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Development and differentiation of the erythroid lineage in mammals

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Summary

The red blood cell (RBC) is responsible for performing the highly specialized function of oxygen transport, making it essential for survival during gestation and postnatal life. Establishment of sufficient RBC numbers, therefore, has evolved to be a major priority of the postimplantation embryo. The “primitive” erythroid lineage is the first to be specified in the developing embryo proper. Significant resources are dedicated to producing RBCs throughout gestation. Two transient and morphologically distinct waves of hematopoietic progenitor-derived erythropoiesis are observed in development before hematopoietic stem cells (HSCs) take over to produce “definitive” RBCs in the fetal liver. Toward the end of gestation, HSCs migrate to the bone marrow, which becomes the primary site of RBC production in the adult. Erythropoiesis is regulated at various stages of erythroid cell maturation to ensure sufficient production of RBCs in response to physiological demands. Here, we highlight key aspects of mammalian erythroid development and maturation as well as differences among the primitive and definitive erythroid cell lineages.

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1. Introduction

Mammalian hematopoiesis produces approximately 10 distinct cell types, the most abundant of which belongs to the erythroid lineage (Seita and Weissman, 2010). Erythropoiesis results in the production of large numbers of RBCs that are responsible for supplying oxygen to the developing embryonic, fetal, and adult tissues. They also help maintain blood viscosity and provide the shear stress required for vascular development and remodeling (Baron, 2013; Lucitti et al., 2007).

In the developing mammalian embryo, hematopoiesis occurs in three sequential waves. The first wave emerges in the yolk sac (YS), with the development of progenitors committed primarily to the primitive erythroid lineage (EryP), as well as to the macrophage and megakaryocyte lineages (Baron et al., 2012). The second wave of hematopoiesis also arises in the YS, producing definitive erythroid, megakaryocyte, and myeloid lineages (Lux et al., 2008). These first two waves are transient and are eventually replaced by RBCs that are derived from a third wave of hematopoiesis, generated from HSCs that arise in the major arteries of the developing embryo, placenta, and YS (Dzierzak and Philipsen, 2013; Speck et al., 2002) and subsequently colonize the fetal liver, where they differentiate to the various hematopoietic cell lineages (Baron et al., 2012). Toward the end of gestation, hematopoiesis transitions to the bone marrow, which becomes the primary site of postnatal blood production in the adult.

The earliest erythroid progenitors, identified in clonogenic colony assays as burst-forming units (BFU-E), give rise to later progenitors known as colony-forming units (CFU-E) that undergo terminal differentiation to enucleated RBCs (reviewed by Hattangadi et al., 2011). In humans, the life span of a RBC averages approximately 120 days (Hattangadi et al., 2011). To maintain circulating RBCs at numbers necessary for sufficient oxygen distribution, approximately 2×10^6 RBC must be generated every second (Palis, 2014). RBC production is regulated primarily by the peptide hormone erythropoietin (EPO) (reviewed by Fried, 2009). Dramatic reductions in RBC numbers lead to compensatory “stress” erythropoiesis through the expansion of BFU-Es (Paulson et al., 2011). This review describes the development of the RBC lineage and how RBC production is regulated in the adult. We highlight some of the key growth factors and genes that regulate mammalian RBC production, as well as differences between erythroid cells at different stages of their development.

2. Emergence of primitive erythroid progenitors in the yolk sac

In the mouse, EryP are first detected around embryonic day (E)7.5 within the “blood islands” of the YS (Ferkowicz and Yoder, 2005). EryP arise from mesodermal progenitors found in close proximity with the visceral endoderm (Baron, 2005). *Gata-4* deficient embryonic stem (ES)-derived embryoid bodies cannot form a visceral endoderm and show defects in primitive erythropoiesis (Bielinska et al., 1996). Explant culture studies using mouse embryos suggested that soluble signals from the visceral endoderm, one of which may be Indian hedgehog, activate primitive hematopoiesis (Belaoussoff et al., 1998; Dyer et al., 2001). Co-culture of Bone Morphogenetic Protein (BMP)-stimulated extraembryonic

endoderm (XEN) cells with EryP progenitors isolated using flow cytometry resulted in progenitor expansion (Artus et al., 2012). Two candidates for the XEN cell factors are Indian hedgehog and Vascular Endothelial Growth Factor (Vegf) (Artus et al., 2012). Together, these studies indicate that secreted signals from the visceral endoderm regulate primitive erythropoiesis.

The close temporal and spatial association of EryP and endothelial cells within the “blood islands” of the YS led to the hypothesis that these two lineages arise from a common progenitor termed the hemangioblast (Baron et al., 2012; reviewed by Ferkowicz and Yoder, 2005; Murray, 1932; Sabin, 1920; Sabin, 1917). Experimental support for the existence of a hemangioblast came from studies of differentiating human and mouse embryonic stem (ES) cells (Choi et al., 1998; Zambidis et al., 2005) and, later, from mouse embryos (Huber et al., 2004). “Blast colony-forming cells” (BL-CFC), derived from ES-cell derived embryoid bodies (EBs), display properties expected of the hemangioblast and are thought to be its *in vitro* equivalent (Choi et al., 1998). However, it is now evident that BL-CFCs are not bipotent but multipotent, giving rise to hematopoietic, endothelial, and mesenchymal cells, including smooth muscle (Ema et al., 2003). Analyses of chimeric mouse embryos expressing four different fluorescent proteins identified polyclonal (not monoclonal) blood islands (Ueno and Weissman, 2006), consistent with the observation that BL-CFC are found primarily in the posterior primitive streak, not in the YS, and that the commitment of these cells takes place as they migrate into the YS (Huber et al., 2004). An early lineage tracing study (Kinder et al., 2001) and a recent clonal analysis (Padron-Barthe et al., 2014) also lend support to the idea that the earliest hematopoietic and endothelial cell populations in the mouse embryo arise from different progenitors. An analysis of antibody-stained embryos using confocal microscopy suggested that “blood bands” rather than the “blood islands” form in the YS (Ferkowicz and Yoder, 2005).

