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The Roles of Krüppel-like Transcription Factors KLF1 and KLF2 in Mouse Embryonic and Human Fetal Erythropoiesis

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THE ROLES OF KRÜPPEL-LIKE TRANSCRIPTION FACTORS KLF1 AND KLF2 IN MOUSE EMBRYONIC AND HUMAN FETAL ERYTHROPOIESIS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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DEDICATION

I would like to dedicate my thesis to my beloved grandparents.

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Late Mrs. Mariakutty Chacko and Mr. V.M. Chacko

They have been a constant source of zestful encouragement throughout my life.
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List of Abbreviations

β-YAC – entire β-globin locus yeast artificial chromosome
3C – chromosome conformation capture
7-AAD – 7-Aminoactinomycin D
APC – Allophycocyanin
Bcl11a – B-Cell lymphoma/leukemia 11a
BFU-E – burst-forming unit erythroid
BrdU – Bromodeoxyuridine
CBP – CREB-binding protein
CD235a – Glycophorin A
Cd24a – CD24 antigen
CD71 – Transferrin receptor
cDNA – complementary DNA
CFU-E – colony-forming unit erythroid
ChIP – Chromatin immunoprecipitation
CO₂ – Carbon dioxide
Cre – Cyclization recombinase
dcKO – double conditional knockout
DPBS – Dulbecco’s phosphate buffered saline
E – embryonic day
EDTA – Ethylenediaminetetraacetic acid
Epo – erythropoietin
Ery cKO – erythroid conditional knockout
EryP-CFC – primitive erythroid colony forming cells
ES cells – embryonic stem cells
FACS – Fluorescence activated cell sorting
FBS – Fetal bovine serum
FoxM1 – Forkhead box M1
Gapdh – Glyceraldehyde 3-phosphate dehydrogenase
HbA – Adult hemoglobin
HbF – Fetal hemoglobin
HbS – sickle hemoglobin
HPFH – Hereditary persistence of fetal hemoglobin
HS – DNase I hypersensitive site
HSC – Hematopoietic stem cell
IMDM – Iscove’s modified Dulbecco’s medium
KLF – Krüppel-like factor
KO – knockout
LCR – locus control region
LMO-2 – LIM domain only 2
MEL – mouse erythroleukemia
MEP – Megakaryocyte / erythroid progenitor
MSC – multispecies conserved sequences
P/CAF – p300/CBP-associated factor
P/S – Penicillin/Streptomycin
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PDS – Fetal bovine plasma derived serum
Pthr – Parathyroid hormone 1 receptor
qPCR – Quantitative PCR
qRT-PCR – Quantitative reverse transcriptase PCR
RNAPol II – RNA polymerase II
SCL/Tal1 – Stem cell leukemia / T-Cell acute lymphocytic leukemia 1
SFEM – Serum free expansion medium
shRNA – short hairpin RNA
Sphk1 – Sphingosine kinase 1
WHO – World Health Organization
YAC – yeast artificial chromosome
μl – microliter
ABSTRACT

Hemoglobinopathies are some of the most common monogenic disorders in the world, affecting millions of people and representing a growing burden on health systems worldwide. Although the pathophysiology of sickle cell anemia and β-thalassemia, two of the most common hemoglobinopathies, have been the focus of much research over the last century, patients affected by these diseases still lack a widely applicable and easily available cure. Sickle cell anemia and β-thalassemia are caused by defects in the structure and production of the β-globin chains that, along with the α-globin chains make up the heterotetrameric hemoglobin molecule. Studies geared towards re-expression of the silenced fetal γ-globin gene in adult erythroid cells as a therapeutic strategy to alleviate the symptoms of β-globin deficiencies have met with some success for the treatment of sickle cell anemia but not for β-thalassemia. A better understanding of normal γ-globin gene regulation will undoubtedly advance the development of more effective therapeutic strategies. Because many of the potential targets that may be modulated to achieve γ-globin re-expression also have functions in erythroid cells other than regulating the γ-globin gene, it is imperative to understand their role in all aspects of erythropoiesis before they are used for therapy.

The current study focuses on the role of two Krüppel-like transcription factors, KLF1 and KLF2, which have known roles in the processes of primitive and definitive erythropoiesis as well as globin gene regulation. The regulation of primitive erythropoiesis by KLF1 and KLF2 is studied using the mouse as a model system.
because it is not possible to study primitive erythropoiesis in humans. Previous studies have shown that KLF1 and KLF2 are essential for and have overlapping roles in primitive erythropoiesis. Simultaneous ablation of KLF1 and KLF2 results in a severely anemic embryonic phenotype that is not evident in KLF1 or KLF2 single knockout embryos. In this study, we show that this anemia is caused by a paucity of blood cells, and exacerbated by diminished β-like globin gene expression. The anemia phenotype is dose-dependent, and interestingly, can be ameliorated by a single copy of the KLF2, but not the KLF1 gene. The roles of KLF1 and KLF2 in maintaining both normal peripheral blood cell numbers and globin mRNA amounts are erythroid cell-specific. It was discovered that KLF2 has an essential function in erythroid precursor maintenance. KLF1 can partially compensate for KLF2 in this role, but is uniquely crucial for erythroid precursor proliferation, through its regulation of G1- to S-phase cell cycle transition. A more drastic impairment of primitive erythroid colony formation from embryonic progenitor cells occurs with simultaneous deficiency of KLF1 and KLF2, than with loss of a single factor.

The regulation of human β-like globin gene expression is studied using a recently developed in vitro system for the production of erythroid cells from umbilical cord blood hematopoietic precursor cells, representing a more “fetal” model of globin gene expression. Previous studies have shown that KLF1 binds to the promoters of the γ- and β-globin genes, while KLF2 binds to the promoter of the γ-globin gene in cord blood-derived erythroid cells. Studies using transgenic mice carrying the entire human β-globin locus had indicated that KLF1 and KLF2 positively regulate γ-globin expression in mouse embryonic erythroid cells. We demonstrate in this study that KLF1 appears to
have dual roles in the regulation of $\gamma$-globin expression in human cord blood-derived definitive erythroid cells. Partial depletion of KLF1 causes elevated $\gamma$-globin expression, while nearly complete depletion of KLF1 results in a down-regulation of $\gamma$-globin expression. Of particular interest was the observation that KLF2 positively regulates $\gamma$-globin expression in cord blood-derived erythroid cells. Surprisingly, KLF2 also positively regulates $\beta$-globin expression in these cells. If regulation of $\gamma$-globin by KLF2 proves to be a direct effect, KLF2 will join a very small group of factors known to directly activate $\gamma$-globin expression.
Chapter 1: Introduction

The blood is an essential body fluid, primarily composed of plasma, red blood cells, white blood cells and platelets. Its principal function is the delivery of oxygen and nutrients to, and the retrieval of waste products from all cells of the body. Blood cells account for 45% of the composition of blood and plasma makes up the remaining 55%. All of the different types of blood cells are derived from a common precursor cell by a process called hematopoiesis (Fig. 1.1). This common precursor is known as the hematopoietic stem cell (HSC). HSCs are capable of long term self-renewal as well as producing all the types of differentiated cells found in blood: red blood cells, white blood cells and platelets. As depicted in Figure 1.1, there are a number of intermediate progenitor and precursor cells between the HSC and the various blood cells. While the exact identity/molecular signature of the HSC is still unknown, the various intermediaries between the HSC and mature cells of each lineage can be identified either by morphology or retrospectively by the types of colonies they produce in colony formation assays. The focus of this study is the process of erythropoiesis or the production of red blood cells, also known as erythrocytes. Erythrocytes are the most abundant cell type present in blood. They get their characteristic red color from hemoglobin, the respiratory substrate.

1.1 Erythropoiesis: When, Where and How?

Erythropoiesis is a complex, multistep developmental process that, in mammals, consists of two discernable phases or “waves” of red blood cell genesis (Palis 2008).
Figure 1.1: Hematopoiesis is the process of formation of all the different types of blood cells from the hematopoietic stem cell (HSC).

These waves are termed primitive and definitive erythropoiesis, and result in the production of primitive and definitive erythroid cells, respectively.

1.1.1 Primitive erythropoiesis

Primitive erythropoiesis is a transient wave of erythropoiesis that occurs during early embryonic life in the yolk sac, in regions called blood islands, and gives rise to the first mammalian erythroid cells (Ferkowicz and Yoder 2005; Haar and Ackerman 1971). These erythroid cells were termed “primitive” since they morphologically resemble the nucleated erythroid cells of non-mammalian vertebrates (McGrath and Palis 2008). Recent studies have shown that the primitive erythroid lineage is more similar to the definitive erythroid lineage than was previously suspected, and mature primitive erythroid cells enucleate in circulation (Baron 2013). Primitive erythroid cells arise as large, nucleated cells from progenitors known as primitive erythroid colony-forming cells (EryP-CFC) (McGrath and Palis 2008). In the mouse, EryP-CFC are detected in the yolk sac at embryonic day 7.25 (E7.25), undergo a brief expansion, and fall off/cease to exist by E9 (Palis et al. 1999). Primitive erythroid cells arise around E7.5 and begin to circulate at E8.25 (Palis 2008). They mature in circulation as a synchronous cohort, from proerythroblasts to basophilic, polychromatophilic and orthochromatic erythroblasts, and eventually undergo enucleation between E12.5 and E16.5 (Fig. 1.2) (Kingsley et al. 2004). In humans, blood islands are observed at 18-20 days of gestation and primitive erythroblasts are the sole erythroid cells in circulation until 6 weeks of gestation (Luckett 1978; Palis 2014).
Figure 1.2: Primitive vs Definitive erythropoiesis. Erythropoiesis in mammals is defined by two successive waves of red blood cell genesis known as primitive and definitive erythropoiesis.


Figure adapted from Palis J. Front Physiol. 2014 (Palis 2014).
1.1.2 Definitive erythropoiesis

The second wave of erythropoiesis in mammals is called definitive erythropoiesis. The liver is the site of definitive erythropoiesis during fetal life. After birth, the site of erythropoiesis switches to the bone marrow. Both of these sites are repositories for hematopoietic stem cells which give rise to definitive erythroid progenitor cells (Palis 2014). There are two types of definitive erythroid progenitor cells, the burst-forming unit erythroid or BFU-E and the colony forming unit erythroid or CFU-E, defined on the basis of the types of colonies they produce in semi-solid media. These progenitors generate nucleated definitive erythroid precursor cells that progressively mature from proerythroblasts to basophilic, polychromatophilic and orthochromatic erythroblasts within either the fetal liver or bone marrow, until they finally undergo enucleation to form reticulocytes which are released into circulation (Fig. 1.2) (Palis 2014). Reticulocytes undergo organelle clearance and cytoskeletal changes to become mature red blood cells (Johnstone 1992). Definitive erythroid cells are seen in mouse fetal circulation starting from E11.5 (McGrath et al. 2011).

1.1.3 Identification of various erythroid progenitor and precursor cells

The three types of erythroid progenitor cells, the primitive EryP-CFC, and the definitive BFU-E and CFU-E, can be identified retrospectively by the types of colonies they produce in semi-solid culture media supplemented with cytokines necessary for erythroid differentiation (Fig. 1.3A) (Palis et al. 1999). Proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatic erythroblasts and mature primitive and definitive erythrocytes can be identified by their morphological characteristics after staining with Wright-Giemsa (Fig. 1.3B).
Figure 1.3: Identification of cells of the erythroid lineage. (A) There are three types of erythroid progenitor cells – primitive erythroid colony forming cells (EryP-CFC), burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) that are identified retrospectively by the morphology of the colonies they produce in semi-solid erythroid differentiation media. (B) Hierarchy of erythroid precursor cells found between erythroid progenitors and mature erythrocytes. (C) Flow cytometry plot showing the distribution of two cell surface markers CD71 (transferrin receptor) and Ter119 on E13.5 fetal liver erythroid cells. At E13.5 fetal livers contain all the different types of erythroid precursor cells that can be identified by the relative amount of CD71 and Ter119 expressed on their surface by this method. The five gates R1 to R5 are defined as follows: R1 (CD71^{LO} Ter119^{NEG}) - early erythroid progenitors, R2 (CD71^{HI} Ter119^{LO}) - erythroid progenitors, R3 (CD71^{HI} Ter119^{HI}) - proerythroblasts and basophilic erythroblasts, R4 (CD71^{MID} Ter119^{HI}) - polychromatic erythroblasts and orthochromatic erythroblasts, and R5 (CD71^{LO} Ter119^{HI}) - reticulocytes. Figure adapted from (A) Palis J, et al. Development 1999 (Palis et al. 1999) (C) Pilon AM, et al. Mol Cell Biol. 2008 (Pilon et al. 2008) and (B) http://medcell.med.yale.edu/histology/blood_bone_marrow_lab/erythropoiesis.php
A commonly used flow-cytometric method to determine the relative numbers of erythroblasts of each of these denominations in a mixed population relies on the changing expression of two surface markers on maturing erythroid cells (Zhang et al. 2003). For mouse cells the markers used are CD71 (transferrin receptor) and Ter119. For human cells the markers used are CD71 (transferrin receptor) and Glycophorin A (GPA or CD235a). Ter119 expression in mouse erythroblasts mirrors Glycophorin A expression in human erythroblasts. Five compartments, designated R1-R5, are defined based on the level of expression of each marker, with R1 consisting of the least mature erythroblasts and R5 consisting of the most mature (Fig. 1.3C). R1 (CD71<sup>LO</sup> Ter119<sup>NEG</sup>) comprises early erythroid progenitors, R2 (CD71<sup>HI</sup> Ter119<sup>LO</sup>) contains erythroid progenitors, R3 (CD71<sup>HI</sup> Ter119<sup>HI</sup>) is made up of proerythroblasts and basophilic erythroblasts, R4 (CD71<sup>MID</sup> Ter119<sup>HI</sup>) consists of polychromatic erythroblasts and orthochromatic erythroblasts, and R5 (CD71<sup>LO</sup> Ter119<sup>HI</sup>) consists of reticulocytes (Pilon et al. 2008).

1.2 Hemoglobin and hemoglobin switching

The most abundant constituent of erythrocytes is hemoglobin, the protein responsible for the high oxygen-carrying capacity of blood. Hemoglobin is a tetrameric molecule, composed of two α-like globin chains and two β-like globin chains, each of which co-ordinate an iron-containing heme group (Fig. 1.4). Each iron ion can bind one molecule of oxygen. The α-globin locus, situated on human chromosome 16 and mouse chromosome 11, consists of three α-like globin genes, ζ- (zeta), α2- and α1-globin (Figs. 1.5 and 1.6).
Figure 1.4: The hemoglobin molecule. Hemoglobin is a tetrameric metallo-protein composed of two α-like globin chains and two β-like globin chains, each of which coordinates an iron-containing heme group. Adapted from http://www.as.miami.edu/chemistry/2086/chap19/newchapter%2019-part1.htm

The β-globin locus differs slightly between humans and mice. The human β-globin locus, situated on chromosome 11, consists of the embryonic ε-globin gene, the fetal γ-globin gene, and the adult δ- and β-globin genes. The mouse β-globin locus, situated on chromosome 7, consists of the embryonic εy- and βh1-globin genes and the adult βmaj- and βmin-globin genes. The mouse β-globin locus does not have a fetal globin gene equivalent of the human γ-globin gene.

The α-like and β-like globin genes are developmentally regulated and expressed in the order in which they are arranged on the chromosome. As a result hemoglobin molecules expressed at different stages during development are composed of different α-like and β-like chains. This phenomenon of progressively changing globin gene expression is known as hemoglobin switching (Weatherall 2001). In humans, these “switches” in the type of hemoglobin expressed are concurrent with changes in the site
of erythropoiesis. In the yolk sac, ζ- and ε-globin are the respective α- and β-like globin genes initially expressed in primitive erythroid cells (ζ₂ε₂ or hemoglobin Gower I). Between 5 and 7 weeks of gestation, primitive erythroid cells switch to expression of hemoglobin Gower II (α₂ε₂). The next switch in hemoglobin expression is observed in the fetal liver. Fetal liver definitive erythroid cells express HbF, composed of 2 α-globin chains and 2 γ-globin chains (α₂γ₂). The final switch in hemoglobin expression is observed shortly after birth, as the site of erythropoiesis moves to the bone marrow. Bone marrow-derived adult definitive erythroid cells predominantly express HbA (α₂β₂, >95%), along with very low levels of HbA₂ (α₂δ₂, ~2%) and HbF (<2%). In mice, primitive erythroid cells initially express ζ-, α- and βh1-globin. By E12.5 ζ-globin is replaced by α-globin as the α-like globin expressed for the remainder of development as well as during adult life (Palis et al. 2010). Mouse primitive erythroid cells at E10.5 express predominantly embryonic εy- and βh1-globins, and very low levels of βmaj- and βmin-globins. A switch in β-like globin gene expression is observed from the embryonic genes to the adult βmaj and βmin genes in mouse fetal liver erythroid cells between E11.5 and E12.5, and these genes continue to be expressed for the remainder of fetal and adult life.

The α- and β-like globin mRNA constitute about 90% of total mRNA in erythroid cells, achieved partly through extremely high levels of globin gene transcription. It was discovered that while proximal promoter sequences were sufficient to endow tissue- and development-specific expression of the globin genes, distal sequences were
Figure 1.5: The human α- and β-globin loci and a depiction of the process of hemoglobin switching in humans. Adapted from (1) Stamatoyannopoulos G. Exp Hematol. 2005 and (2) Weatherall DJ. Nat Rev Genet. 2001.
Figure 1.6: The mouse α- and β-globin loci. (A) The mouse β-globin locus consists of four β-like globin genes: εy, βh1, βmaj and βmin, and an upstream locus control region (LCR). The graph below the locus shows the switching of expression of the mouse β-like globin genes during development. In addition, the graph also shows the pattern of expression of the human β-like globin genes when they are introduced into the mouse as a YAC transgene. Adapted from Kim A, et al. Mol Cells. 2012, and Strouboulis J et.al, Genes Dev. 1992. (B) The mouse α-globin locus consists of three α-like globin genes: ζ, α1 and α2 that display a developmental switch in expression. Adapted from Vernimmen D, et al. EMBO J. 2007, and Palis J, et al. Int J Dev Biol. 2010.
responsible for high-level transcription. These distal sequences are known as the locus control region or LCR, located 6 to 20 kb upstream of the ε-globin gene, in the β-locus and the multispecies conserved sequences or MSC, found 25 to 65 kb upstream of the α-locus (Higgs and Wood 2008; Stamatoyannopoulos 2005). The β-globin LCR was discovered due to the presence of erythroid-specific and developmentally-controlled DNaseI hypersensitive sites that were able to direct high-level human β-globin expression in transgenic mice (Forrester et al. 1987; Grosveld et al. 1987). The LCR consists of 5 such DNaseI hypersensitive sites (HS), designated 5' - HS1 to HS5, with HS1 being the site nearest to the ε-globin gene. Each hypersensitive site is defined by a core sequence of about 250 nucleotides and contains binding motifs for numerous transcription factors. The LCR can activate the expression of only one gene at a time (Wijgerde, Grosveld, Fraser 1995). The LCR is postulated to enhance globin gene expression by interacting with the promoter of the gene through looping, as evidenced by crosslinking of juxtaposed DNA sequences in 3C assays (Fig 1.7) (Tolhuis et al. 2002).

1.3 Hemoglobinopathies

Hemoglobinopathies or the disorders of hemoglobin are some of the most common genetic disorders in the world, with approximately 5% of the world’s population carrying a significant variant (WHO statistics). They result from abnormalities in either the structure or production of hemoglobin. β-hemoglobinopathies are of two main types: sickle cell disease and β-thalassemia.
Figure 1.7: Looping between the LCR and the globin gene promoters at the β-globin chromatin hub. Adapted from Kim A, et al. Mol Cells. 2012 (Kim and Dean 2012)

Figure 1.8: Sickle shaped red blood cells are seen in sickle cell anemia. Arrows point to sickle RBCs. Adapted from American Society of Hematology image bank (Author: Stanley Schrier)
1.3.1 Sickle cell anemia

Pathophysiology: Sickle cell anemia is caused by a point mutation in the adult β-globin gene that causes the substitution of a glutamic acid residue by a valine at the 6th amino acid position in the β-chain. The resulting mutant β-chain is denoted βS and hemoglobin molecules that contain these chains are called sickle-hemoglobin or HbS (α2βS2). In low-oxygen conditions, HbS molecules have a tendency to polymerize within erythroid cells and distort the shape of the erythroid cell from its typical discoidal shape to a sickle shape, giving the disease its name (Fig 1.8). The sickle shaped cells cannot pass through small capillaries with the same ease as normal erythroid cells and thus they cause occlusions in these vessels, which results in a painful condition termed sickle cell crisis. The abnormal shape of sickle cells also targets them for destruction, resulting in anemia.

