2012

REGULATION OF THE MOUSE AND HUMAN β-GLOBIN GENES BY KRÜPPEL LIKE TRANSCRIPTION FACTORS KLF1 AND KLF2

Yousef N. Alhashem
Virginia Commonwealth University

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REGULATION OF THE MOUSE AND HUMAN β-GLOBIN GENES
BY KRÜPEL LIKE TRANSCRIPTION FACTORS KLF1 AND KLF2

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

by

YOUSEF NASSIR ALHASHEM
Bachelor of Science, King Saud University, Saudi Arabia, 1999
Master of Science, Virginia Commonwealth University, 2009

Director: Dr. Joyce A. Lloyd
Professor, Vice Chair of Education
Department of Human and Molecular Genetics
School of Medicine

Virginia Commonwealth University
Richmond, Virginia
December 2012
ACKNOWLEDGEMENT

First I would like to thank Allah for His blessings. I would like to thank my parents for their prayer, care, and exceptional support throughout my life. I express my sincere thanks to my wife, Mariam Alhammad and my son Asama for surrounding me with their love and being with me throughout the past five years. I would like to thank the Ministry of higher education in Saudi Arabia and the Saudi Arabian Cultural Mission in Washington DC for their financial support.

I would like to express my sincerest gratitude to my advisor, Dr. Joyce Lloyd for her enthusiasm and patience in guiding me throughout my academic career. This piece of work will never be accomplished without her advice and comments. Her knowledge, insight, and wisdom inspired me and fueled my attitude to pursue my academic goals.

I would like to show appreciation to the former and current members in Dr. Lloyd’s lab. Special thanks to Mohua Basu for guiding me during the first days in the lab and helping me thereafter. Special thanks to Dr. Latasha Redmond and Tina Lung for their helpful training and technical support. Great thanks to Aditi Chiplunkar and Divya Vinjamur, for the great help and discussion.

I also would like to thank Dr. Gordon Ginder and Dr. Joe Landry and their lab members for the helpful discussion and using their instruments and reagents. I also thank the lab members of Dr. Taylor and Dr. Moran’s lab for using their instruments.

Finally, my best thank goes to my thesis committee members, Dr. Rita Shiang, Dr. David Williams, Dr. Gordon Ginder, and Dr. Jolene Windle for their precious advice and great insights throughout my PhD project.
DEDICATION

To my grandmother Sharifa Alsalih
who could not make it to the end of this endeavor.
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List of Abbreviations

% ....................... percent

α ....................... Alpha

β ....................... Beta

βh1 ................... beta-like embryonic chain (mouse hemoglobin Z)

βLCR .................. β-globin Locus Control Region

βmaj ................... beta major globin

βmin ................... beta minor globin

γ ....................... Gamma

δ ....................... Delta

ε ....................... Epsilon

ζ ....................... Zeta

θ ....................... Theta

μ ....................... micro

μg .................... micro-gram

μl .................... micro-Liter
\[ \chi^2 \] chi-square

2D two-dimensional

3C Chromosome Conformation Capture assays

A adenine

a.a. amino acid

AGM aorta-gonado-mesonephros

AHSP \( \alpha \)-hemoglobin stabilizing protein

BFU-E Burst forming unit erythroid

bHLH basic helix-loop-helix

BL-CFC blast colony forming cells

BrdU Bromodeoxyuridine

BTE Basic Transcription Element

C cytosine

CAT chloramphenicol acetyltransferase

CBP CREB-binding protein

CChIP Carrier ChIP

CD71 transferrin receptor
cDNA .................. complementary DNA

CE ...................... cornified envelope

CFU-E .................. colony-forming unit erythroid

ChIP ..................... Chromatin Immunoprecipitation

CO2 ....................... Carbon Dioxide

CYP1A1 ................. cytochrome P-450IA1

df ......................... degree of freedom

DNA ...................... Deoxyribonucleic acid

DNase .................... deoxyribonuclease

dNTP ....................... deoxyribonucleotides

E ......................... Embryonic day...

EGR1 ...................... early growth response 1 gene

EKLF ....................... Erythroid Krüppel Like Factor (KLF1)

EMSA ...................... electrophoretic mobility shift assay

ES ......................... embryonic stem cells

Ey ........................ beta-like embryonic chain (mouse hemoglobin Y)

FOG ........................ Friend of GATA
G..........................gram

G..................................guanine

GKLF..................Gut enriched Krüppel Like Factor (KLF4)

H.............................Hydrogen

Hb.............................hemoglobin

HbF ..................hemoglobin F (Fetal hemoglobin)

HbS ..................hemoglobin S (Sickle hemoglobin)

HEL....................Human erythroleukemia cell line

hES..................human embryonic stem cell

HLH ..................helix-loop-helix

Hprt ......................hypoxanthine phosphoribosyltransferase

HS ......................hypersensitive site

HSC.........................hematopoietic stem cells

HUVEC..................Human Umbilical Vein Endothelial Cells

IAA ....................Iso-Amyl Alcohol

INF .....................interferon

iPS .....................induced pluripotent stem cells
K562....................chronic myelogenous leukemia cell line

KCl....................Potassium Chloride

kDa....................kilo Dalton

KLF....................Krüppel Like Factor

KO......................knockout

LCR.....................Locus Control Region

LKLF...................Lung Krüppel Like Factor (KLF2)

LSS.....................laminar shear stress

M.........................Molar

MeCP.....................methyl-CpG-binding protein

MEF......................mouse embryonic fibroblasts

MEL......................Mouse erythroleukemia cell line

MgCl₂..................Magnesium Chloride

min......................minute

ml.......................milliliter

mM....................milliMolar

mRNA....................messenger RNA
O$_2$ ..................... Oxygen
° C .......................... degree celcius
OD .......................... optical density
PAS ......................... paraaortic splanchnopleura
PBS .......................... Phosphate Buffered Saline
PDGF ......................... platelet-derived growth factor
qRT-PCR .................... quantitative Real Time Polymerase Chain Reaction
RBC .......................... red blood cells
RNA ......................... Ribonucleic acid
RNase ......................... Ribonuclease
RT-PCR ...................... Reverse Transcriptase Polymerase Chain Reaction
SC ............................ stratum corneum
SCD .......................... Sickle cell disease
SCL .......................... stem cell leukemia gene
siRNA ....................... short interfering RNA
SMC .......................... smooth muscle cells
T ............................... thymine
TAL1 .................... T-cell acute lymphocytic leukemia 1

TER119 ............... Glycophorin A associated protein

TFIID ..................... transcription factor IID

TUNEL ................... Terminal deoxynucleotidyl transferase dUTP nick end labeling

WT ......................... wild type
ABSTRACT

REGULATION OF THE MOUSE AND HUMAN β-GLOBIN GENES

BY KRÜPPEL LIKE TRANSCRIPTION FACTORS KLF1 AND KLF2

By: Yousef Nassir Alhashem, PhD.

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

Virginia Commonwealth University, 2012]

Major Director: Dr. Joyce A. Lloyd
Professor, Vice Chair of Education
Department of Human and Molecular Genetics
School of Medicine

Krüppel-like factors KLF1 and KLF2 are closely related transcription factors with three zinc finger domains in their carboxy-termini. KLF1 (erythroid Krüppel-like factor, or EKLF) plays essential roles in embryonic and adult erythropoiesis. KLF2 is a positive regulator of the mouse and human embryonic β- globin genes. KLF1 and KLF2 have overlapping roles in embryonic erythropoiesis, as demonstrated using single and double knockout (KO) mouse models. Ablation of the KLF1 or KLF2 gene causes embryonic lethality, and double KO embryos are more anemic and die sooner than either single KO. We have shown that KLF1 and KLF2 positively regulate the human ε- (embryonic) and γ-globin (fetal) genes during embryonic erythropoiesis. Chromatin immunoprecipitation assays (ChIP) show that KLF1 and KLF2 bind to the promoters of the human ε- and γ-globin genes, the mouse embryonic Ey- and βh1-globin genes, and also to the β-globin locus control region (LCR) in mouse embryonic erythroid cells. ChIP assays show that KLF1 but not KLF2 ablation results in abnormal histone modifications in the β-globin locus in
mouse embryonic erythroid cells. H3K9Ac and H3K4me3, which correlate with open chromatin and active transcription, are both reduced in KLF1-/- primitive erythroid cells. Human CD34+ hematopoietic stem cells obtained from umbilical cord blood were in vitro differentiated along the erythroid lineage. ChIP assays indicate that both KLF1 and KLF2 bind to the promoter of γ-globin gene in this fetal erythroid model. KLF1 knockdown in these cells affects mainly adult β-globin gene expression. However, the decrease in β-globin gene expression in KLF1 knockdown also affects the ratio of γ- to β-globin in these cells. H3K9Ac and H3K4me3 were decreased only at the β-globin gene which coincides with lower recruitment of RNA polymerase II and its active form, RNA polymerase II phospho-serine 2. In conclusion, we showed using mouse primitive erythroid cells and cord blood definitive cells that KLF1 and KLF2 coordinate the regulation of the mouse and human β-globin genes by direct binding to the promoters and LCR in the β-globin locus. In conclusion, cord blood hematopoietic cells could serve as a complimentary system in addition to the transgenic mouse models to study the regulation of γ-globin gene expression.
CHAPTER ONE:
Introduction

1.1 Erythropoiesis

The process by which blood cells are produced is called hematopoiesis. It is a complicated process during which transcription factors, cell signaling, and chromatin remodeling complexes orchestrate proper and balanced blood cell production (Watkins et al., 2009). Arising from the mesoderm during the gastrulation process, hematopoietic and endothelial cells are considered the first differentiated cells in embryos (Baron and Fraser, 2005). In particular, red blood cells (RBCs, or erythrocytes) are required to ensure normal transportation of O$_2$ and CO$_2$ to and from tissues in mammals and other vertebrates. The O$_2$/CO$_2$ delivering vehicle in the erythrocyte is a tetrameric protein called hemoglobin. The process by which erythrocytes are created is called erythropoiesis, and it is divided into two unique processes: primitive and definitive erythropoieses (Baron and Fraser, 2005, McGrath and Palis, 2005, Fraser et al., 2007, Palis, 2008). Primitive erythropoiesis initiates from the extraembryonic mesoderm of the yolk sac as early as 14 days after conception in human, and as early as embryonic day 7.5 (E7.5) in mice (McGrath and Palis, 2005, Qiu et al., 2008). Definitive erythropoiesis, on the other hand, can be detected in the fetal liver of six-week human and E11.5 mouse embryos (Kingsley et al., 2006, Qiu et al., 2008).

During early mouse embryogenesis, around E7.5, a common precursor known as the hemangioblast differentiates into the endothelial and hematopoietic lineages in the yolk sac (Figure 1.1). The common parental lineage of the endothelial and hematopoietic cells was verified by finding blast colony forming cells (BL-CFC) with the potential to differentiate into...
both lineages (Huber et al., 2004). However, in other studies, primitive erythroblasts were found in the proximal yolk sac, where no endothelial cells were detected, indicating that hemangioblasts might not be the only source of erythroblasts (Fraser et al., 2007). Together, central hematopoietic and peripheral endothelial cells form what is called the blood islands, which develop in later stages into the vasculature and circulating blood of the yolk sac (Palis and Yoder, 2001, Ferkowicz and Yoder, 2005, Palis, 2008). Blood circulation starts upon initiation of the first heartbeat around E8.25 in mice (Ji et al., 2003, Palis, 2008). In mice, the circulating erythroblasts at this time are large nucleated primitive cells which undergo terminal differentiation to proerythroblasts then to enucleated erythrocytes. The terminally differentiated primitive erythrocytes are smaller in size, have higher hemoglobin content, and have less cell division potential than erythroblasts (Kingsley et al., 2006, Fraser et al., 2007, Palis, 2008). The fact that primitive erythroblasts lose their nuclei in the blood stream was verified by seeing extruded nuclei, called pyrenocytes, surrounded by rims of Ey-globin positive cytoplasm. However, primitive erythroblasts cannot lose their nuclei in vitro, signifying that other cells, such as macrophages may play a role in the enucleation process.

In mice, primitive erythroblasts mainly express the embryonic globins, ζ, βh1, and Ey-globins. However, small amounts of adult globins (α1, α2, β1, and β2) could be detected as early as E9.0 (Palis et al., 1999, McGrath and Palis, 2005).

The limited capacity of erythropoiesis in the yolk sac makes it unable to keep up with the exponentially growing embryo. Therefore, starting from day E9.5 in mice, the newly developing fetal liver starts to be the source of definitive erythroid progenitor cells before the emergence of bone marrow as the final organ of erythropoiesis. The first erythroid progenitors that colonize the fetal liver are the burst forming units -erythroid (BFU-E) and they originate from the yolk sac.
(Palis, 2008) and from the paraaortic splanchnopleura and aorta-gonado-mesonephros (PAS/AGM) region of the embryo (Dzierzak et al., 1998). The AGM serves as a hematopoietic site until it begins to degenerate between E11 and E12 (Dzierzak et al., 1998). Definitive erythrocytes are enucleated and about 6-fold smaller than the primitive erythroblast. They express almost exclusively adult globins (α1, α2, β1, and β2) (Palis et al., 1999, McGrath and Palis, 2005).

To assess the maturation pattern of primitive erythroblasts, peripheral embryonic blood samples from E9.5 to E14.5 mouse embryos were studied. It was shown, upon staining with Giemsa dye, that large basophilic proerythroblasts are the predominant blood cells at E9.5-E12.5. At day E13.5, when hemoglobin synthesis increases, the orthochromatophilic erythroblasts become the major cell type. After that, a combination of large nucleated, large enucleated, and small erythrocytes are observed (Fraser et al., 2007). Furthermore, it was demonstrated that cell surface antigens in the enucleating primitive erythrocytes are preferentially redistributed. TER119, a cell membrane antigen associated with glycophorin A, for example, was increased in the newly enucleated primitive erythrocytes. In contrast, CD71 and α-integrin were decreased in these cells, when compared to the nucleated erythroblasts (Fraser et al., 2007).
Hematopoietic stem cells (HSCs) are pluripotential stem cells from which all blood cells arise. They usually express specific cell markers such as CD34, Sca-1, and c-kit (Guo et al., 2003). HSCs are used clinically to treat patients with leukemia and other disease by bone marrow transplantation.

