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Erythroid Development in the Mammalian Embryo

Margaret H. Baron[a,b,c,d,e,f,*], **Andrei Vacaru**[a,d], and **Johnathan Nieves**[a,d]

[a]Departments of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[b]Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[c]Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[d]The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[e]The Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[f]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

Abstract

Erythropoiesis is the process by which progenitors for red blood cells are produced and terminally differentiate. In all vertebrates, two morphologically distinct erythroid lineages (primitive, embryonic, and definitive, fetal/adult) form successively within the yolk sac, fetal liver, and marrow and are essential for normal development. Red blood cells have evolved highly specialized functions in oxygen transport, defense against oxidation, and vascular remodeling. Here we review key features of the ontogeny of red blood cell development in mammals, highlight similarities and differences revealed by genetic and gene expression profiling studies, and discuss methods for identifying erythroid cells at different stages of development and differentiation.

Keywords

primitive erythropoiesis; transgenic mice; mammalian embryo; yolk sac; fetal liver; progenitor; erythroid differentiation; enucleation

Introduction

Erythroid (red blood) cells play an essential role in oxygen delivery and vascular morphogenesis during embryogenesis and throughout postnatal life. Progenitors of the primitive erythroid (EryP) lineage arise early during postimplantation development in the yolk sac of the mammalian embryo. Large, nucleated EryP emerge in great numbers and are the predominant circulating blood cell until a second wave of definitive, enucleated erythrocytes (EryD) is produced by the fetal liver [1; 2; 3; 4]. EryD then rapidly outnumber

*Corresponding author: Margaret H. Baron, MD PhD, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1079, New York, NY 10029-6574, Phone: (212) 241-0825, FAX: (212) 849-2442, margaret.baron@mssm.edu.

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EryP in the fetal blood. Cells of the two erythroid lineages differ in size (EryP are larger than EryD) and express distinct sets of α- and β-like *globin* genes (embryonic/fetal in EryP, adult in EryD) [2; 5; 6]. They also differ in their oxygen-carrying capacity and response to low oxygen tension [7]. It had long been accepted that a key distinguishing feature of circulating primitive and definitive erythroid cells was the presence or absence of a nucleus. It is now known that, like their definitive counterparts, primitive erythroblasts in the mouse embryo also enucleate, but they do so after entering the blood around the time that formation of EryD begins in the fetal liver [8; 9]. Despite their origins from distinct populations of mesodermal progenitors [10], the two erythroid lineages are remarkably similar and both are critical for normal development.

Overview of mammalian hematopoietic development

Mammalian hematopoietic development occurs in successive and partially overlapping waves in the embryo and fetus (Fig. 1). In both mouse and humans, the initial wave of hematopoietic activity (primitive) occurs outside the embryo proper, in the yolk sac, and results primarily in the formation of EryP as well as megakaryocytes and macrophages [11; 12]. EryP progenitors can be identified toward the end of gastrulation in the mouse embryo [2; 13]; they arise from bipotential megakaryocyte/erythroid progenitors (MEPs) [12]. The yolk sac is also the site of a second wave of hematopoiesis that produces definitive erythroid, megakaryocyte, and myeloid cells [2; 12; 14], and possibly also lymphoid [15; 16; 17] cells. The third wave arises from hematopoietic stem cells (HSCs) that emerge from multiple sites within the embryo, including the para-aortic splanchnopleure (P-Sp), the aorta-gonad-mesonephros (AGM) region and other large arteries (vitelline and umbilical), and the placenta (for reviews, see refs. [18; 19; 20; 21]). HSC activity identified following transplantation into newborn (but not adult) mice is also found in the E9.0 yolk sac [22].

The HSCs that form in the P-Sp/AGM do not differentiate there [23] but colonize the fetal liver and, later, the bone marrow, thymus, and (in mouse), the spleen [18; 24; 25; 26].