Prevalence: Sickle cell anemia is an autosomal recessive disorder. People who have one abnormal β-globin gene are said to have sickle cell trait and are asymptomatic. The highest prevalence of sickle cell anemia is in Africa, Central and South America, the Mediterranean countries, India and Saudi Arabia. It is estimated that sickle cell anemia affects approximately 100,000 Americans. Disease occurrence is estimated at about 1 in every 500 African American births and 1 in 36,000 Hispanic American births.

1.3.2 β-thalassemia

Pathophysiology: β-thalassemia is caused by decreased (β+) or absent (β0) production of adult β-globin which results in very low levels or a complete lack of HbA. There is a concurrent accumulation of the excess α-globin chains, which precipitate in cells and cause cellular toxicity. Decreased β-globin production is caused by mutations and
deletions of the β-globin gene and its proximal cis-regulatory elements as well as by mutations in genes encoding trans-regulatory factors such as transcription factors essential for globin gene expression. Clinical symptoms of the disease vary based on residual amounts of β-globin and are classified into three groups:

1. **β-thalassemia minor**: Individuals who are heterozygous for a mutation in the β-globin gene (β⁺/β or β⁰/β) and have mild microcytic anemia.

2. **β-thalassemia intermedia**: Individuals who still produce some β-globin (β⁺/β⁺ or β⁰/β⁺) and have moderate disease severity with varying needs for blood transfusions.

3. **β-thalassemia major**: Individuals who do not produce any β-globin (β⁰/β⁰) and have severe microcytic, hypochromic, transfusion-dependent anemia.

**Prevalence**: The highest occurrence of β-thalassemia is observed in the Mediterranean and Middle Eastern countries, southern Asia including India, China and the Far East and northern Africa.

### 1.3.3 Treatment of β-hemoglobinopathies

Current treatments for sickle cell anemia and β-thalassemia are aimed at management of the disease through alleviation of its symptoms. The only cure available for some patients is through bone marrow transplantation from a closely matched donor. Finding such a donor is rare. In addition, the high level of associated risk and the cost involved limit the accessibility of this form of treatment (Sankaran and Nathan 2010). Thus there is no widely available curative approach for either sickle cell anemia or β-thalassemia.
Treatment of sickle cell anemia aims at management of disease symptoms including pain relief, prevention of infections, organ damage and complications. Many sickle cell anemia patients require frequent blood transfusions to treat anemia and prevent conditions such as stroke. Treatment of β-thalassemia major also involves frequent blood transfusions and monitoring of hemoglobin levels. While blood transfusions allow the affected individuals the ability to lead a more normal life, they are associated with the risk of transmitted infections and have serious side effects including severe iron overload. Advances in iron chelation therapies that remove excess iron from the body have tremendously improved the odds for patients in need of constant transfusions.

Although in most people fetal hemoglobin (HbF) levels decline after birth to less than 2% of total hemoglobin, certain people exhibit constantly elevated levels of HbF throughout their life, an asymptomatic condition known as Hereditary Persistence of Fetal Hemoglobin (HPFH) (Forget 1998). It was observed in clinical evaluations that β-thalassemia patients who had elevated levels of fetal hemoglobin (HbF) manifested less severe disease symptoms (Weatherall 2001), suggesting that re-expression of γ-globin in adult cells can surmount the lack of β-globin. This finding prompted many investigators in the globin field to look for chemical agents capable of inducing the expression of fetal hemoglobin in adult erythroid cells. The earliest of these studies led to the discovery and implementation in treatment regimens of a drug called hydroxyurea, an S-phase inhibitor (Letvin et al. 1984; Platt et al. 1984). The use of hydroxyurea in treating sickle cell disease was discovered through studies of 5-azacytidine, a carcinogenic, demethylating agent, that elevated the levels of fetal
hemoglobin in baboons (DeSimone et al. 1982). The mechanism by which hydroxyurea elevates fetal hemoglobin is not entirely known, although it is speculated that hydroxyurea may stimulate stress erythropoiesis pathways. Hydroxyurea has not proved an equally effective treatment for β-thalassemia (Alebouyeh et al. 2004). Thus the search is still on for pharmacological inducers of fetal hemoglobin. An ideal candidate for treatment should have the following attributes: 1. It should elevate HbF in adult erythroid cells 2. It should not adversely affect erythropoiesis 3. It should preferably act specifically in erythroid cells so as not to affect other hematopoietic / non-hematopoietic lineages 4. It should have minimal side effects. A better understanding of the molecular regulators of γ-globin expression and the underlying mechanisms will undoubtedly accelerate the achievement of this goal.

1.4 Model systems for studying erythropoiesis and globin gene regulation

The knowledge that we have gained thus far about the processes and factors that govern erythropoiesis come from the assimilation of data from studies in numerous model systems. Outlined below are the salient features of some of the more commonly used model systems. Each system has its advantages and drawbacks. No model system in isolation can satisfactorily and conclusively provide answers to all our questions with regards to development and its molecular regulators. However, information pieced together from the combination of multiple model systems has substantively furthered our understanding of developmental biology.

1.4.1 Immortalized cell lines
K562 cell line: K562 is a human erythroleukemia cell line that was established from the pleural effusion of a chronic myelogenous leukemia patient in blast crisis (Lozzio and Lozzio 1975). Cytogenetic studies showed that these cells have the Philadelphia chromosome and other chromosomal aberrations. K562 cells are widely used to study erythropoiesis since they are easy to grow in a suspension culture, represent a human model, show certain features of erythroblasts and can be induced by hemin treatment to express embryonic and fetal hemoglobins (Koeffler and Golde 1980). The major drawback of this cell line is that since it is a leukemia cell line, it is difficult to tease out effects that are downstream of the various chromosomal aberrations.

Mouse erythroleukemia (MEL) cell line: This cell line was derived by immortalization of mouse splenic cells by infection with a virus (FRIEND 1957). MEL cells are erythroid progenitor cells that can be induced to differentiate into erythroid cells by treatment with dimethyl sulfoxide (DMSO) (Antoniou 1991; Friend et al. 1971). They are grown in suspension culture and express mouse adult hemoglobin ($\alpha_2\beta_{maj}^2$) upon DMSO-induced differentiation.

1.4.2 Transgenic globin mice

Erythroid cells mature in very specialized environments within organisms. Since cells grown in culture lack interactions with these developmental niches, it is difficult to study many aspects of erythroid differentiation in tissue culture systems. In order to circumvent this issue and study regulation of the human globin genes in an in vivo system, transgenic mice were developed that carry the entire human $\beta$-globin locus. Mice are an attractive model system due to their relatively short gestation period and because erythropoiesis in mice closely mirrors that in humans. The first $\beta$-locus
transgenic mice were developed using strategies to ligate two cosmid constructs (Strouboulis, Dillon, Grosveld 1992). The use of yeast artificial chromosomes (YAC) enabled researchers to develop transgenic mice with YACs that could incorporate larger DNA fragments than previously available vectors. In this manner, transgenic mice carrying 150-250 kb YACs spanning the entire human β-globin locus and flanking regulatory sequences were generated (Gaensler, Kitamura, Kan 1993; Peterson et al. 1993). The mice are referred to as the β-YAC transgenic mice. β-YAC mice express the human globin genes in a tissue- and developmental-stage specific manner that resembles the expression of the endogenous mouse genes (Fig 1.6 A). The human ε- and γ-globin genes are expressed in yolk sac erythroid cells and the human β-globin gene is expressed in fetal liver-derived and bone marrow-derived erythroid cells. The β-YAC mice have been instrumental in studying numerous aspects of globin gene regulation in vivo. The major drawback of this model is that human γ-globin is expressed as an embryonic gene similar to the mouse embryonic genes, and not as a fetal gene, thus deviating from its expression pattern in humans.

1.4.3 Primary human cell lines

In the last decade, researchers have refined methods of primary human cell culture in order to obtain large numbers of erythroid cells from relatively few CD34+ hematopoietic precursor cells obtained from adult blood or bone marrow and umbilical cord blood (Migliaccio et al. 2002). The development of these methods have enabled the study of various aspects of erythropoiesis in primary human cells unobstructed by the effects of immortalization that were present in previously available cell lines. A
detailed description of how these cells are isolated, cultured and manipulated is presented in the methods section of this dissertation.

1.5 Molecular regulators of erythropoiesis and hemoglobin switching

Research has led to the identification and characterization of numerous transcription factors, both erythroid specific, and more generally expressed, which play important roles in establishing and maintaining an erythroid fate. Some of these factors have dual roles in regulating various aspects of erythroid cell maturation as well as globin gene expression. Others are particularly required for either expression or silencing of the stage specific globin genes in a timely manner. Substantial effort has been devoted to identification and characterization of various repressive complexes that silence \( \gamma \)-globin expression in adult erythroid cells, with the aim of using this information to develop therapeutic strategies to re-express \( \gamma \)-globin in adult erythroid cells (Sankaran and Orkin 2013). Less is known about the factors necessary for activation of \( \gamma \)-globin expression in fetal erythroid cells. A list of some of the factors that have known roles in either activating or silencing \( \gamma \)-globin expression are presented in Table 1.1. Described below are some of the pivotal players of the complex transcriptional network that controls the process of erythropoiesis.

1.5.1 SCL / Tal1 and LMO-2

SCL/Tal1 and LMO-2 are transcription factors necessary for the development of primitive erythropoiesis. SCL/Tal1 is a basic helix-loop-helix transcription factor expressed in hematopoietic progenitor cells as well as in primitive and definitive erythroid cells, mast cells and megakaryocytes (Robb and Begley 1997). SCL knockout
<table>
<thead>
<tr>
<th>Regulator</th>
<th>Direction of modulation needed to increase HbF</th>
<th>Human genetic evidence supporting role in HbF regulation</th>
<th>Human or primate studies modulating factor involved in HbF regulation</th>
<th>Cell culture data supporting a role in HbF regulation</th>
<th>Evidence from mouse models suggesting a role in HbF regulation</th>
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<tr>
<td>BCL11A</td>
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<td>KLF1</td>
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<tr>
<td>MYB</td>
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<td>MicroRNAs 15a/16-1</td>
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<td>SOX6</td>
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<td>HDACs 1/2</td>
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<tr>
<td>DNMT1</td>
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<td>TR2/TR4</td>
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<td>COUP-TFII</td>
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<td>FOP</td>
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<td>NF-E4</td>
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Adapted from Sankaran, et al. CSHP Med 2013.
mice are embryonic lethal and die by E9.5 (Robb et al. 1995). They display a total lack of primitive erythropoiesis, with no discernable blood islands and a failure to produce hematopoietic colonies in colony formation assays. LMO-2 is a LIM domain containing transcription factor. LMO-2 is greatly expressed in the fetal liver of mouse embryos, as well as in the brain and spleen. It is present in both primitive and definitive erythroid cells and absent in cells of other hematopoietic lineages (Warren et al. 1994). LMO-2 knockout mice are embryonic lethal and die around E10.5. LMO-2/- embryos are pale in appearance at E9.75 and lack circulating erythroid cells. They also do not produce erythroid colonies in colony forming assays, indicating a defect in establishment of the primitive erythroid lineage.

1.5.2 Gata1 and Gata2

Gata1 and Gata2 are members of the GATA family of Cys2-Cys2 zinc finger transcription factors. They recognize and bind to the consensus DNA sequence WGATAR. Gata1 and Gata2 are required for mouse viability. Gata1-/- embryos are pale in appearance at E9.5 and die between E10.5 and E11.5 (Fujiwara et al. 1996). Primitive erythroid cells are present in Gata1-/- embryos, but they show defects in maturation and are arrested at the proerythroblast stage of development. Gata1-/- embryos produce definitive erythroid colonies in colony formation assays, but the cells in these colonies are arrested at the proerythroblast stage. Thus Gata1 is required for terminal maturation of both primitive and definitive erythroid cells. Gata2-/- embryos die between E10-E11 and are severely anemic (Tsai et al. 1994). The number of circulating erythroid cells is drastically reduced in E9.5 Gata2-/- embryos, but their maturation is not affected. In vitro differentiation of Gata2-/- ES cells showed that absence of Gata2
greatly impairs development of primitive and definitive erythroid colonies as well as macrophage and mast cell colonies, indicating a defect at the level of hematopoietic stem or progenitor cells. Further investigation revealed that Gata2 is required for the expansion of multipotent hematopoietic progenitors (Tsai and Orkin 1997). Combined absence of Gata1 and Gata2 in Gata1/Gata2 double knockout mice results in a complete loss of the primitive erythroid compartment at E8.5 (Fujiwara et al. 2004).

1.5.3 Runx1 and c-myb

Runx1 and c-myb are transcription factors that are critical for definitive erythropoiesis but not for primitive erythropoiesis. Runx1 (also known as CBFA2 or AML1) codes for the DNA-binding subunit of core binding factor (CBF). Runx1-/- embryos die by E12.5 and are unable to produce both definitive erythroid and myeloid colonies in colony forming assays (Wang et al. 1996). Primitive erythropoiesis appears to proceed normally with mild morphological defects in a small fraction of cells (Yokomizo et al. 2008). C-myb, initially identified as a proto-oncogene, is a transcription factor with pronounced expression in erythroid, myeloid and lymphoid progenitor cells (Mucenski et al. 1991). C-myb-/- embryos develop severe anemia around E15. Primitive erythropoiesis is unaffected in c-myb-/- embryos. Peripheral blood of E15 c-myb-/- embryos contains mostly primitive erythroid cells and very few definitive erythroid cells whereas the opposite is true for wild-type littermates. C-myb-/- fetal livers showed hypocellularity and colony forming assays indicated a drastic reduction in the number of multipotent hematopoietic progenitors, demonstrating that c-myb is essential for definitive erythropoiesis.
1.5.4 Krüppel-like factors (KLFs)

The Krüppel-like factors (KLFs) are a family of Cys$_2$-His$_2$ zinc finger transcription factors, named for the Drosophila protein Krüppel with which they share homology. Each KLF consists of a C-terminal DNA binding domain made up of three zinc fingers and an N-terminal transactivation domain (Fig. 1.9). Since the DNA binding domain of all KLFs are extremely similar to each other they recognize and bind to a common consensus sequence, 5'-CACCC-3', in DNA. While some KLFs show tissue restricted expression patterns, others are ubiquitously expressed. To date, 17 Krüppel-like factors have been discovered with a wide range of roles in development and homeostasis (Fig. 1.10) (McConnell, Physiol Rev 2010). Of these, KLF1, KLF2, KLF3 and KLF8 have known roles in erythropoiesis. KLF1 and KLF2 are discussed in detail since they are central to the research presented in this dissertation.

Figure 1.9: Krüppel-like factors are defined by an N-terminal transactivation domain and a C-terminal DNA binding domain made up of three C2H2 zinc fingers.
Figure 1.10: Phylogenetic mapping of members of the Krüppel-like family of transcription factors.
KLF1

Krüppel-like factor 1 (KLF1) or erythroid Krüppel-like factor (EKLF) was first isolated from MEL cells, by the technique of subtractive hybridization to identify genes preferentially expressed in erythroid cells (Miller and Bieker 1993). The mouse KLF1 protein is 376 amino acids long and contains three Cys2-His2 zinc fingers near its carboxy-terminal (Miller and Bieker 1993). These zinc finger domains share strong homology with the zinc fingers of the Drosophila gene Krüppel and hence this factor was named erythroid Krüppel-like factor (EKLF). The zinc fingers recognize the 9 bp sequence, 5’- CCM-CRC-CCN -3’, and mediate binding of KLF1 to DNA (where R represents A or G and M represents A or C) (Feng, Southwood, Bieker 1994; Miller and Bieker 1993; Tallack et al. 2010).

KLF1 mRNA expression is first detected at E7.5 in the extraembryonic mesoderm of the mouse visceral yolk sac, coincident with the earliest stages of blood island formation (Southwood, Downs, Bieker 1996). KLF1 protein is present in both primitive and definitive erythroid cells in embryos (Southwood, Downs, Bieker 1996). KLF1 expression is up-regulated in primitive erythroid cells between E8.5 and E9.5 and remains high between E9.5 and E11.5, followed by a decline at E12.5 (Isern et al. 2010). KLF1 shows robust expression in fetal liver definitive erythroid cells with 3-fold higher protein amount in definitive compared to primitive cells (Alhashem et al. 2011; Zhou et al. 2006). In adult mice, KLF1 mRNA expression is restricted to bone marrow and spleen tissue (Miller and Bieker 1993). KLF1 is expressed in hematopoietic cells prior to erythroid commitment (Frontelo et al. 2007). KLF1 expression is detected at low levels in multipotent hematopoietic progenitors (MPP) and common myeloid-erythroid
progenitor (CMP) and at higher levels in the common megakaryocyte-erythroid progenitor (MEP) (Frontelo et al. 2007; Lohmann and Bieker 2008). Onset of KLF1 expression is regulated by Gata2 and Smad5 (Lohmann and Bieker 2008). It has also been shown that GATA-1 and CCAAT-binding protein 1 (CP1) bind to and transactivate the KLF1 promoter in MEL cells (Crossley et al. 1994).

KLF1 has emerged as a key regulator of almost all aspects of erythrocyte development (Fig. 1.11). Two research groups independently developed KLF1 knockout mice by gene targeting strategies (Nuez et al. 1995; Perkins, Sharpe, Orkin 1995). KLF1 heterozygous mice survive to adulthood and appear normal. KLF1 knockout mice die in utero by E16. E15 KLF1\(^{-/-}\) embryos show decreased hematocrit and hemoglobin content. Definitive erythroid cells from E15 KLF1\(^{-/-}\) embryos are abnormal in appearance and have reduced amounts of β-globin mRNA and protein compared to wild-type littermates (Perkins, Sharpe, Orkin 1995). Primitive KLF1\(^{-/-}\) erythroid cells show dysregulation of the embryonic βh1- and εy-globin genes and abnormal cellular morphology (Basu et al. 2007; Hodge et al. 2006). In addition to the β-like globins, KLF1 regulates the expression of numerous other erythroid genes, including those encoding cytoskeletal and membrane proteins, and heme synthesis enzymes (Drissen et al. 2005; Hodge et al. 2006; Nilson et al. 2006; Pang et al. 2012).

Primitive and definitive KLF1\(^{-/-}\) blood cells show cell cycle defects (Pilon et al. 2008; Tallack et al. 2009). At E13.5, wild-type fetal livers consist predominantly of R3-R5 erythroid cells, and a small fraction of R1-R2 erythroid cells. In contrast, KLF1\(^{-/-}\) fetal livers consist almost entirely of R1-R2 early erythroid progenitors, demonstrating a role for KLF1 in terminal erythroid differentiation in definitive erythroid cells.
Figure 1.11: KLF1 regulates numerous aspects of erythroid cell development.
(Pilon et al. 2008). Gene expression profiling studies suggested that this maturation defect stemmed from aberrant regulation of genes that control cell cycle progression in KLF1 knockouts. It has been shown that KLF1 directly regulates cell cycle genes, E2f2 and p18INK4c (Pilon et al. 2008; Tallack, Keys, Perkins 2007; Tallack et al. 2009). Furthermore, both primitive and definitive KLF1-/- erythroid cells display hampered G1-to S-phase cell cycle progression (Pilon et al. 2008; Tallack et al. 2009).

Studies using β-YAC mice showed that there was a decrease in human β-globin mRNA amount and elevated γ-globin mRNA expression in compound KLF1+/-, β-YAC transgenic and KLF1-/-, β-YAC transgenic fetal livers (Perkins, Gaensler, Orkin 1996; Wijgerde et al. 1996), suggestive of competition between the γ and β genes. However, β-globin expression was normal and the γ-globin gene was silenced in adult KLF1+/- mouse blood (Wijgerde et al. 1996). Changes in γ- and β- globin gene expression correlate with the percentage of transcriptionally active genes suggesting that changes in expression are not due to changes in rates of transcription (Wijgerde et al. 1996). In addition, loss of DNaseI hypersensitivity was observed at the β-globin promoter and partial loss was observed at HS3 of the LCR in KLF1-/- fetal livers, suggesting that KLF1 is required for normal chromatin configuration (Wijgerde et al. 1996).