1.2 Transcriptional regulation of erythropoiesis

Erythropoiesis is regulated by many cytokines and transcription factors. One of the early hematopoietic transcription factors is the stem cell leukemia gene (SCL, or TAL1), a basic helix-loop-helix (bHLH) DNA-binding protein that binds E-box DNA-sequences (CAGGTG) via its
basic domain and binds other ubiquitously expressed proteins such as E2A protein family members via its HLH domain. SCL is essential for early erythroid proliferation and differentiation (Perry and Soreq, 2002, Ravet et al., 2004). However, the DNA binding domain of SCL is not crucial for its activity because the hematopoietic ability of SCL-null mouse ES cells could be rescued by SCL protein with a mutant DNA-binding domain (Ravet et al., 2004). Ablation of SCL in mice causes embryonic lethality at E8.5 due to the absence of yolk sac blood formation (Perry and Soreq, 2002, Ravet et al., 2004).

GATA-1 is a key transcription factor in erythropoiesis. It is a zinc finger DNA-binding protein that recognizes (T/A)GATA(A/G) sequences found in almost all erythroid specific-genes (Cantor and Orkin, 2002, Perry and Soreq, 2002). GATA-1 null mice die by E10.5-11.5 because of severe anemia that is caused by failure to extend erythroid maturation beyond the proerythroblast stage (Cantor and Orkin, 2002). GATA-1 can act as an activator or suppressor for hematopoietic genes. GATA-1 activates the β-globin minimal promoter in vitro when expressed without FOG-1 (Friend of GATA-1). GATA1-FOG-1-MeCP1 complex was shown, using ChIP assays, to bind inactive genes such as GATA-2 in vivo (Rodriguez et al., 2005). FOG-1 is a protein with nine zinc fingers. It binds to the amino terminal zinc finger domain of GATA-1 and, together, they play a crucial role in terminal erythroid differentiation (Cantor and Orkin, 2002, Perry and Soreq, 2002). Therefore, FOG-1 and other proteins that have the potential to interact with GATA-1 affect the ultimate function of GATA-1 by recruiting other chromatin remodeling complexes such as NuRD, SWI/SNF, and MeCP1. LMO2, LIM-only protein 2, is another hematopoietic/erythropoietic essential protein. Similar to SCL and GATA-1, LMO2 null mice die during early embryogenesis because of severe anemia (Perry and Soreq, 2002).
CREB-binding protein (CBP) is a histone acetyltransferase protein implicated in many cellular processes by binding to different proteins. CBP, for example, binds to GATA-1 and recruits histone acetyltransferase activities that are associated with increased gene expression in general. In addition, GATA-1 is acetylated and activated by CBP (Cantor and Orkin, 2002). CBP is also known for its association with, and activation of Krüppel Like Factor 1 (KLF1), an essential erythroid transcription factor that plays central roles in erythroid commitment and β-globin expression (Zhang and Bieker, 1998).

Nuclear factor erythroid 2 (NF-E2) is a heterodimer protein implicated in erythroid cell development and β-globin regulation. Two subunits, p45 and p18, bind together through their basic leucine zipper domains to make the NF-E2 which binds DNA with (T/C)GCTGA(G/C)TCA(T/C) core sequence (Andrews, 1998). Later research showed that knockout models of NF-E2 do not show erythroid abnormalities but rather show megakaryocytic phenotype. Explanation for that was p45 subunit can be substituted by other similar protein in the erythroid cells. However long range interaction between the mouse adult β-globin promoter and the locus control region (LCR) was described in mouse erythroleukemia (MEL) cells and the overall recruitment of Pol II to the mouse β-globin promoter is decreased when the NF-E2 is ablated (Bulger et al., 2002)

In addition, Krüppel Like Factors 1 and 2 (KLF1, KLF2) are important transcription factors in erythropoiesis. KLF1 is an erythroid-specific transcription factor whose expression is crucial for normal β-globin gene expression. KLF2 is expressed in the endothelial and erythroid cells. Its function in the erythroid cells is similar to that of KLF1.
1.3 The β-globin locus

Hemoglobin, the most abundant protein in erythrocytes, is composed of four polypeptide chains: two α-globin chains and two β-globin chains. Each chain sequesters a heme molecule, to which a molecule of either Oxygen (O₂) or Carbon dioxide (CO₂) attaches (Bank et al., 1980). Each different globin chain is encoded by a different globin gene. The genes are located tandemly in the α-globin and β-globin loci, and are expressed during development in the order they appear on the chromosomes. In humans, the α-globins are encoded by four genes, ζ, α, α, and θ, located on chromosome 16 (Figure 1.2) (Weatherall, 2001, Zhang et al., 2002). β-globins, on the other hand, are encoded by five genes, ε, Λγ, Gγ, δ, and β, located on chromosome 11 (Figure 1.2) (Bank et al., 1980, Weatherall, 2001). Different combinations of α- and β-globins make different types of hemoglobin. Three embryonic hemoglobins, Gower I (ζε₂), Gower II (α₂ε₂), and Portland (ζγ₂), are expressed during early yolk sac erythropoiesis. Fetal hemoglobin, hemoglobin F (α₂γ₂) is expressed during fetal liver erythropoiesis. Adult hemoglobins, hemoglobin A (αβ₂) and hemoglobin A2 (αδ₂), begin to be expressed a few weeks before birth and continue to be the predominant hemoglobin during adult erythropoiesis (Figure 1.2) (Bank et al., 1980, Weatherall, 2001, Qiu et al., 2008). The β-globin locus in the mouse contains four genes, two embryonic (Ey and βh1), and two adult (βmaj and βmin) (Figure 1.3) (Noordermeer and de Laat, 2008).
Figure 1.2: Expression pattern of the human $\alpha$- and $\beta$- globin genes

The $\alpha$- and $\beta$- globin loci in human chromosome 11 and 16 and their spatial/temporal expression during development (Weatherall, 2001).
Figure 1.3: Expression pattern of the β-globin gene in the mouse

The upper part of the chart shows the layout of the mouse β-globin locus including the locus control region. The dotted lines represent the human β-globin genes in transgenic mice (Noordermeer and de Laat, 2008).
Although the mechanisms for control of stage specific expression of β globin genes are not well known, the expression of these genes is regulated by joint action between the regulatory elements within the promoter region of each gene and an upstream enhancer region called the Locus Control Region (LCR). Examples of regulatory elements within the promoters of all β-globin genes are the TATA box, CAAT elements, and CACCC elements (Cao and Moi, 2002). In the γ-globin promoter, two copies of the CAAT element and one copy of the CACCC element are present; whereas two copies of the CACCC element and one copy of the CAAT element are present in the adult β-globin promoter (Cao and Moi, 2002). The TATA box occurs 25-30 bp upstream of the globin transcription sites. It is essential to recruit transcription factor II D (TFIID), which is ultimately essential for the recruitment of RNA polymerase II. A mutation in the TATA box was reported in cases of β-thalassemia (Basran et al., 2008). CACCC elements occur in the promoters of all β-globin genes, and are recognized by KLF transcription factors (Bieker, 2001, Kaczynski et al., 2003, Zhang et al., 2005).

In addition to the promoter elements, the β-globin LCR (βLCR), which is located 6-22 kb upstream the ε-gene and contains seven DNAse I hypersensitive sites (HS1-HS7), plays a crucial role in maintaining high β-globin gene expression (Weatherall, 2001, Chan et al., 2008). Clues about the importance of βLCR came from types of β-thalassemia in which the β-globin genes and promoters are intact though express very low amounts. Upon further investigation, it was revealed that this form of thalassemia is manifested by a large deletion far upstream of the β-globin locus, which identified the βLCR (Weatherall, 2001, Noordermeer and de Laat, 2008). The main constituents of the βLCR are the DNase I hypersensitive sites each of which contains DNA-binding elements such as NF-E2, KLF1, and GATA-1 binding sites. The human βLCR is able to enhance the expression of one β-globin gene at a time (Wijgerde et al., 1995,
Noordermeer and de Laat, 2008), which suggests that a direct interaction between elements in the βLCR and elements in the promoter of one gene is required for appropriate enhancement activity. In contrast, mouse βLCR can drive simultaneous activation of the two embryonic (Ey, βh1) or the two adult (βmaj, βmin) β-globin genes at the same time (Trimborn et al., 1999, Noordermeer and de Laat, 2008). Chromosome Conformation Capture (3C) techniques proved helpful in studying the hypothesis that the βLCR is spatially interacting (looping) with β-globin genes in order to enhance their transcription. Tolhuis et al. showed using the 3C method that the mouse βLCR and active globin genes were indeed in close contact in erythroid cells but not in other cells (Tolhuis et al., 2002).

1.4 Hemoglobin Switching

The sequential switch of gene expression in the β-globin locus has been a field of intensive research in the past few decades. Understanding the mechanism of the switching from embryonic to fetal to adult β-globin gene expression has an important impact on treating or ameliorating sickle cell diseases and thalassemias by preventing or partially inhibiting the switch to adult β-globin gene. Genetic methods are instrumental to identify loci that are associated with increased fetal hemoglobin (HbF) in adults. Three loci have been identified so far that may explain up to 50% of HbF variance (Wilber et al., 2011). The β-globin locus itself is the first locus to be linked to high HbF. β-Thalassemia and variation in the γ-globin promoter known as XmnI-Gγ have shown high association with HbF in linkage disequilibrium studies (Thein et al., 1994, Garner, 2000). A second locus in chromosome 2 was found to be associated with HbF phenotype. This locus contains polymorphisms in the BCL11a gene (Menzel et al., 2007). A
third locus in chromosome 6 has also shown a strong association with HbF and the variations in this locus were mapped to the HBS1L-MYB intergenic area (Thein et al., 2007).

Further investigation of HbF phenotype in HPFH individuals suggested that the hemoglobin switching is orchestrated by different cis-acting elements in the β-globin locus. These cis-acting elements were found in different studies to be occupied by different transcription factors such as KLF1, BCL11a, SOX6, FOP, NF-E4, and others (Wilber et al., 2011). KLF1 is master regulator of erythropoiesis and involved in the hemoglobin switching by directly activating β-globin expression and indirectly inactivating γ-globin expression in adult erythroid cells. BCL11a is zinc finger transcription factor that repress γ-globin expression in adult erythroid cells. SOX6 is another suppressor of γ-globin expression and interacts with BCL11a. FOP is a chromatin-associated protein that directly interacts Protein arginine N-methyltransferase 1 (PRMT1), a histone methyltransferase. NF-E4, or p22, is a fetal erythroid-specific transcription factor that activates γ-globin expression (Wilber et al., 2011). Since hemoglobin switching is coupled with switching in the site of erythropoiesis and transcription factors that have roles in the hemoglobin switching are also important for erythrocyte development and differentiation, it is difficult to pinpoint transcription factors that are only necessary for switching.

In addition to the transcriptional regulation of hemoglobin switching, DNA methylation plays important roles. DNA methylation is important to maintain a closed status of chromatin conformation which by consequence leads to gene silencing. The first hint about a correlation between gene expression and DNA methylation was discovered by studies performed in the chicken β-globin locus (McGhee and Ginder, 1979, Ginder and McGhee, 1981). Further studies performed on the human β-globin locus showed that cells treated with DNA methyltransferase
inhibitors such as 5-azacytidine exhibits decreased DNA methylation and increased \( \gamma \)-globin expression (Charache, 1983). MBD2 is a methyl-CpG binding domain that recruits the NURD complex which leads to the silencing of \( \gamma \)-globin gene expression in transgenic mice (Rupon et al., 2006).

The hemoglobin switching is concomitant with a switch in organs of erythropoiesis. While embryonic hemoglobin is expressed in the yolk sac, fetal and adult hemoglobin are expressed in the fetal liver and the bone marrow (Figure 1.2) (Weatherall, 2001). These two switches could indicate that hemoglobin switching is in fact only a consequence of the switch in organs of erythropoiesis. To explore this idea, the expression of the different \( \beta \)-globin genes was studied in single cell preparations to see whether hemoglobin expression is pancellular or heterocellular. In a study performed on \textit{ex vivo} expanded peripheral blood CD34+, the pancellular expression of \( \gamma \)-globin mRNA was found to be associated with low erythropoietin supplement (Bhanu et al., 2005). This suggests that \( \gamma \)-globin is preferentially expressed in erythroid cells that are less mature and as they go further in maturation, e.g. in the presence of erythropoietin, they lose their ability to express \( \gamma \)-globin. Another study of HbF distribution in single reticulocytes was performed on clinical samples obtained from cord blood, newborn and adult samples. These studies showed that in cord blood samples, a mixture of pancellular \( \gamma \)-globin and \( \beta \)-globin expressing cells are present (Oneal et al., 2006). This suggests that a single cell predominantly express one \( \beta \)-globin gene at a time. However, studies in transgenic mice showed that fetal liver erythrocytes in the mouse express \( \gamma \)- and \( \beta \)-globin mRNA simultaneously (Strouboulis et al., 1992). The presence of \( \gamma \)- and \( \beta \)-globin mRNA and protein in one cell could be due to the prolonged half-life of these globins rather than simultaneous transcription from one allele at the same time. To explore this possibility, RNA fluorescence \textit{in situ} hybridization (FISH) using
probes against the primary transcripts of \(\gamma\)- and \(\beta\)-globin RNA was performed on fetal liver cells of transgenic mice. The study showed that although one cell can express \(\gamma\)- and \(\beta\)-globin at the same time, only one gene could be transcribed at one time (Wijgerde et al., 1995). This is probably because the LCR interacts with the gene promoter through DNA looping at the time of transcription. However, the simultaneous presence of multiple globins in a single cell indicates that the erythropoietic organ is not the sole dictator of which globin is expressed.

### 1.5 \(\beta\)-hemoglobinopathies

Sickle cell disease (SCD) and \(\beta\)-thalassemia are among the most prevalent single gene disorders. SCD is an autosomal recessive disorder caused by a point mutation in the adult \(\beta\)-globin gene by which glutamic acid in position 6 of the \(\beta\)-chain is replaced by valine (Higgs and Wood, 2008). The resulting hemoglobin (HbS, \(\alpha_2\beta^S_2\)) is more likely to polymerize under cellular hypoxic conditions. This polymerization is the main feature responsible for the sickling phenotype in red blood cells (Higgs and Wood, 2008). Consequently, the sickle cells have a shorter life span and are prone to aggregate and occlude the small blood vessels causing tissue hypoxia and severe pain (Higgs and Wood, 2008). SCD is the most common inherited blood disorder, affecting millions of people worldwide. Even though SCD is a monogenic disorder, the phenotypes conferred by it are highly variable. This variability was shown to be correlated with the level of HbF in the SCD patients; the higher HbF, the less severe phenotype manifested (Higgs and Wood, 2008, Lettre et al., 2008).

