Extrinsic and intrinsic control by EKLF (KLF1) within a specialized erythroid niche

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ABSTRACT

The erythroblastic island provides an important nutritional and survival niche for efficient erythropoietic differentiation. Island integrity is reliant on adhesive interactions between erythroid and macrophage cells. We show that erythroblastic islands can be formed from single progenitor cells present in differentiating embryoid bodies, and that these correspond to erythro-myeloid progenitors (EMPs) that first appear in the yolk sac of the early developing embryo. Erythroid Krüppel-like factor (EKLF; KLF1), a crucial zinc finger transcription factor, is expressed in the EMPs, and plays an extrinsic role in erythroid maturation by being expressed in the supportive macrophage of the erythroblastic island and regulating relevant genes important for island integrity within these cells. Together with its well-established intrinsic contributions to erythropoiesis, EKLF thus plays a coordinating role between two different cell types whose interaction provides the optimal environment to generate a mature red blood cell.

KEY WORDS: EKLF/KLF1, Erythroblastic island, Erythroid-myeloid progenitor, Mouse

INTRODUCTION

Central to the homeostasis of the hematopoietic system is the correct balance of progenitor cell proliferation versus lineage-committed differentiation (Orkin and Zon, 2008). The first site of hematopoiesis occurs in the yolk sac and provides the primitive erythrocytes essential for survival of the early embryo until the definitive wave of hematopoiesis begins (McGrath and Palis, 2008). Definitive erythropoiesis in the fetal liver and postnatal bone marrow consists of morphologically identifiable, nucleated precursors that progress from the proerythroblast to enucleated reticulocytes (Granick and Levere, 1964). These events occur while the erythroblasts are in close physical contact with a macrophage in structures known as erythroblastic islands (Chasis and Mohandas, 2008; Manwani and Bieker, 2008).

Erythroblastic islands were described over 50 years ago as specialized microenvironmental compartments within which mammalian erythroblasts proliferate and efficiently differentiate (Bessis, 1958). Erythroblastic islands were first observed in bone marrow, although they are also present in the splenic red pulp and in the fetal liver (Chasis and Mohandas, 2008; Manwani and Bieker, 2008). These islands consist of a central macrophage that extends cytoplasmic protrusions to a ring of 5-30 surrounding erythroid cells encompassing proerythroblast to reticulocyte stages (Gifford et al., 2006). Extensive macrophage-erythroblast and erythroblast-erythroblast adhesive interactions are necessary for a thriving definitive erythropoietic community. As a result, structural proteins that mediate these interactions (Fabricik et al., 2007; Lee et al., 2006; Liu et al., 2007; Mankelow et al., 2004; Sadahira et al., 1995; Soni et al., 2006) as well as transcriptional factors (Gutiérrez et al., 2004; Iavarone et al., 2004; Kusakabe et al., 2011) are both crucial for promoting an effective differentiative environment. Erythroblasts can proliferate, mature and enucleate in vitro in the absence of other cell types; however, this process is typically very inefficient at all stages (Hanspal et al., 1998; Rhodes et al., 2008).

Macrophages provide not only nutrients, but also proliferative and survival signals to the erythroblasts. Macrophages phagocytose extruded erythroblast nuclei at the conclusion of erythroid maturation (Chasis and Mohandas, 2008; de Back et al., 2014; Manwani and Bieker, 2008), and deoxyribonuclease II alpha (DNASE2A) is required for this process (Kawane et al., 2001; Yoshida et al., 2005).

Chemical ablation of splenic macrophage dramatically impairs erythropoiesis (Sadahira et al., 2000), showing the in vivo importance of macrophage in erythroid biology. Powerful additional validation comes from two recent studies (Chow et al., 2013; Ramos et al., 2013) demonstrating that macrophage play a crucial role in stress erythropoiesis, not only after anemia, when efficient erythroid expansion and enucleation are required, but also under pathological conditions, where they provide a supportive niche for the proliferation of altered erythroid cells, such as is observed in polycythemia vera. These studies also corroborate the role of macrophages in supplying iron to assure effective erythropoiesis.

Despite knowledge of their existence for decades, several gaps remain in our understanding of the molecular controls that are important for coordinating the onset and decline of erythroid-macrophage interactions and how they interface with enucleation events that lead to a mature reticulocyte. This becomes particularly important in the context of aberrant repression or expansion of these final maturation steps under disease conditions (Koury, 2014).