In primitive erythroid cells, KLF1 binds to the hypersensitive sites of the LCR, HS1-4, and to the promoters of the εy-, βh1, and βmaj-globin genes (Alhashem et al. 2011; Zhou et al. 2006). This was a surprising discovery since βmaj-globin is expressed at very low levels in primitive erythroid cells. In definitive erythroid cells from fetal liver and bone marrow, KLF1 binds to HS1, HS2, HS3, HS4 and the adult βmaj-globin promoter but not to the embryonic εy- and βh1-globin promoters (Zhou et al. 2006).
Chromatin conformation capture (3C) experiments in wild-type mouse E12.5 fetal liver cells have indicated that the βmaj promoter lies in close proximity to the LCR and the distal 5’HS-62 site in these cells in which the βmaj gene is actively transcribed (Drissen et al. 2004). These locus interactions are severely affected in KLF1-/- fetal livers and are dependent on the presence of KLF1 but not on downstream protein synthesis (Drissen et al. 2004). Thus KLF1 is required for the formation / stabilization of an active chromatin hub (ACH) at the β-globin locus.

Co-transfection experiments revealed that KLF1 protein interacts with three histone acetyl transfersases (HATs), p300, CBP and P/CAF (Zhang and Bieker 1998). CBP and p300 can acetylate KLF1 in vitro and enhance its trans-activation ability in K562 cells (Zhang and Bieker 1998). Histone H3 acetylation and NF-E2 (p45) binding was decreased at HS2 of the LCR and human β-promoter in KLF1-/- hematopoietic progenitor cells from β-YAC transgenic mice (Bottardi et al. 2006). There was also decreased CBP and BRG1 binding to huβ promoter but not to HS2 in KLF1-/- hematopoietic progenitor cells (Bottardi et al. 2006). In E13.5 fetal liver erythroid cells there was decreased binding of NF-E2 (p45), CBP and BRG1 at HS2 of the LCR and human β-promoter in KLF1-/- compared to wild-type (Bottardi et al. 2006).

Actively transcribed genes show preferential intra- and inter-chromosomal co-associations in the nucleus, and the majority of these associations occur within transcription factories that are enriched for RNAPolII-S5P. The β-globin genes display such intra- and inter-chromosomal co-associations with other erythroid-expressed genes in mouse erythroid cells (Schoenfelder et al. 2010). Transcription-related chromosomal interactions between genes that are regulated by KLF1 were shown to be
dependent on KLF1 (Schoenfelder et al. 2010). In addition to decreased interchromosomal associations, KLF1 regulated genes also showed decreased transcription factory occupancy in KLF1-/- erythroid cells (Schoenfelder et al. 2010). Thus KLF1 is a "global" regulator of erythroid gene expression.

**KLF2**

KLF2, also known as lung Krüppel-like factor or LKLF, was discovered due to the similarity of its zinc finger domain to that of KLF1 (Anderson et al. 1995). KLF2 is a 354 amino acid protein in mice and a 355 amino acid protein in humans, with a predicted mass of 37.7 KDa. Within the zinc finger region, KLF1 and KLF2 are 88% homologous to each other. However, outside this region, they share little homology. Due to their highly homologous zinc finger domains, KLF1 and KLF2 are predicted to bind to the same / extremely similar DNA sequences. In addition, KLF2 also contains a 5' proline-rich putative transactivation domain region, similar to KLF1. The KLF2 protein is modular in nature with transactivation potential localized to amino acids 1-110, functionally separable from its zinc finger domain. Amino acids 111-267 of KLF2 confer an inhibitory effect to its transactivation ability (Conkright, Wani, Lingrel 2001). Evidence suggests that, analogous to KLF1 - CBP/p300 interactions, KLF2 interacts with CBP/p300 and this interaction has functional consequences (SenBanerjee et al. 2004).

Human and mouse KLF2 proteins share 90% amino acid similarity (Wani et al. 1999). Of interest is the observation that the proximal promoter regions of human and mouse KLF2 also share an identical sequence of 75 bp near the transcription start site, suggesting that KLF2 may be similarly regulated in both organisms. Studies of the KLF2 promoter region showed that the -243 to -72 bp region upstream of the transcription
start site was required for robust reporter gene expression (Schrick et al. 1999). This 172 bp fragment contains two Sp1 binding sites, which were found to only modestly affect reporter gene expression. Linker scanning mutagenesis identified a 30bp fragment from -138 to -111bp to be important for transcription of the KLF2 gene (Schrick et al. 1999). Heterogeneous nuclear ribonucleoproteins (hnRNPs) hnRNP-U and hnRNP-D, PCAF and p300 bind to this region and can activate transcription of KLF2 in a macrophage cell line (Ahmad and Lingrel 2005). An upstream segment of the KLF2 promoter, from -1643 to -490bp, appears to possess repressive elements (Schrick et al. 1999).

KLF2 expression is detected early during mouse embryonic development, at E7, using RNA prepared from whole embryos (Anderson et al. 1995). KLF2 is also expressed in mouse ES cells and is important for their self-renewal capacity (Jiang et al. 2008). In the adult mouse, KLF2 expression is observed in lung, heart, spleen, skeletal muscle and testis tissues (Anderson et al. 1995). At the cellular level, KLF2 is expressed in erythroid, endothelial, CD4+ and CD8+ quiescent T-cells and bone marrow macrophages (Kuo, Veselits, Leiden 1997; Kuo et al. 1997; Zhang et al. 2005).

Two groups independently developed KLF2/- mice using gene targeting strategies (Kuo et al. 1997; Wani, Means, Lingrel 1998). KLF2+/- mice are phenotypically normal. KLF2 knockout mice are embryonic lethal and die between E11.5 and E14.5 (Chiplunkar et al. 2013; Kuo et al. 1997; Wani, Means, Lingrel 1998). Death of embryos is due to heart failure (Lee et al. 2006). Haemorrhaging has been observed in KLF2/- embryos between E12.5 and E14.5 by some groups but not by others (Kuo et al. 1997; Lee et al. 2006). Detailed studies of mice lacking KLF2 in
specific cell types, revealed multiple roles for KLF2 in different tissues. KLF2 is essential for maintenance of the quiescent state of mature, single positive T-cells (Kuo, Veselits, Leiden 1997). KLF2-/− embryos show abnormalities in vascular tunica media development (Kuo et al. 1997). A role for KLF2 in normal lung development was demonstrated when KLF2-/− mouse embryonic stem cells used to generate chimeric mice contributed to all other organ systems except the lung (Wani, Wert, Lingrel 1999). Animals in which the KLF2-/− ES cells contributed to formation of the lungs died at birth, with deficient lung development (Wani, Wert, Lingrel 1999). KLF2 expression is induced in endothelial cells in response to fluid shear stress, where it is considered to have an atheroprotective effect (Atkins and Jain 2007; Dekker et al. 2002).

We are particularly interested in the role of KLF2 in erythroid cells. Early studies indicated that KLF2 can transactivate reporter gene expression downstream of the human γ- and β-globin gene promoters in in vitro assays (Anderson et al. 1995; Zhang et al. 2005). Studies using KLF2 knockout mice showed that KLF2 regulates the mouse embryonic β-like globin genes, εy- and βh1-globin, but not the adult βmaj- and βmin-globins (Basu et al. 2005). There is an approximate 2-fold decrease in εy- and βh1-globin mRNA amount at E10.5 in KLF2-/− yolk sacs compared to wild-type. No change was observed in adult βmaj- and βmin-globin gene expression in E12.5 fetal livers of KLF2-/− embryos compared to wild-type. Compound β-YAC transgenic / KLF2 null mice were used to determine whether KLF2 regulates the human β-like globin genes. Human embryonic ε-globin mRNA amount was decreased in KLF2-/− mice compared to wild-type in this model. Expression of human fetal γ-globin mRNA in the yolk sac and adult β-globin mRNA in the fetal liver were unaffected. In addition to the observed changes in
globin gene expression, E10.5 KLF2-/− circulating erythroid cells show morphological abnormalities with irregular cell membranes showing attenuated cytoplasmic processes. KLF2-/− embryos also have a higher percentage of apoptotic primitive erythroid cells compared to wild-type (Basu et al. 2005). KLF2-/− E11.5 fetal livers produce fewer CFU-E than wild-type, by in vitro erythroid colony formation assays, indicating that KLF2 may play a role in definitive erythropoiesis (Wani, Means, Lingrel 1998).

**KLF1 and KLF2 gene interactions**

The Krüppel-like factors KLF1 and KLF2 are essential for primitive erythropoiesis. Previous work in our lab has revealed that KLF1 and KLF2 display overlapping roles in many facets of primitive erythropoiesis (Basu et al. 2007). KLF1/KLF2 double knockout embryos have a significantly greater reduction in mouse embryonic Ey- and βh1-globin mRNA at E10.5 than either of the single knockouts. The KLF1/KLF2 double knockout embryos die earlier than either of the single knockouts and are severely anemic in comparison to the visibly normal appearance of KLF1-/− and KLF2-/− embryos (Fig. 1.12). They show a more severe phenotype with regards to erythroid cell morphology and global gene expression, indicating that KLF1 and KLF2 can partially compensate for each other (Basu et al. 2007; Pang et al. 2012). KLF1 has an erythroid specific pattern of expression whereas KLF2 is expressed in many tissues including the lung, lymphocytes, erythroid and endothelial cells. Conditional knockout mouse experiments have demonstrated that the effect of KLF2 on mouse embryonic globin gene expression is erythroid cell-autonomous (Alhashem et al. 2011).
Figure 1.12: E10.5 KLF1-/-KLF2-/- embryos are severely anemic. (A) and (B) E10.5 whole mounts of wild-type embryos surrounded by and dissected out of the yolk sac respectively. (C) and (D) E10.5 KLF1-/-KLF2-/- whole mount embryos surrounded by and dissected out of the yolk sac respectively. Adapted from Basu P, et al. Blood 2007.
Other KLFs

In addition to KLF1 and KLF2, KLF3 and KLF8 have also been shown to have roles in erythropoiesis. KLF3 and KLF8 are transcriptional repressors (Suzuki et al. 2005). KLF3-/− mice are viable, have a mildly anemic phenotype and show reticulocytosis and the presence of abnormal erythroid cells in peripheral blood (Funnell et al. 2012; Sue et al. 2008). KLF8-/− mice are viable and have normal blood counts (Funnell et al. 2013). It was observed that KLF1 positively regulates KLF3 and KLF8 in erythroid cells, whereas KLF3 represses KLF8, forming a transcriptional network that may be important for the normal progression of erythropoiesis (Eaton et al. 2008; Funnell et al. 2007).

1.5.5 BCL11A

Genome wide association studies have recently identified SNPs in the BCL11A gene that correlate with elevated fetal hemoglobin (HbF) in adults (Menzel et al. 2007). Further investigation has shown that stable knockdown of this zinc finger transcription factor in erythroblasts derived from CD34+ human hematopoietic progenitors results in a 3- to 6-fold increase in γ-globin mRNA (Sankaran et al. 2008). BCL11A binds to HS3 of the β-globin LCR as well as to two sites in the γ-δ intergenic region, but not to the promoters of any of the β-like globin genes (Sankaran et al. 2008). 3C assays using fetal liver cells from BCL11A-/− βYAC transgenic mice showed a decreased frequency of loop formation between the LCR and the β-globin gene and slight increase in the frequency of looping between the LCR and γ-globin gene (Xu et al. 2010). These results suggest that BCL11A may be a repressor of γ-globin expression and play a role in the γ-
to β-globin switch. KLF1 positively regulates BCL11A expression in mouse and human definitive erythroid cells and there is evidence suggesting that this may be a direct effect (Borg et al. 2010; Zhou et al. 2010).

Rationale

Hemoglobinopathies currently affect millions of people and represent a growing disease burden worldwide with an estimated 300,000 to 400,000 affected babies born each year (Williams and Weatherall 2012). However, affected patients still lack a widely applicable and easily available cure. Research in the field has been geared towards the elucidation of molecules and mechanisms that control hemoglobin switching, with the aim of re-expressing γ-globin in patients with β-hemoglobinopathies. In order to achieve this goal, it is imperative that we gain a better understanding of two important aspects of normal γ-globin gene regulation: (1) activation of gene expression or the mechanisms by which the γ-globin gene is turned on in the fetus and (2) repression of gene expression or the mechanisms by which the γ-globin gene is silenced in adults. A composite approach that manipulates both these complimentary regulatory mechanisms will likely produce the most effective therapeutic strategy.

A large portion of effort in the field has been devoted to studying derepression of the silenced γ-globin gene in adult erythroid cells and the factors that control repressive mechanisms. These avenues have yielded certain candidates for therapeutic intervention. However, factors involved in silencing of gene expression generally have more global effects on gene expression in erythroid cells as well as other cell types, which may cause unwanted side effects. In addition to releasing repressive
mechanisms it would be beneficial to simultaneously activate γ-globin expression by modulating the levels of factors that positively regulate the γ-globin gene. There is a gap in research with respect to factors that directly activate γ-globin expression in erythroid cells. Evidence from studies in β-YAC transgenic mice has shown that the Krüppel-like factors KLF1 and KLF2 positively regulate γ-globin expression in embryonic erythroid cells (Alhashem et al. 2011). Further, the KLF1 and KLF2 genes affect other aspects of erythropoiesis in the mouse apart from globin gene regulation, individually and in concert with each other. A holistic understanding of the functions of these factors in erythroid cells is imperative before they are targeted for therapeutic applications.

KLF1 and KLF2 are essential for primitive erythropoiesis and previous work in our lab using mouse KLF1/KLF2 double knockout embryos has revealed that KLF1 and KLF2 display overlapping roles in many facets of primitive erythropoiesis (Basu et al. 2007). Simultaneous ablation of these factors results in a severely anemic embryonic phenotype that is not evident in either KLF1 or KLF2 single knockout embryos (Basu et al. 2007). In the first part of the following work, we aim to understand the cell autonomous roles of KLF2 in erythroid cells and the extent of gene interactions between KLF1 and KLF2 in primitive erythroid cells with an emphasis on elucidating the mechanism of these interactions that cause anemia. These studies are carried out using the mouse as a model system since it is not possible to study primitive erythropoiesis in humans. In the second part of this study we employ the recently developed in vitro system for culturing human erythroid cells derived from umbilical cord blood hematopoietic progenitors as a “fetal” model to explore the role of KLF1 and KLF2 in regulating the γ-globin gene in cells where γ-globin is actively expressed. Using this
approach we aim to determine whether KLF1 and KLF2 positively regulate \( \gamma \)-globin expression in human erythroid cells during development.
Chapter 2: Methods

2.1 Studies in the mouse

2.1.1 Generation of mouse models

The KLF1 knockout (KO) mouse model was generated by targeted insertion of the neomycin resistance gene (Fig. 2.1A) (Perkins, Sharpe, Orkin 1995). The KLF2 KO mouse model was developed by interrupting the gene with the hypoxanthine phosphoribosyl-transferase (Hprt) gene (Fig. 2.1B) (Wani, Means, Lingrel 1998). The KLF1 and KLF2 loci are linked on mouse chromosome 8. This made it possible to develop a model with the KLF1 and KLF2 null alleles on the same chromosome, KLF1+/−-KLF2+/−-(R) (Basu et al. 2007). Mice with a floxed KLF2 allele were obtained from Dr. Jerry Lingrel (Fig. 2.1B) (Weinreich et al. 2009). Mice carrying the KLF1 null allele and the KLF2 floxed allele on the same chromosome were obtained by screening for recombinants as previously described (Basu et al. 2007). The ErGFP-Cre mouse model directs erythroid specific expression of the Cre gene (Cyclization recombinase) under the control of the erythropoietin receptor (EpoR) promoter (Heinrich, Pelanda, Klingmuller 2004). The Tie2-Cre mouse model directs erythroid- and endothelial-specific Cre expression under the control of the Tie2 (endothelial-specific receptor tyrosine kinase or Tek) promoter (Kisanuki et al. 2001).

2.1.2 Genotyping

Mice were genotyped using tissue obtained by ear-punching the mice at 3-4 weeks of age. Ear clips were digested for 4-6 hours at 55-60°C in 50μl digestion buffer (10mMTris
Figure 2.1: Graphical representation of wild-type and knockout alleles for KLF1 and wild-type, knockout and floxed alleles for KLF2. (A) Insertion of the neomycin resistance gene (Neo) was used to disrupt the wild-type KLF1 allele. (B) The KLF2 gene was targeted by insertion of the hypoxanthine phosphoribolsyl transferase (Hprt) gene. The KLF2 floxed allele was generated by homologous recombination to incorporate two LoxP sites flanking the second exon of KLF2. E – exon, WT – wild-type, Arrowheads – LoxP sites, Red arrows – position of primers used for genotyping. Figure not drawn to scale.
HCL, pH8.5; 50mM KCl, 40mM MgCl$_2$, 0.45% Tween 20 and 0.45% NP40) containing 1μg/μl proteinase K (Roche, PCR grade). Following digestion, proteinase K was inactivated by 2 cycles of alternate boiling (>95°C) and cooling (4°C) for ten minutes each. 1-2μl of the resulting lysate was used as the test DNA sample in a polymerase chain reaction (PCR). The primers used for genotyping are listed in Table 2.1.

### 2.1.3 Tissue collection

Timed matings were used to obtain embryos at various developmental stages. The observation of a vaginal plug was defined as embryonic day 0.5 (E0.5). Pregnant female mice were sacrificed to obtain embryos between E8.5 and E10.5. Embryos were dissected in PBS in petri dishes. Embryonic blood cells were collected by severing the vitelline and umbilical vessels and allowing embryos to bleed into 1ml PBS in a 12-well plate. The number of blood cells was counted using a hemocytometer and then cells were processed for RNA preparation. Whole-mounts were photographed with an Olympus SZ2-ILST (Olympus America, Center Valley, PA) microscope, using an Olympus Q-Color 3 (Olympus America) camera and QCapture 2.81.0 software (Quantitative Imaging, Surrey, BC). Mouse embryonic yolk sac was collected and processed for embedding and sectioning. The embryo body was collected for genotyping and processed similar to the ear clips, as described above.

### 2.1.4 RNA extraction and cDNA synthesis

Mouse embryonic blood cells, collected in PBS, were pelleted, resuspended in 200μl denaturation solution (Totally RNA total RNA isolation kit, Ambion) and frozen at -80°C. Post genotyping, samples of the required test and control genotypes were processed as follows. Samples were thawed on ice. 3M sodium acetate (Totally RNA kit, Ambion) was
added at 1/10th the volume of the sample, followed by 200μl of acid-phenol:chloroform (pH 4.5, Totally RNA kit, Ambion). Samples were mixed by inverting the tubes 4-5 times and centrifuged at 12,000g for 10 minutes at 4°C. The aqueous phase was transferred to a fresh 1.5ml tube. 2μl of glycogen (5mg/ml stock, Ambion) was added to the aqueous phase and mixed by pipetting. Then an equal volume of cold 100% isopropanol was added to the sample and mixed by inverting the tubes 4-5 times. RNA was allowed to precipitate overnight at -80°C. The following day, samples were thawed on ice and centrifuged at 12,000g for 20-30 minutes at 4°C. The supernatant was discarded, with care not to disturb the RNA pellet. The pellet was washed with 1ml of cold 80% ethanol. The ethanol was discarded and the pellet was air-dried for 2-5 minutes on ice. The pellet was then resuspended in 10μl of RNase-free water (USB Corporation) containing SuperaseIn (1:20 dilution, Ambion). 1μl of this sample was further 1:10 diluted and used to determine RNA integrity and concentration using the Agilent Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies), following the manufacturer’s protocol. 500ng-1μg of RNA per sample was treated with DNasel (Life Technologies) and used to prepare cDNA following the instructions of the iScript cDNA synthesis kit (Biorad). The cDNA thus obtained was diluted as required and used to determine gene expression changes by quantitative reverse transcriptase PCR (qRT-PCR).

2.1.5 Quantitative reverse transcriptase PCR (qRT-PCR)

Changes in gene expression were measured by quantitative PCR (qPCR) using cDNA prepared as described above. Because cDNA is the template for this PCR, and it is obtained by reverse transcription of RNA, this method is actually quantitative reverse
transcriptase PCR (qRT-PCR), though it is abbreviated as qPCR herein. qPCR was carried out using SYBR Green or Taqman reagent (Applied Biosystems) and plates were run on an ABI Prism 7300 analyzer (Applied Biosystems). The absolute quantification method was employed which measures the relative amount of amplified product in a sample compared to a reference standard curve containing 5 known quantities of cDNA (expressed as the amount of RNA used for reverse transcription). Stringent quality control measures are applied to all qPCR results. The R-value of the standard curve must be >0.9. The amount of cDNA in all samples must fall within the limits of the standard curve, i.e. sample readings must fall on the curve. Each sample is run in quadruplets. At least three out of the four readings obtained for each sample must be within 20% of the mean value for all four readings. Cyclophilin A or Gapdh mRNA were used as internal standards for normalization as indicated in the figure legends. Primer sequences used for qRT-PCR analyses of the mouse genes are listed in Table 2.2.