EryP progenitors were initially identified by their ability to form red-pigmented colonies in cultures with semisolid medium supplemented with EPO (Palis et al., 1999). These progenitors are found in the YS only from E7.25 to E9.0 (Isern et al., 2011; Palis et al., 1999). They are the first functionally differentiated mesodermal cell and, at E7.5 and E8.5, represent a considerable proportion (15-20% and 40-50%, respectively) of all cells in the embryo (Isern et al., 2011). The primitive erythroid progenitor is thought to be a bipotent cell that can give rise to unipotent megakaryocyte and EryP progenitors (Tober et al., 2007). Isolation of these progenitors has been challenging, as cell surface markers (e.g. CD31, Tie-2, VE-cadherin, and CD41) that have been used for their enrichment are also expressed on endothelial cells and definitive hematopoietic progenitors (Ema et al., 2006; Ferkowicz et al., 2003). A transgenic mouse model expressing a nuclear histone H2B-GFP fusion protein driven by a human embryonic ε -globin gene promoter and sequences from the β -globin locus control region (LCR) has provided a means to isolate homogenous populations of EryP (Isern et al., 2008; Isern et al., 2010; Isern et al., 2011). In embryos from these mice, green fluorescence was detected as early as E6.75, around the time when the first erythroid cells are specified from mesoderm (Isern et al., 2011). EryP progenitor activity was found exclusively in the GFP-positive population of cells sorted from E7.5 and E8.5 embryos (Isern et al., 2011). Progenitor activity is lost as the cells enter the circulation (Isern et al.,

2011; McGrath et al., 2003). Microarray analysis of GFP-positive populations of EryP isolated at different days of development revealed that several Wnt/ β -catenin pathway genes are expressed in EryP progenitors and are subsequently downregulated (Isern et al., 2011). Wnt/ β -catenin signaling has been shown to regulate specification of the EryP lineage from mesoderm in differentiating cultures of mouse and human ES cells (Nostro et al., 2008; Sturgeon et al., 2014). Study of human EryP progenitors has proven difficult, as the time blood islands emerge in human development (day 16 and 17) is too early to be accessible from elective termination pregnancies (Tavian and Peault, 2005).

3. Maturation of primitive erythroid progenitors

By mid-gestation, the circulatory systems of the mouse embryo, the YS, and the placenta have connected (McGrath et al., 2003). As the heart begins to beat, around E9.0, EryP enter the circulation and begin to mature to the proerythroblasts and on to reticulocytes (enucleated cells) and, finally, to terminally differentiated erythrocytes, in a manner similar to their definitive erythroid cell counterparts (Fraser et al., 2007; Gulliver, 1875; Isern et al., 2011; Morioka and Minamikawa-Tachino, 1993). During this process, the diameter of the cell decreases, the nucleus condenses to approximately 20% of its original size, cell adhesion proteins are expressed, and enucleation ensues (Fraser et al., 2007; Isern et al., 2008). EryP accumulate hemoglobin protein as they differentiate. A summary of “hemoglobin switching,” the sequential change in globin gene expression during development, is beyond the scope of this review and is discussed elsewhere (e.g. see Katsumura et al., 2013; Mahajan et al., 2007; Sankaran et al., 2010; Tallack and Perkins, 2013).

The idea that EryP may enucleate was first proposed in the 1970s (Palis et al., 2010) but was not demonstrated empirically until many years later. An antibody against a mouse embryonic β -like hemoglobin protein was used to demonstrate that EryP begin to enucleate by E12.5 (Kingsley et al., 2004). This finding was confirmed and extended in the *ϵ -globin-H2BGFP* transgenic mouse model (Fraser et al., 2007). A 50% decrease in the number of nucleated EryP was observed from E13.5 to E14.5 and nearly all EryP were found to enucleate by E15.5 (Fraser et al., 2007). EryP persist in the circulation for a much longer time than initially believed: GFP-tagged EryP are found through the end of gestation and, for at least 3 weeks after birth, comprise approximately 0.5% of circulating blood cells (Fraser et al., 2007). The lifespan of EryP is likely similar to that of adult RBCs (Fraser et al., 2007). As in mouse, human EryP have also been observed to enucleate; however, human EryP appear to enucleate in the placenta (Van Handel et al., 2010).

4. First wave of definitive erythropoiesis

By mid-gestation in the mouse embryo, EryP are found circulating with and are outnumbered by the much smaller, definitive EryD. For many years, it was thought that all EryD arise from HSCs that colonize the fetal liver (Ema and Nakauchi, 2000; Orkin and Zon, 2008). However, a transient erythro-myeloid progenitor (EMP) emerges in the YS at approximately E8.25 and can be detected there until E11.5 (Palis et al., 1999). EMPs begin to colonize the fetal liver at E10.5 (Palis et al., 1999). As a result of their origin from a

common progenitor, erythropoiesis and myelopoiesis are synchronized during this wave of hematopoiesis. EMPs arise independently of HSCs (Ema and Nakauchi, 2000; McGrath et al., 2011).

This first wave of definitive hematopoiesis was identified *in vitro* using colony assays for BFU-Es, megakaryocytes and myeloid cells (Bertrand et al., 2005; Palis et al., 1999; Tober et al., 2007). *Ex vivo* maturation of progenitors from E9.5 YS yielded mature erythroid cells expressing the same pattern of β -globin genes as that found in the fetal liver at E11.5 (McGrath et al., 2011). Furthermore, long term reconstitution potential, an essential property of HSC function, is not a feature of cells in the fetal liver until approximately E11-12 (Muller et al., 1994). Therefore, it appears that the first wave of definitive erythropoiesis arises from these multipotent progenitors before HSCs colonize the fetal liver (McGrath et al., 2011).