\(\beta\)-thalassemia is an autosomal recessive disorder caused by an absence (\(\beta^o\)-thalassemia) or poor (\(\beta^+\), \(\beta^{++}\)-thalassemias) synthesis of \(\beta\)-globin chain which leads to anemia and imbalance
in the α- to β- chain ratio (Orkin et al., 1979, Weatherall, 2001). An excess of free α-chain results in premature destruction of erythroid cells because of the abnormal aggregation of the α-chain (Weatherall, 2001). This premature destruction of the red blood cells (RBCs) is the main cause of anemia in β-thalassemia. As a result, the bone marrow is exhausted to compensate for the increased need for new RBCs, thus causing bones deformities (Weatherall, 2001, Thein, 2008). More than 200 mutations have been observed in the β-globin locus that cause β-thalassemia (Weatherall, 2001, Thein, 2008). The molecular basis of β-thalassemia encompasses all sorts of DNA and RNA alterations. Deletions and point mutations in the adult β-globin gene and its promoter, in addition to deletions in the βLCR region are well established causes of β-thalassemia which affect either the gene transcription or RNA processing and translation (Weatherall, 2001, Thein, 2005).

Because SCD and β-thalassemia confer inappropriate structure or amounts of adult β-globin, elucidating the mechanism that underlies the ε- to γ- to β-globin switching is of great clinical significance. Such a mechanism could be exploited in therapeutic strategies to increase the expression of ε- or γ-globin and eliminate or ameliorate the devastating phenotype of SCD and β-thalassemia.

1.6 Krüppel Like Factors (KLFs)

KLFs are a group of DNA-binding transcription factors that bind GC-rich sequences, including CACCC elements. These sequences are bound by the carboxy-terminal Cys2/His2 zinc fingers in the KLFs (Figure 1.4) (Perkins et al., 1995, Bieker, 2001, Kaczynski et al., 2003, Pearson et al., 2008). They are named after the Krüppel gene, a Drosophila gap gene that
encodes a Zinc finger pattern regulator protein (Pearson et al., 2008). Seventeen mammalian proteins have been identified in this family, and they were assigned names from KLF1 to KLF17. The distinguishing characteristics for the KLF family were shown to be three highly conserved zinc fingers near the carboxy-terminal end of the protein and highly conserved sequences in two regions between the fingers (Bieker, 2001, Basu et al., 2004). According to studies of KLF family members and the related SP proteins, the three zinc fingers recognize the consensus sequence (N/C)CN CNC CCN, where “N” denotes any nucleotide (Perkins, 1999, Turner and Crossley, 1999).

KLF proteins are expressed in different tissues and are implicated in many cellular functions such as erythropoiesis, cell differentiation, proliferation regulation, and tissue development (Turner and Crossley, 1999, Bieker, 2001, Kaczynski et al., 2003, Pearson et al., 2008). KLF1, also known as EKLF, is expressed only in erythroid cells and plays essential roles in β-globin gene expression (Miller and Bieker, 1993, Perkins et al., 1995). KLF2, also known as LKLF, on the other hand, plays important roles in T-cell differentiation and blood vessel development (Dang et al., 2000). Moreover, KLF4, also known as GKLF, is essential for normal skin development and loss of it results in lethal dehydration upon birth in KLF4 knockout animal models (Segre et al., 1999).
The KLF transcription factors share three highly conserved C2H2 zinc fingers in the carboxy-terminus. The three fingers are linked by a conserved (TGERP) sequence. The activation/repression domain is located in the amino-terminus of the protein and is highly variable among the family members. (Perkins et al., 1995, Bieker, 2001, Kaczynski et al., 2003, Pearson et al., 2008).

Figure 1.4: Illustration of a KLF protein

Although the 17 members of the KLF family share a conserved zinc finger domain, which means that they bind to relatively similar DNA sequences, they have different functions in different tissues. This functional difference could be explained by the fact that these proteins have different activation/repression domains and have different expression patterns (Turner and Crossley, 1999). For instance, both KLF4 and KLF5 bind to the BTE element of the cytochrome P-450IA1 (CYP1A1) gene but with contrasting effects. KLF4 inhibits the transcription of CYP1A1, while KLF5 induces its transcription (Ghaleb et al., 2005). Another example is KLF8, which is modulated by KLF1 and KLF3 in different ways. Using ChIP assays on E14.5 mouse fetal liver and differentiated MEL cells, it was shown that KLF1 and KLF3 compete with each other in occupying the same CACCC elements in the promoter of KLF8. KLF8 is downregulated when KLF3 is bound to the promoter. In normal situations, KLF3 outcompetes KLF1 in binding to KLF8 promoter. When KLF3 is knocked out, KLF1 binds to the promoter of KLF8 and upregulates its expression. As a result KLF8 is expressed in higher amounts in erythroid cells.
KLF1 (EKLF) was the first KLF member to be discovered (Miller and Bieker, 1993). The KLF1 gene is located on chromosome 19 in human and chromosome 8 in mouse. It has 3 exons

1.7 **Krüppel Like Factor 1**

KLF1 (EKLF) was the first KLF member to be discovered (Miller and Bieker, 1993). The KLF1 gene is located on chromosome 19 in human and chromosome 8 in mouse. It has 3 exons

when KLF3 is absent (Eaton et al., 2008). The regulation of KLFs by KLFs is also observed in the pairs KLF4 and KLF8, KLF1 and KLF3, and KLF2 and KLF1. Using ChIP assays, it was demonstrated that KLF8 represses the expression of KLF4 *in vivo* by binding to a GT-box in its promoter. Mutation in that box eliminates the effect of KLF8 on KLF4 (Wei et al., 2006). ChIP experiments on B.16 fetal liver cells also revealed that KLF1 binds directly to the promoter of KLF3 and activates its expression (Funnell et al., 2007). KLF2 KO mouse embryos exhibit low KLF1 mRNA at E10.5 (Basu et al., 2007).

Using transient transfection assays, KLF11 and KLF13 have been shown to modulate the expression of a reporter genes fused to a γ-globin promoter (Asano et al., 1999, Bulger et al., 2002). Using mouse yolk sacs, fetal liver, and adult spleen at different stages that represent the mouse erythroid development, KLF1, KLF2, KLF3, KLF4, KLF5, KLF8, KLF11, KLF12, and KLF13 were found to be expressed in all the different stages of erythroid development. However, it is difficult to conclude that all these factors are expressed in erythroid cells, since the source of tested samples contain non-erythroid cells (Zhang et al., 2005). However, it was shown, using transient transfection and luciferase assays, that KLFs could activate (KLF2, KLF5, KLF13) or inhibit (KLF8) the γ-globin promoter in erythroid cells. This activation or inhibition is CACCC-dependent since mutating this element abrogates the effect of these KLFs (Zhang et al., 2005).
and encodes 362 amino acids (a.a.). Part of exon 2 and Exon 3 encode the zinc finger domain of the protein. Using a subtractive hybridization method to identify genes that may have roles in erythroid cell differentiation, KLF1 mRNA was initially isolated from MEL cell line. The RNA population in MEL cells was isolated and the RNA population of J774 monocyte-macrophage cells was subtracted to select for erythroid specific mRNAs. It has been found that the protein is erythroid specific and about 38kDa (Miller and Bieker, 1993).

KLF1 is expressed as early as E7.5 in the mouse yolk sac before the blood starts to circulate (Southwood et al., 1996), and has more expression in mouse definitive than in primitive erythroid cells (Zhou et al., 2006, Alhashem et al., 2011). It can also be detected in the erythroid precursor of left and right dorsal aorta and hepatic tissues by E9.5 and E10.5 respectively. At E11, KLF1 can be detected in the liver and throughout the circulatory system. At day E14.5, KLF1 is expressed only in the liver (Southwood et al., 1996).

In chickens, the KLF1 gene (cKLF1) is located on chromosome 9. It has 3 exons and encodes a 405 amino acid protein that is highly similar to mouse and human KLF1 (Chervenak et al., 2006). cKLF1 mRNA could be detected as early as the epiblast stage (stage 4, 16 hrs of development), before hemoglobin is expressed. cKLF1 can be detected in high amounts by stage 8 in the posterior primitive streak (Chervenak et al., 2006). During late developmental stages, cKLF1 levels increase dramatically between day 7 and 14 of development (Chervenak et al., 2006) in a pattern similar to mammalian KLF1.

Using Chromatin Immunoprecipitation (ChIP), it was confirmed that KLF1 binds to the promoters of embryonic β-like globin genes (Ey and βh1), to the DNase I hypersensitive sites HS1, HS2, HS3, and HS5 in primitive erythroid cells, and to the promoter of the adult βmaj-
globin gene in primitive and definitive mouse erythroid cells (Zhou et al., 2006). It was also demonstrated, using Western and Northern blotting, that KLF1 has a temporally-regulated pattern of expression with about a 3-4fold increase of the protein and mRNA amounts in definitive erythropoiesis when compared to primitive erythropoiesis in mouse (Donze et al., 1995, Zhou et al., 2006, Alhashem et al., 2011).

It was shown using band shift analysis (EMSA) that KLF1 binds to the CCACACCCCT sequence located at -90 in the β-globin promoter, and to the CTCCACCCA sequence in the γ-globin promoter. However, the affinity of KLF1 binding to the γ-globin promoter has been shown to be weaker than to the β-globin promoter in vitro (Miller and Bieker, 1993, Donze et al., 1995, Zhang and Bieker, 1998, Dang et al., 2000). This differential affinity could be explained by a possible crowding of cis-elements in the γ- but not in the β-globin promoters, and by the fact that KLF1 is expressed in a higher amount during definitive rather than in primitive erythropoiesis (Dang et al., 2000, Zhou et al., 2006). The promoters of β- and γ-globin genes were studied in an attempt to find the difference in functional elements in the promoters. In addition to the difference in CACCC sequence in β- and γ-globin genes, four CCTTG repeats were found close to the CACCC element of the γ-globin promoter. Further investigations revealed that the CCTTG boxes flanking the CACCC element in the γ-globin promoter attenuates KLF1 recruitment to the promoter. When these repeats were inserted into the β-globin promoter, KLF1 binding to the β-globin CACCC elements was suppressed. Moreover, when these repeats were mutated in the γ-globin promoter, KLF1 could bind easily to the γ-globin promoter (Lee et al., 2000). These results suggest that functional elements surrounding the CACCC element play important roles in modulating KLF1 binding to the CACCC element.
Identifying proteins that specifically bind to the CCTTG elements will help elucidate their role in attenuating KLF1 activity.

Although KLF1 can bind theoretically to any sequence similar to the consensus sequence, (N/C)CN CNC CCN, it has been shown that KLF1 binds with variable affinity to different sequences. KLF1 binds the sequence located in the human β-globin promoter with the strongest affinity (Perkins, 1999). For example, using in vitro EMSA assays of transiently transfected KLF1 in COS7 cells, it was shown that the CACCC element in the erythropoietin promoter (CCACCCCCCT), and in the βh1-globin promoter (CCCCACCCCC) compete poorly with that in the human β-globin promoter for KLF1 binding. In contrast, these elements compete equally for SP1 binding (Perkins, 1999). Genome wide analysis of KLF1 binding revealed more specific DNA sequence elements to which KLF1 binds. The core sequence was narrowed down to 5’ CCM CRC CCN 3’ (R: A/G, M: A/C) (Siatecka et al., 2010, Tallack et al., 2010, Siatecka and Bieker, 2011)

Knockout mouse models were helpful in revealing the global roles of KLF1 in vivo. A KLF1 knockout mouse model was created by interrupting part of the second exon of the KLF1 gene that encodes part of the zinc finger domain by insertion of the neomycin resistance gene using homologous recombination techniques. Mice with the interrupted gene have no KLF1 mRNA, and KLF1+/− mice showed normal development when compared to WT mice (Perkins et al., 1995). Mice lacking KLF1 (KLF1−/−) die by E14.5 to E16 because of severe anemia and β-thalassemia with severe α-β-globin chain imbalance (Perkins et al., 1995). These mice were unaffected during embryonic erythropoiesis that occurs in the yolk sac, but when the switch from embryonic to fetal/adult erythropoiesis, KLF1−/− embryos become anemic, indicating dependency on KLF1 for adult β-globin gene expression (Perkins et al., 1995, Perkins, 1999). Another mouse
model was developed by inserting a LacZ reporter gene and a neomycin resistance cassette in the translation initiation codon (ATG) of the KLF1 gene (Nuez et al., 1995). Similar to the first model discussed, it was shown that the KLF1<sup>−/−</sup> mice of this model die by E14.5 because of anemia that was caused by a defect in the fetal liver erythropoiesis.

In attempt to rescue the hemoglobin chain imbalance, KLF1<sup>+/−</sup> mice were bred with human Aγ-globin transgenic mice to produce KLF1<sup>−/−</sup> Aγ+ embryos. Interestingly, the imbalance of α/β chains was corrected to some extent. However, the survival rate of KLF1<sup>−/−</sup> Aγ+ was not improved when compared to KLF1<sup>−/−</sup> embryos. Moreover, blood cells from KLF1<sup>−/−</sup> Aγ+ were morphologically similar to those from KLF1<sup>−/−</sup> (Perkins et al., 2000), indicating that KLF1 must be required in other cellular functions beyond its known role in β-globin expression.

Ablation of KLF1 has been shown to cause complete absence of formation of the DNase I hypersensitive sites at the β-globin promoters and the Locus control region (Wijgerde et al., 1996, Zhang and Bieker, 1998). The effect of KLF1 on the chromatin structure in E13.5 fetal liver cells containing human β-globin locus was studied. The promoters of human β-globin and mouse β<sup>maj</sup>-globin genes had no DNase I hypersensitive sites in KLF1<sup>−/−</sup> cells. In addition, HS3 was reduced to 50% in KLF1<sup>−/−</sup> compared to WT cells. HS1, 2, and 4 in KLF1<sup>−/−</sup> cells were comparable to WT cells (Wijgerde et al., 1996).

KLF1<sup>−/−</sup> embryos also lack terminally differentiated erythroid cells at day E13.5 (Pilon et al., 2008). This was shown using CD71 (transferrin receptor) and TER119 (Glycophorin A associated protein) markers to sort cells into five different stages of maturation designated R1-R5 as follows: (a) R1, early erythroid progenitor, are CD71<sub>Low</sub>, TER119<sub>Neg</sub>; (b) R2, erythroid progenitor, CD71<sub>Hi</sub>, TER119<sub>Low</sub>; (c) R3, proerythroblast, basophilic erythroblast, CD71<sub>Hi</sub>,
TER119<sup>Hi</sup>; (d) R4, polychromatic erythroblast, orthochromatic erythroblast, CD71<sup>Mid</sup>, TER119<sup>Hi</sup>; (e) R5, reticulocyte, CD71<sup>Low</sup>, TER119<sup>Hi</sup>. The fetal-liver cell population of KLF1<sup>−/−</sup> E13.5 embryos consists of only early erythroid progenitor (R1) and erythroid progenitor (R2). Further investigation of the R1 and R2 cell population in WT and KLF1<sup>−/−</sup> fetal liver revealed that KLF1 is essential for cells to progress from G<sub>1</sub> to S phase in these two populations (Pilon et al., 2008). In correlation with these findings, KLF1 was found to bind CACCC elements in the promoter of E2f2, a key cell cycle regulator (Pilon et al., 2008, Tallack et al., 2009).