Primitive erythropoiesis in the yolk sac

EryP form in the yolk sac, in close temporal and spatial association with endothelial cells. For many years the two lineages were thought to arise from a bipotential progenitor termed the hemangioblast. They have also been described as appearing in "blood islands," clusters of erythroblasts surrounded by endothelial cells. The existence of the hemangioblast in vivo and the concept of the yolk sac blood island have both been challenged [reviewed in ref. 27]. EryP progenitor activity, detected using colony-forming assays in vitro, is present from ~E7.5 until early somite stages (~E8.75) and disappears rather abruptly, by ~E9.0 [2; 5; 13]. Thus, they appear as a nearly synchronous cohort and their progression from proerythroblast to orthochromatic erythroblast to enucleate erythrocyte (reticulocyte) can be followed using Giemsa staining. Morphological hallmarks of their maturation include loss of nucleoli, decreased cell diameter and cross-sectional area, nuclear condensation, changes in expression of cell surface molecules, and, eventually, enucleation [8; 9]. Little is known about primitive erythropoiesis in the human yolk sac, owing to the difficulty of obtaining embryonic tissue at this early time in gestation. However, studies using human embryonic stem (ES) cells suggest that human primitive hematopoiesis is broadly similar to that in the mouse embryo [28].

Mammalian embryonic red blood cells have been designated "primitive" for historical reasons. Like the macrocytic erythroblasts of non-mammalian vertebrate embryos, they form in the yolk sac, enter the circulation as nucleated cells, and their progenitors are produced for only a short time in the embryo [29]. It is now known that EryP continue to mature and do eventually enucleate, several days after entering the bloodstream, where they remain at

least through birth [8; 9]. Like EryD [30], EryP have been found in erythroblastic islands in the fetal liver [31; 32]. EryP enucleation appears to occur in the circulation and in fetal liver [31; 32], though this process remains to be documented using real-time imaging. The expelled nuclei (also known as "pyrenocytes" [31]) are cleared by macrophages in the fetal liver and perhaps other tissues [31; 32].

Human primitive erythroblasts enucleate within the placenta [33]. Immunostaining of this tissue suggests that expelled human EryP nuclei are engulfed by macrophages whose developmental origin may also be the placenta [33].

Biological significance of primitive erythroid cells

Primitive erythroid cells are crucial for the transition from embryo to fetus in developing mammals. In addition to their function in oxygen delivery to cells within the rapidly growing embryo, EryP may scavenge reactive oxygen species [34] and are thought to play a critical role in vascular remodeling during development. The primary capillary plexus of the early yolk sac is remodeled into mature blood vessels that are aligned along the direction of blood flow as cardiac contraction begins [35]. Real-time confocal imaging of ε-*globin*-GFP transgenic mouse embryos cultured ex vivo [36] revealed that hemodynamic parameters such as shear force change during embryogenesis and that the pattern of blood flow can be correlated with the stage of cardiac development [37]. Erythroblasts are required to generate shear forces necessary for vascular remodeling [35]. In the absence of a heartbeat, as observed for *Ncx1* [38] or *Mlc2a* mutant embryos [39], shear forces are not generated and the yolk sac vascular plexus fails to remodel. Moreover, in erythroid-tropomodulin (E-Tmod) mutant embryos, the mechanically weakened EryP are unable to support remodeling of the primary capillary plexus into mature vessels [40]. Therefore, circulation between the yolk sac and the embryo proper cannot be established.