In the mouse, this first wave of definitive hematopoiesis also contains large numbers of highly proliferative, multipotent progenitor colony forming cells (HPP-CFC) identified *in vitro* under specific conditions (Palis et al., 2001). HPP-CFC lack HSC activity and are found in the YS approximately 12-24 hours before the first repopulating HSC can be found in the developing embryo (Yoder and Hiatt, 1997). After prolonged culture, Extensively Self-Renewing Erythroid (ESRE) cells isolated from the YS at E8.5-9.5 and from the fetal liver at E12.5 (first wave of definitive hematopoiesis) can be identified (England et al., 2011). In contrast, cells isolated from fetal liver after E12.5 or from the bone marrow undergo only limited self-renewal (England et al., 2011). Analysis of global gene expression data sets generated for self-renewing and differentiating ESRE erythroblasts identified the polycomb complex protein Bmi-1 as a regulator of ESRE erythroblast self-renewal (Kim et al., 2015). Overexpression of *Bmi-1* induced extensive self-renewal in bone marrow erythroid cell cultures, which normally exhibit limited self-renewal (Kim et al., 2015). ESRE progenitors identified on the basis of their self-renewal during prolonged culture correspond spatially and temporally with the first wave of hematopoiesis; whether they arise from EMPs or HPP-CFCs is not known. The relationship, if any, between HPP-CFCs and EMPs is also unclear. The transient first wave of hematopoiesis functions to bridge the gap between primitive erythropoiesis and colonization of the fetal liver by HSCs.

5. Second wave of definitive erythropoiesis

Primitive hematopoiesis and the first wave definitive hematopoiesis are eventually supplanted by a second wave of definitive hematopoiesis driven by HSC colonization of the fetal liver. In the mouse embryo, functional HSC are first identified in the para-aortic splanchnopleure (E7.5-E9.5); HSC activity can be detected only after culturing of this tissue (Baron et al., 2012). The aorta-gonad-mesonephros (AGM) region develops from the para-aortic splanchnopleure and continues to produce HSCs (E10.5-E11.5), which also emerge within the major blood vessels, the placenta, and, very likely, the YS (reviewed in Dzierzak and Speck, 2008; Ottersbach et al., 2009). At E10.5, cells from intraembryonic sites, but not from the YS, can reconstitute adult recipients (Medvinsky and Dzierzak, 1996; Muller et al., 1994). However, E9.0 YS cells transplanted into “conditioned” newborn mice can repopulate all hematopoietic compartments and the reconstituted bone marrow can be

serially transplanted into secondary recipients (Yoder and Hiatt, 1997; Yoder et al., 1997). These findings suggest that cells from the E9.0 require additional time and/or signals to acquire HSC function. Further support for contribution of the YS to definitive hematopoiesis comes from a mouse model in which ablation of the sodium calcium exchanger, *Ncx1*, results in the development of viable embryos that fail to initiate a heartbeat at E8.25 (Lux et al., 2008). Analysis of the in intra-embryonic and extra-embryonic sites within *Ncx1*-deficient embryos revealed multi-lineage hematopoietic potential in the YS but not in the embryo proper (Lux et al., 2008). The authors concluded that primitive and definitive hematopoietic lineages emerge from the YS before E10.25 and that the majority of hematopoietic cells that seed the fetal liver at this point in development originate in the YS (Lux et al., 2008). Support for a YS origin of early HSCs also comes from a *Runx1* lineage tracing analysis (Samokhvalov et al., 2007).

Considerable evidence now exists for the emergence of HSCs from a specialized “hemogenic endothelium” within a limited region of the ventral wall of the developing aorta (Boisset et al., 2010; Eilken et al., 2009; Hirschi, 2012; Ng et al., 2010; Oberlin et al., 2010; Swiers et al., 2010; Zovein et al., 2010). Around E12.0, HSCs colonize the fetal liver (Muller et al., 1994), where they cycle continuously and begin to differentiate (Godin et al., 1999). Although HSC migration to the bone marrow has generally been thought to occur shortly before birth, the finding that B cell progenitors are abundant in the developing marrow at E15.0 suggests that colonization of the bone marrow may occur earlier in gestation, possibly around the same time that HSCs seed the fetal liver (Delassus and Cumano, 1996). In the bone marrow niche, a majority of HSCs are in a quiescent state and are committed to the production of all hematopoietic lineages throughout postnatal life.

Human hematopoietic development is broadly similar to that of the mouse (Lee et al., 2010; Tavian and Peault, 2005). Human HSCs have been identified in the AGM region, large vessels, and placenta (Ivanovs et al., 2011). HSC activity has been observed to arise in the human AGM by approximately 5 weeks of gestation (Ivanovs et al., 2011). Though the numbers of these cells are small, they display high self-renewal ability (Ivanovs et al., 2011). Sorted human placental CD34⁺ cells can confer multi-lineage reconstitution after transplantation into irradiated NOD-SCID or Rag γ C^{-/-} mice (Robin et al., 2009). The numbers of CD34⁺ cells in the placenta decline around 9 weeks after conception but then remain constant until term (Barcena et al., 2009). Erythroid progenitors are detected at 7-8 weeks, after HSCs have colonized the fetal liver (Tavian et al., 2010).

6. Definitive erythroid progenitors

Murine HSCs are found in a population of cells that lack lineage specific cell surface markers and express Sca1 and c-Kit [i.e. Lineage negative (lin⁻), Sca1⁺, c-Kit⁺ (LSK)] (Chow A, 2014; Ikuta and Weissman, 1992; Spangrude et al., 1988). Within the LSK population, long- and short-term HSCs (CD34⁻ Flt3⁻ and CD34⁺ Flt3⁻, respectively) are functionally identified on the basis of the period of time they can provide full hematopoietic reconstitution after transplantation into irradiated hosts (Osawa et al., 1996). A third (CD34⁺ Flt3⁺) cell type can be identified in the LSK population and is referred to as a multipotent progenitor (MPP) (Adolfsson et al., 2001). The MPP is proposed to give rise to the Common

Lymphoid (CLP) and Myeloid (CMP) progenitors (Akashi et al., 2000). Downstream of the CMP, a megakaryocytic-erythroid progenitor (MEP) gives rise to erythroid progenitors (Akashi et al., 2000) (Figure 1).