To assess the possible non-globin roles of KLF1 in erythroid cells, expression profiling was performed on KLF1<sup>−/−</sup> E14.5 fetal liver cells, and on the B1.6 KLF1<sup>−/−</sup> cell line that is derived from E14.5 KLF1<sup>−/−</sup> fetal liver cells that have an inducible KLF1 transgene (ER-KLF1) (Hodge et al., 2006). As a result, 100 genes showed reproducible KLF1-dependent expression profiles, including α-hemoglobin stabilizing protein (AHSP), heme synthesis enzymes, band 4.9 protein (dematin), and other transcription factors and blood group antigens. Dematin is a trimeric cytoskeleton protein involved in actin bundling and is responsible for the normal erythrocyte shape (Hodge et al., 2006, Mohseni and Chishti, 2008). AHSP is a small protein that neutralizes the oxidant activity of free α-globin chains by binding to them (Hodge et al., 2006, Yu et al., 2007). Another expression profiling study to screen for new KLF1 target genes was also performed. In this study, fetal liver cells from WT and KLF1<sup>−/−</sup> E12.5 embryos were used in addition to in vitro differentiated and expanded cells. As a result more than 40 genes have been shown to be downregulated in KLF1<sup>−/−</sup> cells; most importantly, AHSP and dematin were replicated in this study (Drissen et al., 2005). Besides the non-hemoglobin related role of KLF1, studies showed that KLF1 plays important role in hematopoietic lineage fate. KLF1 starts to express in megakaryocyte-erythroid progenitors (MEP) but its dramatic increase in subset of
MEP cells define the erythroid fate of this subset (Frontelo et al., 2007, Bouilloux et al., 2008, Siatecka and Bieker, 2011). These results suggest that KLF1 acts negatively on megakaryocyte establishing genes.

In addition to the carboxy zinc finger domain, the KLF1 protein has an amino-terminal proline rich transcriptional activation domain between a.a. 20 and 291 (Miller and Bieker, 1993). In another study, it was demonstrated that this domain (a.a. 20-291) in fact contains two subdomains. The first one is located between a.a. 20-124 and found to increase the activity of a reporter gene (chloramphenicol acetyltransferase, CAT) 35-fold greater than full length KLF1; thus, it is considered a stimulatory subdomain. The other subdomain is found to be between a.a. 195-291 and is considered an inhibitory subdomain, since removal of this domain results in a 14-fold increase in reporter gene transactivation when compared to the full length KLF1 (Chen and Bieker, 1996).

Using co-immunoprecipitation techniques, it was demonstrated that KLF1 interacts with CBP, P300, and P/CAF, which are known for their histone acetyltransferase (HAT) activity (Zhang and Bieker, 1998). However, only CBP and P300 were able to acetylate KLF1 at (Lys-288, and Lys-302 of mouse KLF1) and enhance its transactivation property (Zhang and Bieker, 1998, Zhang et al., 2001). It was found that acetylation of KLF1 was not essential for proper DNA binding, but was essential for intact interaction with and recruiting other chromatin remodeling complexes such as the SWI-SNF complex (Zhang et al., 2001); and thus inducing an open chromatin state and active transcription.

Recent genome wide studies aimed to reveal the global occupancy of KLF1, using the ChIP-seq approach, and the global expression signature of KLF1 KO vs WT erythroid cells,
using the RNA-seq approach. These studies were significant in revealing a more complete picture of KLF1 roles in erythropoiesis. In one study, KLF1 ChIP-seq data showed that 945 sites in the genome of E14.5 mouse fetal liver erythroid cells are bound by KLF1 (Tallack et al., 2010). Interestingly, the majority of these sites occur outside of known transcription start sites (TSS). This suggests that KLF1 may act through enhancer rather than promoters, or different splice forms of mRNA present only in erythroid cells and is driven by KLF1 expression. The study also revealed that KLF1 occupancy is usually combined with other known erythroid transcription factors such as GATA-1 and SCL/TAL1 (Cheng et al., 2009, Kassouf et al., 2010, Tallack et al., 2010, Tallack et al., 2012). Another study of global KLF1 occupancy was done in HA-tagged KLF1 transgenic mice (Pilon et al., 2011). In this study, mouse definitive erythroid cells were divided into two categories, erythroid progenitor cells (Ter-119 negative), and erythroblasts (CD71-positive/Ter119-positive). This study revealed more KLF1 binding sites than the previous study. It showed that 13,006 and 15,476 sites are bound by KLF1 in erythroid progenitor and erythroblasts, respectively. The study also confirmed that the majority of KLF1 binding occur outside known promoter regions (Pilon et al., 2011). KLF1 genome wide ChIP accompanied by genome wide RNA sequencing revealed that KLF1 is instrumental in almost all aspects of erythroid development such as α- and β- globin expression, heme synthesis, erythroid proliferation and differentiation (Tallack and Perkins, 2010, Tallack et al., 2010, Pilon et al., 2011, Tallack et al., 2012).

Further studies on the mechanistic roles of KLF1 in erythroid cells revealed that KLF1 regulated genes are transcribed by RNA polymerase II from within what are called transcription factories. A transcription factory is an area of active transcription enriched with activated RNA polymerases and segments of chromatin that carry the genes being transcribed. Multiple
techniques such as chromosome conformation capture (3C), RNA FISH, DNA FISH, and immune-FISH were used to study the contents and dynamic changes of these transcription factories. Investigation revealed that in erythroblasts only a limited number of specialized transcription factories is present in each nucleus. In agreement with KLF1 being essential for the expression of many erythroid genes, these factories are characterized by the presence of KLF1 protein and RNA polymerase II in addition to multiple genes from different chromosomes being transcribed simultaneously. KLF1 is essential for the formation of the factories, which dissolve in its absence. These factories are highly dynamic in a way that genes enter and exit these factories continuously (Eskiw et al., 2010, Schoenfelder et al., 2010).

1.8 Krüppel Like Factor 2

KLF2, also known as lung Krüppel like factor (LKLF), is encoded by a 3-exon gene located on human chromosome 19 and mouse chromosome 8. The protein consists of 355 amino acids in humans and 354 in mice, with a molecular weight of approximately 37.7 kDa. The KLF2 full length protein is about 40% similar to KLF1. However, the amino acid similarity is 85% in the zinc finger domain. KLF2 was first described as a Krüppel like factor predominantly expressed in lung tissue, thus it was given the name Lung KLF (LKLF) (Anderson et al., 1995). The zinc finger domain is located in the carboxy terminus of the protein (from a.a. 274 to 354 in humans), and adjacent to its amino side, a basic sequence serves as a nuclear localization signal. Within the amino terminus, a transcription activation/repression domain occurs. In addition to the high similarity to KLF1, KLF2 resides nearby KLF1 on the same chromosome in human and mouse, suggesting that they originated from a gene duplication event (Basu et al., 2005).

To test the transactivation properties of KLF2, mouse NIH 3T3 fibroblasts were transfected with a CAT reporter gene fused to a human adult β-globin promoter containing two
CACCC elements. The level of CAT was assessed in the presence and absence of KLF2. It was shown in this study that the presence of increased amount of KLF2 resulted in a dose-dependent increase in the level of CAT expression. Furthermore, to test whether this effect is CACCC-dependent, mutation in one or both CACCC elements were performed. Only when both elements were mutated, the level of CAT was diminished near to the basal levels (Anderson et al., 1995). Using transient co-transfection of KLF2 cDNA and a luciferase reporter gene fused to the γ-globin promoter in K562 cells, it was shown that KLF2 increased the γ-globin promoter activation by 1.6-fold when compared with mock transfected cells. This activation is CACCC-dependent because mutating the CACCC element in the promoter diminished the activation by ~2 fold (Zhang et al., 2005). However, in vivo studies in mouse models showed that KLF2 does not regulate adult β-globin genes (Basu et al., 2005).

In contrast to KLF1 being erythroid specific, KLF2 is expressed in many different cell types. Using in situ hybridization techniques, it was shown that KLF2 is expressed in the developing blood vessels and primitive vertebrae in E12.5 mice (Kuo et al., 1997a). At E14.5, KLF2 was also detected in the vertebral column, developing bones, and lung buds. After that, at E18.5, KLF2 expression was prominently seen in the lung and blood vessels (Kuo et al., 1997a). KLF2 was also reported to be expressed in heart, kidney, skeletal muscle, testes, and the lymphoid organs (Kuo et al., 1997b).

In addition, KLF2 is important in T-cell survival and quiescent state maintenance (Kuo et al., 1997b, Buckley et al., 2001). Induction of KLF2 in Jurkat T-cells results in their proliferation inhibition. Using mouse chimeras with WT, KLF2+/−, and KLF2−/− T-cells, it was found that KLF2−/− T-cells have higher proliferation capacity when compared to KLF2+/− and WT T-cells as measured by BrDU incorporation (Buckley et al., 2001).
KLF2 is expressed in primitive as well as definitive erythroid cells in mouse and chicken (Basu et al., 2004, Zhang et al., 2005). Using RT-PCR to study the expression pattern of KLF2, and other family members, it was demonstrated that KLF2 was detected in E10.5 yolk sacs where primitive erythropoiesis takes place; in E14.5 fetal liver where definitive erythropoiesis takes place; and in the spleen of anemic adult mice where definitive erythropoiesis takes place (Zhang et al., 2005). Using a chicken model, it was demonstrated that chicken KLF2 (cKLF2) is expressed at similar levels in the blood cells at embryonic days 5, 7, and 14. Chicken embryonic day 5 represents primitive erythropoiesis, while day 14 represents the definitive erythropoiesis, and day 7 represents the transitional stage between primitive and definitive erythropoiesis. (Basu et al., 2004).

A KLF2 knockout mouse model was developed by replacing the whole KLF2 gene with a neomycin resistance cassette using homologous recombination techniques. In this model E11.5 KLF2−/− mice were not grossly different from WT littermates. However, KLF2−/− mice die by E12.5 to E14.5 due to internal hemorrhaging in the abdomen and around the cardiac outflow tract which was caused by a failure of normal vascular tunica media formation (Kuo et al., 1997a). Having been highly expressed in the developing blood vessels, ablation of KLF2 was investigated to determine the cause of the hemorrhaging observed earlier. PECAM-1, an endothelial cell marker (CD31), was used to stain newly formed blood vessels using whole mount immunohistochemistry techniques. The results indicate that neither vasculature nor angiogenesis were affected by KLF2 deficiency at age E12.5. However, evaluation of the advanced stages of blood vessel morphogenesis revealed that KLF2−/− embryos exhibit severe thinning of the tunica media in the umbilical vessels, which in turn caused aneurysmal dilation. Electron microscopy
analyses revealed that umbilical vessels and aortae in E12.5 KLF2−/− embryos have less extracellular matrix than WT vessels (Kuo et al., 1997a).

Another KLF2 knockout mouse model was developed using homologous recombination techniques to delete the promoter, the transactivation domain, and a part of the zinc finger domain and replace it with a hypoxanthine phosphoribosyltransferase (Hprt) minigene (Wani et al., 1998). A successful disruption of KLF2 was confirmed by a failure to detect KLF2 mRNA using Northern blotting and RT-PCR on E11.5 embryos. KLF2−/− mice die by age E11.5-13.5 due to hemorrhaging. The histological analyses of KLF2−/− embryos at day E11.5 showed that their livers were pale and have reduced hematopoietic precursors. In addition, the peripheral blood cells were of primitive nucleated erythroid cells and reduced in numbers. To elucidate the cause of the hematopoietic defect, erythroid colony forming (CFU-E) assays were conducted on cells from the fetal liver and the yolk sacs of KLF2−/− embryos. Interestingly, the erythroid colonies derived from the yolk sacs were not different in WT and KLF2−/−. On the other hand, the erythroid colonies of the fetal liver were significantly diminished in KLF2−/−. Given that erythroid cells in both yolk sac and fetal liver at day E11.5 are definitive, these results are confusing and suggest that environment-related factors rather than KLF2−/− is the cause of the hematopoietic defect (Wani et al., 1998).

1.8.1 KLF2 in endothelial cells

KLF2 gene expression in endothelial cells is regulated by shear flow stress. In a study using Human Umbilical Vein Endothelial Cells (HUVEC) exposed to shear stress, it was shown that KLF2 is up-regulated by increased shear stress (Wang et al., 2006). In another study, microarray analysis of HUVEC cells exposed to prolonged flow stress identified KLF2 among
those whose expression was up-regulated when compared to control cells. Further investigation on human vascular tissues, using in situ hybridization, indicate that KLF2 is prominently expressed in areas where flow shear stress is increased, i.e. in the aorta wall and in the walls of small branches opposite to branching points (Dekker et al., 2002). A conditional knockout mouse model in which KLF2 knockout is restricted to either endothelial and hematopoietic cells (Tie2-cre) or smooth and cardiac muscles (SM22-cre) was developed. It was shown that only Tie2-cre KLF2−/− mouse embryos die by day E14.5. Even though SM22-cre mice lack KLF2 gene in their smooth and cardiac muscles, they live normally to adulthood. Tie2-cre KLF2−/+ E12.5 embryos show signs of heart failure similar to what was exhibited in conventional KLF2−/−. Using two-dimensional (2D) ultrasonography and Doppler flow studies to further investigate for the causes of the heart failure revealed that it was caused by elevated cardiac output which is caused by lack of KLF2 in the peripheral vascular endothelial cells (Lee et al., 2006).