Origin of the definitive erythroid lineages in the yolk sac and fetal liver

The fetal liver provides a microenvironment for the robust expansion and differentiation of definitive erythroid cells. The earliest definitive erythroid progenitors that colonize the fetal liver likely originate from erythroid/myeloid progenitors (EMPs) produced during the second wave of hematopoiesis in the (yolk sac E8.25-8.5), rather than from HSCs [2; 41; 42]. EMP-derived definitive erythropoiesis has been proposed to bridge the transition between primitive and HSC-derived erythropoiesis [19; 42]. Definitive yolk sac hematopoiesis produces multipotential, highly proliferative progenitors (HPP-CFC) from E8.25 yolk sac [41] and extensively self-renewing erythroid (ESRE) progenitors from E9.5 mouse yolk sac [43], both identified in vitro. ESREs are also found in the E12.5 fetal liver [43]. These remarkable cells expand dramatically (10^6-to-10^{60} fold) upon prolonged culture [43]. Little, if any, ESRE progenitor activity is present in adult bone marrow or spleen under the same conditions [43]. It is possible that the first hepatic colonization described for the human fetus (Figure 1B) corresponds to the second wave of hematopoiesis identified in the mouse embryo [27].

The third wave of hematopoiesis produces HSCs that seed and differentiate within the fetal liver. By ~E14.5 in the mouse [2] and 7–8 weeks in the human embryo [4], definitive erythropoiesis is well underway and EryP are rapidly outnumbered by EryD in the circulation. Throughout postnatal life, definitive erythropoiesis originates from HSCs in the marrow, under normal physiological conditions [19; 20]. Erythroid cells are also produced in the spleen of mice in response to stress or disease [44; 45; 46]. In both mouse and human EryD, the switch to adult β- like *globin* gene expression is completed after birth [reviewed in ref. 47]. This switch marks the final event in erythroid ontogeny.

Transcriptional regulation of erythroid development

Several transcriptional profiles have been reported for a single erythroid stage, heterogeneous populations, or cells differentiated in vitro, in mouse and human [48; 49; 50; 51; 52; 53]. Chronological global gene expression profiling has been performed for primary mouse EryP at 24 hour intervals from E7.5 through E12.5 [13]. This study took advantage of the essentially synchronous maturation of primitive erythroblasts, in combination with sorting of cells from ε-*globin*-H2B-GFP transgenic embryos [32], to analyze gene expression at 6 distinct stages of EryP development, including progenitors [13]. The EryP developmental database, which is available online at Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24127, provides a transcriptional roadmap for primitive erythropoiesis.

A large number of erythroid genes, including *Eklf/Klf1, Gata2, Tal1/Scl*, *Lmo2, Erythrocyte band 4.1* and *Fog-1,* are already expressed at high levels in progenitors at E7.5 [13]. Others, such as ε*Y*-, ζ -, and α-*globin* genes (*Hbb-y*, *Hba-x,* and *Hba-a1*), ε*Y*-, ζ -, and α-*globin* genes (*Hbb-y*, *Hba-x,* and *Hba-a1*), the anion transporter *band 3/Slc4a1*, and *Glycophorin A (Gypa),* increase significantly in expression during the period of progenitor expansion within the yolk sac (E7.5–8.5) [13].

Transcription in maturing EryP is marked by two discrete waves that correlate with key developmental hallmarks. The first wave (E8.5~E9.5) coincides with the transition from progenitor (yolk sac) to circulation stage development [13]. From E9.5 to E11.5, corresponding to proerythroblast through poly- or orthochromophilic erythroblast, transcription decreases globally. The second wave of transcriptional variation (E11.5~E12.5) corresponds to a period of extensive morphological change, decreased cell division rate, cytoskeletal remodeling, and nuclear condensation and extrusion. Gene ontology functions that are enriched in EryP at this period of their maturation (e.g. nuclear organization, DNA packaging and chromatin assembly) reflect these changes in transcript diversity [13]. A nearly reciprocal pattern of gene expression was observed for the two waves of transcriptional variation that may reflect the need to respond to the distinct microenvironmental niches of the yolk sac and circulation [13].

A study of global gene expression in morphologically comparable stages of primitive, fetal definitive and adult mouse definitive erythroid precursors has been reported [34]. Cells of the primitive and definitive erythroid lineages express different genes within shared functional categories based on gene annotation, (for example, genes within the aquaporin family). The authors created a user-friendly website (http://www.cbil.upenn.edu/ErythronDB) that permits comparative searches.