2.1.6 Plastic embedding and sectioning of yolk sac

Histological studies of the yolk sac were carried out by embedding the yolk sac in an eponate resin with guidance and resources from Sue Walker in Dr. John Povlishock’s lab (Dept. of Anatomy and Neurobiology). Briefly, yolk sacs were fixed in 2% paraformaldehyde and 0.25% gluteraldehyde in Millonig’s buffer (Recipe) overnight at 4°C, rinsed 3 times and stored in Millonig’s buffer in the fridge. Post-genotyping, yolk sacs of test and control genotypes were treated with 1% osmium tetroxide in Sorenson buffer for 60 minutes at 4°C with gentle rocking, washed 3 times (5 minutes each) with Millonig’s buffer, followed by serial dehydration in 30%, 40%, 50%, 60% and 70%
ethanol for 5 minutes each and 80%, 95% and 100% ethanol for 10 minutes each. Yolk sacs were then incubated in a 1:1 mixture of 100% ethanol and propylene oxide for 5 minutes and propylene oxide alone for 2 changes of 10 minutes each, followed by resin infiltration in a 1:1 mixture of eponate 12 and propylene oxide overnight with gentle agitation inside a chemical hood. The following day the tissue was transferred to 100% eponate 12 and kept on an agitator for 4 hours, and then transferred to the desired mold containing 100% eponate 12 and incubated in a 55°C oven for 3 days for the resin to solidify. Once the resin solidified, the block was trimmed and 2μm sections containing the tissue were cut using glass knives and a Sorvall JB4 microtome. Sections were placed on microscopy slides (Superfrost Plus slides, Fisher Scientific) in droplets of water and kept on a hot plate to allow adhesion of tissue to the slide as the water evaporates. Once dry, the slides were stained with warm methylene blue stain (1% sodium borate, 1% azure II, 1% toluidine and 1% methylene blue). Sections were mounted in oil using coverslips. Images of yolk sac sections were captured with an Olympus BX41 compound microscope and Olympus DP71 digital camera.

2.1.7 Erythroid progenitor assays

To study primitive erythroid progenitors, E8.25 - E8.5 (6-12 somite pairs) implants including embryo and yolk sac were dissected in PB2 (36). Embryonic tissues were kept cold throughout the dissection procedure by placing them on a metal sheet in a tray containing a mixture of ice and water. At the end of the dissection, each implant was dissociated into single cells by incubating them at 37°C for a total of 20 minutes in 200μl 0.01% trypsin/ 1mM EDTA/ PBS (Worthington Biochemical Corporation, NJ) with mechanical trituration at 10 minutes and 15 minutes. This process was carried out in a
96-well plate to monitor successful dissociation of implants. Once dissociated, 100μl of IMDM containing 20% fetal bovine plasma derived serum (PDS, Animal Technologies, TX) was added to arrest the action of trypsin. The cells were pelleted by centrifugation (0.3rcf, 5 minutes, 4°C), resuspended in 200μl of 20% PDS in IMDM and counted using a hemocytometer. Half of the cells from each implant (100μl) were added to 1.5ml of 1% methylcellulose supplemented with 10% PDS, 5% protein-free hybridoma medium (Gibco/BRL), IL-3 (20ng/ml), IL-6 (20ng/ml), stem cell factor (60ng/ml) (IL-3, IL-6 and SCF – Peprotech, NJ), erythropoietin (2U/ml), MTG and L-glutamine in 1.5ml Eppendorf tubes, vortexed and kept at room temperature for 15-20 minutes to allow bubbles to surface (Table 2.3). Then 1ml of this medium containing cells was plated in 35mm petri dishes and incubated at 37°C, 5% CO₂. The remaining cells were used for genotyping. Erythroid colonies were stained using benzidine and counted on either Day 2 or Day 6 of culture. The benzidine stock solution was made by dissolving 100mg benzidine dihydrochloride in 50ml of 0.5% acetic acid. This stock can be stored in the dark at room temperature for 6 months. When plates were ready to be stained, 5μl of 30% hydrogen peroxide was added to 1ml of benzidine stock solution and 0.5 ml of this mixture was immediately layered on the methylcellulose in the dish. Dishes were incubated with stain for 15 minutes before counting colonies using an inverted microscope. Colony images were captured using an Olympus IX70 microscope and Q-Color 3 camera (Olympus America). Two dimensional colony areas were determined using Image J software.
2.1.8 Cell cycle analysis

Cell cycle profiles were assessed using the Allophycocyanin (APC) BrdU Flow kit (BD Biosciences). The protocol was adapted from Malik et al. (37). Briefly, E9.5 erythroblasts were cultured in 500μl of erythroid maturation medium (see recipe below) containing BrdU for 90 minutes (37°C, 5%CO2), followed by fixation (20 minutes, room temperature) and staining with APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD), according to the manufacturer’s protocol (BD Biosciences). After the final staining step, cells were resuspended in 200μl staining buffer and stored overnight at 4°C. The following day, the BD FACSCanto™ II Analyzer was used to measure total DNA content and BrdU incorporation using 7-AAD and APC fluorescence intensity, respectively. Gating was applied to include only single cells and exclude clumped cells.

Maturation medium recipe: 10% serum replacement (Life Technologies), 10% PFHM-II (Life Technologies), 2 mM glutamax (L-alanyl-L-glutamine, Life Technologies), 150 μM Monothioglycerol (MTG, Sigma), 1% fetal bovine plasma-derived serum (PDS, Animal Technologies, Tyler, TX, USA), 1 U/ml recombinant human EPO (Amgen, Thousand Oaks, CA, USA).

2.1.9 Statistical analysis

The Student’s t-test was used for statistical analyses. p values < 0.05 were considered significant.
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<td></td>
<td></td>
<td>RP: TGCCGAAGTGACTAGCCAAA</td>
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<tr>
<td></td>
<td></td>
<td>Probe: TCAAAACTCTTTGGGAATTGCTGATTG</td>
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</tr>
<tr>
<td>4</td>
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<td>FP: CCTTTGAACGCACGTACCTTTTG</td>
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</tr>
<tr>
<td></td>
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<td>RP: GTAAATCTGCGGTGGTAGGAG</td>
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<td></td>
<td>RP: GTCTTCATTAGTCACCTGCTCG</td>
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<td>ABI Assay ID : Mm02342430_g1 (TaqMan® Gene Expression Assays)</td>
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Table 2.3: Composition of medium for colony forming assay

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<th>Final Concentration</th>
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<td>50%</td>
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</tr>
<tr>
<td>PDS</td>
<td>100%</td>
<td>10%</td>
<td>2ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>100X</td>
<td>1X</td>
<td>200μl</td>
</tr>
<tr>
<td>PFHM</td>
<td>100%</td>
<td>5%</td>
<td>1ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>50ng/μl</td>
<td>20ng/ml</td>
<td>8μl</td>
</tr>
<tr>
<td>IL-3</td>
<td>50ng/μl</td>
<td>20ng/ml</td>
<td>8μl</td>
</tr>
<tr>
<td>Epo</td>
<td>4U/μl</td>
<td>2U/ml</td>
<td>10μl</td>
</tr>
<tr>
<td>SCF</td>
<td>100ng/μl</td>
<td>60ng/ml</td>
<td>12μl</td>
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<tr>
<td>MTG</td>
<td>Dilute 100% MTG 2:26 in IMDM</td>
<td>20μl of diluted MTG in 10ml medium</td>
<td>40μl</td>
</tr>
<tr>
<td>IMDM</td>
<td>1X</td>
<td>-</td>
<td>7ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td></td>
<td></td>
<td>20ml</td>
</tr>
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</table>
2.2 Studies using *in vitro* derived human erythroid cells

2.2.1 Isolation of human CD34+ hematopoietic progenitors from cord blood

Fresh umbilical cord blood (collected within 24-48 hours) was obtained from the St. Louis Cord Blood Bank (SLCBB, St.Louis, MO). We usually receive 80-100ml of cord blood. The cord blood is carefully transferred into 50ml centrifuge tubes and diluted 1:1 with DPBS. Diluted cord blood is layered on top of Ficoll-Paque™ Premium (GE Healthcare) in the ratio 3ml Ficoll : 4ml cord blood in 50ml centrifuge tubes and subjected to density gradient centrifugation and subsequent wash steps, according to the manufacturer’s protocol, to obtain the mononuclear cells. After the final wash step the mononuclear cells are resuspended in PBS containing 2% FBS and 1mM EDTA and processed for isolation of CD34+ cells using the EasySep™ human CD34 positive selection kit (Stem Cell Technologies) following the kit protocol. After the last wash step, the cells obtained are resuspended in expansion medium (see recipe below) at a density of 0.5-1 million cells per ml in 12 well plates and cultured at 37°C in a 5% CO₂ incubator.

Expansion medium: StemSpan SFEM medium (Stem Cell Technologies) containing 1X CC100 cytokine cocktail (Stem Cell Technologies), 8μl/ml LDL (Sigma, L7914) and 2% Penicillin / Streptomycin (P/S, Gibco)

2.2.2 Expansion of CD34+ hematopoietic progenitors

Very few CD34+ hematopoietic progenitors are found in cord blood. On average, from 80-100ml of cord blood we obtain 0.5-2 million CD34+ cells after magnetic enrichment. In order to have enough cells for various experimental endpoints, these CD34+ cells are expanded for 7 days by culturing in expansion medium (see recipe above) in 12 well
tissue culture plates and maintaining cell density at 1-2 million cells / ml. Cells were counted and medium changed on alternate days.

2.2.3 Lentiviral infection of CD34+ hematopoietic progenitors

In order to manipulate the expression of our genes of interest, KLF1 and KLF2, in this system, lentiviral vectors carrying either KLF1- or KLF2-targeted shRNA coding sequences were either acquired from other labs or cloned. Two KLF1-targeted shRNA vectors (pRRL_K1V1 and pRRL_K1V2) as well as a scrambled shRNA vector (pRRL_Scr) were acquired from Dr. François Morlé (Université Claude Bernard Lyon1, Lyon, France) and Dr. Fawzia Louache (INSERM, Villejuif, France). These vectors were constructed on a pRRL backbone (Bouilloux, Blood 2008) with the shRNA-coding sequence under the control of the H1 promoter. In addition to the shRNA-coding sequence they carry a GFP-coding sequence, which aids in identification and recovery of successfully infected cells by FACS. Sheng Zu Zhu from Dr. Gordon Ginder’s laboratory (Virginia Commonwealth University) replaced the GFP gene in the pRRL vector with an RFP gene. Five KLF2-targeted shRNA vectors were constructed by cloning shRNA-coding sequences into the RFP-containing pRRL plasmid.

shRNA sequences aimed at targeting KLF2 mRNA were designed using software from the following sources: Genelink (www.genelink.com), Whitehead institute siRNA selection program (http://sirna.wi.mit.edu/, Bingbing, NAR 2004), Clonetech RNAi target sequence selector (http://bioinfo.clontech.com/rnaidesigner/frontpage.jsp) and siDirect (http://sidirect2.rnai.jp/design.cgi). shRNA sequences were shortlisted based on the following rules: 1. There should be a G/C base at the 5’ end of the sense strand of the shRNA 2. There should be an A/U at the 3’ end of the sense strand of the shRNA 3.
Five out of the last seven bases at the 3’ end of the sense strand of the shRNA should
be A/U. For each shRNA, 4 oligonucleotides were synthesized (Eurofins MWG Operon)
according to the shRNA cassette design depicted in Fig.2.2. The oligonucleotide
sequences are listed in Table 2.4. The naming of the 5 KLF2 shRNAs denotes the base
position in the KLF2 mRNA. Oligonucleotides were annealed and ligated to obtain the
entire shRNA coding sequence which was then cloned into the pRRL vector using MluI
and XhoI restriction sites. Plasmids were transformed into Stbl3 E. coli (Life
Technologies) and plated on LB agar plates containing 100μg / ml Ampicillin (Thermo
Fisher Scientific). Five ampicillin resistant colonies were picked from each
transformation reaction and cultured for mini preps (Qiagen Miniprep Kit), followed by
sequencing to confirm shRNA sequence in each clone. A single colony was then used
for large scale plasmid production (Life Technologies Maxiprep Kit) for use in virus
preparation. Out of the five shRNAs tested, only two, K2sh_206 and K2sh_1529,
successfully knocked down KLF2 mRNA.

Lentiviral particles are produced by co-transfection of three plasmids into 293T cells
(semi-confluent in 10cm tissue culture dishes): packaging plasmid pCMV-dR8.74,
envelope plasmid pMD2G and the pRRL plasmid carrying our shRNA. The packaging
and envelope plasmids were a gift from Dr. Gordon Ginder’s laboratory. Transfected
293T cells are maintained at 37°C, 3% CO2 for 16-18 hours in 10ml of DMEM
containing 10% FBS and 1% P/S, after which culture medium is replaced by fresh
medium (10ml of DMEM containing 2% FBS and 1% P/S) for collection of viral particles
and 293T cells are incubated at 37°C, 10% CO2 until the medium containing viral
particles is harvested for use in infection of CD34+ cells after 32 hours. The medium
Figure 2.2: shRNA cassette template used to design shRNAs targeting the KLF2 mRNA. The sequences of oligonucleotides for K2sh-206 (see Table 2.3) are used in this example. Figure adapted from Ginder laboratory protocols.
containing viral particles is first filtered through a 0.45μm filter, supplemented with polybrene, and then 600μl of virus medium is added to 200,000 to 300,000 cells in 50μl expansion medium. Cells are incubated at 37°C, 5% CO₂. The following afternoon 650μl of 2X expansion medium is added to the cells and the cells are returned to the incubator for another 2 days.

2.2.4 Recovery of infected cells by FACS and differentiation into erythroid cells
On the third day after infection, infected CD34+ cells are washed 3 times with IMDM containing 10% FBS to get rid of all viral particles, resuspended in SFEM (approximately 10 million cells in 500μl medium), and sorted by fluorescence activated cell sorting (FACS) using a BD FACSAria™ II high speed cell sorter (BD Biosciences) in the VCU Flow Cytometry Core Facility to obtain either GFP+ or RFP+ cells, depending on which pRRL plasmid was used. Fig. 2.3 depicts sample FACS data plots for a mock sample that has not been infected and a sample that has been infected with an shRNA (in this case expressing RFP as the reporter gene). Cells are first gated based on forward scatter area (FSC-A) and side scatter area (SSC-A) to select for single cells and discard any doublets (Fig. 2.3, Gate P1). Single cells are then sorted to retrieve cells that are positive for RFP fluorescence (Fig. 2.3, PE channel, red gate), as an indicator that they have been infected with the desired shRNA-containing vector. The table below the FACS plots shows the percentage of cells in each gate as a function of the parent gate and the total number of cells. Infection efficiency ranges from 50% to 90% in our hands for all the different scramble and gene-specific constructs used. Pelleted CD34+ cells are white in color. Sorted cells are transferred to a pro-erythroid growth medium (see composition below) for three days, followed by erythroid
Figure 2.3: FACS is used to retrieve cells that have been successfully transduced. Representative FACS plots for a mock-treated sample and an shRNA-infected sample depicting the method by which cells that have been successfully infected are selected by FACS. Cells are first gated based on forward scatter area (FSC-A) and side scatter area (SSC-A) to select for single cells and discard any doublets (Gate P1). Single cells are then sorted to retrieve cells that are positive for RFP fluorescence PE channel, red gate), as an indicator that they have been infected with the desired shRNA-containing vector. The table below the FACS plots shows the percentage of cells in each gate as a function of the parent gate and the total number of cells.
differentiation medium (see composition below) for five days. On the eighth day after sorting, erythroid cells, which are deep red in color by this day, are harvested for interrogation. Because the cells have been exposed to erythropoietin (Epo) for 8 days, the day of harvest is termed differentiation day 8 (DD8). In order to confirm that the majority of cells harvested are erythroid cells, a small fraction of cells are stained with fluorophore-conjugated antibodies that recognize surface epitopes, on erythroid cells (see below, and as described in section 1.1.3). In addition, cytospins of cells from each sample and treatment level are prepared to study cellular morphological details by Wright-Giemsa staining.
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2.2.5 Cell surface immunofluorescence staining for markers of erythroid differentiation

200,000 to 1 million cells are collected in 1.5ml Eppendorf tubes, rinsed with and resuspended in 100μl of cell staining buffer (10% FBS in PBS). To this is added 5μl (0.06μg) of APC-conjugated anti-CD71 (Transferrin receptor) antibody and 7.5μl (0.015μg) of PE-conjugated anti-CD235a (Glycophorin A) antibody (eBioscience). When RFP is the reporter gene present in transduced cells, the PE-conjugated anti-CD235a antibody is substituted with a PE-Cy7-conjugated antibody (BioLegend). The cells are incubated with the antibodies on ice, in the dark, for 20 minutes. After staining is complete, cells are washed with and resuspended in 300μl of cell staining buffer (10% FBS in PBS). Distribution of CD71 and CD235a on the surface of erythroid cells is analyzed by measuring fluorescence intensity of each epitope by flow cytometry using the BD FACSCanto™ II Analyzer. On day 8 of differentiation most cells are at the erythroblast stage of maturation and display a CD71^{HI}CD235a^{HI} phenotype. These cells are considered “double positive” for these two markers. Cells that are either CD71^{HI}CD235a^{LOW} or CD71^{LOW}CD235a^{HI} are also erythroid cells, but at an earlier or later stage of erythroid maturation respectively. Cells that are CD71^{LOW}CD235a^{LOW} are considered to be non-erythroid cells.

2.2.6 RNA extraction and cDNA synthesis

1-2 million erythroid cells harvested on DD8 are processed for RNA extraction using Trizol reagent (Life Technologies) according to the manufacturer’s protocol. The RNA pellet obtained was resuspended in 20μl of RNase-free water (USB Corporation) containing SuperaseIn (1:20 dilution, Ambion). 1μl of this sample was diluted 1:10 and
used to determine RNA integrity and concentration using the Agilent Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies), following the manufacturer’s protocol. 1μg of RNA per sample was treated with DNase I (Life Technologies) and used to prepare cDNA following the instructions of the iScript cDNA synthesis kit (Biorad). The cDNA thus obtained was diluted as required and used to determine gene expression changes by quantitative reverse transcriptase PCR (qRT-PCR).

2.2.7 Quantitative reverse transcriptase PCR (qRT-PCR)

Changes in gene expression were measured by quantitative PCR (qPCR) using cDNA prepared as described above. qPCR was carried out using SYBR Green or Taqman reagent (Applied Biosystems) and plates were run on an ABI Prism 7900HT analyzer (Applied Biosystems). The relative quantification method (E^Ct_{endogenous\text{ gene}} / E^Ct_{test\text{ gene}}, where E = efficiency of primer set) was employed to analyze changes in gene expression in knockdown samples compared to scramble controls. Additionally, the expression of each gene was set to 100 for scramble-treated cells and fold change over scramble was calculated for KLF1-shRNA or KLF2-shRNA infected cells, to minimize variations observed due to differences between individuals. A standard curve was run for each gene as described earlier and used to calculate the efficiency of the respective primer set.

2.2.8 Statistical analysis

The Student’s t-test was used for statistical analyses. p values < 0.05 were considered significant.
Chapter 3: Krüppel-like transcription factors KLF1 and KLF2 have unique and coordinate roles in regulating embryonic erythroid precursor maturation

3.1 INTRODUCTION

Erythropoiesis in the mouse, and other mammals, proceeds in two distinct waves during development (McGrath and Palis 2008). First, primitive erythroid cells appear in the yolk sac, an extra-embryonic membrane, in regions known as blood islands (Ferkowicz and Yoder 2005; Haar and Ackerman 1971). Large, nucleated primitive erythroid cells begin to circulate at about mouse embryonic day 8.25 (E8.25) (Palis et al. 1999; Palis 2008). Mouse primitive erythroid progenitor cells form colonies (EryP-CFC) in semi-solid culture media (Palis et al. 1999), which can first be detected at E7. Primitive erythroid cells mature in circulation in a synchronous manner and eventually undergo enucleation (Kingsley et al. 2004). The second wave, or definitive erythropoiesis, begins in the fetal liver and results in mature, enucleated definitive erythroid cells by around E12 (McGrath and Palis 2008).

