Erythropoietin guides multipotent hematopoietic progenitor cells toward an erythroid fate

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The erythroid stress cytokine erythropoietin (Epo) supports the development of committed erythroid progenitors, but its ability to act on upstream, multipotent cells remains to be established. We observe that high systemic levels of Epo reprogram the transcriptomes of multi- and bipotent hematopoietic stem/progenitor cells in vivo. This induces erythroid lineage bias at all lineage bifurcations known to exist between hematopoietic stem cells (HSCs) and committed erythroid progenitors, leading to increased erythroid and decreased myeloid HSC output. Epo, therefore, has a lineage instructive role in vivo, through suppression of non-erythroid fate options, demonstrating the ability of a cytokine to systematically bias successive lineage choices in favor of the generation of a specific cell type.

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RESULTS AND DISCUSSION

We developed a model where systemic Epo levels were selectively increased through hydrodynamic tail vein injection (Zhang et al., 1999) of a CMV-based Epo expression vector, leading to increased peripheral blood erythrocyte numbers (unpublished data). To determine any
preceding effect on erythroid progenitor numbers, we isolated the bone marrow Lin⁻Sca-1⁻c-Kit⁺ population, which contains the myelo-erythroid progenitors (Akashi et al., 2000), including CFU-E and proerythroblasts (Pronk et al., 2007), at 2 d after injection, and plated these cells under conditions permissive for both myeloid and erythroid differentiation. Staining of the developed colonies using 2,7-diaminofluorescein (DAF) to detect hemoglobin showed an increase in the proportion of erythroid colonies (Fig. 1 A). However, this was accompanied by a decrease in the total colony number (Fig. 1 B).
This observation was at variance with the notion that Epo acts primarily as a proliferation/survival factor of E lineage–committed cells, attributing the relative increase in erythropoiesis, at least in part, to suppression of alternative differentiation programs. Further analysis showed that elevated systemic Epo did not affect bone marrow cellularity (Fig. 1 E) or the proportion of total myeloid progenitors (Fig. 1 F). However, flow cytometry–based progenitor phenotyping (Fronk et al., 2007) showed that committed E progenitors (preCFU–E and CFU–E) were increased, whereas committed GM progenitors (GMP and preGM) and Mk progenitors (MkPs) were decreased (Fig. 1, G and H). Similar skewing of myelo-erythroid progenitor numbers was observed upon injection of recombinant Epo (unpublished data). To determine if lineage skewing occurred in the upstream Lin− Sca−1−c–Kit+ (LSK) hematopoietic stem cell (HSC)/multipotent progenitor (MPP) compartment, this population was analyzed for Flt3 expression, which is normally associated with loss of Mk/E potential and increased lymphoid potential (Adolfsson et al., 2005). We observed that the proportion of Flt3hi lymphoid-primed MPPs (LMPP; Adolfsson et al., 2005) was decreased upon Epo exposure (Fig. 1 I), consistent with the observed suppression of lymphoid
colony-forming cells (Fig. 1 C). Therefore, at all known lineage bifurcations between HSCs and committed E progenitors, elevated Epo levels impaired the formation of progenitor cells lacking E potential (LMPP, preGM/GMP, and MkP), selectively promoting the formation of E committed progenitors (CFU-E and preCFU-E), and this process is initiated within the HSC/MPP compartment.

Previous studies have shown that the transcriptional priming of HSCs is an indicator of their lineage potential, and that the priming pattern is regulated by the transcription factors required for subsequent lineage commitment (Bereshchenko et al., 2009; Mancini et al., 2012). To determine whether Epo exposure alters the priming pattern of stem and progenitor cell populations, we mined existing global gene profiling data (Pronk et al., 2007) to identify gene sets highly and selectively expressed in preGMs, preCFU-Es, MkPs, and common lymphoid progenitors (CLPs), representing the first GM, E, M, and lymphoid committed progenitors, respectively (Fig. S1).