EKLF (erythroid Krüppel-like factor; KLF1) is a red cell-enriched, zinc finger DNA binding protein that interacts with its cognate 5′-CCMCRCCCN-3′ element at target promoters and enhancers (Miller and Bieker, 1993). Its roles in mouse and human β-like globin gene regulation during terminal erythroid differentiation have been well established using genetic, biochemical and molecular approaches (Bauer and Orkin, 2011; Siatecka and Bieker, 2011; Tallack and Perkins, 2010, 2013; Yien and Bieker, 2013). EKLF is also highly expressed in the megakaryocyte/erythroid progenitor, where it may play a determining role in the bipotential decisions that lead to preferential establishment of erythroid progeny (reviewed by Dore and Crispino, 2011; Siatecka and Bieker, 2011).

EKLF’s activation target repertoire has expanded beyond the classical β-globin gene to include protein-stabilizing, heme biosynthetic pathway, red cell membrane protein, cell cycle and
transcription factor genes in both primitive and definitive erythroid cells (Siatecka and Bieker, 2011; Tallack and Perkins, 2010; Yien and Bieker, 2013). Relatedly, links have been established between mutant or haploinsufficient levels of EKLF and altered human hematology and anemia (Borg et al., 2011; Helias et al., 2013; Siatecka and Bieker, 2011; Singleton et al., 2012).

Comparative analysis of expression arrays between EKLF wild-type and EKLF-null fetal liver cells show that a number of genes involved in execution of the terminal erythroid differentiation program are downregulated in the absence of EKLF (Drissen et al., 2005; Hodgson et al., 2006; Pilone et al., 2006, 2011; Tallack et al., 2012, 2010). Our studies, originating from observations of differentiating erythroid cells from embryoid bodies, have uncovered an erythroid-intrinsic role for EKLF in erythroblastic island biology. However, they also quickly converged on an unanticipated molecular regulatory role for EKLF within the macrophage of the island, revealing an important extrinsic role in erythroid/macrophage biology.

RESULTS

Erythroblastic islands from differentiating embryonic stem cells

We made some unanticipated observations during our analyses of embryoid body (EB) differentiation from murine embryonic stem cells (ESCs) (Frontelo et al., 2007; Manwani et al., 2007). Isolated ESCs were differentiated for 4 days to enrich for the presence of primitive erythroid progenitors. The resultant EBs were then dispersed and plated at low density in methylcellulose under conditions optimal for primitive erythroid colony formation (Kennedy and Keller, 2003). Individual erythroid colonies were picked, cytospun onto glass slides and viewed after staining with May-Grünwald Giemsa. Strikingly, all cytospin preparations showed maturing erythroblasts arranged in a ring around a central macrophage (Fig. 1A), a markedly close resemblance to the classic erythroblastic island morphology (e.g. Hanspal et al., 1998). These surprising results suggest that an erythroblastic island can be formed from a primary erythroid source. These structures will be referred to as embryoid body erythroblastic islands (EBEIs).

To address whether we could also observe the same results from a definitive erythroid source, we developed a protocol to prospectively sort dispersed cells from differentiating EBs at day 6 (and thus enriched for definitive erythroid cell production) into lin−Kit+scα1-FcR−CD34+[presumptive common myeloid progenitor (CMP)] and lin−Kit+scα1-FcR−CD34−[presumptive megakaryocyte/erythrocyte progenitor (MEP)] fractions (based on Akashi et al., 2000; Nakorn et al., 2003; Terszowski et al., 2005), and plated these individually sorted cells at low density onto methylcellulose under conditions optimal for definitive colony analysis. It has been difficult to obtain colonies from such prospectively sorted populations when derived from differentiating EBs (Drissen et al., 2010); indeed, we found that use of an ESC-based medium rather than the standard media conditions was absolutely crucial for success (see Materials and Methods). Cytospins of the resulting robust, red colonies again revealed erythroblasts surrounding a central macrophage (Fig. 1B).

These initial observations imply that EBEIs can be formed from single cells; such an idea has not been examined in prior studies of erythroblastic island formation. As a result, to further substantiate the clonal nature of our observations we isolated single Hoechst−/−Kit+ cells from EBs at day 6 and plated these in methylcellulose at various concentrations (Fig. 2). The results show a strong linear relationship between cells plated and the resultant colonies (~9%) that crosses through zero, supporting the idea that the colonies are clonal.