An alternative model of the hematopoietic hierarchy has been proposed in which CMPs, with the potential to form MEP and GMP, arise from a CD34⁺ Flt3⁻ population (ST-HSC) (Adolfsson et al., 2005; Pronk et al., 2007) (Figure 1). In this model, CD34⁺ Flt3⁺ cells termed lymphoid-primed multipotent progenitors (LMPPs) emerge from CD34⁺ Flt3⁻ ST-HSCs and produce CLPs and GMPs. LMPPs, however, do not give rise to erythroid-restricted progenitors (Adolfsson et al., 2005) (Figure 1). This model is supported by single cell transcriptome analysis of the hematopoietic cell hierarchy: the molecular fingerprint of a CMP appears to be more closely related to that of stem cells within the LSK population than to CD34⁺ Flt3⁺ cells (Guo et al., 2013). Furthermore, EPO has been shown to skew cells in the LSK population toward the erythroid lineage, suggesting that the erythroid progenitor emerges from a cell closer to the LSK population in the hematopoietic cell hierarchy (Grover et al., 2014).

Two other studies have argued that hematopoiesis does not proceed through a hierarchical progression. A paired-daughter analysis suggested that megakaryocytes and erythrocytes arise directly from HSCs without progressing through ST-HSC, MPP or CMP progenitors (Yamamoto et al., 2013). In an analysis of single human CD34⁺ progenitors from adult bone marrow, erythroid cells and megakaryocytes emerged from multipotent progenitors while the remaining lineages seemed to emerge from unipotent cells (Notta et al., 2015). Clearly, this is a controversial area and more work will be required to resolve the discrepancies among these studies.

The earliest restricted erythroid progenitors arise from MEPs and are characterized by the ability to form BFU-Es (large red colonies) after 5-8 days (mouse) and 10-14 days (humans) in semisolid cultures under specific conditions (Dzierzak and Philipsen, 2013; Gregory and Eaves, 1978) (Figure 2A, B). The BFU-E is a large colony containing thousands of hemoglobinized cells (Dzierzak and Philipsen, 2013). BFU-E respond to EPO, Stem cell factor (SCF), Insulin growth factor 1 (IGF1), corticosteroids, Interleukin-3 (IL-3), and Interleukin-6 (IL-6) (Hattangadi et al., 2011), divide slowly and give rise to rapidly proliferating CFU-Es (Figure 2A, C). In the presence of certain factors (e.g. corticosteroids), BFU-Es undergo extended self-renewal before becoming CFU-Es (Flygare et al., 2011). Mouse BFU-E activity is found in the lineage negative, c-Kit⁺, CD16/32⁻, CD34⁻, CD71^{low} cell population (Akashi et al., 2000; Terszowski et al., 2005). In humans, a population with BFU-E activity has been enriched from cultures of peripheral blood derived CD34⁺ progenitors and from human bone marrow based on the phenotype: CD45⁺ GPA⁻ IL-3R⁻ CD34⁺ CD36⁻ CD71^{low} (Li et al., 2014). There are currently no known markers that permit separation of BFU-Es from their bipotent MEP progenitors.

CFU-Es can be recognized in colony assays as clusters (units) of 9-64 cells in mice (scored after 2 days) (Wu et al., 1995) (Figure 3C) and 8-49 cells in humans (scored after 7 days) (Wu et al., 1995). CFU-Es are committed to terminal erythroid differentiation, are dependent on EPO for survival, and are responsive to SCF but not to corticosteroids (Flygare et al.,

2011). Mouse CFU-E activity is found in the IL-3R⁻ c-Kit⁺ CD71^{high} cell population in mice (Terszowski et al., 2005). In humans, CFU-E activity is found in a population of cells isolated from peripheral blood derived CD34⁺ positive progenitors and from bone marrow that displays the phenotype: CD45⁺ GPA⁻ IL-3R⁻ CD34⁻ CD36⁺ CD71^{high} (Li et al., 2014).

7. Maturation of definitive erythroid progenitors

The transition from CFU-E to proerythroblast is accompanied by loss of c-Kit and gain of Ter119 expression. Once the CFU-E commits to differentiation, it proliferates rapidly and is strongly dependent on EPO (Koury and Bondurant, 1988), which initiates expression of erythroid specific genes and protects against apoptosis (Richmond et al., 2005; Sathyanarayana et al., 2008; Socolovsky et al., 1999). With each cell division, the maturing erythroblast becomes progressively smaller, its nucleus condenses, and hemoglobin accumulates in the cytoplasm (Koury and Bondurant, 1988). The G1 phase of the cell cycle shortens but the length of S and G2M phases remain unchanged (Dolznig et al., 1995). Eventually the nucleus is extruded and all remaining ribosomes, mitochondria and other organelles are lost through autophagy (Griffiths et al., 2012; Johnstone, 1992; Kang et al., 2012; Mortensen et al., 2010; Ney, 2011; Waugh et al., 2001; Zhang et al., 2015).

