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Primitive erythropoiesis in the mammalian embryo

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ABSTRACT Erythropoiesis in adult mammals is characterized by the progressive maturation of hematopoietic stem cells to lineage-specific progenitors, to morphologically identifiable precursors which enucleate to form mature erythrocytes. In contrast, primitive erythropoiesis is characterized by the appearance within the yolk sac of a transient, lineage-restricted progenitor population which generates a wave of erythroid precursors. These precursors undergo progressive maturation in the bloodstream, characterized by nuclear condensation and embryonic hemoglobin accumulation. This process is dependent on erythropoietin signaling through its cognate receptor, as well as the function of several erythroid-specific transcription factors, including GATA1 and EKLF. Targeted disruption of genes in the mouse that result in failure of the emergence or maturation of the primitive erythroid lineage leads to early fetal death, indicating that the primitive erythroid lineage is necessary for survival of the mammalian embryo. While it was thought for over a century that primitive erythroid cells were uniquely nucleated mammalian red cells, it is now recognized that they, like their definitive erythroid counterparts, enucleate to form reticulocytes and pyrenocytes. This surprising finding indicates that the primitive erythroid lineage is indeed mammalian, rather than non-mammalian, in character.

KEY WORDS: *erythropoiesis, enucleation, yolk sac*

Emergence of the primitive erythroid lineage from hemangioblast precursors

The first morphological evidence of hematopoiesis in multiple mammalian embryos, including cat, hamster, mouse, gerbil and human, is the appearance of pools of immature erythroid cells within the mesoderm layer of the yolk sac (Sorenson, 1961; Smith, 1977; Tiedemann, 1977; Haar, 1971; Takashina, 1987). These "blood islands" consist of immature primitive erythroid cells that rapidly become enveloped by endothelial cells (reviewed by Ferkowicz, 2005). The close spatial and temporal appearance of blood cells and endothelial cells in the yolk sac suggested to embryologists of the last century that these lineages arise from hemangioblast precursors (Sabin, 1920). This concept has been verified in mouse embryos where cells with hematopoietic, endothelial, and smooth muscle potential arise during gastrulation in the primitive streak (Huber, 2004; reviewed by Park, 2005). Studies of human embryonic stem cells cultured as embryoid bodies provide *in vitro* evidence that similar events occur in the human (Zambidis, 2005; Kennedy, 2007). Emergence of hematopoietic fates from a flk-1-positive hemangioblast involves transition through a "hemogenic" endothelial intermediate (Lancrin, 2009; Eilken, 2009). Further support of this transition comes from flow cytometric studies indicating that primitive erythroid potential emerges from endothelial marker-positive (VE-cadherin-, PECAM-1-, CD34-, endoglin-, and Tie2-positive) cells (Ema, 2006). Recent lineage tracing studies also indicate that primitive and adult blood cells ultimately derive from flk-1-positive cells (Lugus, 2009). While the current data support the concept that hematopoiesis in the yolk sac arises from hemangioblast precursors, it is not clear if the endothelial and smooth muscle fates are realized *in vivo* (Ueno, 2006; Furuta, 2006).

Specification of primitive erythropoiesis

The yolk sac forms as a bilayered structure with mesoderm cells apposed to visceral endoderm cells (Jollie, 1990). Support for the role of visceral endoderm in mammalian blood cell formation comes from GATA-4-null embryonic stem cell-derived embryoid bodies that lack visceral endoderm and display markedly reduced blood island formation (Bielinska, 1996). Tissue recom-

Abbreviations used in this paper: EryP-CFC, primitive erythroid colony-forming cells.

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bination experiments in the mouse suggest that these endoderm-inducing signals act during early gastrulation, are diffusible, and appear to involve Hedgehog signaling (Belaousoff, 1999; Dyer, 2001). While the downstream targets of Hedgehog signaling have not been elucidated, bone morphogenetic protein 4 (BMP4) is one possible candidate. Mouse embryos lacking BMP4 have a paucity of yolk sac blood islands and die in early post-implantation stages because of defects in mesoderm formation (Winnier, 1995). Exogenous BMP4 mediates the development of mesoderm cells in embryonic stem cell-derived embryoid bodies grown under serum free conditions (Johansson, 1995; Pearson, 2008). It has recently been established that specification of the primitive erythroid lineage is controlled by the coordinated interaction of the Wnt pathway with inhibition of the Notch signaling pathway in hemangioblast precursors (Cheng, 2008). However, examination of Notch1-null embryos reveals normal specification of the primitive erythroid lineage (Hadland, 2004) but an apparent expansion of maturing primitive erythroid cells due to decreased apoptosis (Robert-Moreno, 2007). Taken together, these studies indicate that multiple signaling cascades coordinately regulate the spatio-temporal emergence of the primitive erythroid lineage during gastrulation in the mammalian embryo.

