythroblasts were then purified further by CD117 MACS selection (Miltenyi Biotech).

Gene profiling, data analysis, and RT-PCR: Purified Kit\textsuperscript{pos}CD71\textsuperscript{pos} cells were cultured for 6 hours in IMDM containing 0.5% BSA, transferrin (10 µg/mL) (Sigma #T0665), and insulin (15 ng/mL) (Invitrogen #41400-045). Cells then were exposed to Epo (+/- 5 U/mL) for 90 minutes, and RNA was isolated using Trizol reagent (Invitrogen) and robotic extraction (Autogen Prep245) \(^{12}\). Biotin-cRNA syntheses used 3 µg of RNA, and hybridizations were to Affymetrix 430-2.0 arrays. Signals were processed via GeneChip\textsuperscript{®} 3000 scanning, and GCOS software. In data mining, GeneTraffic, exploratory visual analysis (EVA), ChipInspector, BiblioSphere Pathway-Edition software
(Genomatix), and SAM were used. RT utilized TURBO DNase (Ambion) and Superscript III (Invitrogen). PCR primer pairs (SuperArray Bioscience) were: Onco-M, NM001013365; Gdf3, NM008108; Podxl, NM013723; Itga4, NM010576; Cxcr4, NM009911; Cis, NM009895; β-actin, NM007393. Quantitative PCR utilized iQ™ SYBR® Green and an i-Cycler (BIO-RAD).

**Flow cytometry:** In flow cytometry (BD FACScalibur), 1x10⁶ cells were incubated at 4°C with 1 µg of rat IgG in 0.2 mL of PBS, 0.5% BSA (15 minutes) and for 45 minutes with 1 µg of primary antibodies as: APC-Ter119 or APC-anti-Kit; PE-anti-CD71 (BD Biosciences, #557909, #553356 #553267); and biotin anti-PODXL (R&D Systems, #BAF1556) (or biotin– goat IgG as a negative control). Bound PODXL antibodies were detected using either AlexaFluor-488 or AlexaFluor-647 streptavidin (Molecular Probes). FITC-anti-Cxcr4 and PE-CD49d were from BD Biosciences, and Southern Biotech. Nucleated erythroblasts were assayed by co-staining with PE-Ter119 (BD Biosciences, #553673) and DRAQ5 (10 µM) (Alexis Biochemicals). Reticulocytes were co-stained with anti-PODXL, and Retic-COUNT. In all experiments, equivalent numbers of gated events were analyzed.

**Microscopy:** Cytospin analyses (1x10⁵ cells) involved slide-centrifugation (15 minutes, 300 rpm, Hettich Universal-16A cyto-centrifuge) and Dip-Stain reagent staining (Volu-Sol #VSS016). In confocal microscopy (Leica, LTCS-SP), staged erythroblasts were isolated, immuno-stained, washed, fixed in 4% paraformaldehyde and co-stained with Hoechst 34580 (Molecular Probes).

**Biotin-sialyl-Epo cell lysates, and Western blotting:** Bioactive biotin-sialyl-Epo was prepared as detailed by Wojchowski et al.⁴². This reagent was used to assay cell surface EpoR levels on purified Kit⁰CD7¹⁰Ter11⁰KitnegCD7¹⁰Ter11neg erythroblasts as follows. Cells were incubated (at 2°C for 3 hours) with biotin-sialyl-Epo at 10 U/mL in the presence or absence of unlabeled Epo at 300 U/mL. Cells were then washed and lysed as detailed by Menon et al.⁴³ with the exception that Igepal was decreased to 0.3%. Biotin-sialyl-Epo/EpoR complexes were retrieved from cleared lysates
using streptavidin CL4B agarose (Pierce #20347). EpoR levels then were assayed by Western blotting (Santa Cruz anti-EpoR antibody #SC697) 33.
RESULTS

Primary erythroblast system, and array based discovery of Epo-modulated chemokines and cell surface adhesion / migration factors

Experiments first investigated possible Epo regulation of novel response genes in murine bone marrow- derived erythroblasts. This primary target population was generated via short-term expansion of erythroid progenitor cells in an optimized serum-free SP34-EX system. At day 3.5, Kit\textsuperscript{pos}CD71\textsuperscript{high}Ter119\textsuperscript{neg} erythroblasts were isolated (from n=4 mice at ≥ 99% purity) via lin\textsuperscript{pos} depletion, and Kit\textsuperscript{pos} cell selection (Figure 1A). These staged (and maximally Epo- responsive erythroblasts) were then cultured for 6 hours in the absence of hematopoietic cytokines, and stimulated with Epo for 90 minutes. From these, and parallel unstimulated cultures, biotin-cRNAs and DNAs were prepared. Quantitative PCR also was used to confirm high-level induction of Cis1 (Figure 1B), a known Epo-response gene. In Figure 1C, relative differences for microarray outcomes are illustrated. Overall, approximately 200 Epo response genes were identified with high statistical significance.