The erythroid developmental program is regulated by a network of key transcription factors (Baron, Vacaru, Nieves 2013), including Krüppel-like factor 1 (KLF1 or EKLF, Erythroid Krüppel-like factor). Members of the KLF family have three Cys2-His2 zinc fingers near their C-terminus (McConnell and Yang 2010). KLF1 is erythroid-specific and required for the normal expression of the embryonic and adult β-like globin genes (Basu et al. 2007; Nuez et al. 1995; Perkins, Sharpe, Orkin 1995). KLF1-/- mice
develop β-thalassemia and die by E16 (Nuez et al. 1995; Perkins, Sharpe, Orkin 1995). KLF1 regulates the expression of numerous other erythroid genes, including those encoding cytoskeletal and membrane proteins, and heme synthesis enzymes (Hodge et al. 2006; Pang et al. 2012). In addition, KLF1 regulates cell cycle control, proliferation and apoptosis (Pilon et al. 2008; Tallack et al. 2009; Tallack et al. 2012). Pathogenic mutations in KLF1 can cause congenital dyserythropoietic anemia in humans (Arnaud et al. 2010; Jaffray et al. 2013). Interestingly, certain human mutations result in higher than normal amounts of γ-globin, or hereditary persistence of fetal hemoglobin (Borg et al. 2010; Zhou et al. 2010).

To date, 17 mammalian KLFs have been identified, and they have diverse roles in development (McConnell and Yang 2010). The zinc finger domain of KLF2 is 89% similar to KLF1. KLF2 is necessary for primitive erythropoiesis, and positively regulates embryonic globin gene expression, as shown in KLF2 knockout and erythroid-conditional knockout mouse embryos (Alhashem et al. 2011; Basu et al. 2005; Basu et al. 2007). There are a greater percentage of apoptotic erythroid cells in KLF2-/- than in normal E10.5 yolk sacs (Basu et al. 2005). Global gene expression assays indicate that many genes important in the processes of development, differentiation and cell migration are dysregulated in KLF2-/- compared to wild-type primitive erythroid cells (Redmond et al. 2011). Ablation of KLF2 causes lethality between E11.5 and E14.5 (Chiplunkar et al. 2013; Wani, Means, Lingrel 1998), due to severe intra-embryonic hemorrhaging (Kuo et al. 1997) and/or heart failure (Lee et al. 2006).

KLF1 and KLF2 share functional roles in globin gene regulation. KLF1 and KLF2 can partially compensate for each other, as is evident from the reduced embryonic β-
like globin gene expression of KLF1/KLF2 double (KLF1-/-KLF2-/-) compared to single knockout embryos (Basu et al. 2007). There is also more aberrant morphology, consistent with increased apoptosis, in double compared to single knockout primitive red blood cells. In addition, E10.5 KLF1-/-KLF2-/- embryos are strikingly pale and appear anemic, whereas E10.5 KLF1-/- and E10.5 KLF2-/- mice are grossly normal. In addition, KLF1-/-KLF2-/- mice die by E11.5, which is earlier than KLF1-/- or KLF2-/- embryos. Gene expression profiling of E9.5 erythroid cells was used to identify genes that are synergistically regulated by KLF1 and KLF2 (Pang et al. 2012). These genes are principally involved in cellular pathways that control homeostasis, hemopoiesis, apoptosis and proliferation. Erythroid conditional knockout and other functional studies identified Myc as an important downstream target of KLF1 and KLF2. Additional potential targets include other genes governing proliferation and/or apoptosis, such as Cd24a antigen (Nielsen et al. 1997), forkhead box M1 (FoxM1) (Wierstra and Alves 2007), sphingosine kinase 1 (Sphk1) (Le Scolan et al. 2005) and parathyroid hormone 1 receptor (Pthr) (Qian et al. 2003).

In this work, we further assess how KLF1 and KLF2 coordinately control primitive erythropoiesis. Our first aim was to determine whether an erythroid cell-autonomous function of KLF2 is responsible for the development of anemia in KLF1-/-KLF2-/- E10.5 mouse embryos. We were also interested in determining the cause of the anemia, through identification of cellular processes and molecular regulators that are dysregulated in the absence of KLF1 and KLF2. We demonstrate that E10.5 KLF1/KLF2 double erythroid-conditional knockout embryos are anemic like KLF1-/-KLF2-/- mice. A surprising new discovery was made, in that restoring one copy of the KLF2 (KLF1/-/
KLF2+/-) but not the KLF1 gene (KLF1+/-KLF2-/-) ameliorates this phenotype. Anemia in E10.5 KLF1+/-KLF2-/- and KLF1-/-KLF2-/- mice correlates with a drastic reduction in the number of peripheral blood cells. EryP-CFC assays identified a novel role for KLF2 in the maintenance of primitive erythroid progenitor potential. KLF1 can partially compensate for KLF2 in this function, but its pivotal role is in the regulation of cell proliferation. KLF1-/-KLF2-/- embryos have more severely impaired colony forming ability than single knockouts. The data indicate that KLF1 and KLF2 coordinately control the maturation of primitive erythroid precursors.
3.2 RESULTS

3.2.1 KLF1/KLF2 double conditional KO embryos recapitulate the KLF1-/-KLF2-/- anemia phenotype

KLF1 has an erythroid specific pattern of expression. However, KLF2 has a broader tissue distribution, with expression observed in erythroid and endothelial cells, T-cells and lung tissue. We wished to determine whether the anemia phenotype observed in E10.5 KLF1-/-KLF2-/- mouse embryos is a result of the erythroid cell-autonomous functions of KLF2. In order to determine whether the anemia observed in KLF1-/-KLF2-/- embryos results from erythroid cell-specific ablation of KLF2, the ideal mouse model would be an erythroid conditional knockout of KLF2 on a KLF1 null background. Unfortunately, ErGFP-Cre expression (Alhashem et al. 2011; Heinrich, Pelanda, Klingmuller 2004) is dependent on KLF1 (Fig. 3.1). Consequently, there was no reduction in the amount of KLF2 mRNA in KLF1-/-KLF2F/F,ErGFP-Cre blood cells (data not shown), and this model was untenable. The β-Cre transgenic model (Peterson et al. 2004) was also unworkable because the KLF1 and β-Cre genes are apparently linked. In Tie2-Cre mice, Cre is expressed in erythroid cells as early as E7.5 (Tang et al. 2010). Therefore this model was used for conditional deletion of KLF2 on a KLF1 null background, with the caveat that KLF2 is ablated in erythroid and endothelial cells.

KLF1+/KLF2F/+,Tie2-Cre and KLF1+/KLF2F/F mice were mated to obtain E10.5 embryos of the following genotypes: KLF1-/-KLF2F/F,Tie2-Cre (KLF1/KLF2 double conditional KO or dcKO), KLF1-/-KLF2F/F (KLF1 KO) and KLF1+/+KLF2F/F (wild-type control). At E10.5, wild-type (Fig. 3.2A), KLF1 KO (Fig. 3.2B) and KLF2F/F,Tie2-Cre (KLF2 conditional KO, Fig. 3.2C) embryos are surrounded by yolk
Figure 3.1. ErGFP-Cre mRNA expression is regulated by KLF1 in erythroid cells. ErGFP-Cre mRNA expression in KLF2F/F,ErGFP-Cre (n=3) E10.5 peripheral blood compared to KLF1-/- KLF2F/F,ErGFP-Cre (n=5). The ErGFP-Cre-to-Cyclophilin A mRNA ratio for KLF2F/F,ErGFP-Cre was taken as 100%. Cyclophilin A mRNA was used as an internal standard for qRT-PCR. Error bars indicate standard deviation. * p < 0.025
Figure 3.2: KLF1/KLF2 double conditional KO embryos recapitulate the KLF1/-/KLF2/- anemia phenotype. E10.5 whole-mount embryos surrounded by yolk sacs. (A) Wild-type embryos (KLF1+/+KLF2F/+, n=3) have distinct red blood vessels. (B) KLF1 KO (KLF1-/-KLF2F/F, n=4) and (C) KLF2 conditional KO (KLF2F/F, Tie2-Cre, n=1) embryos are similar in appearance to wild-type embryos. (D) KLF1/KLF2 double conditional KO embryos (KLF1-/-KLF2F/F, Tie2-Cre, n=4) appear anemic in contrast to A, B and C. (E) KLF1-/-KLF2F/+, Tie2-Cre embryos with one normal KLF2 gene appear grossly normal (n=2); whereas (F) a KLF1+//-KLF2F/F, Tie2-Cre (n=1) embryo, with one functional KLF1 gene, appears anemic. Photographs were taken at 15X magnification.
sacs with distinctly red blood vessels. In comparison, E10.5 KLF1/KLF2 dcKO mice (Fig. 3.2D) are pale and appear anemic, similar to KLF1-/-KLF2-/- embryos (Basu et al. 2007).

In the course of these experiments, a few recombinant embryos of the genotypes KLF1-/-KLF2F/+Tie2-Cre and KLF1+/-KLF2F/F,Tie2-Cre were obtained. An interesting and surprising observation was that E10.5 KLF1-/-KLF2F/+Tie2-Cre embryos (Fig. 3.2E), having one KLF2 allele, have yolk sacs with visibly red blood vessels similar to wild-type (Fig. 3.2A), but a KLF1+/-KLF2F/F,Tie2-Cre embryo (Fig. 3.2F), with one KLF1 allele, appears anemic like KLF1/KLF2 dcKO embryos (Fig. 3.2D). In order to verify this observation, matings were performed to obtain several E10.5 KLF1-/-KLF2+/- and KLF1+/-KLF2-/- embryos. KLF1-/-KLF2+/- embryos are similar to wild-type embryos with distinct red blood vessels (Fig. 3.3A), while KLF1+/-KLF2-/- embryos appear anemic (Fig. 3.3B). Thus, the complete knockout embryos have gross phenotypes similar to the conditional knockout mice. These findings confirm that one functional KLF2 gene, but not one KLF1 allele, can ameliorate the anemia phenotype.

To determine whether the pale appearance of KLF1/KLF2 dcKO embryos is due to a defect in the development of blood vessels, yolk sac tissue sections from 3 embryos of each genotype were studied. Blood islands consisting of blood cells (Ery) surrounded by endothelial cells (En) are situated between the epithelium (Ep) and mesothelium (Me), as shown for wild-type in Figs. 3.4A and 3.4D. The yolk sac vasculature of KLF2 KO (Basu et al. 2005), KLF1 KO (Figs. 3.4B and 3.4E) and KLF1/KLF2 dcKO embryos (Figs. 3.4C and 3.4F) appears normal, with well-defined
blood vessels lined by endothelial cells. This suggests that aberrant erythroid rather than vascular development causes the anemia phenotype.

Because the yolk sac vasculature of KLF1/KLF2 dcKO mouse embryos appears normal, the globin mRNA amount in the erythroid cells was queried. E10.5 peripheral blood cells were collected from wild-type, KLF1-/- and KLF1/KLF2 dcKO embryos. RNA prepared from these cells was used to quantify βh1- and Ey-globin expression by qRT-PCR. In addition to these three genotypes, previous data for E10.5 KLF2F/F,ErGFP-Cre (KLF2 erythroid conditional knockout or KLF2Ery-cKO) embryos is included (Alhashem et al. 2011; Heinrich, Pelanda, Klingmuller 2004). A direct comparison can be drawn between KLF1/KLF2 dcKO and KLF2 Ery-cKO embryos because there is a similar amount of KLF2 mRNA remaining in the embryonic erythroid cells of each genotype ((Alhashem et al. 2011) and unpublished data).

Compared to wild-type embryos, KLF2 Ery-cKO and KLF1-/- embryos have an approximately 20% and 35% reduction in βh1-globin mRNA, respectively (Fig. 3.5A, p<0.001). The amount of βh1-globin mRNA in KLF1/KLF2 dcKOs is reduced to less than 50% of that in wild-type embryos, significantly lower than either KLF2 Ery-cKO or KLF1-/- (Fig. 3.5A, p<0.025). A similar decreasing trend is observed in the amount of Ey-globin mRNA (Fig. 3.5B). The amount of Ey-globin mRNA in KLF1/KLF2 dcKOs is reduced to approximately 50% of that in wild-type embryos (p<0.001). Although these decreases are significant, they are not great enough to explain the observed anemia phenotype in KLF1/KLF2 dcKO embryos.
Figure 3.3: Gene dosage of KLF1 and KLF2 impacts the anemia phenotype. E10.5 whole-mount embryos surrounded by yolk sacs. (A) Embryos with one normal KLF2 gene, (A) KLF1-/-KLF2 +/- (n=3) appear grossly normal; whereas (B) KLF1+/-KLF2-/- (n=3) embryos, with one functional KLF1 gene, appear anemic. Photographs were taken at 15X magnification.
Figure 3.4: KLF1/KLF2 double conditional KO embryos have normal yolk sac blood vessels. Blood islands from wild-type (A and D), KLF1 KO (B and E) and KLF1/KLF2 double conditional KO (C and F) yolk sacs. n=3 for each genotype. A,B and C are 400X; and D, E and F are 1000X magnification. The abbreviations used are columnar epithelium (Ep), blood cells (Ery) and endothelial/mesothelial layer (En + Me L).
Figure 3.5. KLF1/KLF2 double conditional KO erythroid cells have reduced embryonic βh1- and Ey-globin mRNA. Mouse embryonic (A) βh1-globin and (B) Ey-globin mRNA expression in E10.5 erythroid cells from wild-type (WT), KLF2F/F, ErGFP-Cre; KLF1-/-KLF2F/F and KLF1-/-KLF2F/F, Tie2-Cre. KLF2F/F,ErGFP-Cre, KLF1-/-KLF2F/F and KLF1-/-KLF2F/F, Tie2-Cre blood cells have less βh1-globin mRNA than wild-type (p<0.001). KLF1-/-KLF2F/F and KLF1-/-KLF2F/F, Tie2-Cre blood cells have less Ey-globin mRNA than wild-type (p<0.001). Cyclophilin A mRNA was used as an internal standard for qRT-PCR. The globin-to-cyclophilin A mRNA ratio for WT was taken as 100%. n=6-8 for each genotype. Error bars indicate standard deviation.
3.2.2 E10.5 KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos have fewer peripheral blood cells than KLF1-/-KLF2+/-

We had previously shown that E10.5 KLF1-/-KLF2-/- embryos have fewer peripheral blood cells than wild-type (Pang et al. 2012). In order to understand the contributions of KLF1 and KLF2 to the starkly different erythropoietic phenotypes of E10.5 KLF1+/-KLF2-/- and KLF1-/-KLF2+/- embryos, the number of circulating blood cells in these embryos was determined. KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos have fewer peripheral blood cells than wild-type (Fig. 3.6A, p<0.005). Of particular interest was the observation that KLF1+/-KLF2-/- have significantly fewer peripheral blood cells than KLF1-/-KLF2+/- embryos (Fig. 3.6A, p<0.01). The number of blood cells in the anemic KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos is similar, and is several-fold less than wild-type. KLF1-/-KLF2+/- embryos have an intermediate number of blood cells, between these two classes.

The amount of embryonic βh1- and Eγ-globin mRNA in peripheral blood cells from these embryos was determined using qRT-PCR. E10.5 peripheral blood cells were collected from wild-type, KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos. KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- blood cells all have an approximately 50% reduction in the amount of βh1-globin (Fig. 3.6B) and Eγ-globin mRNA (Fig. 3.6C) compared to wild-type. There are no significant differences in the amounts of βh1- and Eγ-globin mRNA between these 3 mutant genotypes. Therefore, disparities in globin gene expression do not explain the difference between the phenotypes of KLF1+/-KLF2-/- and KLF1-/-KLF2+/- embryos with regard to anemia. The measurements of the amount of globin mRNA per cell and the number of blood cells per
Figure 3.6. E10.5 KLF1+/-KLF2/- and KLF1-/-KLF2-/- embryos have fewer peripheral blood cells than KLF1-/-KLF2+/- embryos. (A) KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2/- embryos have fewer peripheral blood cells than wild-type (WT) embryos (* = p<0.005). Other significant p-values are as shown by brackets. KLF1+/-KLF2-/ and KLF1-/-KLF2/- embryos have fewer peripheral blood cells than KLF1-/-KLF2+/-, correlating with the anemia phenotype. The amount of mouse embryonic (B) βh1-globin and (C) Ey-globin mRNA is reduced in KLF1-/-KLF2+/-, KLF1+/-KLF2-/ and KLF1-/-KLF2/- blood cells compared to wild-type (WT) (* = p<0.05). Gapdh mRNA was used as an internal standard for qRT-PCR. The globin-to-Gapdh mRNA ratio for WT was taken as 100%. n=4-10 for each genotype. Error bars indicate standard deviation.
embryo were multiplied to calculate the total embryonic globin per embryo. KLF1-/-
KLF2+/− embryos have approximately 4-fold less, whereas KLF1+/-KLF2-/- and KLF1-/-
KLF2-/- embryos have approximately 10-fold less total globin per embryo than wild-type.
Based on these findings, we conclude that a combined reduction in globin per cell and
in number of peripheral blood cells contributes to the anemic phenotype of E10.5
KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos.

E10.5 KLF1-/-KLF2+/− embryos may escape anemia because a compensatory
increase in KLF2 mRNA expression ameliorates the phenotype. To test this hypothesis,
the amount of KLF2 mRNA in blood cells of E10.5 wild-type and KLF1-/- embryos was
quantified by qRT-PCR. An approximately 2.5 fold increase in KLF2 mRNA amount was
observed in KLF1-/- blood cells compared to wild-type (Fig. 3.7), supporting this postulate.

3.2.3 KLF1-/- and KLF1-/-KLF2-/- erythroblasts display impaired G1- to S-phase
cell cycle progression

There is prior evidence that primitive and definitive KLF1-/- blood cells show cell
cycle defects (Pilon et al. 2008; Tallack et al. 2009). Furthermore, KLF1 directly
regulates E2f2 and p18, genes that control cell cycle progression (Pilon et al. 2008;
Tallack, Keys, Perkins 2007; Tallack et al. 2009). To determine whether KLF1 and KLF2
have overlapping functions in cell cycle regulation, and whether changes in proliferation
are responsible for the observed reduction in KLF1-/-KLF2-/- blood cells, the cell cycle
profiles of E9.5 erythroblasts from these embryos were compared to KLF1-/-, KLF2-/-
and wild-type littermates. After culturing the cells for 90 minutes in medium containing
Figure 3.7. Compensatory increase in KLF2 mRNA in KLF1-/− blood cells. KLF2 mRNA expression in KLF1 KO (KLF1-/−-KLF2F/F, n=9) E10.5 peripheral blood compared to wild type (n=4). The KLF2-to-cyclophilin mRNA ratio for wild type was taken as 100%. Cyclophilin A mRNA was used as an internal standard for qRT-PCR. Error bars indicate standard deviation. * p < 0.025
BrdU, an average of 18.8% of wild-type erythroblasts are in G0/G1, 79.7% are in S-phase and 1.5% are in G2/M (Fig. 3.8). It was somewhat surprising that such a high percentage of normal E9.5 cells are in S-phase. However, Malik et al. reported similar results for E10.5 erythroblasts (Malik et al. 2013). The cell cycle distribution of KLF2-/- erythroblasts is similar to wild-type, suggesting that KLF2 ablation alone does not affect cell cycle progression. KLF1-/- and KLF1-/-/KLF2-/- embryos have an approximately 20% reduction in the number of cells in S-phase, compared to wild-type. This is accompanied by an approximately 2-fold increase in the percentage of cells in the G0/G1 phase of the cell cycle, suggesting a block in S-phase entry. There were no significant differences between the cell cycle profiles of KLF1-/- and KLF1-/-/KLF2-/- erythroblasts. The data suggest that KLF1 has a unique role in regulating cell cycle progression in E9.5 erythroblasts that is not shared by KLF2. Simultaneous ablation of KLF2 does not exacerbate the cell cycle disruption in KLF1-/- red blood cells. This work with E9.5 KLF1-/- erythroblasts extends and confirms other reports of cell cycle defects in KLF1-/- blood cells (Pilon et al. 2008; Tallack et al. 2009).