Primitive erythroid progenitors (EryP-CFC)

The identification of primitive erythroid progenitors (EryP-CFC), capable of forming colonies of erythroid cells *in vitro*, constitutes the earliest evidence that mesoderm cells have committed to a hematopoietic fate in the mouse conceptus (Wong, 1986). Murine EryP-CFC form compact colonies of large primitive erythroid cells that require 4-5 days to mature *in vitro* (Fig. 1). Examination of carefully staged mouse embryos indicate that EryP-CFC first emerge within the developing yolk sac at the late primitive streak stage (approximately E7.25), after the onset of gastrulation but before morphological evidence of blood island formation (Palis, 1999). These primitive erythroid progenitors express low levels of CD41 on their surface (Ferkowicz, 2003). EryP-CFC expand in number for 48 hours within the developing yolk sac, but then are rapidly extinguished by E9.0 (Palis, 1999). EryP-CFC are not found in any other time or place in the murine embryo. The restricted spatial and temporal appearance of EryP-CFC indicates that primitive erythropoiesis is transient in nature.

Recently, bipotential primitive erythroid/megakaryocyte progenitors have been found in the gastrulating mouse embryo (Tober, 2007). These progenitors emerge and are extinguished with the same kinetics as EryP-CFC. The emergence of the primitive erythroid and megakaryocyte lineages from a common progenitor indicates that "primitive" hematopoiesis in the yolk sac is at least bilineage in character and thus more complex than previously thought.

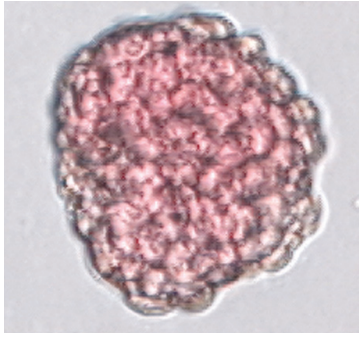

Fig. 1. Primitive erythroid precursor (EryP-CFC)-derived colony of primitive erythroid cells *cultured in semi-solid medium.*

Erythropoietin receptor (EPO-R) transcripts are first detected in nascent yolk sac blood islands of mouse embryos beginning at E7.5 and increase in concentration as primitive erythroid precursors begin to mature (McGann, 1997; Lee, 2001). Decreasing numbers of EPO-R have been detected on the cell surface of terminally differentiating primitive erythroid cells in the fetal hamster (Boussios, 1989). The role of erythropoietin in the differentiation of yolk sac erythroblasts remains somewhat controversial. Initial studies indicated that the addition of exogenous erythropoietin did not increase heme synthesis when whole primitive streak to 20 sp mouse embryos were cultured intact (Cole, 1966). However, a subsequent study from the same laboratory revealed a significant increase in heme synthesis when erythropoietin was added to cultures of disaggregated 0-12 sp embryonic cells (Bateman, 1971). These latter findings were confirmed by culture of E7.5 yolk sac explants where the addition of exogenous erythropoietin increased the number of erythroid cells and the accumulation of embryonic globin transcripts (Palis, 1995). Exogenous erythropoietin abrogates apoptosis of immature primitive erythroid cells cultured *in vitro* (Kimura, 2000). While high levels of erythropoietin transcripts have been found in the fetal liver as early as E10.25 (Makita, 2001), the endogenous source of erythropoietin at earlier developmental time-points remains controversial (Zimmerman, 1997; Lee, 2001).