For Affymetrix 430-2.0 array profiling outcomes, a focus was narrowed to transcripts that were modulated two-fold or more by Epo, and in addition corresponded to chemokine and adhesion/migration factors. This selectively included two secreted cytokines, Gdf3 and Onco-M, together with Cxcr4, Itga4, and PODXL (Figure 1D). Profiling data specifically indicated 10.8- and 3.1- fold induction of Onco-M and Gdf3; 1.3- and 2.2- fold down- modulation of Itga4 and Cxcr4; and 15.3- fold induction of PODXL. Follow-up quantitative RT-PCR analyses confirmed 16.3- and 2.2- fold induction of Onco-M and Gdf3, respectively, 2.3- and 2.9- fold down-modulation of Itga4 and Cxcr4, respectively, and 8.9- fold induction of PODXL (Figure 1E). (In supplemental Figure S-1, several additional functional subsets of Epo-response genes are outlined to further illustrate the robust nature of transcriptome profiling, and utility of this approach).
Gdf3 is a TGF-beta antagonist that inhibits classical TGF-beta and BMP signaling \(^{13}\), and is expressed predominantly in bone marrow, spleen, thymus and adipocytes \(^{13}\). Its expression by erythroid progenitor cells has not previously been described. Onco-M is a pleiotropic cytokine \(^{14}\), but disruption of its receptor selectively decreases erythro-megakaryocytic potentials \(^{19}\). Onco-M and Gdf3 therefore are implicated as uniquely activated chemokine components of an Epo signaling axis. Cxcr4 and Itga4 were each rapidly down-modulated several-fold by Epo (Figure 1D and 1E). Cxcr4 is a seven-transmembrane receptor for stromal derived factor-1 (SDF-1) and can support niche homing by several stem and progenitor cell types \(^{15,35}\). Within bone marrow, SDF-1 further can remain associated with stromal cell surfaces, and recruit Cxcr4\(^{pos}\) cells \(^{15}\). Itga4 preferentially binds VCAM1 and fibronectin (and is also important for early hematopoietic progenitor cell migration and development) \(^{17}\). For both Cxcr4 and Itga4, Epo-dependent down-modulation in Kit\(^{pos}\)CD71\(^{high}\) cells therefore suggests potential roles in promoting erythroblast transit from stromal cell compartments (as does the ensuing down-modulation of Kit per se in Kit\(^{neg}\) CD71\(^{high}\) erythroblasts – see below).

The CD34-related sialomucin, PODXL, in contrast, was strongly up-modulated by Epo in Kit\(^{pos}\)CD71\(^{high}\) erythroblasts (Figure 1D, E). PODXL is best known to be expressed by renal podocytes, and to support filtration slit formation \(^{18}\). However, PODXL also marks developing vascular endothelial cells, and hematopoietic stem cells \(^{18,26}\). For PODXL, Cxcr4 and Itga4, modulation at the erythroblast cell surface was further examined by flow cytometric assays. In keeping with transcript analyses, Epo up-modulated PODXL, and down-modulated Cxcr4 with dose-dependency (Figure 1F). Cell surface levels of Itga4, however, were not significantly affected by Epo over a 24-hour time frame. For Itga4, this might reflect a long half-life (or possibly the opposing actions of factors which promote Itga4 expression).

**Epo-specific induction of PODXL in developing erythroblasts, in part via an EpoR/PY343/Stat5 signaling axis**
The extent to which PODXL’s strong up-modulation might be affected specifically by Epo was next assessed. Kit^{pos}CD71^{high} cells were isolated, cultured in the absence of hematopoietic growth factors, and stimulated with either Epo or SCF. Time-course analyses revealed an approximate 25-fold induction of PODXL by Epo (Figure 2A). No such increase was affected by SCF. Epo’s ability to modulate PODXL expression within a staged series of erythroblasts also was examined. This included Kit^{pos}CD71^{high}, Kit^{neg}CD71^{high} and Kit^{neg}CD71^{high}Ter119^{pos} cells (see Figures 2B and 2C for flow cytometry and cytospin characterizations). (Larger scale images of stained cytospins also are provided in Supplemental Figure S-2). In Kit^{neg}CD71^{high} cells, PODXL expression increased markedly, and was sustained in late-stage Ter119^{pos}CD71^{high} erythroblasts (Figure 2B).