The zinc finger transcriptional regulators GATA1 and EKLF play key roles in both primitive and definitive erythropoiesis [54; 55; 56; 57; 58; 59; 60; 61; 62]. EryP and EryD differ in their requirements for a number of other transcription factors, including Runx1, Sox6, and c-Myb (Table 1). Transcriptional regulators known to have important functions in erythropoiesis are listed in Table 1.

Identification of erythroid cells at different stages of ontogeny and differentiation

Transgenic mouse models for tagging, tracking, and purifying primitive erythroid cells—Primitive and definitive erythroid cells can be partially separated by taking advantage of the difference in their size [e.g. see ref. 8]. However, there are presently no cell surface markers known that uniquely distinguish primitive from definitive erythroid cells. Transgenic mouse lines have been created for tagging and tracking EryP during embryogenesis, using a human ε-*globin* gene promoter and μLCR to target pancellular GFP

[63] or nuclear (histone H2B-) GFP [32] (Fig. 2) to these cells. The bright green fluorescence in ε-*globin*-H2B-GFP transgenic embryos allows the prospective isolation and quantitation of EryP progenitors, which are an abundant cell type at E7.5 (~15% of cells) and E8.5 (40–50%) [13].

The tetraspanin protein CD9 is expressed on nucleated EryP and EryD but not on enucleated cells of either lineage in the mouse [61]. As nucleated EryP are found in the circulation through~E15.5 [8; 9], CD9 is a useful marker for distinguishing between EryP and EryD in peripheral blood past midgestation [61]. CD9 expression has recently been used for this purpose to study membrane remodeling of EryP [64].

Separation of EryD at distinct stages of maturation using flow cytometry—In contrast with the differentiation of primitive erythroid cells as a cohort, definitive erythropoiesis proceeds continuously in the fetal liver and adult bone marrow. Progressive stages of EryD maturation can be separated based on expression of Ter119 and CD71 [65; 66] or CD44 [67; 68]. A method for isolating human bone marrow erythroblasts at distinct stages has been reported recently, using other markers whose expression changes more dramatically on human than on mouse red cells [69].

Two EryD progenitors have been defined functionally using colony assays: the early erythroid burst-forming unit (BFU-E) and later colony-forming unit (CFU-E). Fetal liver BFU-E and CFU-E can be FACS-purified using a combination of negative selection for 9 cell surface proteins and staining for CD71 [70].

Conclusion

Erythroid cells play essential roles in supporting embryonic and fetal development and throughout postnatal life. Mammalians presumably have evolved to produce two, and arguably at least three distinct erythroid lineages during their ontogeny. Primitive erythroid cells appear rapidly in the yolk sac as a large cohort of cells, maturing only after they enter the circulation. As they differentiate, their hemoglobin production increases, underscoring their need to differentiate further. The segregation of primitive erythropoiesis to an extraembryonic site may allow the embryo proper to devote other resources to early organogenesis. A peculiar feature of primitive red cells is that they do not lose their nuclei until around the time that definitive erythropoiesis begins in the fetal liver, raising the question of what function enucleation serves in this lineage. It has been appreciated for many years that mammalian adult erythrocytes are more deformable than the nucleated erythroid cells of birds [71; 72]. Enucleation may, similarly, confer a hydrodynamic advantage on primitive red cells [64]. The large size of mammalian primitive erythroblasts may result in greater shear forces required for rapid vascular remodeling in the embryo.

Red cells at all stages of development have many common features and, not surprisingly, they share expression of many of the same genes. Primitive and definitive erythroid cells differ in their requirements for certain transcription factors and may express distinct genes within multigene families such as those encoding hemoglobin [47], glucose transporters [13], and aquaporins [34]. The red blood cell has served as a model system for understanding a variety of biological problems and is likely to continue to provide surprises and new insights for many years to come.