A number of cell adhesion molecules and their interactions within the erythroblastic islands are crucial for island integrity in definitive populations derived from fetal livers and bone marrow (Chasis and Mohandas, 2008; Manwani and Bieker, 2008). Integrin/actin cytoskeleton interactions may coordinate adhesion and gene expression in the erythroblastic islands by regulating intracellular signaling. We wished to address whether such a mechanism was operant in our primitive EBEI colonies. The erythrocyte-specific isoform of intercellular adhesion molecule 4 (ICAM4) is expressed on erythroid cells and interacts with αv integrin on macrophage cells (Spring et al., 2001). Blocking ICAM4/αv binding with αv synthetic peptides produces a 70% decrease in islands reconstituted in vitro (Lee et al., 2006), and island formation is predominantly defective in ICAM4-null mice (Lee et al., 2006). As a result, we tested the effects on primitive EBEI formation of a synthetic peptide that has previously been used to block ICAM4/αv adhesion and definitive erythroblastic island formation in reconstitution assays (Lee et al., 2006; Mankelow et al., 2004). One synthetic peptide SVPFWVRSMS (FWV) and scrambled peptide (control) were used in increasing concentrations (0.5-2.0 mM) in EBEI assays. The FWV peptide, but not the control, caused a marked, concentration-dependent decrease in the number of colonies per dish (Fig. 3, left). The colonies that did form were smaller and paler (supplementary material Fig. S1). Examination of cytospins from the affected colonies showed complete absence of island-type structures and a reduction in erythroblasts (Fig. 3, right; note that macrophages are still apparent). These data demonstrate that erythroblast ICAM4
binding to macrophage αV integrins is important for proper colony formation even when derived from a primitive erythroid cell.

Collectively, these data strongly suggest that contact between the macrophages and developing erythroblasts is necessary for efficient execution of the primitive and definitive erythroid programs in differentiating EBs. The fact that these arise in a clonal fashion implies that there exists a cell that contains its own intrinsic island-forming capacity.

**EKLF regulation of relevant targets in erythroblastic islands**

Array and RNA-seq data demonstrate that ICAM4 levels are decreased in EKLF-null primitive and definitive erythroid cells (Hodge et al., 2006; Isern et al., 2010; Pilon et al., 2008; Tallack et al., 2012) and in a congenital dyserythropoietic anemia (CDA) patient harboring the EKLF-E325K mutation that exhibits a defect in red cell enucleation (Arnaud et al., 2010). ICAM4 levels also mirror EKLF levels during primitive and definitive erythroid differentiation (Fig. 4A) (Kingsley et al., 2013). We directly verified the array results by comparative qRT-PCR analysis of RNA from embryonic day 13.5 (E13.5) wild-type or EKLF-het or -null fetal liver definitive cells and found that ICAM4 levels are dramatically reduced in the absence of EKLF (Fig. 4B). Although other molecules, such as EMP (MAEA) or α4β1 integrins (ITGA4/ITGB1) could also be contributory in the context of erythroblastic island formation (Chasis and Mohandas, 2008; Manwani and Bieker, 2008), these are not affected by changes in EKLF levels (Pilon et al., 2011, 2008; Tallack et al., 2012).

Perusal of EKLF chromatin immunoprecipitation sequencing (ChIP-seq) data (Pilon et al., 2011) shows EKLF binding to a region upstream of the Icam4 gene (Fig. 4C). This overlaps with GATA1, TAL1 and p300 binding, consistent with the ‘core’ transcription factor observations that have been noted at a subset of EKLF binding sites (Li et al., 2013; Su et al., 2013; Tallack et al., 2012; Wontakal et al., 2012). Inspection of the EKLF DNA binding cognate sequence shows that it falls into the ‘class I’ site category within 5’-CCMCRCCCN, and thus should bind the neonatal anemia (Nan) EKLF variant based on our analysis of Nan-EKLF biochemical properties (Siatecka and Bieker, 2011; Siatecka et al., 2013). Consistent with this, ICAM4 RNA levels are not affected in Nan mouse fetal livers (data not shown).

As the colony preparations that gave rise to EBEI were clonal (being from dispersed single cells seeded in methylcellulose), we considered the possibility that EKLF might be expressed in both the erythroid cell and macrophage. There have been hints of EKLF expression in macrophage (Luo et al., 2004), although our analysis of adult bone marrow hematopoietic material had shown no evidence (Frontelo et al., 2007). To address this we used the mouse strain derived from ESCs that contain a single copy of the EKLF promoter directly upstream of a GFP reporter, integrated into the HPRT locus (Fig. 5A) (Lohmann and Bieker, 2008). This EKLF promoter region contains erythroid cell-specific DNase hypersensitive sites and is sufficient to confer tissue-specific expression on a linked reporter in transgenic mice (Chenis et al., 1998; Xue et al., 2004; Yien and Bieker, 2013). Our published studies have shown the high cell specificity of GFP expression that results from this pEKLF/GFP construct and, importantly, that GFP onset mirrors EKLF onset (Lohmann and Bieker, 2008). We isolated E13.5 fetal liver cells from pEKLF/GFP mice and monitored, by fluorescence-activated cell sorting (FACS),
EKLF/GFP+ population was also F4/80+/Ter119 to minimize erythroid expansion, and found that 34-55% of the we cultured the fetal liver cells in the absence of erythropoietin an additional selection for non-clumped cells with single nuclei and erythroid cells or from engulfed erythroid nuclei. First, we included the possibility that the macrophage GFP signal came from adherent express EKLF (Fig. 5B). Two analyses were performed to exclude basophilic erythroblast; O, orthochromatic erythroblast; P, proerythroblast; 0, reticulocyte.