To more mechanistically consider Epo-regulated PODXL expression, induction via knocked-in EpoR-HM and EpoR-H alleles was studied. EpoR-HM retains a membrane proximal box-1 motif, activates JAK2, couples to MEK1/2 and ERK1/2, but otherwise lacks cytoplasmic PY signal transduction factor docking sites. EpoR-H is related, but possesses a selectively restored PY343 Stat5 binding site (Figure 3A). Erythroid cells were expanded from wt-EpoR, EpoR-HM and EpoR-H marrow preparations. Kit^{pos}CD71^{high} erythroblasts then were isolated and analyzed for PODXL expression at the transcript and cell surface levels (Figure 3B). Each analysis revealed deficient expression in EpoR-HM erythroblasts (PY-null allele), together with a substantial rescue of PODXL expression upon PY343 site restoration in EpoR-H erythroblasts.

Epo dosing effects on PODXL expression also were examined. Primary erythroblasts from wt-EpoR, EpoR-HM, and EpoR-H were isolated and expanded. At day 2.5, cells were transferred to SP34-EX medium with Epo at 0.1, 0.4, or 1.6 U/mL (without SCF). After 24 hours, lin^{pos}-depleted cell populations were analyzed. In wt-EpoR cells, PODXL expression in low-level Epo (0.1 U/mL) was visible via confocal microscopy, and in flow cytometry was detected on ~ 12% of erythroblasts (Figure 3B-1 and 3B-3). Higher dose Epo (1.6 U/mL) boosted frequencies of positive erythroblasts (to ~ 35%) (Figure 3B-2), as well as PODXL cell surface densities. In EpoR-HM cells, in contrast, little PODXL
was detectable. For EpoR-H cells, confocal images and flow cytometry revealed a substantial (yet partial) restoration of PODXL expression. These experiments, together with PODXL transcript analyses (Figure 3-B4), indicate that EpoR PY343 (and Stat5) signals are important for Epo regulation of PODXL, but that expression is enhanced by EpoR C-terminal signals. Finally, in silico analyses indicated the occurrence of two consensus STAT elements within the PODXL proximal promoter (Figure 3C). One further occurred within a STAT/ETS module which was also represented within the Cis 1 gene promoter.

Epo dose-dependent PODXL expression in vivo is sustained among immature circulating and marrow-resident reticulocytes, and rapidly induced at R5 and R4 stages

Based on Epo induction of PODXL by late-stage erythroblasts ex vivo, whether PODXL expression might persist among reticulocytes in vivo next was tested. Specifically, an Epo dose-response relationship for reticulocyte production first was defined, and peripheral red cells from Epo-injected mice were analyzed for PODXL expression (Figure 4A). In Epo-injected mice, a low percentage of RBCs (~13%) stained as PODXL pos; 20% of mature reticulocytes (R4 stage) were PODXL pos and stained up to five-fold brighter; and 74% of immature reticulocytes (R5 stage) were PODXL pos and stained at high-intensity (IRF, immature reticulocyte fraction) (Figure 4B). Within the IRF, PODXL densities interestingly also increased in a sharply Epo dose-dependent fashion (Figure 4C).

In vivo findings for PODXL pos expression among circulating reticulocytes prompted analyses of Epo-induced PODXL-positive erythroid progenitor cell production in marrow. Here, Epo was observed to stimulate sequential increases in the production of PODXL posKit posCD71 high proerythroblasts, NH4Cl-resistant early stage PODXL posCD71 highTer119 pos erythroblasts, and maturing PODXL posCD71 highTer119 pos erythroblasts (Figure 5A, upper left panels). For these PODXL pos cohorts, peak representation occurred sequentially at days 1.5, 1.5-2.5, and 2.5, respectively. These analyses therefore establish these (pro)erythroblast populations as in vivo targets for Epo-modulation of PODXL. Overall levels of total Kit posCD71 high, NH4Cl-resistant CD71 highTer119 pos and total
CD71highTer119pos erythroblasts also were analyzed (Figure 5A, upper right panels). For total populations of R4 reticulocytes (lower right panel), overall increases in frequencies post-day 2.5 were consistent with a wave of Epo induced erythroblast production (and PODXL expression), as were parallel decreases in early stage R5-reticulocyte pools (lower left panel). Based on a transient pulse of PODXLpos reticulocytes at day 0.5 (Figure 5B, upper panels), frequencies of Epo-induced PODXLpos subpopulations of R5 and R4 reticulocytes, however, suggested a perhaps less straightforward relationship. This Epo response is not so well understood, but could reflect rapid effects on the release of a late-stage erythroblast pool, and/or possible Epo-modulation of PODXL among late-stage cells. This prospect that Epo might modulate PODXL expression at late stages prompted analyses of EpoR expression levels in Ter119pos erythroblasts. This was accomplished by using biotin-sialyl-Epo to retrieve cell surface EpoR's from purified KitposCD71highTer119neg, and KitnegCD71highTer119pos cells. Ter119pos cells, in fact, proved to express EpoR at levels comparable to Kitpos pro-erythroblasts (see Supplemental Figure S-3).