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Abbreviations

AGM	aorta-gonad-mesonephros
BFU-E	burst-forming unit erythroid
E#	embryonic day post-fertilization
CFU-E	colony-forming cells erythroid
EryD	definitive, enucleated erythrocytes
EMP	erythroid-myeloid progenitor
EryP	primitive (nucleated) erythrocytes
ES	embryonic stem
ESRE	extensively self-renewing erythroid
E-Tmod	erythroid-tropomodulin
GFP	green fluorescent protein
HPP-CFC	high proliferating progenitors-colony forming cell
MEP	bipotential megakaryocyte/erythroid progenitor

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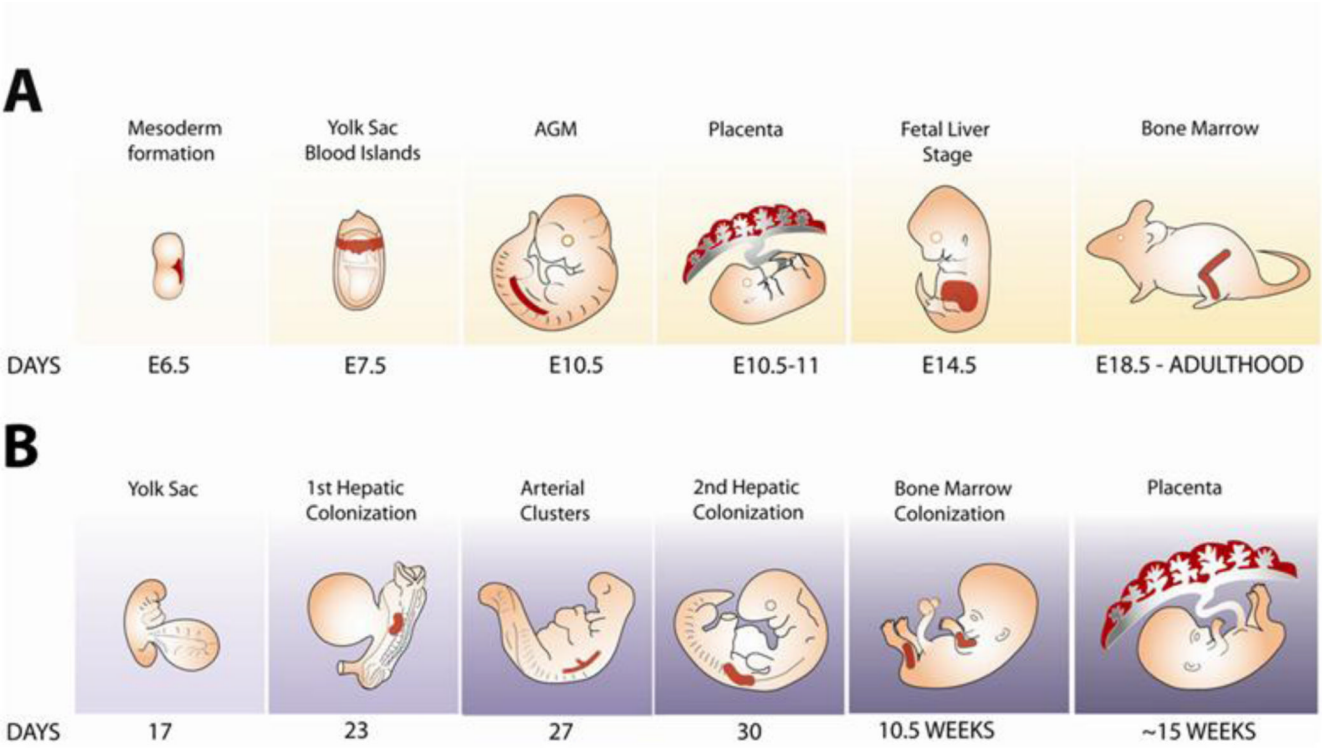