Engagement of the homodimeric EPO-R by erythropoietin leads to autophosphorylation of the Janus kinase 2 (JAK2) and subsequent phosphorylation of several tyrosine residues of the cytoplasmic tail of EPO-R (reviewed by Richmond, 2005). The critical role of JAK2 in EPO-R signaling was established by examination of the phenotype of JAK2-null mice, which phenocopy the severe fetal anemia and in utero death of the erythropoietin-null and EPO-R-null embryos (Neubauer, 1998). Phosphorylation of tyrosine residues in the cytoplasmic region of EPO-R leads to the recruitment of multiple signaling molecules, including the signal transducer and activator of transcription 5 (STAT5). STAT5 subsequently translocates to the nucleus to regulate the transcription of the antiapoptotic gene Bcl-x(L) and enhance definitive erythroid cell survival (Silva, 1999). Robust STAT5 signaling has been detected in primitive erythroid cells derived from the *in vitro* culture of murine embryonic stem cells (Tsuji-Takayama, 2005). Bcl-x(L) protects primitive erythroid cells from apoptosis (Motoyama, 1999).

The functional role of erythropoietin signaling through its cognate receptor has been demonstrated by the similar phenotype of mice with targeted disruption of erythropoietin compared to disruption of EPO-R (Wu, 1995; Lin, 1996; Kieran, 1996). The loss of either gene leads to a 5 to 20-fold reduction in number of circulating primitive erythroblasts by E11.5 and the mutant embryos die with severe anemia by E13.5. These results indicate that signaling through the erythropoietin receptor is critically important for the normal maturation of primitive erythroid cells. However, unlike the complete dependence of definitive erythropoiesis on erythropoietin signaling, some EPO-R-null primitive erythroid cells do appear to complete their maturation, suggesting

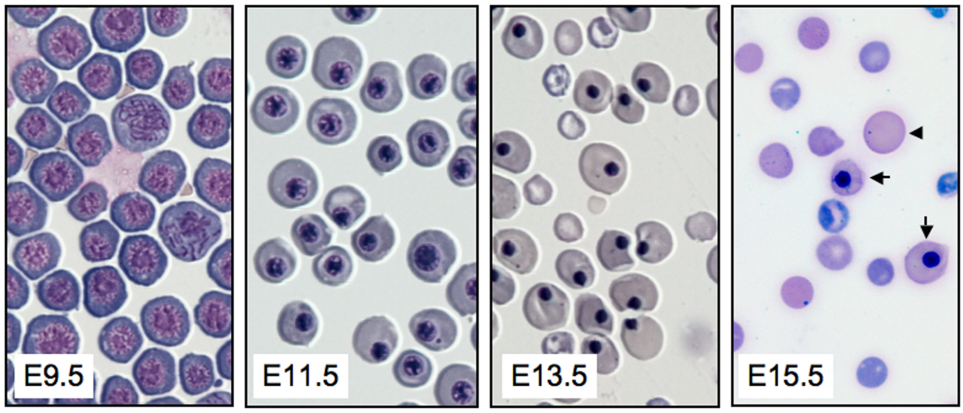

Fig. 2. Peripheral blood cells from E9.5, E11.5, E13.5 and E15.5 of mouse development. *The synchronous, progressive maturation of primitive erythroid cells from immature erythroblasts to enucleated erythrocytes (arrowhead) is evident.*

that other cytokine signaling cascades also function to support the maturation of primitive erythroid precursors.

Maturation of primitive erythroid precursors

The transient appearance of EryP-CFC leads to the generation of a wave of synchronously maturing erythroid precursors. Immature primitive erythroblasts begin to enter the bloodstream with the onset of cardiac contractions (McGrath, 2003; Ji, 2003). These cells are extremely large in size, attaining volumes between 400-700 femtoliters (Kingsley, 2004). Evaluation of blood smears from progressive times of hamster and of mouse embryogenesis reveals the morphological changes classically associated with definitive erythroid precursor maturation in the adult marrow, including decrease in erythroblast cell size, nuclear condensation with loss of euchromatin, and hemoglobin accumulation (Fig. 2; Sasaki, 1985; Kingsley, 2004; Fraser, 2007). The latter, in association with decreased RNA levels, results in loss of cytoplasmic basophilia observed following Wright-Giemsa staining.