for overlap of GFP (i.e. EKLF expression) and the F4/80 macrophage marker. Our results show that ~36% of F4/80+ macrophage cells express EKLF (Fig. 5B). Two analyses were performed to exclude the possibility that the macrophage GFP signal came from adherent erythroid cells or from engulfed erythroid nuclei. First, we included an additional selection for non-clumped cells with single nuclei and obtained similar results (supplementary material Fig. S2A). Second, we cultured the fetal liver cells in the absence of erythropoietin to minimize erythroid expansion, and found that 34-55% of the EKLF/GFP+ population was also F4/80+/Ter119+ (supplementary material Fig. S2B).

We next investigated whether expression levels of macrophage genes that express proteins important for island integrity are dependent on EKLF. We compared RNA expression levels of α integrin (Lee et al., 2006; Mankelow et al., 2004), VCAM1 (Sadahira et al., 1995), EMP (MAEA) (Soni et al., 2006), palladin (Liu et al., 2007), MAF (Kusakabe et al., 2011) and DNASE2A (Kawane et al., 2001) between EKLF/GFP+/F4/80+ and EKLF/ GFP-F4/80+ sorted singlet cells (supplementary material Fig. S3).

Other than EMP/MAEA, none are appreciably expressed in the erythroid cell. The controls confirm that EKLF is preferentially expressed in the EKLF/GFP+ population (Fig. 5C). The test samples show that there is no difference in αv integrin, EMP/MAEA, MAF or palladin expression in the two cell populations; on the other hand, DNASE2A [recently shown to be an EKLF target in macrophages (Porcu et al., 2011)] and VCAM1 are more highly expressed in EKLF/GFP+ cells (Fig. 5C). In support of this observation, surface VCAM1 protein is expressed in ~30% of the EKLF/GFP+ cells (Fig. 5D), and VCAM1 levels are decreased in total fetal liver cell RNA from EKLF-null compared with wild-type embryos (Tallack et al., 2012). These results demonstrate that although αv integrin (the macrophage partner of erythroid ICAM4) is not differentially regulated by the presence of EKLF, a different adhesion molecule (VCAM1, the macrophage partner of erythroid α4β1 integrin) is expressed at a higher level in EKLF/GFP+/F4/80+ cells, suggesting that EKLF regulates two erythroid-macrophage adhesive interactions together.

Role for EKLF in erythroblastic island integrity

E13.5 fetal livers from the pEKLF/GFP mouse were then used to enrich for erythroblastic island clusters, which were stained for F4/80 and monitored for GFP. These show a typical island structure, with a central F4/80+ macrophage surrounded by 10-15 GFP+ cells (Fig. 5E). These results demonstrate that EKLF+ cells are associated with the island macrophages when isolated directly from fetal livers, and support the previous observations derived from differentiating ESCs.

If ICAM4 and VCAM1 are downstream targets of EKLF, then a prediction is that island integrity should be compromised in the absence of EKLF. For this study we compared cells in wild-type and EKLF-null fetal livers (E13.5). FACS analyses show that the percentage (prevalence) of F4/80+ cells is ~2-fold lower in the EKLF-null fetal liver (Fig. 6A). As it is already known that EKLF is required for erythroid production in vivo, this shows that F4/80 macrophage production is also compromised in the absence of EKLF.

To address whether definitive erythroblastic islands are affected in the absence of EKLF, we required a globin-independent readout for the red cell component, as adult β-globin levels are virtually nonexistent. This is further complicated by the fact that EKLF is a global regulator of erythroid gene expression, so many standard cell surface markers (e.g. Ter119, CD44) cannot be used. In this context, although CD9 is incompletely affected (~50%) by the absence of EKLF (Isen et al., 2010), we found that too many (30%) F4/80 macrophage cells also express CD9 (data not shown). As a result we simply stained island clusters from wild-type and EKLF-null E13.5 fetal livers and examined their morphology after May-Grünwald Giemsa stain and after F4/80-DAPI co-stain. We find that clusters from EKLF-null cells are aberrant and consist of macrophages (Kawane et al., 2001). Consistent with observations in EKLF-null erythroid cells (Pilon et al., 2008), analysis of DNA content shows that the cell cycle is deregulated in EKLF-null F4/80+ cells, as these accumulate at G1 (Fig. 6C); at the same time, there is no evidence for apoptosis (no sub-G1 cells).