Figure 1. Ontogeny of mouse and human hematopoiesis

(A) Hematopoietic development in the mouse. The panels represent, from left to right, formation of mesoderm during gastrulation (E6.5), development of blood islands within the yolk sac (~E7.5), emergence of HSCs in the AGM region (E10.5) and placenta (E10.5–11), active fetal liver hematopoiesis (E14.5), and hematopoiesis in the bone marrow (~E18.5 in the late gestation fetus and throughout postnatal life). Myeloid and definitive erythroid potentials are found in the allantois and chorion (not shown) prior to their fusion to form the placenta [106]. The formation of HSCs is completed by mid-gestation. Lymphopoiesis is not represented in this figure. Cardiac function begins as early as ~E8.25, with active circulation by ~E9.0[107]. For a detailed review of mammalian hematopoiesis, see ref. [19]. (B) Hematopoietic development in the human embryo. The panels represent, from left to right, hematopoiesis at the yolk sac stage (day 17), at the time of the first hepatic colonization by HSCs (day 23), arterial cluster formation (day 27), the second hepatic colonization (day 30), and bone marrow colonization (10.5 weeks). For a review, see ref. [21]. In contrast with the mouse embryo, where HSCs are found in the placenta at around the same time as in the AGM region [108; 109], well before colonization of the bone marrow (~10.5 weeks [110]), human HSC activity is not detected in the placenta until several weeks after bone marrow colonization, at ~15 weeks [111]. Active circulation begins by ~day 21 [4].