Like definitive erythropoiesis, the maturation of primitive erythroid precursors is associated with several cell divisions that result in amplified numbers of erythroblasts. We have found that primitive erythroblast numbers expand 100-fold between E8.5 and E10.5 of mouse gestation. The presence of mitotic cells in the bloodstream (Bethlemfalvay, 1970), thymidine incorporation studies (de la Chapelle, 1969), and cell cycle analysis (Sangiorgi, 1990) indicate that circulating murine primitive erythroblasts cease dividing byE13.5. Primitive erythroblasts rapidly accumulate hemoglobin. As early as E9, they contain 22 pg/cell of hemoglobin, which is the same amount present in fully mature adult erythrocytes (Steiner, 1973). Consistent with their large size, primitive erythroblasts continue to accumulate hemoglobin, ultimately achieving steady state levels of 80-100 pg/cell (Fantoni, 1969; Steiner, 1973). It is not known when transcription ceases in maturing erythroid precursors. However, synthesis of non-heme proteins ceases by E12.5, while hemoglobin synthesis directed by stable globin transcripts continues until E14.5 (Fantoni, 1968).

"Maturational" globin switching

It was recognized several decades ago that embryonic (primitive) erythroblasts contained different hemoglobins when compared with adult (definitive) erythrocytes (Barker, 1968). Primitive erythroid cells in the mouse express not only embryonic (βH1 and εy) globins but also low levels of adult-type (β1 and β2) globins (Brotherton, 1979). Embryonic hemoglobins in the mouse have a high affinity for oxygen, presumably to facilitate oxygen exchange across the placenta (Wells, 1979). Initial studies suggested that the levels of βH1- and εy-globin transcripts were dynamically regulated during embryogenesis (Farace, 1984; Whitelaw, 1991). A more recent analysis of globin gene expression during the maturation of primitive erythroid precursors has confirmed and extended these findings by specifically examining primitive versus definitive erythroid cells (Kingsley, 2006). Surprisingly, βH1-globin transcripts were detected prior to εy-globin transcripts as blood islands begin to form in the murine yolk sac at E7.5. By E10.5, maturing primitive erythroid cells contained approximately equal quantities of βH1- and εy-globin transcripts as well as low levels of the "adult" β1- and β2-globin transcripts. By E15.5, εy-globin was the predominant β-globin transcript in primitive erythroid reticulocytes (Kingsley, 2006). Thus, there is a βH1- to εy-globin "switch" as primitive erythroid cells mature (Fig. 3). Switching within the α-globin locus was also found, with a transition from ζ- plus α-globin to entirely α-globin transcript expression in primitive erythroid cells (Fig. 3; Kingsley, 2006). These findings indicate that the α- and β-globin loci are actively regulated in the primitive erythroid lineage as the cells transition from progenitors to increasingly more mature precursors.

The study of primary human primitive erythroblasts indicate that they also undergo a ζ- to α-globin switch between 5 and 7 weeks gestation (Peschle, 1985). The culture of human embryonic stem cells leads to the generation of primitive erythroblasts, thus permitting access to cells that would otherwise be difficult or impossible to study due to ethical and practical concerns. Recent studies of embryonic stem cell-derived human primitive erythroblasts indicate that they, like their primary counterparts, undergo a "maturational" switch from hemoglobin Gower I ($\zeta_2\varepsilon_2$) to hemoglobin Gower II ($\alpha_2\varepsilon_2$) (Qiu, 2008).

Enucleation of primitive erythroblasts

The red cells of mammals circulate as enucleated erythrocytes. In contrast, the red cells of birds, amphibians and fish circulate as nucleated cells throughout their life span. The presence of nucleated primitive erythroid cells in mammalian embryos has raised the possibility that this lineage shares many features with their non-mammalian counterparts. However, in the early 1970's, enucleated «megalocytes» having the same size and hemoglobin content as nucleated yolk sac erythroblasts were detected at later times of mouse gestation (Fig. 2, arrowhead; Bethlemfalvay, 1970; Steiner, 1973). These cells are 3-fold larger than the fetal liver-derived «macrocytes» that enter the bloodstream beginning at E12.5 (Steiner, 1973), and were postulated

to be primitive erythroblasts that had undergone enucleation. While nucleated yolk sac-derived erythroid cells are no longer present in the bloodstream after E16.5 (Brotherton, 1979), megalocytes were found in the bloodstream until E18.5 (Bethlemfalvay, 1970; Steiner, 1973). Primitive erythroblasts also undergo other changes during their maturation consistent with enucleation, including the loss of the intermediate filament vimentin (Sangiorgi, 1990) and the loss of histone proteins (Morioka, 1998). Furthermore, murine primitive erythroblasts examined at E12.5 contain little evidence of marginal bands (Koury, 1987), the circumferential set of microtubules found in avian and amphibian erythrocytes.