In both mouse and humans, five distinct cell morphologies of maturing erythroblasts are identified: proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatophilic erythroblast, and reticulocyte (see cartoon in Figure 3A) (Chen et al., 2009). Flow cytometric approaches have been developed to phenotypically identify the distinct stages of erythroid maturation. In the mouse, CD71 and Ter119 expression have been used to segregate erythroid cells from fetal liver or bone marrow into 5 gates (R1-R5) (Zhang et al., 2003). Erythroid cells within the R1 (CD71^{low}, Ter119-negative) and R2 (CD71^{high}, Ter119-negative) gates contain erythroid progenitors (BFU-E and CFU-E) (Zhang et al., 2003). Populations R3-R5 are Ter119⁺ and contain terminally differentiating erythroblasts. The maturation of proerythroblasts to reticulocytes can be tracked by the loss of CD71 expression from gates R3-R5 (Zhang et al., 2003). A different scheme for identification and separation of the different stages of erythroid maturation is based on expression of Ter119 and CD44 as well as cell size (as measured by FSC) (Chen et al., 2009). The loss of CD44 expression and decrease in cell size within the Ter119⁺ population can be used to track 5 stages of erythroid maturation from erythroblast to reticulocyte (Chen et al., 2009; Liu et al., 2013). The proerythroblast undergoes three mitoses, sequentially generating 2 basophilic, 4 polychromatic, 8 orthochromatic erythroblasts, and 16 reticulocytes (therefore at a ratio of 1:2:4:8:16), a sequence that can be tracked and quantified using flow cytometry (Liu et al., 2013). Using this approach, it was possible to demonstrate an altered ratio of maturing erythroblasts in animal models of phlebotomy induced acute anemia and chronic hemolytic anemia (Liu et al., 2013). The five stages of human erythroblast maturation, and the same 1:2:4:8:16 ratio, were identified on the basis of loss of band3 expression and gain of alpha 4 integrin expression in the CD235a⁺ population of bone marrow (Hu et al., 2013). This quantitative scheme was used to show that bone marrow cells from patients with myelodysplastic syndrome (MDS) have altered ratios of maturing erythroblasts undergoing terminal erythroid maturation (Hu et al., 2013).

Terminally differentiated RBCs contain a highly specialized cytoskeletal network that is tethered to the cell membrane. The organization of the RBC membrane has evolved to enable the cell to withstand significant deformation without losing structural integrity (Mohandas and Gallagher, 2008). As the erythroid cell matures, cytoskeletal proteins assemble into a two-dimensional elastic network that is tethered to cytoplasmic domains of transmembrane proteins within the membrane (Chasis et al., 1989; Mohandas and Evans, 1994; Mohandas and Gallagher, 2008). The principal protein constituents of the RBC cytoskeleton network are spectrin, actin, protein 4.1R, adducin, dematin, tropomyosin and tropomodulin (Bennett and Baines, 2001; Mohandas and Gallagher, 2008). The assembly of these proteins gives the RBC its unique oval biconcave disk shape and endows the cell with a high surface area to volume ratio, cytoplasmic viscosity, and membrane deformability necessary for it to withstand physical stressors encountered during circulation. Mutations that either directly or indirectly disturb the RBC skeletal network can cause anemia (Mohandas and Gallagher, 2008; Ney, 2011). A classic example of the latter type of mutation is sickle cell anemia (Frenette and Atweh, 2007).

At the orthochromatophilic stage of maturation, the erythroid cell has exited the cell cycle (Hsieh et al., 2000; Pop et al., 2010). The condensed nucleus is polarized to one side of the cytoplasm and is eventually extruded, resulting in the formation of a reticulocyte and a nucleus surrounded by a small amount of cytoplasm and a plasma membrane (Fraser et al., 2007; McGrath et al., 2008). The membrane surrounding the extruded nucleus is marked with phosphatidylserine, a signal for engulfment by erythroblastic island (EBI) macrophages (Yoshida et al., 2005). Erythroblasts in birds, reptiles, and other “lower” vertebrates do not undergo enucleation. Why mammals evolved mechanisms to remove their nucleus during erythroid maturation is not understood. A potential explanation may lie in the observation that mammalian adult erythrocytes are more deformable than the nucleated erythroid cells of birds (Gaehtgens et al., 1981a; Gaehtgens et al., 1981b). Enucleation may, therefore, be an adaptation to allow the mammalian erythrocyte to circulate through narrow capillaries beds. It has also been suggested that loss of the nucleus increases the intracellular volume available for hemoglobin protein (Ji et al., 2011).

While erythroid progenitors can differentiate in the absence of other cell types in culture, *in vivo* they undergo terminal maturation within EBIs. EBIs provide a specialized niche for erythropoiesis and have been identified in bone marrow, fetal liver, and spleen. They are morphologically distinct, three-dimensional structures comprising a central macrophage surrounded by erythroblasts at various stages of maturation (for reviews, see Chasis, 2006; Socolovsky, 2013). Scanning electron microscopy revealed macrophage extensions that surround peripheral erythroblasts and provide tight membrane contact between these cells. The central macrophages of the EBI are thought to function as nurse cells and as scavengers, engulfing nuclei expelled from the maturing erythroblasts (reviewed in Chasis, 2006; Socolovsky, 2013; Spike et al., 2007; Spike and Macleod, 2007). Two studies have identified a crucial role for the EBI macrophage in erythropoietic recovery from stress conditions such as acute blood loss, myeloablation, hemolytic anemia, or β -thalassemia (Chow et al., 2013; Ramos et al., 2013).

Global gene expression profiling of human and mouse erythroblasts sorted at different stages of maturation revealed distinct molecular profiles (An et al., 2014; Kingsley et al., 2013; Merryweather-Clarke et al., 2011), indicating, not surprisingly, that erythropoiesis involves the expression of specific sets of genes required at each stage of maturation. Analysis of the transcriptomes of these defined populations of cells revealed genes not previously known to be differentially expressed during erythroid maturation.