Using a combination of Draq5 staining and light scatter properties (in parallel with thiazole orange), it also was possible to assay the formation of bone marrow-resident early R5- and late R4 -stage reticulocytes. In this compartment, two unexpected Epo-induced events were observed. First, in both R5 and R4 populations, frequencies of PODXLpos reticulocytes increased several fold within 0.5 days of Epo exposure (Figure 5B, upper panels). (For details on flow cytometry and gating of reticulocytes, also see Supplemental Figure S-4). Second, this was paralleled by an apparent decrease in overall numbers of marrow-resident R4 reticulocytes. This latter event is consistent with rapid Epo- (and possibly PODXL-) dependent effects on R4 reticulocyte release to blood. Epo’s apparent ability to modulate PODXL expression in marrow reticulocytes was analyzed further based on levels of Epo-induced expression (as assayed via relative fluorescence intensities of PODXL staining) (Figure 6A). In R5, and to a lesser yet significant extent in R4 reticulocytes, surface cell levels of PODXL expression were up-modulated several fold by Epo within 0.5 to 1.5 days. In parallel analyses of blood, frequencies of circulating PODXLpos R5 and R4 reticulocytes increased overall by ~ 6 fold and
~ 4 fold, respectively (Figure 6B) – and this included a rapid pulse in PODXL pos R4 reticulocyte levels at day 0.5 (upper right panel, arrow).

**EpoR-HM mice exhibit deficient Epo - induced PODXL expression and circulating reticulocyte production, and accumulate mature erythroblasts within the bone marrow compartment**

Finally, Epo-induced PODXL pos erythroid cell formation was examined in wt-EpoR, EpoR-HM and EpoR-H mice. As described by Zang et al. 31, EpoR-HM mice possess approximately wild-type BFUe and CFUe levels, and generate a near normal red cell mass at steady-state. As described by Menon et al. 12, however, this EpoR allele selectively fails to support efficient stress erythropoiesis. During Epo-induced reticulocyte formation, possible in vivo correlations with diminished PODXL expression levels in EpoR-HM mice therefore were sought. Interestingly, EpoR-HM mice failed to generate normal levels of circulating reticulocytes, even at high Epo doses (1800 U/kg) – and this paralleled a deficient representation of PODXL pos reticulocytes within an IRF compartment (Figure 7A).

Furthermore, EpoR-H mice exhibited clear Epo dose-dependent PODXL expression in the IRF while in EpoR-HM mice, the percentage of PODXL- expressing immature reticulocytes remained largely unchanged in response to increasing Epo doses (1200U/kg and 1500U/kg) (Figure 7B). Beyond this, when frequencies of nucleated marrow-resident red cells were analyzed (via Draq5 and Ter119 co-staining), abnormally elevated levels were observed in EpoR-HM marrow (Figure 7C). This latter finding is consistent with an aberrant retention of late-stage red cells, and correlates well with deficiencies in PODXL expression.

**DISCUSSION**

The present investigations reveal that Epo’s effects on bone marrow erythroblasts include a rapid and substantial modulation of a novel set of cell migration and/or adhesion factors. In part, the discovery of these Epo-modulated factors was accomplished through the use of a unique ex vivo system for bone marrow derived erythroblast development. In profiling experiments, a Kit pos CD71 high population
specifically was used based on analyses which demonstrated this maximum Epo responsiveness for this (pro)erythroblast cohort. In this regard, and based on the colony-forming properties of this progenitor pool, these Kit<sup>pos</sup>CD71<sup>high</sup> erythroblasts correspond most closely to CFUe. In this system, the ability of progenitor cells to develop to Kit<sup>neg</sup>CD71<sup>high</sup>Ter119<sup>neg</sup> and Kit<sup>neg</sup>CD71<sup>high</sup>Ter119<sup>pos</sup> erythroblast stages further enabled developmental analyses.