3.2.4 KLF1-/-/KLF2-/- embryos have defects in primitive erythroid precursor maturation

The paucity of peripheral blood cells in KLF1-/-/KLF2-/- embryos may result from a decrease in the number or a defect in the differentiation of erythroid progenitors. To determine whether KLF1 and KLF2 control the primitive erythroid progenitor (EryP-CFC) compartment, we performed colony forming assays on E8.25 - E8.5 wild-type, KLF1-/-,
Figure 3.8. KLF1/− and KLF1/−-KLF2/− blood cells show aberrations in G1- to S-phase progression during cell division. (A) Representative flow cytometry plots measuring DNA content (7-AAD) and BrdU incorporation (APC-BrdU) are shown for WT and KLF1/−-KLF2/− embryos. The graph below depicts analyses of the percentage of cells in G0/G1, S and G2/M phases of the cell cycle in wild-type (WT), KLF2/−, KLF1/− and KLF1/−-KLF2/− E9.5 erythroblasts. n = 3-9 for each genotype. Error bars indicate standard error. * = p-value <0.01 compared to wild-type. This set of experiments was done with assistance from Kristen Wade.
KL2-/- and KLF1-/-KLF2-/- embryos (Fig. 3.9A). At this stage of development, EryP-CFC colony numbers reflect the number of erythroid progenitors present in the yolk sac. On day 4 of culture, visibly red primitive erythroid colonies are observed in wild-type (n=10) cultures (Fig. 3.9A); however, no red colonies are visible in KLF1-/-KLF2-/- (n=4) cultures, indicating a defect in the erythroid compartment.

To further assess this defect, colonies in methylcellulose were stained with benzidine for hemoglobin, and observed on day 6 of culture. KLF1-/- embryos gave rise to normal numbers of EryP-CFCs (Fig. 3.9B), but the colonies are approximately 10-fold smaller than wild-type (Fig. 3.9C). Interestingly, KLF2-/- embryos produce significantly fewer EryP-CFCs than wild-type at day 6 (Fig. 3.9B, p<0.016), but colony size is not distinguishable from wild-type (Fig. 3.9C). The number of EryP-CFCs derived from KLF1-/-KLF2-/- embryos is significantly less than wild-type (Fig. 3.9B, p<0.001), KLF2-/- (p<0.028) and KLF1-/- (p<0.02). The KLF1-/-KLF2-/- colonies are also smaller than wild-type, although similar in size to KLF1-/--. It was of interest to distinguish whether there are fewer than normal progenitors in KLF1-/-KLF2-/- embryos, or if they are unable to produce colonies that survive to day 6 of culture. Therefore the method of Malik et al. was used to count nascent colonies on day 2 of culture (Malik et al. 2013). There are similar numbers of EryP-CFC in KLF1-/-KLF2-/- (n=3) and KLF1+/+KLF2+/+ (n=3) on day 2 (Fig. 3.9D). This suggests that normal numbers of primitive erythroid progenitors are specified in KLF1-/-KLF2-/- embryos, but the simultaneous ablation of KLF1 and KLF2 leads to a stochastic defect in maturation of erythroid precursors, such that fewer colonies are observed on day 6 of culture. The KLF1 and KLF2 genes interact to synergistically control colony number. Thus aberrant precursor potential and
Figure 3.9. KLF1-/-KLF2-/- embryos have defects in primitive erythroid precursor maturation. E8.25 - E8.5 implants were dissociated into single cells using trypsin and plated in methylcellulose supplemented with growth factors. Erythroid colonies were stained with benzidine and counted on day 2 or day 6 of culture. (A) Schematic diagram of experimental procedure (not drawn to scale). (B) Comparison of number of EryP-CFC colonies formed per 10^4 cells plated from wild-type (WT), KLF2-/-, KLF1-/- and KLF1-/-KLF2-/- implants on day 6 of culture. The average of number of colonies formed per 10^4 cells plated from wild type implants was set to 100% for each litter. 'n' indicates number of embryos. (C) The average colony size was determined by measuring the two-dimensional area of colonies from captured images. Average colony size of KLF2-/-, KLF1-/- and KLF1-/-KLF2-/- colonies was compared to size of wild-type colonies, and significant differences and p-values are shown by brackets. 'n' indicates number of colonies analyzed from 2 to 6 embryos of each genotype. Representative colonies from each genotype are shown below the respective genotype (100X magnification, scale bar is 50μm). (D) Comparison of number of EryP-CFC colonies formed per 10^4 cells plated from KLF1+/KLF2+/+ and KLF1-/-KLF2-/- implants on day 2 of culture. The average of number of colonies formed per 10^4 cells plated from KLF1+/KLF2+/+ implants was set to 100. 'n' indicates number of embryos. Error bars indicate standard deviation.
proliferation capacity likely contribute to the reduction in the number of circulating blood cells in E10.5 KLF1-/-KLF2-/-embryos.

3.2.5 KLF1 and KLF2 modulate the expression of genes controlling proliferation

Microarray analyses had been performed to demonstrate that the expression of five proliferation-associated genes, Foxm1, Cd24a, Myc, Sphk1 and Pthr, is progressively reduced in wild-type, KLF1-/- and KLF1-/-KLF2-/- E10.5 blood cells (Pang et al. 2012). Therefore, qRT-PCR was used to assess expression of these genes in wild-type, KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 blood cells. The amounts of all five mRNAs are significantly reduced in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- blood cells compared to wild-type, verifying the microarray analyses. Foxm1 mRNA is the only mRNA that is significantly reduced in KLF1+/-KLF2-/- and KLF1-/-KLF2-/- compared to KLF1-/-KLF2+/- (Fig. 3.10A), specifically correlating with the anemia phenotype and reduced peripheral blood cell number. Sphk1 mRNA amounts are significantly lower in KLF1-/-KLF2-/- than in KLF1-/-KLF2+/-, but not in KLF1+/-KLF2-/- compared to KLF1-/-KLF2+/- (Fig. 3.10B). Myc mRNA amounts are not significantly different between KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- (Fig. 3.10C). Cd24a and Pthr mRNA amounts are reduced in KLF1-/-KLF2-/- compared to KLF1+/-KLF2-/-, negatively correlating with anemia phenotype (Fig. 3.10D and E). Thus, reduced expression of all 5 genes may contribute to the decreased number of erythroid cells in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos compared to wild-type. However, FoxM1 is the only gene that correlates with the E10.5 anemia phenotype, having lower expression in KLF1+/-KLF2-/- than in KLF1-/-KLF2-/- blood cells.
Figure 3.10. KLF1 and KLF2 modulate the expression of genes involved in proliferation. qRT-PCR was used to determine the amount of (A) Foxm1, (B) Sphk1, (C) cMyc mRNA, (D) Cd24a and (B) Pthr mRNA in wild-type (WT), KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 blood cells. Gapdh mRNA was used as an internal standard. Wild-type was taken as 100%. n= 4-10 per genotype. All of the genotypes (KLF1-/-KLF2+/-, KLF1+/+KLF2-/- and KLF1-/-KLF2-/-) were significantly different from the wild-type (* = p< 0.0001). Other significant p-values are as shown by brackets. Error bars indicate standard deviation. This work was done with assistance from Safa Mohamad.
3.3 DISCUSSION

The KLF1 and KLF2 genes interact to control globin gene regulation and primitive erythroid precursor maturation. Interactions between related transcription factor genes in erythroid cells have previously been reported. There are normal numbers of primitive erythroid cells in Gata1 null E9.5 yolk sac blood islands, although these cells are less differentiated than wild-type (Fujiwara et al. 1996). E9.5 Gata2 knockout embryos have fewer erythroid cells than wild-type (Tsai et al. 1994). Gata1/Gata2 double knockout embryos show a more severe defect in primitive erythropoiesis than either Gata1 or Gata2 knockout embryos, with yolk sac blood islands almost completely devoid of erythroid cells (Fujiwara et al. 2004). Functional overlap has recently been described for KLF3 and KLF8, which act as transcriptional repressors. KLF3 knockout mice are viable with a mild erythroid phenotype (Funnell et al. 2012; Sue et al. 2008). KLF8 knockout mice are viable with normal erythroid parameters (Funnell et al. 2013). The KLF3/KLF8 double knockout causes embryonic lethality, and greater dysregulation of fetal liver gene expression than either single knockout (Funnell et al. 2013).

We have demonstrated that the reduced number of E10.5 KLF1-/-/KLF2-/- peripheral blood cells stems from a defect in erythroid precursor maintenance and maturation. Primitive erythroid progenitor colonies (EryP-CFC) derived from E8.5 KLF1-/- embryos are similar in frequency but significantly smaller in size than wild-type. Previous studies of definitive fetal liver erythroid progenitors produced somewhat variable results with respect to the frequency of BFU-E and CFU-E colony forming units. Perkins et al. found a similar number of BFU-E and CFU-E in E15 KLF1-/- and wild-type
mice (Perkins, Sharpe, Orkin 1995). Pilon et al. used sorted erythroid progenitors (CD71\textsuperscript{LO}, Ter119\textsuperscript{NEG}) from E13.5 KLF1-/- fetal livers, and also found wild-type frequencies of BFU-E (Pilon et al. 2008). However, Pilon et al. observed an increased frequency of CFU-E in KLF1-/- compared to wild-type embryos, which was attributed to a block in erythropoiesis. Interestingly, they observed that KLF1-/- fetal livers have significantly fewer cells than wild-type (Pilon et al. 2008). In addition, BFU-E and CFU-E colonies from KLF1-/- embryos contain less hemoglobin and require 28-48 hours longer to reach the same size as wild-type BFU-E and CFU-E, suggesting a defect in proliferation, analogous to our findings for embryonic progenitors (16). This suggests that KLF1, though present in a lower amount in primitive than in definitive erythroid cells (Alhashem et al. 2011; Zhou et al. 2006), plays a similar role in proliferation at both stages.

A novel role for KLF2 in the maintenance of mouse primitive erythroid precursors has been identified in this work. This correlates with ENCODE data from the Ross Hardison laboratory, which indicates that KLF2 is robustly expressed in mouse adult megakaryocyte-erythroid progenitors (MEPs) (UCSC Accession: wgEncodeEM003184). Investigations into the role of KLF2 in definitive erythroid progenitors have produced discordant results. Wani et al. reported a severe reduction in the number of CFU-E derived from E11.5 KLF2-/- fetal liver progenitors compared to wild-type (Wani, Means, Lingrel 1998). However, Kuo et al. found normal numbers of erythroid colonies in KLF2-/- fetal liver cultures (Kuo et al. 1997). Our studies reveal that KLF2-/- embryos have fewer primitive erythroid progenitor colonies than wild-type embryos on day 6 of culture. KLF1 can partially compensate for the lack of KLF2, because fewer EryP-CFC are
obtained from KLF1-/-KLF2-/- than from KLF2-/- embryos. Interestingly, there are normal numbers of erythroid colonies derived from KLF1-/-KLF2-/- embryos on day 2 of culture. A model consistent with these observations is that only some KLF1-/-KLF2-/- colonies are maintained beyond day 2, due to a stochastic selection event. It has been established that KLF2 ablation leads to increased apoptosis in E10.5 yolk sac erythroid cells (Basu et al. 2005), and there is some evidence of a modest increase in apoptosis in KLF1-/- definitive erythroid cells (Tallack et al. 2012). Although it is not possible to study apoptosis directly in E8.5 erythroid progenitor cells in this model system, the stochastic event in KLF1-/-KLF2-/- cultures might logically involve apoptosis.

The importance of KLF1 and KLF2 gene dosage for the regulation of several proliferation genes has been demonstrated. The transcription factor FoxM1 stimulates proliferation by promoting S-phase and M-phase entry in the cell cycle (Wierstra 2013). FoxM1-/- embryos have approximately 3-fold fewer cardiomyocytes, with defects in DNA replication and mitosis (Ramakrishna et al. 2007), but no increase in apoptosis was noted in these cells or in other FoxM1-/- mouse models (Ramakrishna et al. 2007; Wierstra and Alves 2007). FoxM1 mRNA is present in lower amounts in KLF1+/-KLF2-/- than in KLF1-/-KLF2+/- embryonic erythroid cells, suggesting that it is more responsive to KLF2 than KLF1, and correlating with the anemia phenotype in E10.5 KLF1+/-KLF2-/- but not KLF1-/-KLF2+/- embryos. Although the difference in FoxM1 expression between KLF1-/-KLF2+/- and KLF1+/-KLF2-/- embryos is modest, we speculate that small changes could have large effects in a milieu where the expression of multiple proliferation genes is dysregulated. A requirement for KLF2 in the regulation of a set of genes, including Foxm1, may contribute to the reduced number of circulating blood cells.
in KLF1+/-KLF2-/- compared to KLF1-/-KLF2+/- embryos. In addition, there is a modest increase in KLF2 mRNA in KLF1-/- blood cells, possibly contributing to the KLF1-/-KLF2+/- phenotype.

In summary, the Krüppel-like transcription factors KLF1 and KLF2 are critical for embryonic erythropoiesis. They are expressed at similar amounts and display functional compensation in primitive erythroid cells. This is evident in the dramatic anemia phenotype observed in KLF1/KLF2 double knockout but not in KLF1 or KLF2 single knockout embryos at E10.5. In this study, we show that the anemia is primarily due to a reduction in the number of peripheral blood cells. Surprisingly, the anemia and the number of erythroid cells observed in E10.5 embryos is dose dependent, with more peripheral blood cells in KLF1-/-KLF2+/- than in KLF1+/-KLF2-/- embryos. KLF1 and KLF2 have a greater than additive positive effect on maintaining the colony forming ability of E8.5 erythroid progenitor cells, but KLF2 depletion alone leads to a reduced number of colonies compared to wild-type. These data suggest that certain genes involved in the maturation or proliferation of red blood cells are preferentially responsive to regulation by KLF2, as opposed to KLF1. Further studies of the roles of KLF1 and KLF2 in erythroid precursor maturation may lead to novel therapeutic strategies for the anemias.
Chapter 4: Control of β-like globin gene expression in an *in vitro* model of human fetal erythropoiesis

4.1 INTRODUCTION

Hemoglobinopathies are a group of genetic disorders that affect millions of people worldwide (Weatherall 2006). They are caused by abnormalities in the structure or amount of hemoglobin produced in red blood cells. Hemoglobin, the protein responsible for transport of oxygen from the lungs to the various tissues of the body, is a tetramer composed of two α-globin chains and two β-globin chains, each of which is tightly linked to an iron-containing heme group. Sickle cell anemia and β-thalassemia are two of the most common hemoglobinopathies, and they result from defects in the structure and amount of β-globin chains, respectively. The year 2010 marked a century since the initial description of sickle cell disease (Herrick 2001). This period has been an era of intense research aimed at elucidating the molecular pathophysiology of sickle cell disease and other hemoglobinopathies (Orkin and Higgs 2010; Sankaran and Nathan 2010). The advent of molecular biology in particular has greatly advanced our understanding of disease mechanisms. However, affected patients still lack a widely applicable and easily available cure. It is hoped that by 2025, the year that marks the centenary of the initial reports of thalassemia, new therapies for the hemoglobinopathies will be on the horizon.

The human β-globin locus is situated on chromosome 11 and consists of 5 β-like globin genes: ε, Gγ, Aγ, δ and β-globin. These genes are developmentally regulated and expressed in the order in which they are present on the chromosome. Thus ε-globin is
expressed during early embryonic development in yolk sac-derived primitive erythroid cells, \( \gamma \)-globin during the fetal period in fetal liver-derived definitive erythroid cells and \( \delta \)- (\(<2\%\)) and \( \beta \)-globin (\(~98\%) during adult life in bone-marrow derived definitive erythroid cells. This sequential change in \( \beta \)-like globin gene expression is termed “hemoglobin switching”. In the majority of people, fetal \( \gamma \)-globin expression is silenced after birth to barely detectable levels (\(<2\%\)). However in some people \( \gamma \)-globin continues to be expressed throughout the person’s lifetime. The level of \( \gamma \)-globin expressed varies greatly from case to case. This phenomenon is known as hereditary persistence of fetal hemoglobin (HPFH) and is clinically asymptomatic (Forget 1998). It was observed that patients with sickle cell anemia or \( \beta \)-thalassemia who also have HPFH manifest less severe disease symptoms, suggesting that \( \gamma \)-globin can substitute for \( \beta \)-globin in adult erythrocytes (Weatherall 2001). This discovery prompted researchers to study the molecules and mechanisms that control hemoglobin switching, with the aim of re-expressing \( \gamma \)-globin in patients with \( \beta \)-hemoglobinopathies. In order to achieve this goal, it is imperative that we gain a better understanding of two important aspects of \( \gamma \)-globin gene regulation: (1) activation of gene expression or the mechanisms by which the \( \gamma \)-globin gene is turned on in the fetus and (2) repression of gene expression or the mechanisms by which the \( \gamma \)-globin gene is silenced in adults. A composite approach that encompasses both these complimentary regulatory mechanisms will likely produce the most effective therapeutic strategy.

Recent studies have implicated Krüppel-like factor 1 (KLF1), a member of the Krüppel-like factor family of Cys2-His2 zinc finger transcription factors, as having a substantial role in the \( \gamma \)- to \( \beta \)-globin switch (Borg et al. 2010; Zhou et al. 2010). Borg et
al determined through linkage analysis that a heterozygous nonsense mutation in KLF1 was responsible for HPFH in members of a Maltese family (Borg et al. 2010). Knockdown of KLF1 in erythroid cells derived from adult CD34+ hematopoietic progenitors resulted in increased expression of γ-globin (Borg et al. 2010; Zhou et al. 2010). It has been proposed that this negative regulation of γ-globin by KLF1 is an indirect effect through Bcl11a, a repressor of γ-globin, which shows decreased expression in this system (Borg et al. 2010; Zhou et al. 2010). KLF1 regulation of Bcl11a may be direct since KLF1 binds to the Bcl11a promoter (Sankaran et al. 2008).

In contrast to these results, studies using compound KLF1-/- / β-YAC transgenic mice have shown that there is decreased expression of γ-globin in yolk sacs of E10.5 embryos (Alhashem et al. 2011). Furthermore, KLF1 binds to the γ-globin promoter in primitive erythroid cells from wild-type β-YAC transgenic mice. Of particular interest was the observation that KLF2 also binds to the γ-globin promoter in these cells and compound KLF2-/- / β-YAC transgenic mice show a modest decrease in γ-globin expression (Alhashem et al. 2011). While the mouse is an invaluable model system in many respects, one drawback of studying human globin gene regulation in a mouse model is that γ-globin is expressed in the fetal liver stage in definitive erythroid cells in humans, whereas transgenic γ-globin is expressed in the primitive stage in yolk sac erythroid cells in mice. This difference in regulation may reflect a difference in the availability or function of various trans-acting factors in the mouse compared to humans.

In order to delineate the role of KLF1 in human fetal globin gene regulation we used erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitor cells, as a model more representative of the fetal milieu than analogous
models derived from adult peripheral blood CD34+ cells. In these cells, γ- and β-globin are expressed at similar levels on day 8 of erythroid differentiation (Fig 4.1A), confirming that these in vitro cultured cells are similar to the erythroid cells of a new-born in their hemoglobin phenotype, and γ-globin expression is not yet silenced as it is in adult erythroid cells (Alhashem 2012, Weatherall 2001). The pattern of KLF1 and KLF2 expression in these cells is similar to the pattern observed in mouse fetal liver definitive erythroid cells, with ten-fold higher expression of KLF1 compared to KLF2 (Fig. 4.1B) (Alhashem 2012, Alhashem et al. 2011). Both KLF1 and KLF2 bind to the γ-globin promoter in these cells. However, there is greater enrichment of KLF1 binding to the β-globin promoter than to the γ-globin promoter (Fig. 4.2A). Furthermore, KLF2 displays an impressive enrichment at the γ-globin promoter, whereas it does not bind to the β-globin promoter (Fig. 4.2B) (Alhashem 2012).

These initial studies suggest that KLF1 may have a more influential role in β-globin gene regulation, and KLF2 may be important for γ-globin gene regulation. Based on these results, we wished to determine first whether the role of KLF1 in regulating the expression of γ-globin in fetal erythroid cells is similar to or divergent from its role in adult erythrocytes. Our second aim was to investigate whether KLF2 participates in the regulation of globin gene expression in definitive erythroid cells, particularly with respect to control of γ-globin expression. Since KLF2-/- mice die early in development around the time of onset of definitive erythropoiesis, it is difficult to study its role in definitive
Figure 4.1: Expression patterns of (A) $\gamma$- and $\beta$-globin and (B) KLF1 and KLF2 in erythroid cells derived from cord blood hematopoietic precursors.
Adapted from Alhashem 2012.
Figure 4.2: Binding of (A) KLF1 and (B) KLF2 to various sites in the β-globin locus in erythroid cells derived from cord blood hematopoietic precursors. Adapted from Alhashem 2012.
erythroid cells in the mouse. Our previous research has revealed that KLF1 and KLF2 have overlapping roles in globin gene regulation in the mouse. It was therefore of interest to determine whether KLF1 and KLF2 have similar functions in human globin gene regulation.
4.2 RESULTS

*Sections 4.2.1 to 4.2.4 of the following work were done in collaboration with Dr. Yousef Alhashem.