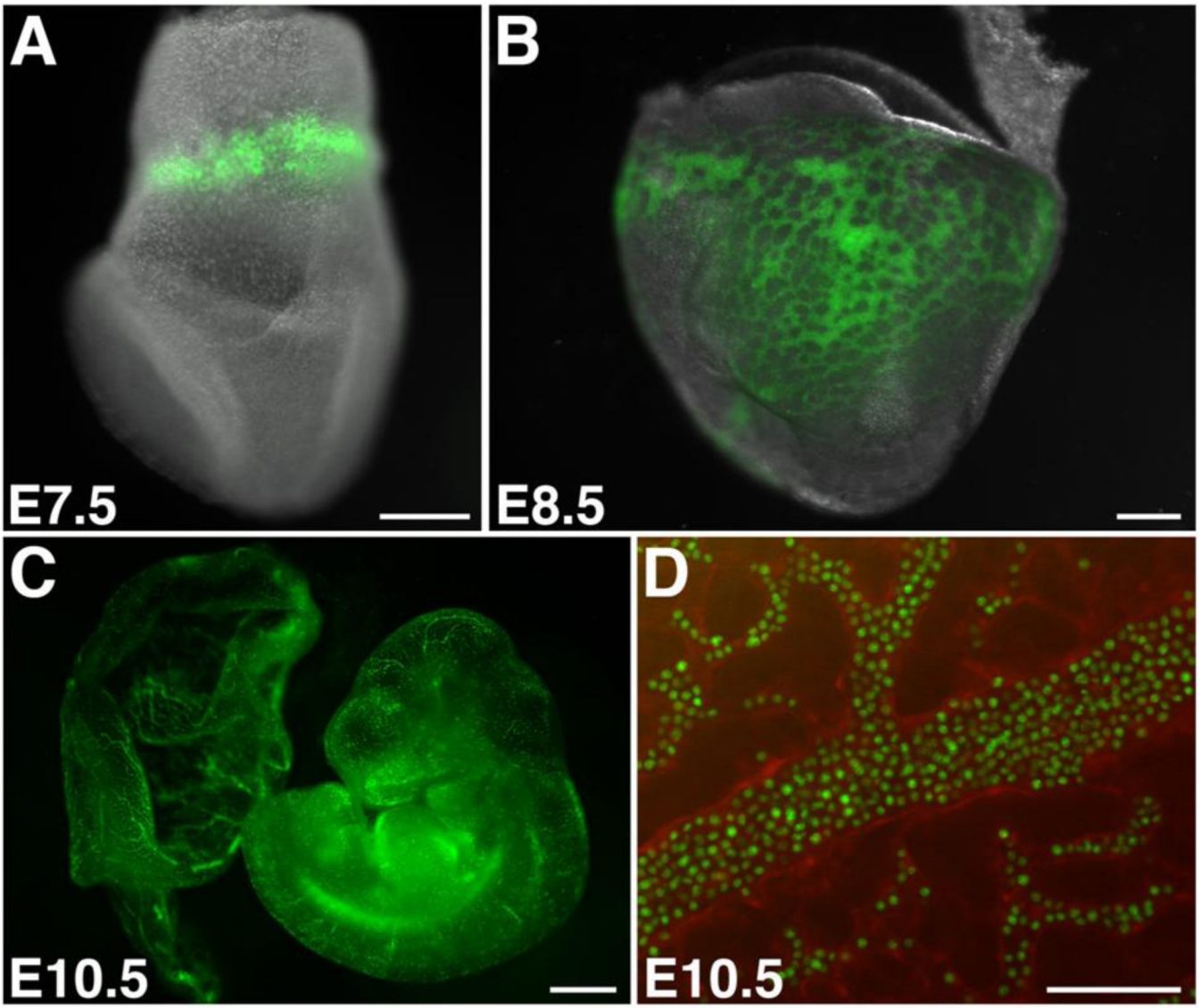

Figure 2. Photographs of ε-*globin*-H2B-GFP transgenic mouse embryos at different developmental stages

Overlay of bright field and GFP channel of an ~E7.5 (A) and of an ~E8.5 embryo (B). Scale bars, 200μm. (C) GFP expression in an E10.5 embryo. The yolk sac (left) has been opened. Scale bar, 1mm. (D) Detail of yolk sac from an E10.5 ε-*globin*-H2BGFP; *Flk1*-Cre; *Rosa26*-tdTom transgenic embryo. Red fluorescence (tdTomato) is seen in endothelial cells of the yolk sac vasculature and results from the excision of a STOP cassette from the *Rosa26*-tdTom transgene [112] by *Flk1* promoter-driven Cre. Scale bar, 100 μm.

Table 1
Transcription factors and other regulatory proteins in erythropoiesis

This table focuses on functions in erythroid cells but many of these genes/proteins are also known to play roles in other lineages. References are primarily for studies on mouse mutant phenotypes