In Kit<sup>pos</sup>CD71<sup>high</sup> erythroblasts, profiling selectively identified five novel Epo response factors that may affect progenitor cell migration. Two are Epo-modulated cytokines, Onco-M and Gdf3. For Onco-M, this pleiotropic cytokine is known to induce bFGF-independent endothelial cell migration, as well as leukocyte migration across endothelial cell monolayers. In Kit<sup>pos</sup>CD71<sup>high</sup> erythroblasts, Onco-M notably was among the most highly induced Epo-response genes. Disruption of the Onco-M receptor-beta (OSMR) leads to specific defects in erythropoiesis, including decreased BFUe and CFUe and mild anemia. Reconstitution studies also suggest possible stromal cell effects in that transplantation of control hematopoietic cells into an OSMR<sup>-/-</sup> background yields decreased erythroid cell pools. As secreted by Epo-stimulated erythroid progenitors, Onco-M therefore might also modulate stromal components (or possibly later stage erythroid blood islands). Due to its potential multiple effects on diverse target cells, however, the nature of Onco-M’s candidate effects on erythroblast migration and/or development are presently speculative. For growth/differentiation factor 3 (Gdf3), this TGF-beta and BMP antagonist has not previously been associated with erythropoiesis, or Epo’s actions. TGF-beta, however has been shown to modulate human erythroblast proliferation, and promote differentiation. Gdf3 therefore may dampen this TGF-beta effect. Alternatively, BMP4 appears to be important selectively for stress splenic erythropoiesis and Gdf3 therefore may limit BMP4- dependent proerythroblast pools within this distinct niche. As illustrated by red cell- derived TGFbeta-1 inhibition of neutrophil chemotaxis, TGF-beta ligands can also affect cell migration, and Epo-induced Gdf3 therefore might more directly modulate developing erythroblasts.
In Kit\textsuperscript{pos}CD\textsubscript{71}\textsuperscript{high} erythroblasts, two cell surface adhesion and/or migration factors, Cxcr\textsubscript{4} and Itga\textsubscript{4}, were rapidly down-modulated by Epo. Cxcr\textsubscript{4} is a seven-transmembrane receptor for SDF-1, and is known to facilitate homing for hematopoietic, neural, smooth muscle and mesenchymal progenitors \textsuperscript{35,43-45}. Studies of bone marrow compartments furthermore have shown that SDF-1 can remain associated with stromal cell surfaces, and act to recruit Cxcr\textsubscript{4}pos cells \textsuperscript{46}. For early stage erythroid cells, Cxcr\textsubscript{4} therefore may support niche homing, and adhesion. Epo- mediated down modulation of Cxcr\textsubscript{4} then is predicted to facilitate erythroblast release from a stromal niche. Notably, this appears to occur in parallel with an observed rapid loss of Kit expression, which similarly is predicted to promote release from SCF-positive stromal cells \textsuperscript{47-49}. During the preparation of this manuscript, Kim et al. \textsuperscript{50} interestingly reported that GCSF-induced mobilization of myeloid Gr1\textsuperscript{pos} progenitors similarly may involve an active repression of Cxcr\textsubscript{4} expression \textsuperscript{50}. In Kit\textsuperscript{pos}CD\textsubscript{71}\textsuperscript{high} erythroblasts, Epo also down-modulated the expression of integrin-alpha4 (Itga\textsubscript{4}/VLA\textsubscript{4}/cd\textsubscript{49d}). As expressed in multiple hematopoietic lineages, Itga\textsubscript{4} heterodimerizes with beta-1 integrin and preferentially binds fibronectin, VCAM-1 and paxillin. Via these routes, Itga\textsubscript{4} can regulate hematopoietic progenitor cell adhesion to stroma \textsuperscript{51}, and Itga\textsubscript{4} deletion in hematopoietic tissues mobilizes hematopoietic progenitor cells \textsuperscript{16}. The presently indicated Epo down-modulation of Itga\textsubscript{4} therefore might also contribute to erythroblast release from a stromal niche.

In contrast to Cxcr\textsubscript{4} and Itga\textsubscript{4}, PODXL expression was sharply up-modulated by Epo. To our knowledge, connections between Epo and PODXL have not previously been reported (nor has cytokine-regulation of PODXL). Analyses also indicated specificity in that Epo, but not SCF rapidly up-modulated PODXL up to 25-fold in Kit\textsuperscript{pos}CD\textsubscript{71}\textsuperscript{high} marrow- derived erythroblasts. Investigations using erythroblasts with knocked-in EpoR-H or EpoR-HM alleles further indicated an important role for PY343- dependent signals for PODXL induction. Within the EpoR, PY343 previously has been shown to specifically mediate Stat5 binding and activation \textsuperscript{52,53}. PODXL induction by Epo therefore appears to require EpoR/PY343/Stat5 signals. Enhanced PODXL expression via the full length EpoR, however, indicates that additional circuits to PODXL gene activation exist. Previously, PODXL gene expression
has been shown in embryonic kidney cells to be stimulated by Wilms tumor-1 via a conserved -1615 element \(^{54}\), and to be repressed by p53 \(^{55}\). Otherwise, \textit{PODXL} gene expression is not well studied, and Stat5 has not previously been implicated as a \textit{PODXL} regulator. Correlations between elevated Stat5 activity and increased PODXL expression, however, do exist in the contexts of blood, breast and prostate cancers \(^{56-64}\). Among leukemias, abnormally elevated PODXL expression has been described in blasts from 77% of acute myelogenous leukemia (AML) patients with t(8;21)(q22;q22), inv(16)(p13q22) or t(15;17)(q22;q21) events; and in 81% of blasts from acute lymphoblastic leukemia (ALL) patients with t(9;22)(q34;q11.2) translocations \(^{62}\). In AML and ALL, evidence also exists to indicate that chronic Stat5 activation is of causative importance \(^{56,57}\). Direct correlations between Stat5 and PODXL in AML and ALL progression, however, remain to be tested. In invasive breast carcinoma, increased PODXL expression is an indicator of poor outcomes \(^{58,59}\), and Stat5 deregulation also may affect tumorigenesis \(^{58,59}\). While controversial, outcomes of certain breast cancer trials with Epo have raised questions concerning possible disease progression effects \(^{65,66}\). Finally, Epo recently has been demonstrated to stimulate Stat5 expression in prostate cancer cells \(^{67}\), and \textit{PODXL} germ line variants within a 7q32-q33 region of allelic imbalance are also associated with prostate tumor risk \(^{64}\).