Using antibodies specific for embryonic globins to identify primitive erythroid cells, it was determined that primitive erythroblasts in the mouse fetus enucleate between E12.5-E16.5 of gestation (Kingsley, 2004). Enucleated primitive erythrocytes have been identified in the bloodstream of mice even several days after birth (Kingsley, 2004). These surprising findings have been confirmed more recently using mice containing a GFP transgene driven by the human ε-globin promoter to identify primitive erythroid cells (Fraser, 2007). Enucleation of erythroid cells in the marrow results in the formation of two daughter cells. The first is the anucleate reticulocyte that completes its maturation by removing internal organelles and reorganizing its cytoskeleton. The second consists of the condensed nucleus surrounded by a thin rim of cytoplasm and an intact cell membrane. This second cell, recently termed "pyrenocyte", is rapidly ingested by macrophages (McGrath, 2008; Yoshida, 2005). Primitive pyrenocytes that appear to have temporarily escaped this fate have been detected in the bloodstream of mouse embryos between E12.5-E16.5 of gestation, that is, during the time when primitive erythroid cells enucleate (McGrath, 2008). Interestingly, primitive erythroblasts are capable of physically interacting with macrophage cells *in vitro* and are thought to do so within the fetal liver *in vivo* (McGrath, 2008; Isern, 2008). These interactions are mediated in part through α4β1 integrins present on the surface of primitive erythroid cells and are presumed to involve VCAM1 expressed by macrophage cells in the fetal liver (McGrath, 2008; Isern, 2008). These observations, taken together, indicate that terminal differentiation of mammalian primitive erythroid cells results in erythrocytes more similar to the enucleated red cells of mammals rather than the nucleated red cells of birds, fish and amphibians.

Transcriptional regulation of primitive erythropoiesis

Lineage differentiation in the hematopoietic system and terminal maturation of erythroid cells are regulated in part by the differential expression and combinatorial action of transcription factors. Two transcription factors in particular, GATA1 and KLF1, play central roles in erythroid-specific transcription by forming complexes with multiple other proteins to upregulate the expression of erythroid-specific genes.

GATA1 is the founding member of the GATA transcription factor family of dual zinc finger proteins that bind a WGATAR consensus motif present in essentially all erythroid-specific genes. GATA1 is expressed in nascent blood islands of the yolk sac (Fig. 4; Silver, 1997). Targeted disruption of GATA1 in the mouse leads to a block of primitive erythroid cell maturation at the proerythroblast stage of maturation. This block of primitive erythropoiesis results in embryonic death between E9.5-10.5, prior to the shift of hematopoiesis from the yolk sac to the fetal liver (Fujiwara, 1996). Different functional domains of GATA1 are required for activation of target genes in primitive versus definitive erythroid cells suggesting that different transcriptional complexes may form in these lineages (Shimizu, 2001). A screen of proteins that bind to GATA1 led to the identification of Friend of GATA (FOG1), a zinc finger protein that binds the amino zinc finger of GATA1 (Tsang, 1997). Like mice lacking GATA1, FOG1-null primitive erythroid cells also arrest at the proerythroblast stage of maturation and the embryos die of severe anemia at E10.5-E11 (Tsang, 1998).

Studies of definitive erythroid cells lacking GATA1 indicate that GATA2, which is present on GATA-binding sites, is displaced by GATA1 as erythroid lineage differentiation proceeds (Grass, 2003; Grass, 2006). Interestingly, between E7.5 and E8.5 a reciprocal downregulation of GATA2 and upregulation of GATA1 has been detected in yolk sac blood islands (Silver, 1997), suggesting that a GATA switch may occur as primitive erythroid cells transition from progenitors to precursors *in vivo*. Recent genome-wide approaches are defining the genes potentially up- and down-regulated by GATA1 in definitive erythroid cells (Yu, 2009; Chang, 2009; Fujiwara, 2009). Similar studies in primitive erythroid cells have not yet been reported.