1.8.2 KLF2 in erythroid cells

Being highly similar to KLF1, it was suggested that the hemorrhagic phenotype seen in KLF2−/− embryos was caused partially by defects in the development of red blood cells and platelets. To assess this hypothesis, Kuo et al. tested whether KLF2−/− embryonic stem cells (ES) can differentiate normally into hematopoietic stem cells (HSC) under proper conditions. In addition, fetal liver cells from WT and KLF2−/− embryos were grown in vitro and the resultant erythroid and myeloid colonies were compared in both groups. These two experiments showed no differences in hematopoiesis in WT and KLF2−/− cells. Also, blood smears from WT and KLF2−/− embryos at E12.5 showed normal blood cell populations (Kuo et al., 1997a). Another study by Lee et al showed that ablating KLF2 only in definitive hematopoietic cells (using Vav-cre transgene) neither cause anemia nor cardiovascular defects since the animals live to
These results disagree with what was shown by Wani et al. that erythropoiesis is compromised in the fetal livers of KLF2-/- embryos at day E11.5 (Wani et al., 1998). An explanation of the normal phenotype in Vav-cre KLF2-/- mice is that KLF2 is required only in embryonic erythropoiesis and that Vav-promoter is not activated early in embryogenesis, i.e. KLF2 function is accomplished before it has been excised by cre. Although it was shown that KLF2 is not important for normal adult erythropoiesis, a careful assessment of Vav-cre expression during early embryogenesis and correlating it with that of KLF2 is necessary to rule out that the hemorrhaging phenotype in KLF2-/- embryos is caused by a defect in hematopoiesis.

The importance of KLF2 during the early stages of erythropoiesis was studied using the KLF2 knockout model developed by Wani et al. (Wani et al., 1998). KLF2 was shown to be important in primitive but not in definitive erythropoiesis (Basu et al., 2005). E10.5 mouse yolk sacs lacking KLF2 had an approximately 50% decrease in the expression level of the embryonic β-like globin genes, Ey and βh1, when compared to the expression in WT yolk sacs. However, KLF2 does not modulate the expression level of adult β-globin genes, βmaj and βmin, in the fetal lever at E12.5. Likewise, KLF2 showed a more significant effect on the expression level of human ε-globin than on γ-globin when mice carrying the human β-globin locus were studied (Basu et al., 2005). In addition, the morphology of primitive erythroid cells in the yolk sacs of E10.5 KLF2-/- embryos was investigated using light and electron microscopic analyses. KLF2-/- primitive erythroid cells are highly irregular and have pseudopodia-like appendages, and features correlated with cell apoptosis, such as nuclear membrane dissolution and enlarged perinuclear space which has been confirmed using TUNEL assays (Basu et al., 2005).
Study of KLF2 target genes in mouse erythroid cells was done by isolating erythroid cells of E9.5 embryos using a laser microdissection (LCM) technique (Redmond et al., 2011). This study identified 196 genes as differentially expressed in KLF2-/- yolk sac erythroid cells vs. WT. 89 genes out of these genes were downregulated in KLF2 -/- cells, few of them were found to be erythroid enriched based on another microarray used to study primitive erythroid enriched genes (Redmond et al., 2008). Examples of erythroid enriched genes that are downregulated by KLF2 are cell signaling factors CD24a antigen, cytotoxic T-lymphocyte associated protein 2 α-(Ctla2a), adenylate cyclase 7 and reelin (Redmond et al., 2011).

1.8.3 KLF2 in T-cells

To further understand the mechanistic role of KLF2 in blood vessel development and T-cell quiescent state maintenance, one needs to study KLF2 target genes and elucidate the pathways in which these genes are implicated. Because KLF2 is important in T-cell quiescence and IL-2 is critical for T-cell activation, it was suggested by Wu and Lingrel that KLF2 may regulate, directly or indirectly, the induction of IL-2. Using transient transfection and luciferase assays in activated T-cells, it was shown that IL-2 expression was enhanced by 5-fold in the presence of KLF2. Upon searching for consensus KLF binding motifs in the promoter of IL-2, a CTCCACCC element was found between -290 and -310bp from the transcription initiation site. Mutating this element to CATGT caused inhibition of IL-2 induction by KLF2 thus confirming the functional importance of this element. Ultimately, chromatin immunoprecipitation (ChIP) using anti HA-tagged KLF2 on activated T-cells was performed to verify that KLF2 physically occupies the CACCC element in the promoter of IL-2 (Wu and Lingrel, 2005). It was demonstrated in another study, using luciferase assays, that KLF2 activates the promoter of CD62L, also known as L-selectin, and S1PR1, sphingosine-1-phosphate receptor 1 (Bai et al.,
CD62L is a T-cell surface protein important in the localization of lymphocyte to lymph nodes (Wirth et al., 2009). S1PR1 is a cell membrane receptor that binds to a lipid mediator S1P and also enhances the localization of lymphocytes to the lymph nodes and improves endothelial barrier function (Bai et al., 2007, Tauseef et al., 2008).

1.8.4 KLF2 in mouse embryonic stem cells

Because KLF2 is closely related to KLF4, it was tested for its ability to induce pluripotency in mouse embryonic fibroblasts (MEFs). It was demonstrated that KLF2 can produce induced pluripotent stem (iPS) cells. KLF1 and KLF5 were also found to produce iPS cells, but with decreased efficiency (Nakagawa et al., 2008).

A genome-wide chromatin immunoprecipitation (ChIP on chip) was performed to identify the binding sites of KLF2, KLF4, and KLF5 in mouse ES cells. These three KLFs were selected because they are downregulated when ES cells differentiate (Jiang et al., 2008). Surprisingly, the three KLFs showed 205 similar binding sites across the genome. They bind known critical cell regulator genes such as Nanog, Sox2, Oct3/4, Mycn, Tcf3 (transcription factor 3), Sall4 (sal-like 4), and Esrb (estrogen-receptor-related receptor β). The binding profiles on the promoters of these genes were validated using RNAi and qPCR. Furthermore, it was investigated, using an EMSA approach, whether mutating the CACCC element prevents these KLFs from binding to the DNA and activating a reporter gene. The distal CACCC element in the Nanog promoter (CCCCACCC) was used with 13 mutant versions within and flanking its sequence in EMSA. It was shown that CCACCC or CCGGCC are necessary sequences KLF2 and KLF4 to which KLF2 and KLF4 can bind (Jiang et al., 2008).
To determine whether regulation of the mouse embryonic globin genes by KLF2 is erythroid-cell autonomous, a conditional KO mouse model was used in which the KLF2 gene is deleted in erythroid cells. Erythroid cell-autonomous effects are defined here as being due to loss of KLF2 in erythroid cells, and not indirectly due to KLF2 ablation in another cell type.

KLF2F/+, ErGFP-Cre and KLF2F/F mice were mated to obtain E10.5 KLF2F/F, ErGFP-Cre (test) and KLF2F/F (control) embryos. The ErGFP-Cre transgene is active in primitive erythroid cells (Heinrich et al., 2004, Maetens et al., 2007). qRT-PCR assays showed that there is significantly less βh1-globin mRNA in KLF2F/F, ErGFP-Cre compared to KLF2F/F (without Cre) embryos (Alhashem et al., 2011). The βh1-globin mRNA was reduced by approximately 25% in KLF2F/F, ErGFP-Cre embryonic blood cells (Alhashem et al., 2011). There is a similar decreasing trend in Ey-globin mRNA in KLF2F/F, ErGFP-Cre compared to KLF2F/F embryos, although this difference is not statistically significant (Alhashem et al., 2011). These modest differences are consistent with the fact that there is approximately 40% less KLF2 mRNA in KLF2F/F, ErGFP-Cre than in KLF2F/F embryos, making these embryos more comparable to KLF2+/− than to KLF2−/− (17). The modest effect of the conditional knockout on globin gene regulation is likely due to inefficient excision of the KLF2 gene by the Cre recombinase. KLF2 very likely has an erythroid-cell autonomous role in the regulation of the βh1- and Ey-globin genes. However, the possibility that KLF2 also has non-cell autonomous functions in globin gene expression cannot be excluded.

1.9 KLF1 and KLF2 have compensatory roles in primitive erythropoiesis

KLF1+/−KLF2−/− mouse embryos and yolk sacs are pale and anemic at day E10.5 when compared to WT and single knockout (KLF1+/− or KLF2+/−) embryos and yolk sacs (Basu et al.,
This phenotype was less obvious at E9.5. This implies that the cause of the anemia could be a failure to expand the erythroid cell population and/or a defect in normal hemoglobin synthesis in a timely manner (Basu et al., 2007). It was shown also, using the same embryos to measure the level of embryonic β-like gene expression (Ey and βh1), that the level of Ey and βh1 mRNA are significantly less than in WT, KLF1−/−, and KLF2−/− embryos (Basu et al., 2007).

Furthermore, erythroid cell morphology was assessed at E9.5 and E10.5 using WT, KLF1−/−, KLF2−/−, and KLF1−/−KLF2−/− blood (Basu et al., 2007). Cytospins from WT and KLF2−/− showed similar morphology in erythroid cells at E9.5. KLF2−/− blood cells have abnormal morphology at day E10.5. Abnormal phenotypes such as cytoplasmic blebbing and nuclear atypia were seen in KLF1−/− and KLF1−/−KLF2−/− at E9.5. Semi-quantitative analyses indicated that KLF1−/−KLF2−/− erythroid cells have a significantly more abnormal phenotype than WT, KLF1−/−, and KLF2−/−. Moreover, the level of erythroid maturation at day E9.5 was assessed in the aforementioned genotypes. Using FACS techniques to sort erythroid cells based on their CD71 and TER119 cell markers, it was shown that KLF1−/− and KLF1−/−KLF2−/− erythroid cells exhibit delayed maturation progress when compared to WT and KLF2−/− erythroid cells (Basu et al., 2007).

The mouse KLF1 and KLF2 also regulate the human β-globin genes in transgenic mouse models. Using dual human β-globin locus transgenic (Tg-HBB) and KO mice revealed that ε-globin mRNA is significantly reduced to 15% and 49% in KLF1−/− Tg-HBB and KLF2−/− Tg-HBB yolk sacs, respectively, compared to Tg-HBB (Alhashem et al., 2011). γ-globin mRNA was reduced to 31% of Tg-HBB in KLF1−/− Tg-HBB yolk sacs (Alhashem et al., 2011). KLF2 has a more modest effect on γ-globin gene expression (Basu et al., 2005), which is reduced to 73% of
Tg-HBB in KLF2/- Tg-HBB yolk sacs. KLF1 appears to have a greater effect on ε- and γ-globin gene expression, but KLF2 also contributes. The quantity of ε- and γ-globin mRNA in KLF1/- Tg-HBB yolk sacs may be marginally underestimated compared to KLF1/-KLF2/- Tg-HBB. Thus, possible synergistic regulation of the human ε- and γ-globin genes by KLF1 and KLF2 in the transgenic mouse model cannot be ruled out (Alhashem et al., 2011).

Expression profiling microarrays were used to study the compensatory role of KLF1 and KLF2 in E9.5 primitive erythroid cells (Pang et al., 2012). The study was focused mainly on discovering the genes that have a decreasing trend of mRNA amounts in WT, KLF1−/−, and KLF1−/− KLF2−/− E9.5 erythroid cells. Myc gene was one of the genes that were regulalted by both KLF1 and KLF2. Luceferase and ChIP assays showed that Myc is directly regulated by KLF1 and KLF2(Pang et al., 2012).
CHAPTER TWO:
Methods

2.1 Generation of Knockout and Transgenic mice

The KLF1 knockout (KO) mouse model was developed by targeting the gene with the neomycin resistance gene (Perkins et al., 1995). The KLF2 KO mouse model was developed by targeting the gene with the hypoxanthine phosphoribosyl-transferase (Hprt) gene (Wani et al., 1998). Because KLF1 and KLF2 are located in close proximity on mouse chromosome 8, recombination between the two genes is rare. Therefore, a mouse model that has the KLF1 and KLF2 KO alleles on the same DNA homolog was generated (KLF1+/-KLF2+/-) (Basu et al., 2007). Transgenic mice that carry the entire human β-globin locus (Tg-HBB) were previously described (Stroubloulis et al., 1992, Gaensler et al., 1993).

2.2 Mouse Dissection and Collection of Erythroid Cells

Tg-HBB mice were bred with KLF1+/-, KLF2+/-, or KLF1+/-KLF2+/- mice to obtain KLF1+/- Tg-HBB, KLF2+/- Tg-HBB, and KLF1+/-KLF2+/- Tg-HBB mice. Mouse embryonic yolk sacs and blood cells were collected as described (Basu et al., 2007). Females in the timed mating were checked every morning for the presence of a vaginal plug, indicating a successful mating, after which the plugged females were separated from the males and that day was considered E0.5. At scheduled embryonic day (E9.5, E10.5, E11.5, or E12.5) plugged females were dissected after being anesthetized using 2.5% Avertin, followed by cervical dislocation. The uterine horns were dissected out and whole embryos were placed in Petri dishes containing
1X PBS. Processing one embryo at a time, maternal tissues were separated from yolk sac and embryo making sure that yolk sacs do not rupture and loose blood. Yolk sacs were washed in 1X PBS to minimize contamination with maternal tissues, and then transferred into a 12-well plate contains 1 ml 1X PBS. To collect embryonic blood, mouse embryos let bleed through their main blood vessels into the 1X PBS for few minutes by tearing off the yolk sac. To collect fetal liver, E12.5 embryo is dissected out of their yolk sacs before fetal liver is dissected out of other embryonic tissues. Fetal liver then transferred into a clean well filled with 1X PBS and pushed or pipetted to make single cell suspension. Part of the embryo tail was collected for genotyping.

2.3 Genotyping

DNA was prepared for genotyping by digesting adult mouse ear clips or embryo tails in digestion buffer (10mM Tris HCL (pH8.5), 50mM KCl, 40mM MgCl₂, 0.45% Tween 20, and 0.45% NP-40) and 1µg/µl proteinase K for at least 3 hours at 55-60°C followed by 2 cycles of enzyme inactivation at 95°C for 10 minutes each. Once the DNA was prepared, PCR was performed by adding an appropriate volume of DNA solution to a PCR grand mix that contains 0.2mM dNTPs, 1.5mM MgCl₂, PCR buffer (Invitrogen), 0.04 U/ul *taq* polymerase (Invitrogen), 1.75 ug/ul RNase A (Invitrogen), and the appropriate pairs of WT and knockout primers (refer to *Table*). Samples were amplified under the following conditions: 3 min at 94°C, 35 cycles of 40 sec at 94°C, 45 sec at 58°C, 75 sec at 72°C, followed by 5 min at 72°C. In addition to running each sample twice, at least two previously genotyped samples and H₂O negative control were added to each genotyping run. The PCR product was visualized on a 2% Agarose gel run at 100 volts until the two expected bands are clearly separated. Pictures of the agarose gels were taken.
using A- Innotech instrument (San Leandro, CA). The primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) and are listed in Table 2.1.