Within erythropoietic contexts, the discovered stage specificity of Epo-induced PODXL expression, as well as PODXL’s proposed functions next are considered. Previously, and at embryonic day 10, predominant PODXL expression was described among the majority of hematopoietic progenitors, and for nucleated RBCs \(^{26}\). At subsequent neonatal stages, however, PODXL expression was progressively restricted to early Kit\(^{pos}\) progenitors. By day 7 post-partum, PODXL expression appeared to be restricted further to early Lin\(^{neg}\)Sca-1\(^{pos}\)Kit\(^{pos}\) progenitors. These analyses used largely unfractionated bone marrow cells (in which erythroid progenitor cell representation is limited), and gated on overall PODXL\(^{pos}\) cells. By comparison, the present ex vivo system provided for sensitive analyses of PODXL expression at discrete sub-stages of erythroid cell development – and revealed substantial frequencies of PODXL\(^{pos}\) Kit\(^{pos}\)CD71\(^{high}\), Kit\(^{neg}\)CD71\(^{high}\) and CD71\(^{high}\)Ter119\(^{pos}\).
erythroblasts. Moreover, levels of PODXL expression were discovered to depend sharply upon Epo levels. In previous analyses, PODXL expression in adult bone marrow erythroblasts likely was not detected due to this apparent need for increased Epo levels. This finding therefore is consistent with a select role for increased PODXL expression during anemia. Induction of anemia with phenylhydrazine, in fact, recently has been shown to lead to increases in frequencies of PODXL\textsuperscript{pos}-Ter119\textsuperscript{pos} erythroblasts\textsuperscript{26}. The observed dependence of efficient PODXL induction on an intact EpoR PY343 Stat5 binding site likewise implicates prime roles for PODXL selectively during stress erythropoiesis\textsuperscript{12}.

Somewhat unexpectedly, Epo’s effects on PODXL expression proved to also be exerted in late-stage erythroblasts, and perhaps within reticulocyte populations. In stage R4 and R5 reticulocytes, increases in PODXL expression levels were rapidly affected by Epo (i.e. within ≤12 hours). Epo is thought to act primarily on late CFUe stage erythroblasts, but apparent effects in derived reticulocytes could involve (for example) a stabilization of PODXL transcripts and/or protein. In addition, Epo appeared to induce a release of mature R4 reticulocytes from marrow in a parallel time-course. The notion that immature reticulocytes might comprise a direct target for this (and possibly additional) Epo responses therefore merits consideration. The overall impact of this released red cell population on the erythron, however, is presently undetermined. Finally, in mice expressing a minimal PY-null EpoR allele (EpoR-HM), Epo failed to efficiently stimulate circulating reticulocyte production. In addition, EpoR-HM reticulocytes exhibited only low-level PODXL expression, and were abnormally represented in bone marrow as apparently unreleased late Draq5\textsuperscript{neg} Ter119\textsuperscript{pos} cells. At limiting Epo concentrations or during anemia, EpoR-HM erythroblasts exhibit defects in survival and/or growth\textsuperscript{12,33,68}. In the present experiments, however, these potentially complicating features should not be major factors in that anemia was not induced, and high-dose Epo was administered (1500 U/kg). This result, together with discovered effects of Epo on a select set of adhesion factors (and cytokines) in bone marrow erythroblasts, supports the case that a novel action mode exists via which Epo acts dynamically (especially during anemia) to modulate (pro)erythroblast surfaces within a hypothesized
stromal niche, and to promote their migration (possibly to blood islands) (see model, Figure 7D).

Whether Epo’s actions in other tissues might involve similar responses is of significant interest, as is the prospect that PODXL or its expression might be altered in the contexts of myelodysplasia, resistance to Epo therapy, and/or diseases involving altered red cell adhesion (e.g. sickle cell disease).