The helix-loop-helix stem cell leukemia gene (Scl, Tal1) and the Lim domain-containing transcription factor Lmo2 are also found in a multiprotein complex with GATA1 in adult erythroid

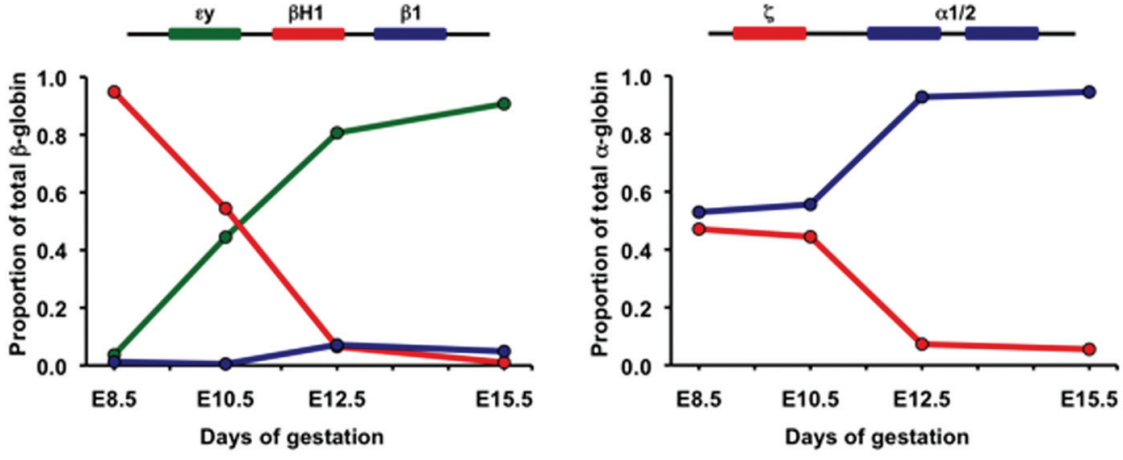

Fig. 3. Primitive erythroid cells undergo "maturational" switches of their β- and α-globin genes as they mature *in vivo*. *Relative levels of the various globin transcripts in primitive erythroid cells at various developmental times are shown. Schematic diagrams of the β- and α-globin loci are also shown.*

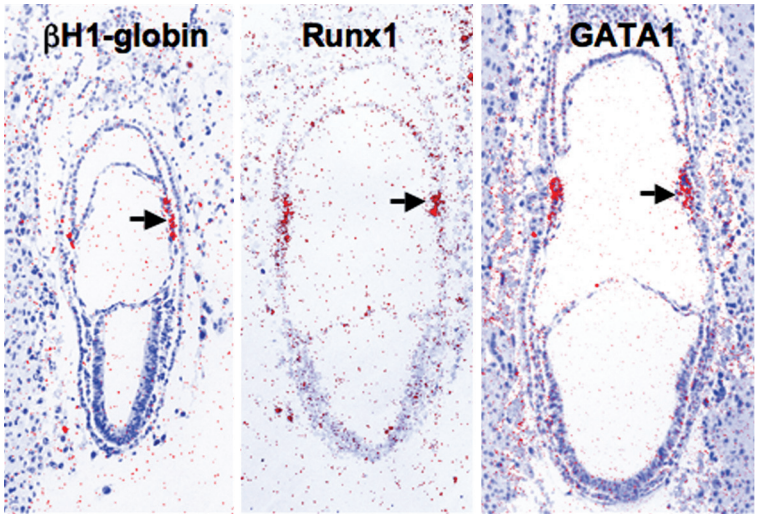

Fig. 4. Spatial expression patterns of *βH1-globin, Runx1* and *GATA1* transcripts in neural plate stage (E7.5) mouse embryos. *All three of these genes are expressed in emerging yolk sac blood islands (arrows).*

cells (Wadman, 1997). Both Scl and Lmo2 are expressed throughout the extraembryonic mesoderm layer of the early yolk sac in mouse embryos and continue to be expressed in forming blood islands (Kallianpour, 1994; Silver, 1997). Targeted disruption of Scl and of Lmo2 in mice each leads to a complete block in the emergence of embryonic hematopoietic cells and to embryonic death in utero at E9.5-10.5 (Warren, 1994; Shivdasani, 1995; Robb, 1995). Consistent with these findings, Scl is required for hemangioblast cell transition to hemogenic endothelium (Lancrin, 2009). Interestingly, *in vivo* marking of scl-expressing cells labels primitive erythroid cells but fails to mark endothelial cells in the developing mouse embryo (Bockamp, 2009). The cis-regulatory domains responsible for Lmo2 expression in endothelial versus yolk sac blood cells have come under recent scrutiny (Landry, 2009). These studies, taken together, indicate that Scl and Lmo2 are central to the emergence of primitive hematopoiesis and highlight the ongoing difficulties in untangling the emergence of blood and endothelium in the mammalian embryo.