8. Growth Factor Requirements for Erythropoiesis

The growth factors SCF and EPO are pivotal for normal erythropoiesis. *Scf* (*Steel*) mouse mutants die prenatally of severe anemia because of a failure in definitive erythropoiesis (Metcalf, 2008; Tan et al., 1990). Analysis of conditional *Scf* mouse knockouts has established that SCF expression is vital for maintaining normal RBC numbers in the adult (Ding et al., 2012). Macrocytic anemia in the *Steel-Dickie* mouse has provided evidence for the importance of membrane bound SCF in erythropoiesis (Brannan et al., 1991). In this mouse model, exon 6 is deleted from the *Scf* gene locus and, as a result, only soluble SCF is produced (Brannan et al., 1991). In the absence of membrane bound SCF, normal rates of erythropoiesis cannot be sustained and the mouse develops anemia (Brannan et al., 1991). Primitive erythropoiesis is not affected by mutations in either the c-Kit receptor or its ligand, SCF (Ema et al., 2006). Instead, the embryos die later in development due to a failure in definitive erythropoiesis. Although, like BFU-E, EryP progenitors express c-Kit, they do not respond to SCF *in vitro* (Isern et al., 2011).

The Erythropoietin Receptor (EpoR) is responsible for providing a key survival signal for CFU-E (Bunn, 2013; Lodish et al., 2010). Mouse embryos deficient in EpoR have defects in proliferation and maturation at the primitive erythroblast stage (approximately E10.5), but EryP progenitors are not affected and the embryo develops to the definitive stage of erythropoiesis despite these defects (Lin et al., 1996). In definitive erythropoiesis, the generation of BFU-E and CFU-E is not dependent on either EpoR or EPO (Wu et al., 1995). Mice deficient in EpoR are anemic due to a nearly complete block in the terminal maturation of erythroid progenitors, with embryonic death occurring around E13.5. The different responses to EPO and SCF by cells of the primitive and definitive erythroid lineages suggest that other factors are responsible for regulating primitive erythropoiesis.

9. Regulation of erythroid development

Transcriptional regulation of erythroid development has been extensively studied (reviewed in Baron et al., 2013; Dore and Crispino, 2011; Dzierzak and Philipsen, 2013). A core set of transcription factors dictates lineage specificity and maturation of erythroid cells in mammals. Detailed discussion of the many transcription factors that have been demonstrated to function in erythroid development and differentiation is beyond the scope of this review. Here, we highlight the GATA proteins GATA-1 and GATA-2 and KLF1 (also known as EKLF), central transcription factors that regulate these processes in primitive and definitive erythropoiesis. We also discuss some of the differences in gene expression between the two lineages.

Expression of *Klf1* is restricted to the erythroid lineage (Southwood et al., 1996). Fetuses with a null mutation in *Klf1* develop anemia and die at approximately E14 due to a deficiency in β -globin expression (Nuez et al., 1995; Perkins et al., 1995). Humans heterozygous for the *Klf1* gene have a healthy phenotype but hereditary persistence of fetal hemoglobin is observed (Borg et al., 2010). *Klf1* also has a role in regulating commitment of MEPs to the erythroid or megakaryocyte lineage, as it was found to suppress megakaryopoiesis (Frontelo et al., 2007; Siatecka et al., 2007). Null mutations of *Klf1* in the EryP lineage result in a partial “identity crisis” in which circulating EryP display an upregulation of select megakaryocyte-related proteins and transcription factors (Isern et al., 2010). In addition to regulating *globin* genes, *Klf1* controls the expression of other transcription factors, cytoskeletal components, and many other proteins involved in primitive and definitive erythroid maturation (Hodge et al., 2006; Isern et al., 2010; Tallack et al., 2009; Tallack et al., 2012; Tallack et al., 2010). Therefore, *Klf1* functions as a master transcriptional regulator of erythropoiesis (reviewed in Siatecka and Bieker, 2011; Yien and Bieker, 2013).

The GATA transcription factors GATA-1 and GATA-2 are critical regulators of hematopoiesis (Reviewed in Bresnick et al., 2012). These two transcription factors display reciprocal expression profiles during erythropoiesis and play different roles in erythroid development: GATA-2 regulates proliferation and maintenance of erythroid and hematopoietic progenitors, while GATA-1 promotes differentiation (Bresnick et al., 2012; Fujiwara et al., 2004; Moriguchi and Yamamoto, 2014; Tsai et al., 1994). GATA-2 is a transactivator of *Gata-1* during erythroid progenitor expansion (Bresnick et al., 2012; Cantor, 2005; Ferreira et al., 2005). A biphasic GATA transition known as “GATA Factor Switching” then ensues in which *Gata-2* expression is suppressed by GATA-1 during erythroblast maturation (Kaneko et al., 2010). GATA factor switching, initially discovered in mice, has recently been demonstrated to occur in human erythropoiesis as well (Li et al., 2014). Global gene expression studies comparing sorted human BFU-E and CFU-E from differentiating cultures of CD34⁺ cells revealed co-expression of GATA-1 and GATA-2 in BFU-Es but expression of only GATA-1 in CFU-Es (Li et al., 2014). GATA-1 activates genes that transcribe a cohort of components required to assemble the autophagy machinery that removes organelles in the late stages of erythroid maturation (Kang et al., 2012), a finding that adds to a growing list of maturational programs regulated by GATA-1 during erythropoiesis (Bresnick et al., 2012). *Gata-1* and *Gata-2* null mutant embryos die between E10 and E11 of gestation from severe anemia (Fujiwara et al., 1996; Tsai et al., 1994). Only the combined knockout of *Gata-1* and *Gata-2* completely ablates the EryP lineage in the YS (Fujiwara et al., 2004). Therefore, there is some overlap in GATA factor function in EryP such that at least one is necessary to initiate red cell formation in the YS (Fujiwara et al., 2004). GATA-1 works in concert with other transcription factors and co-factors, including *Tal-1*, *Lmo-2*, and *Ldb-1*, to activate erythroid genes in what have been termed “Ldb-1 Complexes” (Vyas et al., 1999). FOG-1 is another cofactor for GATA-1 (Mancini et al., 2011). Conditional knockout studies of *Gata-1* and *Zfpml* (which encodes FOG-1) demonstrate that these factors work sequentially to establish lineage commitment in hematopoiesis (Mancini et al., 2011). *Fog-1* deletion results in increased myelopoiesis from CMPs, indicating that *Fog-1* expression is indispensable for establishing the MEP during

hematopoiesis. Within the MEP, GATA-1 binds FOG-1 at specific binding sites to drive commitment to the erythroid lineage (Mancini et al., 2011).