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AUTHORSHIP CONTRIBUTIONS – P.S., M.P.M., O.B., O.O., and D.M.W. each contributed in major ways to the design and execution of essentially all experiments. W.K., E.H., and J.F. assisted in the development, and use of our SP34-EX expansion system, and in most primary culture experiments. W.K. also performed most RT and quantitative PCR analyses. All authors, including J.F. and K.N., contributed to figure construction, data analyses and interpretations, as well as manuscript writing and assembly.
REFERENCES


FIGURE LEGENDS

Figure 1. Gene array- based discovery of Epo-modulated cytokines and cell surface adhesion factors in murine bone marrow derived erythroblasts. A] Illustrated are steps used to expand and isolate Kit$^{pos}$CD71$^{high}$Ter119$^{neg}$ erythroblasts, together with a representative flow cytometric profile. B] For erythroblasts prepared from n=4 independent bone marrow preparations, hematopoietic cytokines were withdrawn for 6 hours, and cells then were exposed to Epo (+/- 5 U/mL). At 90 minutes, RNA was prepared and used in transcriptome analyses. As a control, levels of Epo-induced $Cis1$ transcript levels (right panel) were analyzed by quantitative RT-PCR. C] For Epo-regulated genes, genome-wide outcomes are illustrated by relative difference analyses. *.Cel files were analyzed using ChipInspector. Significantly changed probes were then defined by SAM analysis (left panel) at a false discovery rate of 0.0% (and corresponding significantly changed transcripts needed to be covered by at least three such probes). The SAM analysis method is described by Tusher et al., 69 and Storey and Tibshirani 70. The false discovery rate is a measure for discrimination of significant features. D] Affymetrix 430-2.0 array based analyses of Epo-regulated cytokine and cell surface adhesion factors. Values are mean fold-modulation by Epo (+/- SE, n=4). E] Quantitative RT-PCR analyses of Epo regulation of $Onco-M$, $Gdf3$, $Cxcr4$, $Itga4$ and $PODXL$. Values are means (+/- SE) and are normalized to beta-actin. F] For PODXL, $Cxcr4$, and $Itga4$, cell surface levels also were assayed (by flow cytometry) among Kit$^{pos}$CD71$^{high}$ erythroblasts following their isolation, and subsequent 24-hour culture in Epo at 0.1, 0.4 and 1.6 U/mL in the presence or absence of SCF (50 ng/mL).

Figure 2. Epo-specific PODXL induction in developmentally staged marrow erythroblasts. A] In MACS purified Kit$^{pos}$CD71$^{high}$ erythroblasts, time- courses of EPO, and SCF induction of $PODXL$ expression (following cytokine withdrawal) were assessed by quantitative RT-PCR. B] In SP34-EX expansion cultures, cell surface PODXL expression among Kit$^{pos}$CD71$^{high}$, Kit$^{neg}$CD71$^{high}$, and Kit$^{neg}$CD71$^{high}$Ter119$^{pos}$ erythroblasts was analyzed by flow cytometry. Frequencies of PODXL$^{pos}$ cells are indicated for bisected PODXL$^{low}$ and PODXL$^{high}$ subpopulations. C] For the above stages of
maturing erythroblasts, cytospin morphologies for MACS and/or FACS-purified populations also are shown.

**Figure 3. Epo- induced PODXL expression depends on EpoR/PY343/Stat5 signaling.**  
A] wt-EpoR and minimal knocked-in EpoR-HM and EpoR-H alleles are diagrammed. B] EpoR-HM fails to support efficient Epo-induced *PODXL* expression – Kit<sup>pos</sup>CD71<sup>high</sup> erythroblasts from wild-type, EpoR-HM, and EpoR-H marrow, were expanded. At day 2.5, expansion cultures were shifted to SP34-EX medium lacking SCF, and containing Epo at 0.1, 0.4, and 1.6 U/mL. At day 3.5, lin<sup>pos</sup>-depleted cultures were analyzed for PODXL expression by flow cytometry, (B-1, B-2) and confocal microscopy (B-3). Also graphed for wt-EpoR, EpoR-HM and EpoR-H erythroblasts is the fold-induction of *PODXL* due to Epo (1.6U/mL) (B-4). C] *In silico* analyses of predicted STAT elements and STAT/ETS modules in murine *PODXL* and *Cis1* promoters. The occurrences of consensus elements were predicted using Genomatix ChipInspector software.