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<th>Gene</th>
<th>Allele</th>
<th>Primer sequence 5’ – 3’</th>
<th>Amplicon size (bp)</th>
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</thead>
</table>
| KLF1                  | WT     | F: GGT GAA CCC CAA AGG TAC AA  
R: CTG GGA CCT CTG TCA GTA GTC | 170 |
|                       | KO     | F: GCC AGA GGC CAC TTG TGT AG  
R: CTG GGA CCT CTG TCA GTA GTC | 220 |
| KLF2                  | WT     | F: TTG CCG TCC TTT GCC ACT TTC G  
R: TTG TTT AGG TCC TCA TCC GTG CCG | 400 |
|                       | KO     | F: CGG TCT CTT GTA GCC AAA GGG  
R: CCT ACC CGC TTC CAT TGC TC | 450 |
| Human β-globin locus transgene |   | F: TGG CTC ACT TTC TCA GAA GCC AGT  
R: AGG GAG AAG CCA TAC CCT TGA AGT | 300 |

**Table 2.1: Primer sequences for genotyping of mouse KLF1, KLF2, human β-globin locus transgene**

2.4 Purification of RNA

Two different kits were used to prepare RNA from yolk sacs, embryonic blood, or cord blood CD34+ cells. One is TōTALLY RNA™ Kit (Ambion®, Life Technologies Corporation, Catalog Number AM1910). The other one is TRIzol® Reagent (Ambion®, Life Technologies Corporation, Catalog Number 15596-026).

2.5 TōTALLY RNA™ Kit

200 μl of Denaturation Solution was added to the frozen yolk sac followed by vortexing for two minutes to make a homogenized lysate. Then, 200 μl of Phenol: Chloroform: IAA was added to the lysate and vortexed for one minute, incubated on ice for 5 minutes, and then centrifuged at 12000xg for 5 minutes at 4°C. The aqueous layer was transferred to a new 1.5 ml tube. Then, 20 μl of sodium acetate (3M, pH4.5) and 200 μl of Acid-Phenol: Chloroform were
added and the sample and it was vortexed for 1 minute, incubated for 5 minutes, and centrifuged for 5 minutes at 12000xg at 4°C. The aqueous layer was transferred to a new tube, to which an equal volume of cold isopropanol and ~2 µl of 5 mg/ml glycogen were added. The RNA was kept at -20°C to precipitate for at least 2 hrs. Following precipitation, the tube was centrifuged at 12000xg for 20 min at 4°C, the isopropanol removed, and the pellet was washed by 75% ethanol and dried for 15 min before it was reconstituted in 20 µl of RNase free water containing 1:20 Superase•In (Applied Biosystems, Foster City, CA). RNA concentration was assessed by measuring the absorbance at wavelength 260 and 280 using a Nanodrop spectrophotometer (Thermo scientific, Wilmington, DE). OD_{260}/OD_{280} was used as a measure of RNA purity which is optimal at 2.0. The integrity of RNA was assessed by running ~0.1 µg RNA on a 1% agarose gel for 30 min at 150 volts. Two clear bands of ribosomal RNA with minimal smearing indicate good RNA integrity.

2.6 **TRIzol® Reagent**

One 0.5-1 ml of Trizol® reagent was added to pelleted cells to lyse them. Cell lyses enhanced by pipetting up and down or by brief vortexing. Lysed cells incubated at RT for 5 minutes for complete lysis. At this stage cell lysate could be stored at -80°C or proceeded with RNA isolation. To continue, 0.2 ml chloroform per 1 ml Trizol is added to the cells. Trizol/Chloroform is vortexed for 30 seconds then centrifuged at 12000 Xg speed at 4°C centrifuge for 15 minutes. The aqueous top layer then transferred to a new tube. It is recommended to leave few microliters of the aqueous phase to avoid the interface which is rich in DNA. 1-4 ul of glycogen (~10 ug) is added to enhance RNA visualization after pelleting. 0.5 ml of isopropanol then added to the aqueous phase to precipitate RNA. RNA kept to precipitate at -20°C for overnight. Precipitated RNA recovered by centrifugation at 12000 Xg for 20 minutes
at 4°C. A pellet of RNA should be seen at the bottom of the tube. The supernatant is discarded carefully to avoid losing the pellet. The pellet then washed with 75% ethanol, diluted in DEPC treated water and dried for 15 min. After wash, the RNA pellet dissolved in 30 ul RNase free water containing 1:20 Superase•In. RNA concentration was assessed by measuring the absorbance at wavelength 260 and 280 using a Nanodrop spectrophotometer (Thermo scientific, Wilmington, DE). OD$_{260}$/OD$_{280}$ was used as a measure of RNA purity which is optimal at 2.0. The integrity of RNA was assessed by running ~0.1 μg RNA on a 1% agarose gel for 30 min at 150 volts. Two clear bands of ribosomal RNA with minimal smearing indicate good RNA integrity.

2.7 Synthesis of cDNA

RNA is treated with DNase I to eliminate traces of DNA that could possibly precipitate with RNA. 1 ug of RNA was treated with I unit of DNase I (Invitrogen™, Life Technologies Corporation, Catalog Number 18068-015) in a reaction tube containing 1 ul of DNase I buffer and total volume of 10 ul. The reaction is stopped by adding EDTA to final concentration 2 uM. The DNase I enzyme is deactivated by incubating the samples at 65°C for 10 minutes. cDNA was synthesized using iScript™ cDNA synthesis kit (Biorad, Hercules, CA). In a reaction tube, 4ul of 5X iScript Reaction Mix, and 1ul of iScript Reverse Transcriptase were added to 1ug of total RNA. The total volume was then adjusted to 20 ul. The reaction tube was incubated under the following conditions: 5 min at 25°C, 30 min at 42°C, then 5 min at 85°C.
2.8 Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Mouse KLF1, KLF2, cyclophilin A, glycophorin A (GPA), Eγ- and βh1-globin, and human ε- and γ-globin mRNA amounts were quantified using qRT-PCR with SYBR Green or Taqman reagents (Applied Biosystems, Foster City, CA). Mouse cyclophilin A and GPA mRNA were used as internal standards for normalization as indicated in the figure legends. GPA is erythroid-specific. For reactions with Sybr green, the following reagents were added to each reaction tube: 1X Power Sybr Green master mix (Applied Biosystems®, Cat. 4367659), 4μM of forward and 4μM of reverse primers and diluted cDNA in total volumes of 25 μl or 12.5 μl. For Taqman technology, the following reagents were added: 1X Taqman® Universal PCR Master Mix (Applied Biosystems®, Cat. 4304437) 4μM of probe, 4μM forward primer, and 4μM reverse primer and diluted cDNA in total volumes of 25 μl or 12.5 μl. Each cDNA sample was run in triplicate. qRT-PCR was performed using an ABI Prism 7300 or 7900HT analyzer (Applied Biosystems, Foster City, CA). For quantification using SYBR Green chemistry, a dissociation step was performed, and it was verified that only one product was amplified. A standard curve from pooled cDNA samples was included in each run and used either to measure the relative amounts of unknown samples or to obtain the efficiency of specific primers (Efficiency[E]= 10^(-1/slope)). Fold change (relative expression) was calculated as $E^{Ct_{endogenous\ gene}} / E^{Ct_{test\ gene}}$. Primer and probe sequences are indicated in Table 2.2. Statistical significance was calculated using the Student’s t-test.
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</table>

Table 2.2: qRT-PCR primer and probe sequences
2.9 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed essentially as previously described (Hou et al., 2008). Briefly, for each biological replicate, ~5×10⁶ blood cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped by adding 125 mM glycine and incubating at RT for 5 minutes. Cells centrifuged at 300 Xg for 5 minutes and supernatant discarded. Pelleted cells could be stored at -80°C at this stage. Cell lysis buffer (10 mM Tris, 10 mM NaCl, 0.2 % NP-40 [pH 8.0], protease inhibitors) is added to lyse cells. Cell lysate was then centrifuged at 500 Xg for 5 minutes. The supernatant is discarded and the pelleted nuclei are lysed by adding 0.5 ml Nuclei lysis buffer (50 mM Tris, 10 mM EDTA, 1% SDS, protease inhibitors). Nuclei being lysed are kept on ice for 10 minutes to complete the lysis process after which chromatin will be released and in the nuclei lysis buffer. The volume of chromatin then adjusted to 2X10⁶ cells per 100 ul with IP dilution buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.01% SDS, 1% Triton X-100, protease inhibitors) and distributed to 300 ul per 1.5 ml tube. Chromatin was sheared to approximately 500 bp using a Bioruptor sonicator (Diagenode, Sparta, NJ) using the following settings: 3 cycles of: “H” level, 30 sec ON, 30 sec OFF, for 10 min (Total time 30 mins, 15 mins sonication time). Cold water and Crushed ice was added to the water bath before starting each cycle to keep sonication at low temp. During sonication 50 ul of Protein G Agarose (Millipore, Cat. 16-266) per IP was washed three times using IP dilution buffer then kept at 50 ul per IP in the same buffer. The sheared chromatin then centrifuged for 10 minutes at 10000 Xg at 4°C. The soluble chromatin in the supernatant is removed into new tubes. Chromatin is diluted to 1 ml per antibody using the IP dilution buffer (e.g. if using only 2 antibodies, one specific and one non-specific, the chromatin is diluted to 2
ml). 10% of chromatin is taken into new tubes and kept at -20°C. Chromatin is distributed to one tube per antibody with 1 ml chromatin per tube. 1-10 ug of antibody (equal ug of specific and non-specific antibodies should be added) is added to the chromatin. For KLF2 ChIP, 3 mg total protein was added of either the KLF2 anti-serum or the pre-immune serum. 50 ul of washed protein G agarose is added to each tube. Chromatin/antibody is incubated at 4°C for overnight. Chromatin then washed as following: twice with 500 ul of IP wash buffer 1 (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 [pH 8.1]), once with IP wash buffer 2 (10 mM Tris, 0.25 M LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% desoxycholate [pH 8.1]), twice with TE (pH 8.0). The chromatin/ antibody is eluted by adding 400 ul of freshly prepared IP elution buffer (0.1 M NaHCO3, 1% SDS (10ml: 1M NaHCO3 1ml, 20% SDS 0.5ml, water 8.5 ml). The eluted chromatin then centrifuged to separate chromatin and protein G agarose. The eluted and input chromatin then reverse crosslinked by adding NaCl (final concentration 0.3 M) and proteinase K (0.25 mg/ml) and incubating at 65°C for 6 hours. The proteinase K is deactivated by incubating the chromatin at 95°C for 10 minutes. DNA now is free of crosslinked protein. DNA then purified using phenol chloroform phase extraction. Purified DNA is quantitated using quantitative PCR (qPCR) and SYBR Green chemistry. Fold enrichment was calculated as $2^{\Delta C_{\text{input}} - C_{\text{test}}}$ and expressed relative to the IgG control. Antibodies used were: Anti-H3K9Ac (Abcam, cat. Ab4441); Anti-H3K4me3 (Upstate-Millipore, cat. 07-473); Anti-KLF1 (Abcam, cat. AB-2483); Anti-KLF2 (KLF2_Ng (Jiang et al., 2008); Anti-KLF2 (KLF2-SC from Santa Cruz Biotechnology, cat. sc-18690); non-specific IgG (rabbit, Abcam ab46540, or goat, Santa Cruz Biotechnology, sc-2028). For optimal results, the KLF2_Ng antibody is recommended. Primer sequences for qPCR are indicated in Table 2.3.
<table>
<thead>
<tr>
<th>Site</th>
<th>Primer sequence 5’ − 3’</th>
</tr>
</thead>
</table>
| mβActin         | F: ACCCCATTTGAACATGGCATT  
                   | R: TGTAGAAGGTTGTGGTGCCAGAT                                                |
| m5’HS2           | F: AGGGTTTGGTGGCCAGATGTT  
                   | R: ACCCAGATAGCACTGTACGATCAGTCAC                                           |
| m5’HS3           | F: CTTGAGACTGAGAGGCTGCTT  
                   | R: ATGGGACCTCCTGATAGACACATCTTT                                            |
| mEy promoter     | F: TGCTTCTGACACCTCTGTGATCA  
                   | R: GGTCTTTTCCTCAGCAGTAAGG                                                 |
| mβh1 promoter    | F: GGACACGTCTCCAGCCTCTTGA  
                   | R: CATATGCTTGTGATAGCTGCT                                                  |
| mβmaj promoter   | F: GCTTCTGACATAGTTGTGACTCA  
                   | R: CACCGACCTTCTCAGCATCA                                                   |
| mEy Exon3        | F: GGCCTAGTCACTCCTGGAATT  
                   | R: GGCATAGGGACACACAGGAT                                                   |
| mβh1 Exon3       | F: TTGGCAAGTGGTGTGATTTGGA  
                   | R: TGGACTCAAGAGGGCATCATAG                                                 |
| mβmaj Exon3      | F: GAAAGTGGTGGCTGAGTGG   
                   | R: TGTTCACAGGCAAGACAGG                                                     |
| h5’HS2           | F: GGCTCAAGCAGCAATGC  
                   | R: CATCACTCTAGGCTGAGAACATCTG                                              |
| h5’HS3           | F: CTCTAGAACCTTGAGTTCTCAGGATT  
                   | R: CCCCTGTTCTCCATGCTGATTTAAG                                               |
| h ε-promoter     | F: CACAAAATTAGTGTCATCCATCAC  
                   | R: CACATGGTACCGGTCAAGG                                                    |
| h γ-promoter     | F: CAATATCTGGATGAAACGTCCCT  
                   | R: TGCTTCTGCAAGGCTATTGG                                                   |
| h β-promoter     | F: GAGGGTTTGAAGTCCACTTAAA  
                   | R: CAGGGTGAGGTCTAGTGGA                                                     |
| h ε-Exon2        | F: CAGGGCCGCCTTTGCTAAG  
                   | R: CACCTGGAAGTCTCCAGGATCCA                                                  |
| h γ-Exon2        | F: TGCCAAAGAGTGTCAGCTTCC  
                   | R: GCAAGGTTGCGCTTTGAGATC                                                   |
| h β-Exon2        | F: TGCCCAAGGAGTGTCAGCTTAC  
                   | R: GTGAGCCAGGCCCATCCTAAG                                                  |
| Necdin           | F: TTGTGCCAGCAGAACATCTGGAAG  
                   | R: GACCCCCAGAAGAAGGCTGTA                                                   |

**Table 2.3: Primer sequences used in ChIP assays**

Primers for the human -globin locus acquired from Kim et al., 2007 (1). Primers for the mouse -globin locus acquired from Kingsley et al., 2006 (2)
2.10 Generation of Custom KLF2 antibody