Collectively, these studies suggest that in combination with defective red cell interactions following the drop in erythroid ICAM4 and macrophage VCAM1 expression, loss of EKLF also leads to a deficiency in digestion of internalized erythroid nuclei due to the low level of EKLF-dependent DNASE2A in macrophages.
The end result is flawed production of mature, enucleated red blood cells from the erythroblastic island, an effect superimposed on the already profound \( \beta \)-thalassemia and global expression defects observed in EKLF-null red cells.

**EKLF onset during erythroid-myeloid generation in early development**

Waves of hematopoietic progenitors emerge from the developing mouse yolk sac (Bertrand et al., 2005; McGrath et al., 2011; Palis, 2014; Palis et al., 2001, 1999). One arises early (beginning at E7.25) and produces primitive erythroid cells (EryP-CFC). Another arises later (beginning at E8.25) and yields definitive erythroid as well as a varied set of myeloid progeny from a cell termed the erythro-myeloid progenitor (EMP). These transient waves precede the emergence of long-term hematopoietic stem cells during mammalian development from other sites of the embryo (Baron, 2013; Chen et al., 2011; Dzierzak and Speck, 2008; McGrath and Palis, 2005). Our observations on the clonal emergence of erythroid-macrophage islands that arise during primitive or definitive hematopoietic stages of ESC differentiation, the co-expression of EKLF in erythroid and an F4/80 subset of macrophage cells during definitive hematopoiesis, and the availability of the marked GFP mice prompted us to determine the specific cell-surface phenotype of EKLF/GFP+ cells. Previous studies have shown that EKLF is expressed by the neural plate stage (\( \sim \)E7.5) within the blood islands of the yolk sac (Southwood et al., 1996), and that the transgenic pEKLF construct used here recapitulates this early expression (Xue et al., 2004). However, these studies were focused on the primitive erythroid onset and did not encompass the possibility of a role in EMP onset in the yolk sac.

In the E8.0-E10.0 embryonic yolk sac, the sequential pattern of CD45, Kit and F4/80 expression can be used to establish a temporal developmental sequence (Bertrand et al., 2005; Kierdorf et al., 2013). In particular, CD45⁻/Kit⁺/F4/80⁻ cells from the early yolk sac are thought to be EMPs, as they uniquely give rise to cells that express macrophage and erythroid markers. As a result, we isolated GFP+ cells from yolk sacs of E8.5 and E10.5 pEKLF/GFP mice and examined their cell surface expression. Analyses of E8.5 yolk sacs
reveal that all stages in the developmental sequence are present at high levels within the EKLF/GFP+ cell population (Fig. 7A). Of particular interest is that ∼20% of the EKLF/GFP+ cells are CD45−/Kit+ EMP progenitors and that this population is significantly enriched ∼4-fold compared with EKLF−/GFP− cells. All subsets are much less apparent in the yolk sac by E10.5 (Fig. 7B), when the increased EKLF/GFP cell numbers retain a small but reproducible high-level of CD45+/Kit− expression (∼2%). These data (along with the F4/80 data of Fig. 4B) suggest that EKLF/GFP is expressed in the EMP progenitor and its more mature progeny before E9.0 (Bertrand et al., 2005; Kierdorf et al., 2013) and before generation of F4/80+ macrophages.

DISCUSSION
Cellular and mechanistic aspects of erythroid/macrophage biology in early development

We propose a model that encompasses novel aspects of erythroblastic island biology uncovered by our analyses (summarized in Fig. 8). Surprising is the observation that single cells derived from differentiating EBs can give rise to erythroblastic islands, whether isolated from stages enriched for primitive or definitive erythroid cell production. This progenitor cell is thus able to set up its own island environment. In vivo, these are most likely to arise from the EMP population present at early developmental stages (Fig. 8, top). This EMP population arises in the E8.25 yolk sac then seeds the fetal liver by E10.5, providing the first source of definitive erythroid progenitors prior to or coincident with colonization by distinct hematopoietic stem cell (HSC) sources (Chen et al., 2011; Lux et al., 2008; McGrath et al., 2011; Tober et al., 2013).