Gene symbol(s)	Name(s)/Protein	Mutant Phenotype	Comments	Refs.
Gata1	GATA1	Maturation block in EryP and EryD	Erythroid progenitors form but fail to mature	[57; 58]
Gata2	GATA2	Embryonic lethality in knockout at E10.5–E11.5 due to failure in progenitor expansion	Double KO revealed functional overlap between Gata1 and Gata2 in EryP	[61; 67]
Fog1	Friend of GATA1; FOG1	Fog1 knockout mice die between E10.5 and E12.5 of severe anemia and defective megakaryopoiesis	Acts sequentially with GATA1 to specify definitive erythroid and megakaryocytic progenitors	[68; 69]
Klf1/Eklf	Erythroid Kruppel-Like Zinc Finger Protein	Embryonic lethality by ~E13.5 due to defective fetal liver erythropoiesis	Defects in EryP and EryD maturation	[49; 59; 60; 62; 63; 64]
Scl/Tal	Basic helix-loop-helix SCL/tal-1	Embryonic lethality at E9–10.5 due to hematopoietic and vascular defects	Master regulator of hematopoiesis, forms complexes with Gata1 and Lmo2	[70; 71; 72]
Lmo2	Rbtn2; LIM domain only 2, Cysteine-rich LIM domain protein	Embryonic lethality at E10.5 due to failure in primitive erythropoiesis		[73]
Runx1	Runt-related transcription factor 1; Core Binding Factor-a2 (Cbfa2), AML1	Embryonic lethality at E11.5–12.5 due to failure in definitive erythropoiesis and hemorrhaging	Some evidence for a (nonessential) function in primitive erythropoiesis	[74; 75; 76]
Ldb1	LIM domain-binding protein 1	Embryonic lethality after E9.5 (defective yolk sac development)	Mutant displays various other patterning defects; positive regulator of late steps in erythroid maturation. ES cell studies: decreased hemangioblast numbers and block in differentiation	[77; 78; 79]
c-Myb	c-MYB; myeloblastosis oncogene	Absence of EryD progenitors	Required for definitive but not primitive erythropoiesis; reduced megakaryocyte progenitor numbers	[12; 80]
c-Myc	c-MYC; myelocytomatosis oncogene	Epiblast-restricted c-Myc-null embryos severely anemic and die before E12, largely due to defects in primitive erythropoiesis	Physiologic levels critical; downregulation required for terminal maturation of fetal liver EryD	[81]
Klf2	Kruppel-Like Zinc Finger Protein 2	Critical for primitive erythropoiesis	Redundant roles with Klf1/Eklf	[82]
Klf6	Kruppel-Like Zinc Finger Protein 6	Knockouts embryonic lethal by E12	May function primarily in mesoderm specification rather than in primitive erythropoiesis	[83]
BRG1	Brahma Related Gene 1, SMARCA4	Embryonic lethal E10.5–11; severe EryP defect by E9.5	Tie2-Cre conditional KO	[84]
c-Maf	Proto-oncogene	Knockouts die from severe anemia E15–18	Important function in fetal liver macrophages; decreased EryD in EBIs of fetal liver, effect is non cell-autonomous	[85]

Gene symbol(s)	Name(s)/Protein	Mutant Phenotype	Comments	Refs.
ZFAT	Zinc finger and AT hook domain	Embryonic lethal E9.5, required in EryP	Regulates Tal, Lmo2, Gata1	[86]
P400/Do mino	E1A binding protein p400	Embryonic lethality E11.5 due to defects in primitive erythropoiesis	SWI2/SNF2 family ATPase; Nterminal domain deletion	[87]
Zbp89	ZBP-89; Zinc finger protein 148 (ZFP-148)	Embryonic lethal between E8.5 and E10.5 due to placental insufficiency	Part of complex with GATA1 and FOG1. Required for definitive but not primitive erythropoiesis.	[88]
Gfi1b	Growth factor independent 1b; Gfi1b	Embryonic lethal at E14.5 as a result of deficiencies in erythroid and megakaryocyte development	Target of Runx1 and regulator of endothelial to hematopoietic transition	[89; 90]
Sox6	SRY-box containing gene 6	Necessary for efficient physiological and stress erythropoiesis in mouse EryD; silencer of embryonic and fetal globin genes in EryD	Sox6, Gata1 and Bcl11A co-occupy human β-globin cluster; Sox6 expressed in mouse EryP progenitors but then downregulated	[13; 91; 92]
Bcl11A	B cell CLL/lymphoma 11A; Bcl11a	Knockout mice die perinatally from unknown causes. Disruption of embryonic/fetal to adult *globin* gene switch	Embryonic *globin* gene expression not silenced in knockout mice; regulated by Eklf/Klf1. Essential for normal lymphoid development	[93; 94; 95; 96]
NuRD	Nucleosome remodelling histone deacetylase	Cofactor for Fog1; mutations in Fog1 that disrupt interactions with NuRD results in anemia and macrothrombocytopenia	Context-dependent repressor and activator functions. Complex phenotype with similarities to Gata1 or Fog1 mutations	[97]
PIT1	PIT1/SLC20A1	Embryonic lethality at E12.5, erythroid maturation defects	Defects in vitro and in vivo; target of Eklf/Klf1	[98; 99]