Generation of KLF2 antibody is described in Chapter 4

2.11 Selection and Expansion of Cord blood CD34+

Cord blood cells were purchased from the St. Louis Cord Blood Bank (SLCBB, St. Louis, MO). Upon receipt of cord blood units, Ficoll-Paque™ (GE Healthcare, Cat. 17-5442-02) density gradient media was used to isolate mononuclear cells (MNCs) following the manufacturer instructions. Briefly, about 90 ml of cord blood was diluted 2-4X with PBS containing 2% FBS. Two volumes of diluted cord blood are layered on top of 1.5 volumes of Ficoll in 50 ml tubes. Tubes were centrifuged at 400X at 18°C for 35 minutes with zero brakes and acceleration. The middle MNC layer was transferred to new tube and washed two times with at least 3 volumes PBS containing 2% FBS. The washed MNCs undergo CD34+ selection using the EasySep, Human CD34 selection kit (Stem Cell Technologies, Cat. 18056) following the manufacturer protocol. Briefly, MNCs were diluted to 5X10⁸ cells per ml with PBS containing 2% FBS and 1mM EDTA. Then 100 µL/mL EasySep® Positive Selection Cocktail was added to the cells and incubated for 15 minutes at RT. Then 50 µL/mL EasySep® Magnetic Nanoparticles were added and incubated for 10 minutes at RT. MNCs were then diluted with PBS containing 2% FBS and 1mM EDTA to final volume of 2.5 ml and added into EasySep® Magnet (Stem Cell Technologies, Cat. 18000) for five minutes. Then non CD34+ cells were discarded by inverting the tubes while it is in the magnet. Selected cells then washed 4 times and put for 5 minutes in the magnet at each time. At the end purified CD34+ resuspended in the Expansion medium (StemSpan SFEM medium (Stem Cell technologies, Cat. 09680) containing 1X CC100 (Stem Cell technologies, Cat. 02690), 8 µl/ml LDL (Sigma, L7914), and 2% Penicillin/Streptomycin (P/S, Gibco®, Cat. 15140-122)) at 1.5 million cells per ml. Cells expanded for one week and kept at density of 1-2 million cells per ml with same media as above.
Media was changed every 2-3 days. Cells are grown in 12 well plates with media not more than 1 ml per well.

2.12 *In vitro* Differentiation of CD34+ Cells into Erythroid Lineage

At the end of CD34+ expansion and/or gene knockdown periods, cells were transferred into differentiation medium at two consecutive stages. First, cells were kept in Growth/Differentiation medium for three days (IMDM; 20% FBS, Thermo Scientific, SH30071; 10 ng/ml SCF, R&D systems, 255-SC-10; 1 ng/ml IL-3, R&D systems, 203-IL-10; 1μM Dexamethasone, Sigma, D1756; 1μM Estradiol, Sigma, E1024; 1U/ml Erythropoietin; 2% P/S). Then cells were transferred to full differentiation medium (IMDM; 20% FBS;1U/ml Erythropoietin; 10 ng/ml insulin; 2% P/S) for 5 days. In both stages cells were kept in 12-well plates at density of 1-2 X 10^6 cells/ml. The growth and differentiation media is replaced every 2-3 days by spinning down cells at 300 xg for five minutes and removing the supernatant media and reconstituting the cells at 1-2 X 10^6 cells/ml. Cells were counted using a hemacytometer; at least one four small square in the four large squares are counted to make up total of 16 small square. The cells are multiplied by the factor 10^4 to give the final cell count per ml.

Cytospins were prepared by diluting about 5 X 10^4 in 100 ul PBS or the same media they were in. The cells were transferred in a cytospin funnel and assembled on a superfrost glass slide then spun for 5 minutes at 600 rpm using Shandon cytospin instrument.

Generation of Knockdown plasmids

The shRNA target sequence against human KLF1, in addition to the scramble sequence were subcloned into a pRRLSIN.cPPT.PGK-GFP.WPRE backbone as described (Bouilloux et
The plasmid was a gift from Dr. François Morle (Université de Lyon, Lyon, France) and Fawzia Louache (University of Paris, INSERM U981, France). The KLF2 shRNA target sequence was adopted from a previously published article (Dekker et al., 2005). Briefly for each of the KLF2 shRNA prepared, 4 oligonucleotides (Table 2.4) that assemble that shRNA were ordered from Eurofins MWG Operon (Huntsville, AL) as shown in (Figure 2.1). Probes were kinased, annealed, and ligated to make a complete shRNA. H1 promoter was PCR amplified from material of KLF1-shRNA plasmid using primers that have MluI and XhoI restriction sites at the 3’ and 5’ ends respectively. The PCR amplified H1 promoter was digested with MluI and XhoI then ligated to KLF2-shRNA. The ligated H1-KLF2-shRNA was subcloned to a precut pRRL backbone plasmid. Plasmids were transformed into stbl3 E.Coli (Invitrogen™, Cat. C7373-03). Transformed bacteria plated on LB agar supplemented with 100 μg/ml Ampicillin (Thermo Fisher Scientific Inc., Catalog Number BP1760-25). Few ampicillin resistant colonies were picked for mini preps followed by plasmid sequencing and diagnostic restriction digestion. One colony that passes the sequencing and restriction digestion inspections is used for large scale plasmid preparation. Figure 2.2 summarizes the whole process.
Figure 2.1: Illustration of probes that assemble KLF2 shRNAs
Figure 2.2: Schematic overview of the strategy followed to generate KLF2 shRNA KD plasmids
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
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<td>KLF1-shRNA</td>
<td>GATCCCGCGGCAAGAGCTACACCAATTTCAAGAGATTGTTGATGCTCTTGCCGCGTTTTTGGAAA</td>
</tr>
<tr>
<td>Scramble-shRNA</td>
<td>AGCTTTTCTAAAAAGCGGCAAGCTACACCAATCTCTTTGAATTGCTGTTAAGAGATCATTTTTCGGCGGG</td>
</tr>
<tr>
<td>KLF2 shRNA (a) Probe 1</td>
<td>CGCGTCCCCCATACACCAAGAGTTTCGTTCAAGAGA</td>
</tr>
<tr>
<td>KLF2 shRNA (a) Probe 2</td>
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</tr>
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<td>KLF2 shRNA (a) Probe 3</td>
<td>TCGAGCAAAAAAGACCTCACACCAAGAGTTTCGTTCAAGAGAA</td>
</tr>
<tr>
<td>KLF2 shRNA (a) Probe 4</td>
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<tr>
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<td>pRRL sequencing F</td>
<td>AGGGCTGACCTTTGGAAAAG</td>
</tr>
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</table>

**Table 2.4: Sequences of shRNAs and oligonucleotides used for sequencing and making shRNA**
2.13 Lentiviral Transduction and KLF Knockdown

Lentiviruses that carry the shRNAs were made by cotransfection of 293T cells with two packaging plasmids (pCMV-R, and pMD2G, gift from Gordon Ginder, VCU) and a specific pRRL plasmid that have one shRNA against KLF1 or KLF2 and a GFP gene. The packaged viral particles were secreted in the media of 293T cells and collected after 36 hours of cotransfection. Expanded CD34+ cells were seeded at 0.3X10^6 cells per 50 μl and distributed into 12 well plates at 50 μl per well. 600 μl of viral particles were added to each well and kept in a 37°C incubator with 5% CO₂ for 12 hours before 350 μl of expansion media was added. Cells were kept a total three days before they were washed and submitted for flowcytometry to select for GFP positive cells.

2.14 Flowcytometry

CD34+ cells that were positively infected with the lentiviral particles were isolated based on their GFP expression by using a FACSaria flowcytometer running FACSDiva software (BD Biosciences, San Jose, CA). The flowsorting was done using the flowcytometry core facility at the Virginia Commonwealth University, Massey Cancer Center (Richmond, VA). Occasionally, sorted cells were resorted again to measure the specificity of the instrument. Usually 60-90% of cells were positive for GFP and the instrument is more than 95% specific based on re-sorting results (Figure 2.3)
Figure 2.3: Flow cytometry data sample

Expanded CD34+ cells were washed at least two times with PBS containing 2% FBS then submitted to the flowsorting core facility to sort based on the GFP expression. (A) Cells were sorted based on their size to exclude cell debris and doublets as shown in gate P1. FSC: forward scatter, SSC: side scatter. (B-D) show the intensity of GFP signal in cells sorted through P1, green (P13) are GFP+ cells, grey (P7) are GFP negative cells. Most cells fall in the middle of the GFP range. (B) GFP negative cells, (C) cells infected with an shRNA lentivirus, (D) cells recovered from (C) were re-sorted to detect GFP sorting specificity.
CHAPTER THREE:
Regulation of β-globin genes by KLF1 and KLF2 in the mouse

Contents of this chapter are published in (Alhashem et al., 2011) or (Pang et al., 2012).

3.1 Introduction:

Erythroid cells are one of the first differentiated cell types in embryos (Baron and Fraser, 2005). There are two unique processes in development: primitive and definitive erythropoiesis. Primitive erythropoiesis initiates from the extraembryonic mesoderm of the yolk sac as early as embryonic day 7.5 (E7.5) in mice (McGrath and Palis, 2005, Qiu et al., 2008). Definitive erythropoiesis is detected in the mouse fetal liver by E11.5 (Brotherton et al., 1979). The human β-globins are encoded by four major genes, ε (embryonic), Gγ- and Aγ- (fetal), and β- (adult), located on chromosome 11. The mouse β-globin locus contains four genes, two embryonic (Ey and βh1), and two adult (βmaj and βmin).

The expression of the β-globin genes is jointly regulated by elements in the promoter regions and an upstream enhancer region, the Locus Control Region (LCR). The human β-globin LCR, located 6-22 kb upstream of the ε-globin gene, contains multiple erythroid-specific DNase I hypersensitive sites (HS) and plays a crucial role in maintaining β-globin gene expression (Grosveld et al., 1987, Fraser et al., 1990). Examples of regulatory elements within the promoters of all β-globin genes are TATA, CAAT, and CACCC (Lloyd et al., 1989).

Krüppel-like factors (KLFs) are a family of transcription factors that bind GC-rich sequences such as CACCC elements. The KLFs bind DNA via three carboxy-terminal Cys2/His2 zinc fingers (Bieker, 2001). Seventeen mammalian proteins have been identified in
this family and are designated KLF1 to KLF17. KLFs are implicated in many cellular functions such as erythropoiesis, cell differentiation, proliferation, and tissue development (McConnell and Yang, 2010). The human and mouse KLF proteins are highly conserved. For example, KLF1 is 73% similar in the two species (though 90% similar within the zinc finger domain), and KLF2 is 90% similar in mouse and man.

KLF1, also known as erythroid Krüppel-like factor or EKLF, is expressed only in erythroid cells and plays essential roles in embryonic and adult β-globin gene expression (Nuez et al., 1995, Perkins et al., 1995, Basu et al., 2007). KLF1 is a master regulator of adult β-globin gene expression (Nuez et al., 1995, Perkins et al., 1995). Semi-quantitative chromatin immunoprecipitation (ChIP) assays revealed that HA-tagged KLF1 binds to the promoters of the embryonic β-like globin genes (Ey and βh1) and to HS1, HS2, HS3, and HS5 in mouse primitive erythroid cells, and to the promoter of mouse adult βmaj-globin gene in primitive and definitive cells (Zhou et al., 2006). KLF1 interacts with CBP, p300, and PCAF, which have histone acetyltransferase (HAT) activity (Zhang and Bieker, 1998). KLF1 is also implicated in erythroid processes other than β-globin gene regulation such as cell maturation and cell membrane integrity (Hodge et al., 2006, Pilon et al., 2008).

KLF2, originally known as Lung KLF or LKLF, has important roles in T-cell differentiation and blood vessel development (McConnell and Yang, 2010). KLF2 is a positive regulator of the mouse and human embryonic β-globin genes (Basu et al., 2005). KLF1 and KLF2 have high homology within their DNA-binding domains and reside close to each other on the same chromosome in human and mouse, suggesting that they originated from a gene duplication event (Basu et al., 2005). KLF1 and KLF2 can partially functionally compensate for each other in regulating the mouse embryonic β-globin genes. When both KLF1 and KLF2 are
simultaneously ablated in mice, the amounts of Ey- and βh1-globin mRNA are reduced more
than in KLF1 or KLF2 single knockouts (Basu et al., 2007). Like KLF1, KLF2 recruits proteins
with HAT activity such as CBP, p300 and PCAF (SenBanerjee et al., 2004).