Gene profiling of in vivo Epo-exposed LSKFlt3− cells showed that genes associated with E lineage commitment were up-regulated, whereas those associated with M and GM lineage commitment were down-regulated (Fig. 2 A). This occurred without any change to the size or composition of the HSC compartment (Fig. 2, B and C), to the cell cycle status of HSC/MPPs (Fig. 2 D), or to HSC repopulating activity (not depicted). Downstream of HSCs, bipotent preMegEs had up-regulated preCFU-E genes, whereas most MkP gene expression was suppressed (Fig. 2 E), consistent with the observed erythroid bias at the MkP/preCFU-E bifurcation. LMPPs showed general down-regulation of CLP and preGM genes (Fig. 2 F).

The decrease in LMPP lymphoid gene expression was accompanied by suppressed LMPP B cell differentiation potential (Fig. 2 G), and a decreased number of downstream CLPs (Fig. 2 H). The effect of Epo on GM and T cell potential of LMPPs remains to be determined. Lineage programming of stem/progenitor cells was therefore regulated by Epo, and correlated with altered lineage potential and progenitor output.

To address if Epo acts directly and instructively on the HSC/MPP compartment, we isolated LSKCD150+Flt3− cells and treated these with recombinant Epo in vitro. We observed a virtually identical pattern of regulation of lineage programming as seen upon in vivo Epo exposure (Fig. 3 A). To determine if endogenous erythropoietic stress would cause similar transcriptional HSC reprogramming, CD45.2 *Gata1* conditional null (or control) cells (CD45.2) were co-transplanted with wild-type CD45.1 cells into lethally irradiated CD45.1/2 hosts at a 10:1 ratio, followed by *Gata1* deletion through poly(I-C) induction of the *Mx1-Cre* and forcing the wild-type CD45.1 HSCs present along with wild-type CD45.1 cells into lethally irradiated CD45.1/2 recipients. Transplanted HSCs were isolated from CD45.2 miR144/451−/− GATA-1−/− eGFP/+ mice. This strain contains an EGFP knockin into a microRNA locus uniformly expressed in E lineage cells, including circulating mature erythrocytes, allowing for EGFP-based tracking of E lineage output (Rasmussen and O’Carroll, 2011; Mancini et al., 2012; Fig. 4 A). Analysis of peripheral blood showed that Epo–exposed HSCs generated significantly higher numbers of erythrocytes (Fig. 4, B and C) and fewer myeloid cells (Fig. 4 D). Overall, a fourfold increase of the erythrocyte/myeloid output ratio was observed.

There are several signaling pathways that emanate from the Epo receptor, including the phosphoinositols (PI3) kinase/Akt, Erk/MAPK, and JAK–STAT pathways (Richmond et al., 2005). To determine their individual contributions to Epo-induced transcriptional reprogramming, LSKFlt3−CD150+ HSCs were exposed to Epo in vitro in the presence of inhibitors specific for these pathways (Fig. 3 D). We observed that up-regulation of preCFU-E genes was consistently dependent on PI3 kinase activation, whereas JAK/STAT and Erk activation was involved in a gene-specific manner. In contrast, preGM gene expression was not significantly affected by PI3 kinase inhibition, whereas, remarkably, Erk inhibition converted the normal inhibition by Epo to robust up-regulation. The major Epo-activated signaling pathways therefore play distinct and lineage-specific roles in reprogramming of the HSC transcriptome. We have previously shown that loss of C/EBPα function leads to loss of myeloid programming in LSKFlt3−CD150+ HSC/MPPs (Bereshchenko et al., 2009). Direct regulation of myeloid genes by C/EBPα may therefore explain the highly coordinated regulation of preGM genes upon inhibitor treatment.

To further investigate the cellular mechanism underlying Epo-induced transcriptional reprogramming, we performed single cell gene expression profiling of control and Epo exposed LSKFlt3− HSCs. We investigated the expression of GM (*Mpo, Eng, Gsf1, Csf1r, and Csf2rn*)-, E (*Gata1* and *Klf1*), and HSC (*Hoxb4, Bmi1, Meis1*, and *Cxxr4*)-associated genes. We observed a 50% increase in proportion of cells expressing one or more E genes (Fig. 3 E). In addition, the average expression of E genes in these cells was increased (1.8-fold, *P* = 0.10), with a similar decrease in the average expression of GM genes (1.7-fold, *P* = 0.008; Fig. 3 F). Importantly, we consistently observed coexpression of E genes with one or more GM genes, as well as HSC-associated genes, consistent with the increase in E programming occurring in multipotent stem/progenitor cells, rather than through the expansion of a subpopulation of committed erythroid progenitors.