Crucial for integrity of this erythroblastic island is EKLF. EKLF is expressed at the right time and place in development (the CD45−/Kit+ EMP cell) to exert an effect. Within the island progeny it directly activates ICAM4 in the erythroid compartment, and activates VCAM1 in the macrophage compartment (Fig. 8, bottom left). These enable a two-pronged adhesive intercellular interaction to occur with their respective integrin partners on the opposite cell type. In the absence of EKLF or when the human E325K mutant is expressed, these interactions decrease and contribute to an abundance of nucleated, unprocessed cells in circulation.

In addition, there is a second crucial role for EKLF: activation of DNASE2A in the macrophage (Fig. 8, bottom right). In its absence, macrophages become engorged with undigested erythroid nuclei from any cells that did manage to enter, triggering an inflammatory response such that high levels of IFNβ are released into circulation, leading to anemia and embryonic lethality (Kawane et al., 2001; Nagata, 2007; Yoshida et al., 2005). Circulating erythroid cell numbers in DNASE2A-null embryos are only 1/10th that of wild type (Kawane et al., 2001), and many of these remain nucleated. The Ristaldi lab has also published data showing that EKLF is expressed in F4/80 macrophage and directly regulates DNASE2A expression (Porcu et al., 2011). Consistent with the downstream effects in DNASE2A-null embryos, IFNβ levels are increased in EKLF-null fetal liver cells (Porcu et al., 2011).

Abnormal numbers of nucleated red cells are observed in EKLF-null embryos (Perkins et al., 1995), and are also a characteristic of human CDA type IV patients who contain a mutation in one allele of KLF1 (Arnaud et al., 2010; Jaffray et al., 2013). This variant is altered within the second zinc finger (E325K), changing its cognate binding specificity and probably converting it to an interfering protein (Singleton et al., 2011). In this context, it is interesting to compare these observations to that of the Nan mouse, which harbors a mutation at the same site but with a different substitution (E339D) (Heruth et al., 2010; Siatecka et al., 2010). These mice, although anemic, do not present with any nucleated circulating erythroid cells,
Enriched in splenic
(Sadahira et al., 1995). In addition, VCAM1 and CD31 are selectively parallels that of EKLF, suggesting that changes in EKLF levels dynamic nature of ICAM4 expression during differentiation dependent on the maturation stage (An and Mohandas, 2011). The such changes can directly affect adhesiveness within the island tissue macrophage subsets (e.g. lung, peritoneal, microglia), and phases, including ICAM4 and fashions during expansion and terminal erythroid differentiation membranes protein dynamics and complexity

Membrane protein dynamics and complexity
Cell surface proteins are dynamically regulated in a temporal fashion during expansion and terminal erythroid differentiation phases, including ICAM4 and β1 integrin, supporting the idea that such changes can directly affect adheriveness within the island dependent on the maturation stage (An and Mohandas, 2011). The dynamic nature of ICAM4 expression during differentiation parallels that of EKLF, suggesting that changes in EKLF levels exert a finely tuned transcriptional control over these events.

VCAM1 plays a crucial role within the erythroblastic island (Sadahira et al., 1995). In addition, VCAM1 and CD31 are selectively enriched in splenic ‘red pulp’ macrophages compared with other tissue macrophage subsets (e.g. lung, peritoneal, microglia), and consistent with the idea that its mechanism leading to anemia is different in detail from that of the CDA type IV patients.

EKLF is expressed within CMPs and plays a role in bipotential decisions emanating from MEPs (Frontelo et al., 2007). Our studies further support the concept that EKLF action is important at pre-erythroid stages, in this case within the EMP. During erythropoiesis, EKLF plays a role in all maturation stages; however, our studies now bring in the novel concept that the latter maturational steps are also aided by EKLF regulation of select genes within the macrophage component of the specialized erythroblastic island niche.

Regulatory cross-connections

Supplying iron to the maturing erythroid cells is an important in vivo function of island macrophages (Chasis and Mohandas, 2008; Chow et al., 2013; Manwani and Bieker, 2008; Ramos et al., 2013), and can also be demonstrated in co-culture systems [e.g. transport of ferritin (Leimberg et al., 2008)]. In this context, and given the importance of EKLF in controlling a number of steps in the red cell heme biosynthetic pathway (Tallack et al., 2010) (particularly the transferrin receptor and mitoferrin), it is tempting to speculate that EKLF may also play an analogous regulatory role for iron transfer in the macrophage. EKLF expression is regulated by the BMP4 pathway during early mammalian development, particularly via Smad5 (Adelman et al., 2002; Kang et al., 2012; Lohmann and Bieker, 2008). This same pathway plays a significant role in stress erythropoiesis (Porayette and Paulson, 2008). As macrophage depletion leads to a reduction of BMP4 activity (Chow et al., 2013), part of the explanation for the lower erythroid numbers after stress induction under these conditions is likely to be that EKLF levels are compromised, leading to a global negative effect on red cell expansion and maturation. Conversely, increased EKLF levels lead to precocious erythroid differentiation (Frontelo et al., 2007); thus secreted BMP4 from the adherent macrophages (Milion et al., 2010; Paulson et al., 2011) can use this molecular pathway to support normal or accelerated red cell production. This suggests that EKLF expression levels in the red cell can be dynamically adjusted based on signals from adjacent macrophage both in normal and stress conditions.