4.2.1 KLF1 is a positive regulator of β-globin expression in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors

In order to determine whether KLF1 positively regulates β-globin expression in human fetal erythroid cells, CD34+ hematopoietic progenitor cells were isolated from umbilical cord blood samples infected with lentiviral particles carrying KLF1-targeted shRNA-coding sequences, and differentiated along the erythroid lineage as described in Methods section 2.2. This system is considered to be more representative of a fetal erythroid milieu in comparison to a parallel system that uses CD34+ hematopoietic progenitors isolated from adult mobilized peripheral blood. On day 8 of differentiation (DD8), erythroid cells were harvested and processed for RNA extraction to analyze gene expression. Two different shRNAs, K1V1 and K1V2, that target different seed sequences in the KLF1 mRNA, were used to knock down KLF1 by RNA interference. K1V1 produced a knockdown efficiency of approximately 50% (Yousef Alhashem, Fig 4.3). K1V2 produced a more efficient knockdown, ranging between 50% and 90% knockdown efficiency (Fig 4.3).

Because KLF1 is known to regulate numerous aspects of erythroid cell differentiation, it is important to confirm that modulation of KLF1 levels does not adversely affect maturation of erythroid cells and to ensure that test and control samples being compared are composed of similarly staged erythroid cells.
Figure 4.3: KLF1 knockdown efficiency. KLF1 mRNA amount was measured by qRT-PCR and normalized to Cyclophilin A mRNA as the internal standard for qRT-PCR. The amount of KLF1 in scramble-treated cells after normalization to Cyclophilin A was set to 100 for each individual/sample. $n = 3$ for K1V1 shRNA and $n = 11$ for K1V2 shRNA.

Figure 4.4: KLF1 knockdown does not adversely affect erythroid differentiation on differentiation day 8 (DD8). Representative flow cytometry plots for scramble shRNA-infected and K1V2 shRNA-infected cells showing that the majority of erythroid cells are double positive for CD71 and CD235a in both scramble-treated and K1V2 shRNA-infected cells on day 8 of differentiation. $n=3$ for each treatment level.
(Siatecka and Bieker 2011). In order to assess the stage of erythroid differentiation, cells were stained with APC-conjugated anti-CD71 (transferrin receptor) antibody and PE-conjugated anti-CD235a (Glycophorin A) antibody, as described in Chapter 2 (Section 2.2.5). The majority of cells infected with the control scramble shRNA (SCR) fall in the CD71⁺CD235a⁺ or double positive quadrant (79% ± 27.6, n = 3). Cells infected with the KLF1-targeted shRNA K1V2 also populate the CD71⁺CD235a⁺ quadrant (78% ± 21.7, n = 3), indicating that KLF1 knockdown does not affect erythroid maturation at day 8 of *in vitro* differentiation (DD8). Additionally, the stage of differentiation of scramble shRNA-infected cells was compared to the stage of differentiation of “mock” infected cells (cells that are not infected with a virus but which are cultured in the same manner as virus-infected cells) to ensure that viral infection does not compromise erythroid differentiation. The stage of differentiation of scramble shRNA-infected cells (79% ± 27.6 CD71⁺CD235a⁺, n = 3) is similar to that of mock-treated cells (70% ± 24.4 CD71⁺CD235a⁺, n = 3), confirming that viral infection does not alter erythroid differentiation in these cells. Representative flow cytometry plots for scramble shRNA-infected and K1V2 shRNA-infected cells are shown in Figure 4.4.

KLF1 knockdown resulted in decreased expression of β-globin mRNA compared to scramble shRNA controls, resembling observations in adult erythroid cells (Fig 4.5). The reduction in the amount of β-globin mRNA correlates positively and significantly with the residual amount of KLF1 mRNA in the cells (r² = 0.64, Prob>F = 0.0006, Fig 4.5). Thus the role of KLF1 in regulation of β-globin expression is similar in erythroid cells derived from CD34⁺ hematopoietic progenitors of both umbilical cord blood as well mobilized adult peripheral blood (Zhou et al. 2010).
Figure 4.5: KLF1 is a positive regulator of β-globin expression in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors. The amount of KLF1 mRNA and β-globin mRNA was measured by qRT-PCR and normalized to Cyclophilin A mRNA. The fold change in KLF1 expression and β-globin expression in each sample was calculated by setting the value for each gene in scramble controls to 100. A correlation was observed between the amount of KLF1 mRNA present in cells and the corresponding amount of β-globin expression by linear regression analysis. n = 14, $r^2 = 0.64$, Prob>F = 0.0006. Each dot on the correlation plot represents a biological sample, i.e. cells obtained from one umbilical cord. Samples that were infected with the K1V1 shRNA are represented as blue dots and samples that were infected with the K1V2 shRNA are represented as red dots.
Figure 4.6: Regulation of γ-globin expression by KLF1 in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors follows a non-linear pattern. The amount of KLF1 mRNA and γ-globin mRNA was measured by qRT-PCR and normalized to Cyclophilin A mRNA. The fold change in KLF1 expression and γ-globin expression in each sample was calculated by setting the value for each gene in scramble controls to 100. No correlation was observed between the amount of KLF1 mRNA present in cells and the corresponding amount of γ-globin expression by linear regression analysis. When the data was analyzed by allowing the statistical program JMP to fit a non-linear model to the available data in an unbiased manner, the smooth curve shown in the figure was obtained. The non-linear model supports our interpretation of the data with regard to sensitivity of the regulation of γ-globin gene expression to the amount of KLF1 available in cells. n = 14. Each dot on the scatter-plot represents a biological sample, i.e., cells obtained from one umbilical cord. Samples that were infected with the K1V1 shRNA are represented as blue dots and samples that were infected with the K1V2 shRNA are represented as red dots.
4.2.2 Regulation of γ-globin expression by KLF1 in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors follows a non-linear pattern

We next analyzed expression of γ-globin after knockdown of KLF1. We observed that with partial KLF1 knockdown, when there is between 20-60% residual KLF1 in cells, there is a 1.5 to 2-fold increase in γ-globin expression compared to scrambled shRNA controls (Fig 4.6). This result concurs with previous observations in KLF1-depleted adult erythroid cells as well as in people with heterozygous mutations in the KLF1 gene with respect to the negative regulation of γ-globin expression by KLF1 (Borg et al. 2010; Zhou et al. 2010). However, with robust knockdown, when there is less than 10% residual KLF1 in cells, an approximately 40% reduction in γ-globin expression is observed (Fig 4.6). This result is in agreement with data obtained from KLF1 knockout mouse studies (Alhashem et al. 2011). Thus the relationship between the amount of KLF1mRNA present in cells and the corresponding amount of γ-globin expression appears to be non-linear in nature and not as straight-forward as previously believed.

4.2.3 KLF1 positively regulates Bcl11a expression

Studies in adult human erythroid cells have shown that Bcl11a is a repressor of γ-globin expression (Sankaran et al. 2008), and that Bcl11a is positively regulated by KLF1 (Borg et al. 2010; Zhou et al. 2010). We studied the expression of Bcl11a in umbilical cord blood derived erythroid cells to determine whether changes in Bcl11a expression may be an intermediate determining factor in the observed non-linear pattern of γ-globin regulation by KLF1. Bcl11a mRNA is decreased in KLF1 knockdown
Figure 4.7: KLF1 positively regulates Bcl11a expression. The amount of KLF1 mRNA and Bcl11a mRNA was measured by qRT-PCR and normalized to Cyclophilin A mRNA. The fold change in KLF1 expression and Bcl11a expression in each sample was calculated by setting the value for each gene in scramble controls to 100. A correlation was observed between the amount of KLF1 mRNA present in cells and the corresponding amount of Bcl11a expression by linear regression analysis. n = 14, \( r^2 = 0.67 \), Prob>F = 0.0012. Each dot on the correlation plot represents a biological sample, i.e. cells obtained from one umbilical cord. Samples that were infected with the K1V1 shRNA are represented as blue dots and samples that were infected with the K1V2 shRNA are represented as red dots.
Figure 4.8: KLF2 expression is upregulated in KLF1 knockdown erythroid cells. The amount of KLF2 mRNA was measured by qRT-PCR in scramble-treated and K1V2 shRNA-treated cells and normalized to Cyclophilin A mRNA. The fold change in KLF2 expression in each sample was calculated by setting the value for the amount of KLF2 mRNA in scramble controls to 100. n = 4, * = p<0.05.
cells compared to scramble controls, and the reduction in Bcl11a mRNA amounts positively correlates with the reduction in KLF1 mRNA in the respective sample \( (r^2 = 0.67, \text{Prob}>F = 0.0012, \text{Fig 4.7}) \). The pattern of Bcl11a expression obtained with KLF1 knockdown is more similar to the pattern of \( \beta \)-globin expression than to the pattern of \( \gamma \)-globin expression observed with KLF1 knockdown in erythroid cells derived from cord blood hematopoietic progenitors, suggesting that regulation of \( \gamma \)-globin expression by Bcl11a may not be a direct effect.

**4.2.4 KLF2 expression is upregulated in KLF1 knockdown erythroid cells**

Gene expression analyses in KLF1\(-/-\) mouse embryonic erythroid cells revealed that KLF2 mRNA expression is upregulated in the absence of KLF1. Since KLF1 and KLF2 have overlapping functions in erythroid gene regulation, this increase in KLF2 expression is suggestive of a compensatory mechanism. In order to determine whether a similar upregulation of KLF2 is observed when KLF1 mRNA amounts are diminished in human erythroid cells, we determined the amount of KLF2 mRNA in KLF1 knockdown cells compared to scramble control cells. KLF2 mRNA amount is approximately 4-fold elevated in KLF1 knockdown (K1V2) human erythroid cells compared to scramble (SCR) control cells \( (p\text{-value} = 0.023, n = 4) \), similar to observations in KLF1\(-/-\) mouse embryonic erythroid cells \( (\text{Fig 4.8}) \). This increase, although significant, is extremely variable between samples as is evident from the large standard deviation, perhaps indicating that KLF1 does not directly regulate KLF2 \( (\text{Fig 4.8}) \).
4.2.5 KLF2 positively regulates γ- and β-globin expression in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors

We hypothesized that KLF2 may positively regulate γ-globin expression and that an increased amount of KLF2 in KLF1 knockdown erythroid cells may be a critical factor in activating γ-globin expression in these cells. To determine whether KLF2 regulates γ-globin expression in human erythroid cells derived from cord blood CD34+ hematopoietic progenitors, we knocked down KLF2 mRNA in these cells by RNA interference. Two different shRNAs, K2sh_206 and K2sh_1529 that target different seed sequences in the KLF2 mRNA were used. The transduction efficiency was >70% with both KLF2-targeting shRNAs as well as with a control, scramble (SCR) shRNA (n=3 for each shRNA). K2sh_206 produced a knockdown efficiency of approximately 65% and K2sh_1529 produced a knockdown efficiency of approximately 50% (Fig 4.9).

In order to assess the stage of erythroid differentiation, cells were stained with APC-conjugated anti-CD71 (transferrin receptor) antibody and PE-conjugated anti-CD235a (Glycophorin A) antibody, as described above. The majority of cells infected with the control scramble shRNA (SCR) fall in the CD71+CD235a+ or double positive quadrant (80.5±0.35%, n = 2). Cells infected with both KLF2-targeted shRNAs, K2sh_206 (79.6±2.12%, n = 2) and K2sh_1529 (83.5±1.63%, n = 2), also populate the CD71+CD235a+ quadrant, indicating that KLF2 knockdown does not affect erythroid
Figure 4.9: KLF2 knockdown efficiency in erythroid cells derived from cord blood precursors. KLF2 mRNA amount was measured by qRT-PCR and normalized to Cyclophilin A mRNA as the internal standard for qRT-PCR. The amount of KLF2 in scramble-treated cells (SCR) after normalization to Cyclophilin A was set to 100 for each sample. n = 3 for each treatment level.
Figure 4.10: KLF2 knockdown does not adversely affect erythroid differentiation on differentiation day 8 (DD8). Representative flow cytometry plots for mock, scramble shRNA-infected, K2_sh206 shRNA-infected and K2_sh1529 shRNA-infected cells showing that the majority of erythroid cells are double positive for CD71 and CD235a in mock cells, scramble-treated cells and in both KLF2 shRNA-infected groups on day 8 of differentiation. n=3 for each treatment level.
maturation at day 8 of *in vitro* differentiation (DD8). Additionally, the stage of differentiation of scramble shRNA-infected cells is compared to the stage of differentiation of mock infected cells to ensure that viral infection does not compromise erythroid differentiation. The stage of differentiation of scramble shRNA-infected cells (80.5±0.35% CD71⁺CD235a⁺, n = 2) is similar to that of mock-treated cells (77.1±4.7% CD71⁺CD235a⁺, n = 2), confirming that viral infection does not alter erythroid differentiation in these cells. Representative flow cytometry plots for mock-treated, scramble shRNA-infected, K2sh_206 and K2sh_1529 shRNA-infected cells are shown in Figure 4.10.

KLF2 knockdown resulted in decreased expression of γ-globin mRNA compared to scramble shRNA controls, with both the KLF2-targeting shRNAs, K2sh_206 and K2sh_1529 (Fig 4.11). There was an approximately 25% reduction in the amount of γ-globin mRNA in erythroid cells infected with K2sh_206 compared to scramble-infected cells. Infection with the second KLF2 shRNA construct, K2sh_1529, resulted in an approximately 40% decrease in the amount of γ-globin mRNA compared to scramble-infected cells. Surprisingly, knocking down KLF2 also resulted in a decrease in the amount of β-globin mRNA in these cells compared to scramble controls (Fig 4.12). Knockdown of KLF2 with both K2sh_206 and K2sh_1529 resulted in approximately 40% less β-globin mRNA compared to scramble shRNA-treated cells.
Figure 4.11: KLF2 positively regulates γ-globin expression in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors. The amount of γ-globin mRNA was measured by qRT-PCR in scramble-treated and KLF2 shRNA-treated cells and normalized to Cyclophilin A mRNA. The fold change in γ-globin expression in each sample was calculated by setting the value for the amount of γ-globin mRNA in scramble controls to 100. n = 3 for each treatment level, * = p<0.05.

Figure 4.12: KLF2 positively regulates β-globin expression in erythroid cells derived from human umbilical cord blood CD34+ hematopoietic progenitors. The amount of β-globin mRNA was measured by qRT-PCR in scramble-treated and KLF2 shRNA-treated cells and normalized to Cyclophilin A mRNA. The fold change in β-globin expression in each sample was calculated by setting the value for the amount of β-globin mRNA in scramble controls to 100. n = 3 for each treatment level, * = p<0.05.
4.3 DISCUSSION

The recent development of a human *in vitro* primary cell culture system has enabled researchers to study mechanisms that control regulation of the human \( \beta \)-globin genes, particularly regulation of the \( \gamma \)-globin gene, in their natural environment, i.e. in human erythroid cells, as opposed to studying their regulation in foreign cells such as mouse cells or compromised cells such as erythroleukemia cells (Migliaccio et al. 2002). Using this model, erythroid cells are obtained for study by isolation of CD34+ hematopoietic progenitor cells from mobilized adult peripheral blood, umbilical cord blood, or bone marrow, followed by expansion and differentiation along the erythroid lineage. A further advantage of this model is the ability to manipulate the expression of various genes of interest by RNA interference and study downstream effects. The focus of our research is to understand the role of Krüppel-like transcription factors, KLF1 and KLF2, in regulation of the \( \gamma \)-globin gene. We are particularly interested in mechanisms that control activation of \( \gamma \)-globin expression. As \( \gamma \)-globin is normally expressed in fetal liver erythroid cells in humans, we isolated CD34+ hematopoietic progenitors from umbilical cord blood and differentiated them into erythroid cells, representing a more “fetal” than “adult” model system for the study of globin gene regulation (Alhashem 2012). We have employed this model to study the effect of depletion of either KLF1 or KLF2 mRNA on regulation of the \( \beta \)-like globin genes. This led to the identification of novel roles for both KLF1 and KLF2 in regulating human \( \beta \)-like globin gene expression.

Studies using erythroid cells derived from adult hematopoietic progenitors had previously demonstrated that depletion of KLF1 results in derepression of the \( \gamma \)-globin
gene in these cells (Borg et al. 2010; Zhou et al. 2010). We have shown here that γ-globin expression is sensitive to KLF1 dosage. When KLF1 is depleted in the fetal model such that there is between 20% and 60% remaining KLF1 in cells, γ-globin expression elevated approximately 1.5- to 2.5-fold higher than expression in control cells. The degree of up-regulation in γ-globin expression varies greatly from sample to sample, or in other words, between people. For example, out of two samples with 55-60% remaining KLF1, one showed a 2-fold increase in γ-globin expression whereas the other showed a 1.3-fold increase. Similarly, out of four samples that had 15-20% remaining KLF1, two showed a 1.4-fold and 1.6-fold increase in γ-globin expression while the other two showed no change in γ-globin expression compared to control. This variation in the γ-globin up-regulation is also observed in people with heterozygous mutations in KLF1. Ten individuals from a Maltese family who have the same KLF1 mutation varied in HbF levels between 3% and 19.5% (Borg et al. 2010). Fluctuations in γ-globin up-regulation are probably the result of differences between people at certain loci that behave as genetic modifiers. For example, heterozygosity at SNP rs766432 in the Bcl11a locus may contribute to some of the observed variation in the Maltese HPFH pedigree (Borg et al. 2010). Additionally, the increase in γ-globin expression observed in our experiments with cells of cord blood origin, with KLF1 knockdown, is less pronounced than the corresponding 6- to 10-fold increase seen in adult erythroid cells in which KLF1 levels are depleted (Borg et al. 2010; Zhou et al. 2010). These differences may reflect inherent limitations in the capacity of gene activation, since γ-globin is already actively expressed in erythroid cells of the fetal model whereas it is almost completely silenced in the adult model.
We have shown here for the first time that depletion of KLF1 mRNA amount to less than 10% of normal levels results in decreased expression of the γ-globin gene. This finding corroborates observations in KLF1−/− β-YAC mice in which KLF1 ablation led to decreased γ-globin expression in mouse embryos (Alhashem et al. 2011). Interestingly, in both KLF1 knockdown human erythroid cells as well as KLF1−/− β-YAC mice, lack of KLF1 reduced γ-globin expression only by 50%, whereas β-globin expression is more severely affected, indicating that regulation of the β-globin gene is more dependent on KLF1 than regulation of the γ-globin gene. We hypothesize that elevated γ-globin expression in cells with partial KLF1 depletion is caused by dysregulation of modifiers of γ-globin expression, such as Bcl11a and KLF2, that are intermediate between KLF1 and γ-globin in the natural order of regulatory events and that may or may not be directly regulated by KLF1. Elevation in γ-globin expression may also result from reduced ability of the β-globin promoter to compete for the LCR due to a reduction in KLF1 binding to its promoter. We hypothesize that decreased γ- and β-globin expression in cells with almost complete KLF1 depletion may result from loss of LCR interactions with both the γ- and β-globin gene promoters due to diminished active chromatin hub formation at the β-locus and diminished recruitment of the γ- and β-globin genes to transcription factories, all of which require KLF1 (Drissen et al. 2004; Schoenfelder et al. 2010).

We demonstrate in this study that KLF2 plays a positive role in γ-globin gene regulation in human erythroid cells. Earlier evidence from β-YAC mice indicated that deletion of the KLF2 gene causes decreased γ-globin expression during primitive
erythropoiesis in mouse embryos (Alhashem et al. 2011). It has not been possible to study the effect of KLF2 ablation on definitive erythropoiesis in mice due to early embryonic lethality (Kuo et al. 1997; Wani, Means, Lingrel 1998). Partial depletion of KLF2 in our in vitro human fetal erythroid cell model resulted in a modest decrease in $\gamma$-globin expression. KLF2 binds robustly to the $\gamma$-globin promoter in these cells (Alhashem 2012). It will be interesting to determine whether the amount of the decrease in $\gamma$-globin expression correlates with the amount of the decrease of KLF2 in these cells. KLF2 depletion also resulted in reduced $\beta$-globin expression. This observation came as a surprise because KLF2 neither regulates the mouse $\beta^{maj}$-globin gene, nor does it bind to the adult $\beta$-globin promoter in either mouse or cord blood derived human erythroid precursor cells. A decrease in the amount of both $\gamma$- and $\beta$-globin expression in erythroid cells is often indicative of an inhibition of erythroid differentiation. We have assessed the levels of CD71 and CD235a by flow cytometry and found that the distribution of these erythroid cell surface markers is not altered in KLF2 knockdown cells compared to control cells. It is possible that marginal defects in the differentiation of erythroid cells may not be detected by this method because each gate of the flow cytometry analysis corresponds to more than one population of erythroid precursor cells (see section 1.1.3) (Pilon, Mol Cell Biol 2008). To address this possibility the relative number of proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatific erythroblasts will be determined in KLF2 knockdown samples compared to scramble-treated controls using Giemsa-stained cytospins (Alhashem 2012). The expression of $\alpha$-globin will also be compared in KLF2 knockdown and control samples.
If differentiation is unperturbed, the amount of α-globin expression should not vary between KLF2 knockdown and control samples.