The primitive and definitive erythroid lineages differ in their requirements for a number of transcriptional regulators. For instance, the function of c-Myb, Runx1, and ZBP-89 are critical for normal EryD but not for EryP maturation (Mucenski et al., 1991; Okuda et al., 1996; Wang et al., 1996; Woo et al., 2008). Recent transcriptome studies have revealed several transcriptional regulators that are expressed at higher levels in EryP than in EryD, with large differences in *Pbx-1*, *Foxh-1*, *Arid-3a*, *Pdlim-7*, and *Cited-2* (Kingsley et al., 2013). By contrast, a number of other genes, including *Sox-6*, *Nr3c-1*, *Cebpa*, and *Irf-9*, are highly enriched only in EryD (Kingsley et al., 2013). Differences in expression of genes within shared functional categories have also been observed in these lineages. For example aquaporins 3 and 8 are expressed in EryP, while aquaporins 1 and 9 are expressed in EryD (Kingsley et al., 2013). However, with the exception of *Sox-6*, which regulates globin gene expression (Yi et al., 2006), the significance of these differences in transcription factors in the two erythroid lineages is not understood.

Although mouse and human erythropoiesis are broadly similar, the differences that have been identified in their ontogeny (Baron, 2013; Baron et al., 2012; Lensch and Daley, 2004) and regulation of erythroid specific genes (most notably, but not exclusively, *globin* genes) indicate that they have partially diverged. A comparative study (Pishesha et al., 2014) of stage-matched erythroid precursors based on several published databases demonstrated that control of the expression of key transcriptional regulators, transmembrane and cytoskeletal proteins, and erythroid cell enzymes is largely conserved between mouse and human. However, when analyzed at a global level, they show extensive divergence at comparable stages of maturation. A similar conclusion was reached in another study of human and mouse erythroid cells that were purified by cell sorting at distinct stages of maturation (An et al., 2014).

Non-transcriptional mechanisms also play a role in the regulation of erythropoiesis and erythroid-specific gene expression. These include translational control by microRNAs (Listowski et al., 2013) and long noncoding RNAs (lncRNAs) (Alvarez-Dominguez et al., 2014) as well as epigenetic mechanisms (Ginder, 2015; Ginder et al., 2008; Wozniak and Bresnick, 2008).

10. Regulation of steady state erythropoiesis

EPO regulates the steady state output of RBCs into circulation (Fried, 2009). EPO is synthesized primarily in the kidney by peritubular fibroblasts (Jelkmann, 2011). The *Epo* gene locus has an enhancer that is activated by hypoxia inducible transcription factors (Hifs) (Jelkmann, 2011). A Hif binding site in the EPO gene locus permits transcriptional regulation in response to the local oxygen levels in the peripheral blood. *Hif2* is the primary transcription factor responsible for increasing *Epo* expression (Jelkmann, 2011). If the hematocrit decreases, and subsequently hemoglobin levels fall, the kidney begins to sense low oxygen levels. As a result, *Hif2* is activated in the peritubular fibroblasts and binds the *Epo* enhancer to increase transcription. In response to hypoxic conditions in the peripheral

blood, serum EPO levels are elevated transiently. Once hemoglobin levels and red cell numbers are restored, *Hif2* is deactivated and EPO production falls to steady state levels. In adult mice, EPO levels regulate RBC output in both bone marrow and spleen. In humans, however, RBC production is primarily restricted to the bone marrow (for more information on hypoxia and erythropoiesis see Haase, 2010; Rogers et al., 2008).

Under steady state conditions, RBC output is regulated at the CFU-E stage of erythropoiesis (Hattangadi et al., 2011). In mice, 10-20% of CFU-Es in the bone marrow and 40-60% in the spleen are annexin V⁺ (Koulis et al., 2011; Liu et al., 2006). An increase in serum EPO levels stimulates a sharp increase in the pro-survival factor BCL-X_L that rescues annexin V⁺ CFU-Es from apoptosis and provides an immediate supply of RBCs and stabilization of hemoglobin levels (Koulis et al., 2012). Prolonged elevation of EPO levels further stimulates erythropoiesis by downregulating the pro-apoptotic factors Fas/FasL, Bim, and Bam (Koulis et al., 2011). Once hemoglobin levels return to baseline, EPO levels drop and pro-apoptotic factors re-establish a reservoir of annexin V⁺ CFU-Es (Koulis et al., 2011). This system most probably evolved to mitigate fluctuations in hemoglobin levels without exhausting hematopoiesis.

11. Erythropoiesis under conditions of stress

Under steady state conditions, an increase in CFU-E survival is sufficient to normalize RBC numbers and hemoglobin levels in the peripheral blood. Bone marrow-derived CFU-Es can undergo 3-5 divisions before they complete terminal maturation (Hattangadi et al., 2011). As a result, elevated EPO levels can stimulate only a limited increase in RBC numbers from the CFU-E population (Figure 4A). Therefore, the mechanisms that regulate steady state erythropoiesis are not sufficient to correct RBC deficiency under extreme conditions of stress (see above). Under these circumstances, BFU-Es undergo increased proliferation before becoming CFU-Es, to provide a boost in RBC production (Hattangadi et al., 2011).