Our studies support the notion that an increased understanding of the in vivo steps important for generation of erythroblast islands, accompanied by an understanding of the molecular regulation of the relevant intercellular components within the macrophage and erythroid compartments, will be important for the design of efficient protocols to generate blood cells in vitro, as well as for illuminating how these steps may be tweaked in vivo as needed to alleviate patient morbidity during dysregulated erythropoiesis and anemia.

Fig. 7. Analysis of EMP and progeny in sorted EKLF/GFP+ or EKLF/GFP− yolk sac cells in early development. Yolk sacs from E8.5 (A; n=5) or E10.5 (B; n=5) embryos were dispersed and analyzed for EKLF/GFP, CD45 and Kit (CD117) expression by FACS. The statistically significant different percentages of CD45−Kit+ levels between EKLF/GFP+ and EKLF/GFP− cells (i.e. lower right quadrant in each case) are shown for E8.5 (A).

F4/80+/VCAM1+ double positivity is coordinately expressed in these cells (Gautier et al., 2012; Kohyama et al., 2009). Red pulp macrophages are closely associated with erythroid cells in the spleen. Our studies suggest that VCAM1 is similarly enriched in fetal liver macrophages that are associated with the erythroblastic island, and is regulated by EKLF.

Definitive red cell interaction with the macrophage is crucial for optimal nutrient access, survival, proliferation and differentiation, but there are additional contributors to effective enucleation (Socolovsky, 2013). Although an abnormal level of circulating nucleated definitive cells are observed in embryos deficient for the DNASE2A, MAF or palladin macrophage proteins (Kawane et al., 2001; Kusakabe et al., 2011; Liu et al., 2007), ablating the majority of macrophage cells does not automatically yield an abundance of nucleated red cells in circulation (Chow et al., 2013; Ramos et al., 2013). In addition, there are a number of structural, enzymatic and chromatin-associated erythroid cell factors that play an intrinsic role in morphologically preparing the cell for proper nuclear extrusion (Ji et al., 2011; Keerthivasan et al., 2011; Konstantinidis et al., 2012; Ney, 2011; Ubukawa et al., 2012; von Lindern, 2006). In the case of EKLF, the abundance of nucleated red cells in circulation seen in its absence or in the CDA KLF1/E325K patients may not only result from defective function in the island macrophage, but also as part of the global erythroid-specific panoply of EKLF targets that include ones important for these final maturation steps.

Definitive red cell interaction with the macrophage is crucial for optimal nutrient access, survival, proliferation and differentiation, but there are additional contributors to effective enucleation (Socolovsky, 2013). Although an abnormal level of circulating nucleated definitive cells are observed in embryos deficient for the DNASE2A, MAF or palladin macrophage proteins (Kawane et al., 2001; Kusakabe et al., 2011; Liu et al., 2007), ablating the majority of macrophage cells does not automatically yield an abundance of nucleated red cells in circulation (Chow et al., 2013; Ramos et al., 2013). In addition, there are a number of structural, enzymatic and chromatin-associated erythroid cell factors that play an intrinsic role in morphologically preparing the cell for proper nuclear extrusion (Ji et al., 2011; Keerthivasan et al., 2011; Konstantinidis et al., 2012; Ney, 2011; Ubukawa et al., 2012; von Lindern, 2006). In the case of EKLF, the abundance of nucleated red cells in circulation seen in its absence or in the CDA KLF1/E325K patients may not only result from defective function in the island macrophage, but also as part of the global erythroid-specific panoply of EKLF targets that include ones important for these final maturation steps.