An alternative explanation for reduced γ- and β-globin expression in KLF2 knockdown erythroid cells is that KLF2 may be required for normal chromatin structure at the β-locus. Although there is no prior evidence that KLF2 has a role in β-locus organization, it is possible that KLF2 may have a function similar to KLF1 in this respect. Changes in locus organization in the absence of KLF2 would explain the observed reduction in β-globin gene expression despite the fact that KLF2 does not bind to the β-globin promoter. 3C assays can be used to determine whether KLF2 is required for interactions between the LCR and the globin gene promoters. Another possibility is that KLF2 may regulate gene expression through interactions with histone acetyl transferases, p300/CBP and PCAF. There is evidence that the KLF1 and KLF2 proteins physically interact with p300/CBP and PCAF in cells (Das et al. 2006; SenBanerjee et al. 2004; Zhang and Bieker 1998). A reduction in activating histone marks, H3K4Me3 and H3K9Ac, and a reduction in RNA Polymerase II (RNAPol II) recruitment was observed at the β-globin promoter in KLF1 knockdown erythroid cells compared to control cells derived from cord blood precursors (Alhashem 2012). H3K4 trimethylation, H3K9 acetylation and RNAPol II recruitment will be assessed in KLF2 knockdown erythroid cells compared to controls to determine whether KLF2 affects activating histone marks across the β-locus. A third possibility is that KLF2 may indirectly regulate the β-globin gene by controlling the expression of other factors required for optimal β-globin expression. KLF1 expression was slightly reduced in KLF2-/- mouse embryonic yolk sacs (Basu P, unpublished data). The expression of KLF1 will be quantitated in
KLF2 knockdown human erythroid cells to determine whether KLF2 positively regulates KLF1 expression. If KLF2 positively regulates KLF1 expression, that would directly affect β-globin expression. Thus this study begins to address the role of KLF1 and KLF2 in human erythroid cells with a “fetal” pattern of β-like globin gene expression and offers many exciting avenues for further exploration. Figure 4.13 is a schematic that consolidates our current data obtained using the cord-blood derived erythroid cells with the existing model for globin gene regulation, centered on KLF1 and KLF2.
Figure 4.13: A schematic outlining β-like globin gene regulation by KLF1 and KLF2. KLF2 positively regulates KLF1 in mouse embryonic yolk sac cells. The question mark between KLF1 and KLF2 in the figure indicates that it is yet to be determined whether KLF2 regulates KLF1 expression in human erythroid cells.
Chapter 5: Discussion and Future Directions

The mouse has proved an invaluable system for the study of biological processes especially with regard to identifying gene function using gene knockout models. Striking advantages of the mouse as a model organism include the high degree of similarity between mouse and human genomes (>99%) and physiology, short gestation period and breeding cycle, large litter size, ease of genetic manipulation and relatively smaller space requirements than other mammals (Peters et al. 2007; Rosenthal and Brown 2007). Advances in our understanding of the control of globin gene regulation and the molecular pathophysiology of hemoglobinopathies have depended upon access to a plethora of mouse genetic tools (Bauer, Kamran, Orkin 2012; Tsiftsoglou, Vizirianakis, Strouboulis 2009). In particular, the mouse is a great system to study developmental processes like primitive erythropoiesis that are not as easy to study in humans due to ethical concerns and a lack of accessibility to relevant tissues during early embryonic development.

Like humans, mice show two waves of erythropoiesis, primitive erythropoiesis that originates in the yolk sac and definitive erythropoiesis that occurs at multiple sites within the embryo proper. The human β-globin locus consists of five β-like globin genes: ε, Gγ, Aγ, δ and β globin. Similar to humans, the mouse β-globin locus consists of four temporally and spatially regulated β-like globin genes: Ey, βh1, βmaj and βmin. Both the human and mouse β-globin genes are dependent on the presence of an upstream locus control region (LCR) for robust expression. Studies using gene-targeting strategies in mice to abolish the expression of specific genes have led to the elucidation of a network
of transcription factors that are important for erythropoiesis (Fig. 5.1) (Loose and Patient 2006; Swiers, Patient, Loose 2006). An added layer of specificity has been provided by the development of conditional knockout mice which allow cell-type-specific ablation of genes (Mikkola and Orkin 2005). Conditional knockout mice have been instrumental in dissecting cell-autonomous and non-cell-autonomous roles of factors that are expressed in multiple cell types. The primary advantage of a mouse model is that the effect of gene ablation can be studied in vivo, in the context of the entire organism replete with various intercellular and systemic interactions, as opposed to ex vivo cell culture systems in which cells are grown in relative isolation.

In this study we have used knockout models that ablate the expression of KLF1 and KLF2 in mice to study the overlapping roles of these related transcription factors in primitive erythropoiesis. We have also used a conditional knockout model to determine the erythroid cell-autonomous role of KLF2, which is expressed in lymphocytes and endothelial cells in addition to erythroid cells. Previous studies have shown that simultaneous ablation of KLF1 and KLF2 results in early embryonic lethality and severe anemia in mouse embryos (Basu et al. 2007). In this study, we show that this anemia is caused by a paucity of blood cells, and exacerbated by diminished β-like globin gene expression. The anemia phenotype is dose-dependent, and interestingly, can be ameliorated by a single copy of the KLF2, but not the KLF1 gene. The roles of KLF1 and KLF2 in maintaining both normal peripheral blood cell numbers and globin mRNA amounts are erythroid cell-specific. It was discovered that KLF2 has an essential function in erythroid precursor maintenance. KLF1 can partially compensate for KLF2 in this role, but is uniquely crucial for erythroid precursor proliferation, through its
Figure 5.1: A model genetic network that regulates erythroid development based on the results of gene-targeting studies in mice. Adapted from (Loose and Patient 2006; Swiers, Patient, Loose 2006)
regulation of G1- to S-phase cell cycle transition. A more drastic impairment of primitive erythroid colony formation from embryonic progenitor cells occurs with simultaneous loss of KLF1 and KLF2, than with loss of a single factor. The results of this study have been instrumental in designing experiments to explore the roles of KLF1 and KLF2 in human fetal and adult erythropoiesis. These discoveries are also pertinent to the development of therapeutic strategies to elevate $\gamma$-globin expression in adult erythroid cells. It is important that the results of this study and others similar to it, that delineate the basic biological processes controlled by various transcription factors, inform attempts to modulate the levels of these factors (including KLF1 and BCL11a) to elevate $\gamma$-globin expression.

The study of the overlapping roles of KLF1 and KLF2 in mouse embryonic erythropoiesis has provided us with clues regarding how these factors interact to regulate downstream gene expression and what processes they may co-regulate in mouse and human erythroid cells. However, it is not known how the gene interactions between KLF1 and KLF2 are functionally played out in cells. There is no evidence for dimerization of these factors. KLF1 and KLF2 bind to DNA via recognition of a CACCC sequence, which is the common binding motif for both factors, as well as for other KLFs. While some gene promoters have multiple CACCC binding sites, others have only one CACCC site. Additionally, enrichment of both KLF1 and KLF2 is often observed at the same CACCC site. This observation indicates that in a population of cells, KLF1 is bound to a certain CACCC site in a portion of alleles, whereas KLF2 is bound to the same site in a portion of alleles. Thus if both KLF1 and KLF2 have equal affinity to a particular CACCC binding site and compete for binding at that site, the respective level
of each protein in a cell may have functional significance to the cell’s transcriptional output. Furthermore, if either factor is evaluated as a potential therapeutic target or is downstream of a potential therapeutic target, the function of the other may also be dysregulated in this paradigm. In an endeavor to further characterize the functional overlap between KLF1 and KLF2, studies from our laboratory identified the subset of genes that are synergistically down-regulated in KLF1⁻/⁻-KLF2⁻/⁻ embryonic erythroid cells compared to KLF1⁻/⁻ and wild-type erythroid cells using microarray technology (Pang et al. 2012). These studies identified c-myc as a central factor in the network of genes co-regulated by KLF1 and KLF2. They also identified groups of genes belonging to certain functional categories including cellular proliferation, apoptosis and homeostasis, as significantly down-regulated in KLF1⁻/⁻-KLF2⁻/⁻ erythroid cells. For future experiments, it will be interesting to determine the genome-wide binding profile of KLF2 in erythroid cells by ChIP-seq and compare the binding profiles of KLF1 and KLF2 to each other and to the expression profiling results from KLF1/KLF2 double knockouts. To further our understanding of the functional redundancy of KLF1 and KLF2, it will be intriguing to determine whether expression of the KLF2 gene in a pattern similar to the KLF1 gene in erythroid cells can rescue lethality of KLF1⁻/⁻ embryos by generating a transgenic mouse model in which the KLF2 gene is expressed under the control of the KLF1 promoter. Gene interactions amongst related members of transcription factor families that are functionally relevant to hematopoiesis have been discovered for multiple groups of genes including the GATA family members, Gata1 and Gata2 (Fujiwara et al. 2004), the Runx family members Runx1, Runx2 and Runx3 (Wang et al. 2010), and more recently for the Snail family members Snai2 and Snai3 (Pioli and Weis
However, the mechanisms governing these interactions are not well understood. Observations from the study of bHLH transcription factors Hand1 and Hand 2 belonging to the Twist gene family, that display gene interactions and functional redundancy in cardiovascular development, have highlighted the importance of level of expression (gene dosage) of interacting genes and the effect of post-translational modifications on the outcome of gene interactions (Conway, Firulli, Firulli 2010). It is known that KLF1 function is regulated by various post-translational modifications including acetylation, phosphorylation and sumoylation (Siatecka and Bieker 2011). KLF2 is known to interact with E3 ubiquitin ligase WWP1, which suppresses its transactivation potential (Conkright, Wani, Lingrel 2001). It will be of interest to determine how these post-translational modifications affect KLF1 and KLF2 gene interactions by generating mice with point mutations of the relevant residues.

The major difference between humans and mice with respect to the β-globin locus is that in humans ε-globin is expressed in yolk sac primitive erythroid cells and γ-globin is expressed predominantly in fetal liver erythroid cells, whereas in mice both Ey and βh1 are expressed in embryonic erythroid cells and there is no fetal gene equivalent to the human γ-globin gene. In an effort to directly study regulation of the human β-globin locus, transgenic mice were generated that carry the entire human β-globin locus in addition to the endogenous mouse globin genes (Gaensler, Kitamura, Kan 1993; Peterson et al. 1993). These mice are popularly referred to as the β-YAC mice. Although the human β-like globin genes are expressed in a tissue- and developmental stage-specific manner in the β-YAC mice, γ-globin is expressed in yolk sac erythroid cells coincident with ε-globin and the endogenous mouse embryonic
globin genes and not in mouse fetal liver erythroid cells. This discrepancy in γ-globin expression is the main drawback of the β-YAC model, which has otherwise been instrumental in deciphering developmental control of globin gene regulation including elucidation of interactions between the LCR and the promoters of the globin genes.

Research in the last two decades has unveiled multifaceted roles for KLF1 in the control of globin gene regulation (reviewed in (Tallack and Perkins 2013). KLF1 directly regulates the expression of the β-globin gene by binding to its promoter at a CACCC site. KLF1 is required for physical interactions between the LCR and the β-globin promoter, as well as for the formation of an active chromatin hub at the β-locus (Drissen et al. 2004). In addition to its "local" functions at the β-globin locus, KLF1 also has more global regulatory portfolio. Intra- and inter-chromosomal associations between the β-globin gene and other KLF1-regulated genes, as well as recruitment of these genes to transcription factories were shown to be KLF1-dependent in erythroid cells (Schoenfelder et al. 2010).

There is dichotomy in the literature with respect to the role of KLF1 in γ-globin gene regulation. Evidence from studies in adult erythroid cells suggested that KLF1 negatively regulates the expression of the γ-globin gene (Borg et al. 2010; Zhou et al. 2010). These observations gained credence from the fact that certain people with heterozygous mutations in the KLF1 gene show elevated levels of γ-globin and HbF (Arnaud et al. 2010; Borg et al. 2010). However, studies using compound KLF1-/- / β-YAC transgenic mice had divergent results. At the embryonic stage, when γ-globin is actively expressed in these mice, KLF1 ablation caused a decrease in γ-globin expression (Alhashem et al. 2011). Furthermore, KLF1 was detected bound to the γ-
globin promoter in primitive erythroid cells from wild-type β-YAC transgenic mice. At the fetal liver stage, when γ-globin is silenced in the β-YAC mice, elevated γ-globin mRNA expression was observed in KLF1+/− and KLF1−/− fetal livers (Perkins, Gaensler, Orkin 1996; Wijgerde et al. 1996). However, in adult KLF1+/− mice expression of the γ-globin gene was silenced (Wijgerde et al. 1996). Results from our investigations into the role of KLF1 in erythroid cells derived from cord blood hematopoietic progenitors indicate that both increased γ-globin expression and decreased γ-globin expression can result from down-regulation of KLF1. Expression of the γ-globin gene appears to be sensitive to small changes in the amount of KLF1. Partial depletion of KLF1 (corresponding to haploinsufficiency) causes elevated γ-globin expression, while nearly complete depletion of KLF1 (similar to ablation) results in a down-regulation of γ-globin expression. It will be of interest to determine whether this dual mode of γ-globin gene regulation is accompanied by changes in LCR interactions with the γ-globin promoter, i.e. is there an increased frequency of interactions between the LCR and the γ-globin promoter in cells with elevated γ-globin expression and is there a decrease in the frequency of interaction between the LCR and the γ-globin promoter in cells with decreased γ-globin expression compared to control cells. Changes in the frequency of interaction between the LCR and the γ-globin promoter can be assessed using chromosome conformation capture (3C) assays.

Thus depletion of KLF1 in erythroid cells may have the dual advantage of down-regulating the expression of defective adult β-globin in patients with β-hemoglobinopathies while at the same time upregulating the expression of γ-globin.
Being an erythroid-specific transcription factor adds to the attraction of modulating levels of KLF1 as a therapeutic target for β-hemoglobinopathies. However, our current results, as well as extensive studies in KLF1 knockout mice, would advise caution. Numerous groups have shown that KLF1 regulates many other aspects of erythropoiesis in addition to globin gene expression (reviewed in Siatecka and Bieker 2011). KLF1−/− erythroid cells display morphological abnormalities including cell membrane and nuclear irregularities (Basu et al. 2007; Drissen et al. 2005), defective erythroid maturation (Basu et al. 2007; Pilon et al. 2008) and dysregulation of global gene expression in erythroid cells (Drissen, MCB 2005; Hodge, Blood 2006; Nilson, Exp Hem 2006; Pilon, MCB 2008; Tallack, Genome Res 2010; Pang, Mol Cell Biol 2012)(Drissen et al. 2005; Hodge et al. 2006; Nilson et al. 2006; Pang et al. 2012; Pilon et al. 2008; Tallack et al. 2010). Hence therapeutic modulation of KLF1 levels should be approached with careful consideration to possible repercussions.

Negative regulation of γ-globin by KLF1 is an indirect effect mediated partially by the repressor Bcl11a, although other factors are probably involved (Borg et al. 2010; Xu et al. 2013; Zhou et al. 2010). Evidence suggests that KLF1 directly regulates Bcl11a. There is much advocacy for the pharmaceutical targeting of Bcl11a to elevate γ-globin expression. While KLF1 expression appears to share a linear relationship with Bcl11a expression, this does not translate to a linear relationship between Bcl11a expression and γ-globin expression, suggesting that both KLF1 and Bcl11a are indirect negative regulators of γ-globin expression. This premise is supported by the fact that Bcl11a binds not to the promoter of the γ-globin gene but to an intergenic region between the γ- and δ-globin genes and interacts with numerous chromatin remodeling factors,
suggesting that it may play a role in maintaining locus configuration rather than having a direct effect on γ-globin repression (Sankaran et al. 2008). Thus modulation of Bcl11a levels alone may not prove to be an effective therapeutic strategy. Recent studies have identified Mi2-β, a component of the nucleosome remodeling and histone deacetylase (NURD) complex, as a potent negative regulator of γ-globin expression, that may prove to be an alternative therapeutic target (Amaya et al. 2013; Costa et al. 2012).

KLF2 is phylogenetically closely related to KLF1. Mouse KLF1 and KLF2 proteins are 83% identical to each other within their zinc finger binding domains, while human KLF1 and KLF2 are 84% identical to each other within this region. Due to this high degree of similarity, KLF1 and KLF2 are predicted to bind to the same CACCC consensus sequences. This observation led to the hypothesis and subsequent experimental confirmation that KLF1 and KLF2 have overlapping roles in regulating embryonic β-like globin gene expression in the mouse (Basu et al. 2007). Additionally, KLF2 binds to the γ-globin promoter in primitive erythroid cells from β-YAC transgenic mice and compound KLF2-/- / β-YAC transgenic mice show a modest decrease in γ-globin expression (Alhashem et al. 2011). The development of new in vitro culture techniques for the production and manipulation of human erythroid cells from mobilized adult peripheral blood and umbilical cord blood hematopoietic progenitors have enabled researchers to study the regulation of the human β-like globin genes in their natural cellular environment as opposed to earlier studies that depended on murine trans-acting factors (Migliaccio et al. 2002). We have used this system to study the effect of KLF2 depletion on the regulation of the γ- and β-globin genes in erythroid cells derived from cord blood hematopoietic progenitors. KLF2 positively regulates γ-globin expression in
these cells, analogous to observations in the β-YAC mice. Moreover KLF2 binds robustly to the γ-globin promoter in these cells, suggesting that regulation of γ-globin expression may be a direct effect (Alhashem 2012). The decrease in γ-globin expression was modest, ranging between 30% and 40% depending on which shRNA was used. KLF2 was knocked down by approximately 50% compared to the scramble control in these initial samples. Further experiments with more efficient knockdowns need to be done to determine whether decreases in the amount of γ-globin expression correlate with reductions in the amount of KLF2 in cells. In order to obtain better knockdown efficiency, two shRNAs targeting different seed sequences in the KLF2 mRNA will be used simultaneously. If regulation of γ-globin by KLF2 is a direct effect, KLF2 will be one of a very small group of factors, including NF-E4, TR2 and TR4, known to directly activate γ-globin expression (Sankaran and Orkin 2013). KLF1 and KLF2 are expressed at similar levels in mouse primitive erythroid cells, which is the stage at which γ-globin is expressed in the transgenic β-YAC mice. In β-YAC fetal liver erythroid cells, where γ-globin expression is silenced and β-globin is actively expressed, KLF1 expression is ten-fold higher than KLF2 expression (Alhashem et al. 2011). It will be particularly interesting to determine whether KLF2 is expressed at different levels in human “fetal” erythroid cells derived from cord blood precursors compared to “adult” erythroid cells derived from mobilized peripheral blood.

The CD34+ hematopoietic precursor derived erythroid cells have provided us with a new human model system in which we can study KLF1 and KLF2 gene interactions. KLF1 and KLF2 mRNA will be simultaneously depleted in this system by RNA interference. Because the KLF1-targeting shRNA plasmid has a GFP reporter
gene and the KLF2-targeting shRNA plasmid has an RFP reporter gene, it will be possible to retrieve cells that have been virally transduced with shRNAs targeting both KLF1 and KLF2 by FACS. The expression of the globin genes as well as other genes that are co-regulated by KLF1 and KLF2 will be assessed in KLF1/KLF2 double knockdown cells compared to KLF1 knockdown, KLF2 knockdown and scramble-treated cells. Additionally the stage of erythroid differentiation and definitive erythroid colony-forming ability of KLF1/KLF2 double knockdown cells will be determined. It will be of interest to determine whether KLF1 and KLF2 have overlapping functions in human definitive erythropoiesis, akin to their roles in mouse embryonic erythropoiesis.
Literature Cited


Vita

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