Glucocorticoid (GC) nuclear hormone receptor signaling regulates stress erythropoiesis. Mice lacking the GC receptor or harbor a defect in the DNA binding domain have normal steady state erythropoiesis, whereas their ability to respond to stress is significantly impaired (Bauer et al., 1999; Reichardt et al., 1998). This defect is manifest in the spleen, as evidenced by a reduction in CFU-Es formed in semisolid cultures (Bauer et al., 1999). The bone marrow does not exhibit a GC receptor dependent defect under stress (Bauer et al., 1999). GC receptor ligands induce significant expansion of BFU-Es (Figure 4B) *in vitro* and have been the major factor explored in the hope of establishing large scale expansion *ex vivo* of erythroid progenitors for transfusion (Wessely et al., 1997). The genes *Zfp36l2*, *Hopx*, *Bmi-1* and *Nlrp6* have all been shown to regulate induction of erythroid progenitor self-renewal by dexamethasone (Kim et al., 2015; Zhang et al., 2013). Knockdown of these genes using shRNAs resulted in inhibition of dexamethasone-induced progenitor self-renewal (Kim et al., 2015; Zhang et al., 2013). GC receptor signaling induces expression of Hif-1 α -regulated genes in BFU-Es (Flygare et al., 2011) and Hif-1 α activation synergizes with GC signaling to enhance BFU-E proliferation *in vitro* (Flygare et al., 2011), suggesting that hypoxia and stress signaling cooperate *in vivo* to restore RBC numbers. The Peroxisome Proliferator-Activated Receptor- α (PPAR- α) synergizes with GC receptor signaling to

stimulate the expansion of early mouse or human erythroid progenitors in culture (Lee et al., 2015). Although *PPAR-α* null mice show no hematological differences in recovery from acute anemia compared to wild type mice, *PPAR-α* agonists enhance recovery (Lee et al., 2015).

SCF (c-Kit ligand) is a significant player in both steady state erythropoiesis and recovery from stress erythropoiesis. The recovery from phenylhydrazine-induced anemia was severely impaired in mice treated with anti-c-Kit antibodies, which compete for SCF binding (Broudy et al., 1996). *In vitro* expansion of erythroid progenitors with dexamethasone, a synthetic GC receptor ligand, is dependent on c-Kit signaling (von Lindern et al., 1999). In the absence of c-Kit signaling, GC receptor signaling cannot induce prolonged proliferation of the BFU-E population (von Lindern et al., 1999).

BMP4 is also involved in stress erythropoiesis. The *flexed tail* mouse, which expresses a dominant-negative *Smad5* mutant protein that inhibits BMP4 signaling, does not display a steady state erythroid phenotype. However, for two weeks after birth, the mice exhibit anemia (Hegde et al., 2007; Lenox et al., 2005). BMP4 signaling through *Smad5* regulates the erythroid response to acute anemia (Lenox et al., 2005; Lenox et al., 2009). BFU-Es must be primed with Sonic hedgehog in order to respond to BMP4 (Perry et al., 2009). Therefore, Hypoxia, SCF, and BMP4 signaling synergize to increase proliferation of BFU-E progenitors (Perry et al., 2007).

13. Conclusion

Great strides have been made in our understanding of mammalian erythropoiesis. However, the prevalence of anemias and insufficient supply of RBCs for transfusion continue to be problems worldwide (Migliaccio et al., 2012) and many important questions remain to be addressed. Species-specific differences in gene expression patterns can be exploited to make testable predictions that will lead to the development of better mouse models for human disease (Pishesha et al., 2014). A better understanding of the critical regulators of erythroid cell maturation may suggest new options for the development of more effective therapies for human erythroid cell disorders.

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- In the developing embryo mouse hematopoiesis emerges in three waves
- Erythroid cell maturation occurs through five distinct stages.
- Erythropoietin and stem cell factor are essential for sufficient red cell production.
- Erythropoietin rescues erythroid progenitors from apoptosis to maintain red cell numbers.
- Erythroid progenitors undergo increased self-renewal in response to stress conditions.

Figure 1. Two models of the mouse hematopoietic cell hierarchy

In model 1, all adult blood lineages arise from a common multipotent cell within the lineage negative (lin^-), Sca1^+ , c-Kit^+ (LSK) population (Akashi et al., 2000; Kondo et al., 1997). The hematopoietic cell hierarchy bifurcates at the level of multipotent progenitors (MPP), which give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). Model 2 depicts a revised version of the mouse hematopoietic hierarchy where MPPs do not produce CMPs; instead, CMP progenitors form directly from short term HSC (ST-HSC). ST-HSCs give rise to lymphoid-primed multipotent progenitors (LMPP) that produce granulocyte monocyte progenitors (GMP) and CLPs. GMPs, therefore, arise through commitment of both LMPP and CMPs. Megakaryocyte erythroid progenitors are still derived from CMPs but are produced through a different pathway. (This figure was modified from (Adolfsson et al., 2005) with permission).

Figure 2. Commitment and maturation of erythroid progenitors

(A) Cartoon depicting the cellular pathway from MEP to proerythroblast, with some of the distinguishing cell surface markers indicated. (B) Photograph of a mouse BFU-E colony. A BFU-E progenitor forms a large, pigmented colony containing several thousand cells. With close observation, one can appreciate that the BFU-E colony is composed of several smaller CFU-Es. Scale bar, 100 μ m. (C) Photograph of a mouse CFU-E colony. CFU-Es from mouse are typically non-pigmented, but can be identified by their size and by the appearance of cell borders, which are not well defined. The presence of hemoglobin can be confirmed by staining colonies with a dye such as benzidine. Scale bar, 20 μ m.

Figure 3. The stages of erythoid cell maturation

Cartoon depicting the stages of erythroid cell maturation.

Figure 4. Red blood cell output under steady state and stress conditions

(A) Under steady state conditions, progenitors committed to the erythroid lineage undergo several cell divisions before they begin to differentiate. EPO and SCF are the major growth factors that control whether a progenitor will mature or self-renew under steady state conditions. (B) Under stress conditions, early progenitors undergo increased self-renewal before they begin to differentiate. Factors such as BMP (Lenox et al., 2009) and corticosteroids (Bauer et al., 1999) work in concert with EPO and SCF to increase RBC production.