Regulatory cross-connections

Supplying iron to the maturing erythroid cells is an important in vivo function of island macrophages (Chasis and Mohandas, 2008; Chow et al., 2013; Manwani and Bieker, 2008; Ramos et al., 2013), and can also be demonstrated in co-culture systems [e.g. transport of ferritin (Leimberg et al., 2008)]. In this context, and given the importance of EKLF in controlling a number of steps in the red cell heme biosynthetic pathway (Tallack et al., 2010) (particularly the transferrin receptor and mitoferrin), it is tempting to speculate that EKLF may also play an analogous regulatory role for iron transfer in the macrophage. EKLF expression is regulated by the BMP4 pathway during early mammalian development, particularly via Smad5 (Adelman et al., 2002; Kang et al., 2012; Lohmann and Bieker, 2008). This same pathway plays a significant role in stress erythropoiesis (Porayette and Paulson, 2008). As macrophage depletion leads to a reduction of BMP4 activity (Chow et al., 2013), part of the explanation for the lower erythroid numbers after stress induction under these conditions is likely to be that EKLF levels are compromised, leading to a global negative effect on red cell expansion and maturation. Conversely, increased EKLF levels lead to precocious erythroid differentiation (Frontelo et al., 2007); thus secreted BMP4 from the adherent macrophages (Milion et al., 2010; Paulson et al., 2011) can use this molecular pathway to support normal or accelerated red cell production. This suggests that EKLF expression levels in the red cell can be dynamically adjusted based on signals from adjacent macrophage both in normal and stress conditions.

Our studies support the notion that an increased understanding of the in vivo steps important for generation of erythroblast islands, accompanied by an understanding of the molecular regulation of the relevant intercellular components within the macrophage and erythroid compartments, will be important for the design of efficient protocols to generate blood cells in vitro, as well as for illuminating how these steps may be tweaked in vivo as needed to alleviate patient morbidity during dysregulated erythropoiesis and anemia.
MATERIALS AND METHODS

Cell isolation and culture
Murine ESC lines were maintained and differentiated to EBs according to established protocols (Adelman et al., 2002; Choi et al., 2005; Kennedy and Keller, 2003; Manwani et al., 2007; Zafonte et al., 2007). ESCs that contain a site-specific integrated GFP driven by the EKLF/KLF1 promoter have been described (Lohmann and Bieker, 2008). Judiciously timed harvesting of EBs enabled enrichment of primitive or definitive erythroid progenitors that were then dispersed and followed with secondary platings for generation of isolated erythroid colonies. Semisolid media (methylcellulose) were used in all ESC and EB colony platings, using a low cell concentration (no more than 50-100 cells per 35 mm dish) to maximize cell separation and EB colony formation (Adelman et al., 2002). Synthetic peptides (BioSynthesis or ElimBio) were premixed into the methylcellulose as needed.

Fetal liver cells harvested from staged embryos were mechanically dispersed to single cells (Lohmann and Bieker, 2008). Yolk sac cells were disaggregated with collagenase (Palis et al., 2001, 1999), and EBs with trypsin. Cytospins were fixed with May-Grünwald solution (Fluka) and stained with Giemsa solution (Sigma-Aldrich), and pictures were taken using a Nikon Microphot-FX fluorescence microscope equipped with a Q-Imaging camera.

FACS analysis
Single-cell suspensions of murine fetal livers, yolk sacs, or EBs were stained for FACS with the following antibodies: Ter119-APC, F4/80-PE, VCAM1-APC and CD45-PE from eBiosciences, Kit-APC from Invitrogen, Ter119-PE from BD Biosciences and F4/80-FITC from Serotec.

Multicolor FACS sorting for CMP or MEP populations was performed as previously described (Frontelo et al., 2007). Dispersed and sorted cells were cultured in MethoCult M3120 (StemCell Technologies) that contained IMDM, 10% PDS or FBS, 5% PFHM-II, 300 μM monothioglycerol, 25 ng/ml ascorbic acid, 2 mM glutamine, 300 μg/ml transferrin, 100 ng/ml SCF, 2 U/ml erythropoietin, 5 ng/ml thrombopoietin and 5 ng/ml IL11 (Kennedy and Keller, 2003). Importantly, colonies from these cell sorts did not form when using culture conditions that typically work well with fetal liver or bone marrow cells (Terszowski et al., 2005).

Flow cytometry data were analyzed using FlowJo (TreeStar). In all FACS analyses, gates are drawn based on negative controls from the same samples within the same experiment; mean±s.d. are shown for each quadrant of interest. Least squares fitting was performed as described (http://www.physics.csbsju.edu/stats/).

Island analysis
Island clusters were enriched and analyzed morphologically or by immunofluorescence as described previously (Fraser et al., 2010; Iavarone et al., 2004; McGrath et al., 2008). Total RNA was isolated and processed for quantitative analysis as described (Frontelo et al., 2007; Lohmann and Bieker, 2008; Siatecka et al., 2007). Primer sequences used for qPCR analysis are listed in supplementary material Table S1.

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Competing interests
The authors declare no competing financial interests.

Author contributions
L.X., M.G., M.N.G. and D.M. performed experiments, D.M. and J.J.B. directed experiments, and J.J.B. wrote the paper with input from the other authors.

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Supplementary material
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