**Figure 4. In vivo Epo dose-dependent expression of PODXL by immature reticulocytes.**  
A] Epo dose-dependent increases in reticulocytes, PODXL<sup>pos</sup> immature reticulocytes, and PODXL expression levels in wild-type mice - At day-3 post Epo injection (0, 600, 1800U/kg), increases in peripheral blood reticulocytes, and in PODXL<sup>pos</sup> immature reticulocytes (IRF, immature reticulocyte fraction) were assayed by flow cytometry (left and center panels). Average PODXL expression levels within the IRF also were assayed based on fluorescence intensity (right panels). B] PODXL staining intensities specifically within the IRF compartment, and among stage mature R4 and immature R5 reticulocytes are illustrated. Here, wild-type mice were injected with Epo (1200 U/Kg). For peripheral blood sampled at day-3, frequencies of PODXL<sup>pos</sup> cells within reticulocyte compartments then were determined. C] Epo dose-dependent increases in PODXL<sup>pos</sup> expression levels (cell surface densities) by R5-stage reticulocytes also are illustrated.
Figure 5. Epo rapidly induces the formation of PODXL^pos (pro)erythroblasts, and reticulocytes within bone marrow. A] Epo induction of the sequential formation of PODXL^pos bone marrow Kit^posCD71^{high} and CD71^{high}Ter119^pos erythroblasts - Wild-type mice were injected with Epo (1500 U/kg) at 1 and 24 hours. At days 0.5, 1.5, 2.5, 3.5 and 4.5, levels of marrow resident PODXL^posKit^posCD71^{high} (pro)erythroblasts, NH_4Cl-resistant PODXL^posCD71^{high}Ter119^pos erythroblasts, and PODXL^posCD71^{high}Ter119^pos erythroblasts were determined (left panels). For these populations, note the sequential waves of Epo-induced PODXL^pos erythroblast formation (arrows). Total levels of these erythroblast cohorts also were determined (right panels). B] Epo-induced formation of marrow-resident R5- and R4- PODXL^pos reticulocytes (upper panels). For R4 reticulocytes, also note the rapid Epo-induced decrease of this cohort within marrow (arrow, lower right panel).

Figure 6. Epo rapidly increases cell surface densities of PODXL expression in R5 and R4 bone marrow reticulocytes. A] In Epo-injected wild-type mice, cell surface levels (i.e. densities) of PODXL expression were assayed among bone marrow resident R5 and R4 reticulocytes. Values (relative fluorescent intensities) are means (+/- SE) for n=3 independent mice. Lower panels illustrate representative flow cytometry profiles at days 0, 0.5 and 1.5. B] R5 and R4 reticulocytes in peripheral blood also were assayed in Epo-treated mice, including total and PODXL^pos reticulocytes populations. For R4 reticulocytes, note the rapid pulse of PODXL positivity at day 0.5 (upper right panel, arrow).

Figure 7. Deficient EpoR- HM reticulocyte production in response to Epo, and abnormal representation of anucleate red cells in EpoR-HM marrow. A] For wt-EpoR, EpoR-HM and EpoR-H mice, time courses of Epo- induced in vivo reticulocyte production are graphed (left panel) (means +/- SE, n=5 per group, 1200 U/kg). Frequencies of PODXL^pos immature reticulocytes (IRF, day-3 post Epo) also are illustrated (right panel). B] Wild-type (wt-EpoR), EpoR-HM and EpoR-H mice were treated with Epo at 0, 1200 and 1800 U/kg. At day-3.5, levels of PODXL expression among immature reticulocytes were determined. C] In bone marrow of wt-EpoR, EpoR-HM and EpoR-H mice (at day-3 post-Epo injection, 1500 U/kg) relative frequencies (ratios) of anucleated vs. nucleated Ter119^pos cells
were determined based on DRAQ5 staining of Ter119<sup>pos</sup> cells. **Model for Epo- regulation of erythroid progenitor cell adhesion and migration within a proposed stromal niche.** Epo’s actions on Kit<sup>pos</sup>CD<sub>71</sub><sup>high</sup> proerythroblasts are depicted to involve an Epo dose-dependent repression of Cxcr4 expression and an induction of PODXL. This Epo response is sustained as progenitors advance to a Kit<sup>neg</sup>CD<sub>71</sub><sup>high</sup> erythroblast stage and exit a proposed stromal niche. Epo-dependent PODXL expression further persists among immature reticulocytes, and is hypothesized to enhance their release to blood.
(A) Graph showing fold-induction of PODXL TX over time with cytokine exposure.

(B) Flow cytometry analysis with CD71 and APC markers for Kit and Ter119.

<table>
<thead>
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<th>Kit</th>
<th>PODXL$^{pos}$</th>
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*(among gated PODXL$^{pos}$ cells)*

(C) Micrographs showing cell development with Kit and CD71 markers.

Figure 2
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Figure 5

(A) PODXL \textsuperscript{pos} / Kit \textsuperscript{pos} / CD71 \textsuperscript{high} \\

PODXL \textsuperscript{pos} / Kit \textsuperscript{pos} / CD71 \textsuperscript{high} (NH4) \\

PODXL \textsuperscript{pos} / CD71 \textsuperscript{high} / Ter119 \textsuperscript{pos} \\

Days Post Epo Injection

(B) PODXL \textsuperscript{pos} R5 Reticulocytes \\

PODXL \textsuperscript{pos} R4 Reticulocytes \\

R5 Reticulocytes \\

R4 Reticulocytes \\

Days Post Epo Injection
Figure 6

(A) BONE MARROW

(B) BLOOD

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Erythropoietin modulation of podocalyxin, and a proposed erythroblast niche

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