Several members of the Kruppel-like transcription factor family are known to activate erythroid-specific genes. The prototypical member, KLF1 (EKLF) was originally cloned from erythroid cells and is expressed not only in definitive but also in primitive erythroid precursors (Miller, 1993; Southwood, 1996). Targeted disruption of KLF1 in the mouse leads to severe abnormalities of definitive erythroid cells and in fetal death in utero at E15.5 (Perkins, 1995; Nuez, 1995). While it was initially thought that KLF1 primarily regulated the adult β-globin gene through interactions with its CACC motif, it is now recognized that KLF1 also regulates the expression of multiple erythroid-specific genes, including cytoskeletal proteins and alpha hemoglobin stabilizing protein (Hodge, 2006; Pilon, 2006). Consensus binding sites for KLF1 and GATA1 are found near the cis-regulatory regions of many erythroid-specific genes.

Three other KLF family members have been implicated in the regulation of globin genes in primitive erythroid cells. Studies of KLF2-null mouse embryos revealed significant decreases in βH1- and εy-globin transcripts in primitive erythroid cells (Basu, 2005). Human ε-globin transgenes were also reduced in mice lacking KLF2, suggesting that this transcription factor may play a similar role in humans. KLF4 knockdown in zebrafish embryos leads to decreased embryonic globin gene expression (Gardiner, 2007). Consistent with this finding, KLF4 preferentially binds the CACC sites in the promoters of the embryonic compared to the adult β-globin genes. Finally, KLF6-null mouse embryos display severe anemia at E10.5 consistent with an important role in the regulation of yolk sac hematopoiesis (Matsumoto, 2006).

The transcription factor Runx1 has been associated with the emergence of intraembryonic hematopoietic stem cells, since targeted disruption of Runx1 leads to a lack of aortic clusters at E10.5 and fetal hemorrhage and death by E12.5 (Wang, 1996; North, 1998). However, Runx1 is expressed not only in intraembryonic clusters but also earlier in development within nascent yolk sac blood islands (Fig. 4; Lacaud, 2002) and in circulating primitive erythroid cells (North, 1999). Recent evidence indicates that targeted disruption of Runx1 in mice leads to decreased expression of GATA1 and KLF1 transcription factors as well as to mild abnormalities in the morphology of primitive erythroid cells (Yokomizo, 2008). Runx1 expression in the yolk sac is directly regulated by SCL, providing evidence that complex transcriptional networks are central to the emergence and maturation of primitive erythroid cells (Landry, 2008).

Transcription factors function within the context of modified chromatin domains. Unlike definitive erythroid cells, the βH1- and εy-globin genes in primary primitive erythroid cells of the mouse lie within a large hyperacetylated domain (Kingsley, 2006). The importance of chromatin remodeling in primitive erythroid cells has recently been established by studies of the chromatin remodeling enzyme brahma related gene 1 (BRG1) as well as p400/mDomino. Targeted disruption of BRG1 in mouse embryonic blood and endothelium results in the failure of primitive erythroid cells to transcribe embryonic and adult β-globin genes as well as the ζ-globin gene (Griffen, 2008). This leads to a severe thalassemic syndrome with apoptosis of primitive erythroid cells and death in utero at E10.5-E11. Similarly, disruption of the SWI2/SNF2 family ATPase, p400/mDomino, which is a core subunit of a large chromatin-remodeling complex, displayed marked anemia and fetal death at E11.5 (Ueda, 2007).

Summary

As surmised by Maximov one hundred years ago (1909), primitive erythroid cells constitute a unique erythroid lineage distinct from later definitive erythroid cells. Primitive erythroblasts are the first blood cells to differentiate within the mammalian embryo and failure of this lineage to differentiate leads to early fetal demise. Features unique to primitive erythroid cells include their immense size and hemoglobin content, their expression of predominantly embryonic hemoglobins, and their differentiation within the vascular space. However, primitive erythroid cells share many similarities with their definitive counterparts, including a maturational process characterized by progressive hemoglobin accumulation, nuclear condensation, and ultimately enucleation. While it has long been thought that primitive erythroblasts share many similarities with the red cells of non-mammalian

species which remain nucleated throughout their lifespan, it is now recognized that primitive erythropoiesis is in fact mammalian in character and serves as a useful model for the study of erythroid cell maturation.

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