To establish if Epo-induced reprogramming of stem/progenitor cells resulted in skewing of their lineage output, we transplanted control and Epo–exposed HSCs (defined as LSKFlt3−CD150+; CD45.2 allotype) into sublethally irradiated CD45.1 recipients. Transplanted HSCs were isolated from CD45.2 miR144/451−/− GATA-1−/− eGFP/+ mice. This strain contains an EGFP knockin into a microRNA locus uniformly expressed in E lineage cells, including circulating mature erythrocytes, allowing for EGFP-based tracking of E lineage output (Rasmussen and O’Carroll, 2011; Mancini et al., 2012; Fig. 4 A). Analysis of peripheral blood showed that Epo–exposed HSCs generated significantly higher numbers of erythrocytes (Fig. 4, B and C) and fewer myeloid cells (Fig. 4 D). Overall, a fourfold increase of the erythrocyte/myeloid output ratio was observed.

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Figure 3. Cellular and molecular mechanism of Epo action on HSCs. (A) LSKFlt3−CD150+ cells were cultured in the presence (Epo) or absence (Control) of Epo. After 24 h, cells were harvested and analyzed for gene expression. Data are represented as in Fig. 2. Values are means, n = 2, from 2 experiments with triplicate measurements. (B) Bar graph showing different serum cytokines levels in CD45.1/2 mice competitively transplanted with $10^5$ CD45.1 wild-type cells and either $10^6$ CD45.2 Gata1fl/fl; Mx1-Cre$^{tg/+}$ (cKO) or $10^6$ Gata1fl/fl BM cells (Con). Values are mean ± SD, n = 9 (control) and 8 (cKO), from 2 experiments. (C) CD45.1/2 mice were competitively transplanted with $10^5$ CD45.1 wild-type and either $10^6$ CD45.2 Gata1fl/fl; Mx1-Cre$^{tg/+}$ (cKO) or $10^6$ Gata1fl/fl BM cells (Con). Cre recombination was induced by three poly(I-C) injections at 2-d intervals, and CD45.1+CD45.2−LSKFlt3−CD150+ cells were isolated 6 wk after the first poly(I-C) injection and subjected to gene expression profiling. Data are represented as the log2 of the ratio between gene expression in WT/cKO and WT/Control co-transplanted mice after normalization to Hprt. Values are means, n = 2, from 2 experiments with triplicate measurements. (D) LSKFlt3−CD150+ cells were cultured in the presence (Epo) or absence (Control) of Epo. For both conditions, individual cultures were supplied with either vehicle (DMSO) or inhibitors of one of the following kinases: 25 µM Jak2 (AG490), 25 µM PI3K (LY294002), or 25 µM ERK1/2 (PD98059). After 24 h, cells were harvested and analyzed for gene expression. Values are means, n = 2, from 2 experiments with triplicate measurements. (E) Heat map of gene expression in single sorted LSKFlt3− HSCs analyzed by microfluidics-based real time PCR. Expression values were normalized using Kit expression and are shown as deviation from the mean expression value of each individual gene. n = 3 from 3 experiments. (F) Bar graph of mean expression of GM−, E−, and HSC-associated genes in single cells from E. Values represent the mean ΔΔCt(gene)−ΔΔCt(Kit) for each of the genes indicated, including only cells where expression was detected. ***, P < 0.0005.
Exposure of HSC/MPPs to Epo is therefore sufficient to bias lineage output away from the myeloid lineage and toward erythropoiesis, even in the absence of exogenous Epo during their further lineage commitment and differentiation. These results challenge our current view of lineage-specific cytokine action in two ways. First, they show that Epo can act on HSC/MPPs, in addition to E lineage–committed cells, to simultaneously increase E and decrease GM output. Second, we find that this occurs through transcriptional reprogramming, leading to increased E lineage priming and decreased GM, Mk, and lymphoid priming, and the systematic suppression of alternative lymphoid and myeloid lineage options at all lineage bifurcations examined. Up-regulation of erythroid gene expression showed a strong dependence on PI3 kinase signaling. In contrast, Erk signaling was critical for myeloid gene suppression; if Erk activity was blocked, Epo stimulation led to a general up-regulation of myeloid gene expression. This dissociation of lineage reprogramming is consistent with an instructive, rather than selective, effect of Epo on the HSC compartment; Epo exposure could lead to the selective expansion or survival of cells expressing high levels of preCFU-E and low levels of MkP and preGM genes. However, this model is not readily reconciled with the ability of simultaneous Epo stimulation and Erk inhibition to up-regulate CFU-E and preGM genes and suppress MkP genes. Our results therefore show that the high systemic levels of Epo associated with severe anemia can act in a direct and instructive manner on the HSC/MPP compartment, leading to a reduction of GM, Mk, and lymphoid phenotypic progenitors and colony-forming cells. The overall result is the creation of enhanced throughput from HSCs to the E compartment, an “erythroid superhighway,” allowing the focused generation of E-lineage progenitors (Fig. 4E), upon which Epo may then further act through its well-characterized proliferative and pro-survival effects.

MATERIALS AND METHODS

Mouse lines. C57BL/6J (CD45.2+) mice and congenic B6.SJL-PtprcaPep3b/BoyJ (CD45.1+ congenic C57BL/6J) mice were bred at the University of Edinburgh. The Gata1 conditional allele (Lindeboom et al., 2003), Mx1-Cre transgenic mice (Kühn et al., 1995), and mir144/451-eGFP reporter mice (Rasmussen and O’Carroll, 2011) were previously described. Mouse strains were backcrossed to C57Bl6/J for six or more generations before use, and mice were aged 8–12 wk at the start of experiments. All animal procedures were approved by the University of Oxford Clinical Medicine Ethical Review Committee and were maintained in accordance with Institutional and UK Home Office guidelines.

Hydrodynamic gene transduction and ELISA. Mice were tail vein–injected, as described previously (Hirai et al., 2006), in Krebs-Ringer buffer with 1 µg of pCMV6-Entry vector containing Epo cDNA (OriGene) or empty pCMV6-Entry vector. Epo, Thrombopoietin, and G-CSF serum levels were determined using Quantikine immunoassays (R&D Systems).
Flow cytometry, cell separation, and cell sorting. Flow cytometry was performed essentially as previously described (Bereschenko et al., 2009). A list of antibodies used is provided in Table S3. Cells were sorted using FACS Aria II cell sorters and analyzed using LSRFortessa (BD). All FACS data were analyzed with Flowjo software (Tree Star).

In vitro culture assays. Media for colony-forming assays were from STEMCELL Technologies and used according to the manufacturer’s instructions. 500 cells were seeded per culture in M3343, M3434, and Megacult media and 1,000 cells in M3436 and M3630 media. The colonies generated in methylcellulose were scored as BFU-E, CFU-GM, and CFU-B by morphometry 7–10 d after seeding, and as CFU-Mk (in MegaCult assay) by acetylatedinesterase staining. To score E colonies in multilineage culture (M3434), colonies were stained with 2,7-diaminofluorene (DAF) staining solution (10 mg/ml DAF in 90% glacial acetic acid, 50% H2O2, and 200 mM Tri-HCl, pH 7.5) and DAF-positive cells were identified as cells with intracellular blue granules. Cells were plated in duplicate or triplicate for each assay. B cell potential of LMPPs was evaluated as previously described (Adolfsson et al., 2005) with B-lineage cells defined as Mac-1

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levels of GFP+Ter119+ erythrocytes that were identified by their distinct cated time point, blood samples were evaluated by flow cytometry for the

gene by subtraction of the mean value of all positive cells for the gene. These normalized values were used to generate heat maps, and to calculate the
cumulative significance of Epo regulation of E and GM genes (i.e., all E genes without Epo against all E genes with Epo, and the same comparison for GM genes) using Student’s t test.

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