KLF1 and KLF2 coordinately regulate the mouse embryonic β-globin genes. In the
current studies, we wished to determine whether KLF1 and KLF2 also control the human
embryonic and fetal β-globin genes. This knowledge could facilitate therapeutic strategies to
express these genes in adults with β-hemoglobinopathies. A second goal was to further establish
the mechanistic roles of KLF1 and KLF2 in globin gene regulation. In this work, it was
determined that KLF1 and KLF2 mRNA are expressed in similar amounts in mouse primitive
erthroid cells. The expression of KLF1 but not KLF2 mRNA is greatly increased in definitive
erthroid cells. Transgenic mice that harbor the complete human β-globin locus (Strouboulis et
al., 1992, Gaensler et al., 1993), in conjunction with gene knockouts, were used to determine that
KLF1 and KLF2 positively regulate human ε- and γ-globin gene expression in the embryo. KLF2
has an erythroid cell autonomous role in embryonic globin gene regulation, though it may also
have non-cell autonomous functions. In quantitative ChIP assays, KLF1 and KLF2 bind to the
promoters of the mouse embryonic Ey- and βh1-, and the human ε- and γ-globin genes in mouse
primitive erythroid cells. KLF1, but not KLF2, is required to establish the normal histone
modification status in the mouse and human β-globin loci in mouse primitive erythroid cells.
Although both KLF1 and KLF2 directly affect globin gene regulation through the same DNA
binding sites, their mechanisms of action appear to be somewhat different.
3.2 KLF1 and KLF2 mRNA amounts are similar in primitive but not definitive erythroid cells

KLF1 is a major regulator of the human and mouse adult β-globin genes (Nuez et al., 1995, Perkins et al., 1995), while KLF2 regulates embryonic but not adult β-globin gene expression (Basu et al., 2005). The relative amounts of KLF1 and KLF2 mRNA were compared in primitive and definitive erythroid cells matched for similar stages of differentiation. Embryonic day 9.5 (E9.5) blood (primitive) and E12.5 fetal liver (definitive) mRNA were used for the comparison because both contain mostly basophilic erythroblasts (Zhang et al., 2003, Fraser et al., 2007). The vast majority of E12.5 fetal liver cells are erythroid (Zhang et al., 2003, Fraser et al., 2007). Quantitative reverse transcriptase-PCR (qRT-PCR) results indicate that KLF1 and KLF2 mRNAs are expressed in similar amounts at E9.5 (Figure 3.1). The amount of KLF1 mRNA dramatically increases by E12.5, while KLF2 mRNA remains relatively unchanged. The ratio of KLF1 to KLF2 mRNA at E9.5 is less than two, but it is more than 16 at E12.5. The developmental expression pattern of KLF1 and KLF2 mRNA correlates with globin gene expression. KLF1 and KLF2 regulate the mouse Ey- and βh1-globin genes which are expressed at E9.5, whereas KLF1 but not KLF2 regulates the adult β-globin genes expressed at E12.5 (Basu et al., 2005, Basu et al., 2007).
Figure 3.1: Developmental expression patterns of KLF1 and KLF2 mRNA

Expression of KLF1 and KLF2 mRNA during primitive (E9.5 blood) and definitive (E12.5 fetal liver) erythropoiesis. Erythroid cells from E9.5 circulating blood and E12.5 fetal liver are in similar stages of differentiation. The amounts of mouse KLF1 and KLF2 mRNA were measured using qRT-PCR and normalized to Cyclophilin A. Fold change was calculated using the $2^{ΔCT}$ method, after correcting for different primer efficiency. At least 4 biological replicates were tested at each time point. “*” indicate statistical significance (p-value <0.05). The error bars indicate standard deviation. WT: wild type, K1-/-: KLF1-/-, K2-/-: KLF2-/-.
3.3 KLF1 and KLF2 occupy the embryonic and fetal β-globin promoters and the LCR

KLF1 and KLF2 control human and mouse embryonic β-globin gene expression. To better understand the mechanism for this control, chromatin immunoprecipitation (ChIP) assays using polyclonal antibodies against KLF1 and KLF2 were performed on Tg-HBB transgenic mice, which have the human β-globin locus. Unlike definitive erythroid cells which are enucleated, circulating primitive erythroid cells are nucleated at E10.5 and E11.5, making them amenable to ChIP analyses. The amounts of Ey-, γ-, and ε-globin mRNAs do not change appreciably between E10.5 and E11.5 in mouse embryos, although γ-globin is expressed more than ε-globin mRNA. However, the amount of βh1-globin mRNA at E11.5 is approximately 25% lower than at E10.5 (Strouboulis et al., 1992). To capture data for the βh1-globin gene, ChIP assays were performed at E10.5 where feasible, though less cells per embryo are available than at E11.5.

First, the specificity of the commercial KLF1 antibody was confirmed in ChIP assays using WT and KLF1-/- E13.5 fetal liver cells. The antibody is specific and binds to KLF1 in the promoters of the adult β-globin genes in the mouse and human β-globin loci in WT, but not in KLF1-/- fetal livers (Figure 3.2). At E10.5, KLF1 is significantly enriched at the promoters of the Ey- and βh1-globin genes, and at mouse 5’HS2 in the LCR (Figure 3.3A, right). In the human β-globin locus, KLF1 is significantly enriched at the promoter of the γ-globin gene, 5’HS2 and 5’HS3 (Figure 3.3A, left). At E11.5, the pattern of KLF1 enrichment at the mouse and human β-globin loci is similar to that at E10.5, except that binding of KLF1 to the ε-globin promoter is also evident at E11.5, perhaps due to increased sensitivity of the assay due to more available cells at this time point (Figure 3.3B). As a negative control, KLF1 does not bind to the
promoter of β-actin at E10.5 and E11.5. The ChIP assays indicate that KLF1 binds to all of the mouse and human embryonic and fetal β-globin gene promoters, and to the LCR.

At E11.5, KLF2 is detected at the Ey- but not the βh1-globin promoter (Figure 3.3C, right), which is consistent with higher expression of the Ey- than the βh1-globin gene at this time point (Strouboulis et al., 1992). There was no evidence that KLF2 binds to 5’HS2 or 5’HS3 in the mouse β-globin LCR. In the human β-globin locus, KLF2 is enriched by about 2-fold at the γ-globin promoter and at 5’HS2 and 5’HS3 (Figure 3.3C, left). Although the KLF2 binding measured at the ε-globin promoter was not statistically different from the negative control, it is approaching significance. The lower amount of expression of the ε- compared to the γ-globin gene in transgenic mice at E11.5 may have decreased the sensitivity of the ChIP assay. KLF2 binds to 5’HS2 and 5’HS3 in the human β-globin locus, which differs from data obtained by examining the mouse locus. These results strongly suggest that regulation of the embryonic and fetal β-globin genes by KLF1 and KLF2 is achieved by direct binding to the CACCC elements in the promoters and LCR. Binding of KLF1 and KLF2 to the LCR could be necessary for direct contact between the LCR and the β-globin gene promoters, as in adult erythroid cells (Drissen et al., 2004).
Figure 3.2: Testing the specificity of the KLF1 antibody

ChIP assays were performed on E13.5 fetal liver cells from WT or KLF1-/- (K1KO) human -globin transgenic mice. The Y-axis represents the relative fold enrichment. The mean IgG enrichment was set as 1.0 and the enrichment in KLF1-/- mice was scaled appropriately. The X-axis shows the location of the primers used (Pr: promoter, m: mouse, h: human). Necdin was used as a negative control to which KLF1 is not known to be bound. The -Actin promoter contains a CACCC element that apparently binds KLF1 in the fetal liver. n= 3 per genotype. Error bars: standard error. *; significant difference (p-value <0.05); NS: not significant.
Figure 3.3 A
Figure 3.3 B
Figure 3.3 C
Figure 3.3: KLF1 and KLF2 bind the mouse and human β-globin loci in primitive erythroid cells

ChIP assays were performed on E10.5 (A) or E11.5 (B,C) primitive erythroid cells of normal mice or transgenic mice that carry the entire human β-globin locus. Polyclonal antibodies specific for KLF1 (A,B) or KLF2 (C), and non-specific IgG control antibody were used. The Y-axis represents the relative fold enrichment. The mean IgG enrichment was set as 1.0 and the enrichment of KLF1 was scaled appropriately. The X-axis shows the location of the primers used for qPCR (Pr: promoter). The primers were specific to the DNase I hypersensitive sites 5’HS2 and 5’HS3, the promoters of the mouse (Ey-, βh1-, βmaj) and the human β-globin genes (ε, γ, and β). Primers specific to β-actin were used as negative controls. “**” indicates significant enrichment compared to IgG (p-value <0.05). (A) KLF1 ChIP on E10.5 erythroid cells, n=3; (B) KLF1 ChIP on E11.5 erythroid cells, n=3; (C) KLF2 ChIP on E11.5 erythroid cells. Two KLF2 antibodies were used on the mouse β-globin locus (one from Santa Cruz, and the other was a gift from Dr. Ng). Mouse locus and β-Actin: n=4 for IgG and KLF2_Ng, n=2 for KLF2_SC; human locus: n=2. The error bars indicate standard error mean (SEM).
3.4  KLF1 affects histone modifications at the β-globin locus in embryos

Histone modifications correlate with the state of gene transcription. In general, acetylated histones mark actively transcribed loci (Kiefer et al., 2008). More specifically, histone 3 lysine 9 acetylation (H3K9Ac) marks chromatin regions of open conformation, while histone 3 lysine 4 trimethylation (H3K4me3) marks chromatin regions of active transcription (Kiefer et al., 2008, Hosey et al., 2010). KLF1 and KLF2 interact with histone acetyltransferase cofactors such as CBP, p300, and PCAF (Zhang and Bieker, 1998, SenBanerjee et al., 2004). To determine whether the absence of KLF1 and KLF2 in the blood cells from mutant embryos disrupts the normal deposition of histone marks at the mouse and human β-globin loci, ChIP assays were performed with antibodies specific to H3K9Ac and H3K4me3. These histone marks are mostly enriched within the second or third exons of the β-globin genes (Demers et al., 2007, Kim et al., 2007b).

At E10.5, the mouse Ey- and βh1- and human ε- and γ-globin mRNAs are expressed. As expected, in normal cells H3K9Ac (Figure 3.4 A) and H3K4me3 (Figure 3.4 B) marks were relatively enriched at the expressed genes compared to non-specific antibodies. Surprisingly, H3K4me3 is also enriched in the adult mouse β-globin gene, βmaj, even though it is not yet expressed (Figure 3.4 B). It is possible that H3K4me3 marks this gene prior to transcription. At the LCR, the amount of H3K9Ac and H3K4me3 is generally less than at the globin genes, but is enriched compared to IgG.

When KLF1 is ablated, the enrichment of H3K9Ac is significantly reduced in the mouse Ey- and βh1-, and human ε- and γ-globin genes (Figure 3.4A). Similarly, the enrichment of H3K4me3 is significantly reduced in the Ey-, βh1-, and ε-globin genes when KLF1 is ablated (Figure 3.4 B). The amount of H3K4me3 in the γ-globin gene is modestly but not significantly
reduced in the absence of KLF1, even though γ-globin mRNA is significantly reduced in KLF1-/- compared to WT primitive erythroid cells. In the absence of KLF1, the amount of H3K9Ac and H3K4me3 at mouse and human 5’HS2 and 5’HS3 is also significantly decreased compared to WT (Figure 3.4 A,B). The mouse necdin gene is a neuron-specific gene that is not expressed in erythroid cells (Sengupta et al., 2008). The amounts of H3K9Ac and H3K4me3 at the necdin gene are low and not different in WT and KLF1-/- primitive blood cells. The lower abundance of H3K9Ac and H3K4me3 at the mouse and human β-globin genes and LCR in KLF1-/- compared to WT cells correlates with reduced transcription.

KLF2 does not affect histone modifications to the same extent as KLF1. Most of the sites in the β-globin locus that were tested do not exhibit differences in H3K9Ac and H3K4me3 marks between WT and KLF2-/- primitive erythroid cells (Figure 3.4 C,D). However, the H3K9Ac and H3K4me3 enrichment is reduced in KLF2-/- compared to WT at the adult human β-globin gene. Unexpectedly, H3K4me3 enrichment is higher at the Ey-globin gene in KLF2-/- compared to WT erythroid cells (Figure 3.4 D).
Figure 3.4 A, B
Figure 3.4 A, B
Figure 3.4: Differential enrichment of H3K9Ac and H3K4me3 at the mouse and human β-globin loci in WT, KLF1/- and KLF2/- primitive erythroid cells

ChIP assays using anti-H3K9Ac (A, C) or anti-H3K4me3 (B, D) were performed on E10.5 erythroid cells from WT, KLF1/- (A, B), or KLF2/- (C, D) embryos with the human β-globin locus. H3K9Ac generally indicates open chromatin conformation, whereas H3K4me3 indicates active transcription. The mean IgG enrichment was set as 1.0 and the enrichment of H3K9Ac or H3K4me3 were scaled appropriately. Necdin was used as a negative control. WT: wild type, Ex: exonic region. N=3. Error bars: standard error. “*” indicates significant enrichment compared to IgG (p-value <0.05).
3.5 Discussion

It was originally believed that KLF1 is required only for adult $\beta$-globin gene expression, because KLF1 KO mice die just after the embryonic to adult $\beta$–globin gene switch (Nuez et al., 1995, Perkins et al., 1995). However, more quantitative mRNA analyses in KLF1 KO mouse embryos established that KLF1 regulates mouse Ey- and $\beta$h1-globin gene expression in primitive erythroid cells (Basu et al., 2007). KLF2 also has a role in mouse Ey- and $\beta$h1-globin gene regulation (Basu et al., 2005). Furthermore, in transgenic mouse models, KLF2 regulates the human $\varepsilon$- and $\gamma$-globin genes in E10.5 yolk sac (Basu et al., 2005). Here, we established that KLF1 is also required for normal expression of the human embryonic $\varepsilon$- and fetal $\gamma$-globin genes during mouse primitive erythropoiesis.

The ChIP data on mouse primitive erythroid cells provides the first quantitative evidence that KLF1 binds to the $\beta$-globin promoters during primitive erythropoiesis. We did not detect KLF1 at the promoter of the $\beta^{\text{maj}}$–globin gene, as was reported in semi-quantitative assays using a tagged KLF1 knock-in in primitive erythroid cells (Zhou et al., 2006). KLF2 directly binds to the promoters/enhancers of key regulators of stem cell pluripotency in ES cells (Jiang et al., 2008). Our data shows that KLF2 is directly recruited to the promoters of the murine and human embryonic and fetal $\beta$-globin genes in native primitive erythroid cells.

Our work indicates that KLF1 acts directly as a positive regulator of the human $\varepsilon$- and $\gamma$-, and mouse Ey- and $\beta$h1-globin genes during embryonic erythropoiesis. This is in contrast to increasing evidence that has recently emerged, supporting an indirect negative role for KLF1 in $\gamma$-globin gene regulation during adult erythropoiesis. Certain mutations in the human KLF1 gene have been correlated with hereditary persistence of fetal hemoglobin (HPFH) (Arnaud et al.,
2010, Borg et al., 2010, Siatecka et al., 2010, Satta et al., 2011). An E325K mutation was detected in patients with congenital dyserythropoietic anemia (CDA) who express increased amounts of HbF (Arnaud et al., 2010). A mutation in the analogous residue, E339D, occurs in the Nan mouse, which has an increase in βh1-globin expression in the fetal liver and adult spleen (Siatecka et al., 2010). A heterozygous K288X mutation is found in a Maltese family with HPFH. The mutation eliminates the KLF1 zinc fingers and hence abrogates DNA binding (Borg et al., 2010). Interestingly, an S270X mutation in a Sardinian family does not cause an increase in HbF even though it eliminates the zinc fingers (Satta et al., 2011). A compound heterozygote with the S270X and a K332Q mutation does have HPFH (Satta et al., 2011). The mechanism for the negative effect of KLF1 on γ-globin gene regulation in the adult is most probably indirect, via upregulation of BCL11A (Borg et al., 2010, Zhou et al., 2010). Apparently, KLF1 can positively or negatively affect γ-globin gene regulation, depending on the erythroid cell milieu.

The expression patterns of KLF1 and KLF2 in primitive and definitive erythroid cells were analyzed, and reveal a possible explanation for the different milieu at the two stages. The ratio of KLF1 to KLF2 mRNA increases dramatically as erythroid cells switch from the primitive to the definitive stage. KLF1 acts as a repressor of megakaryocytic differentiation genes and therefore drives megakaryocyte-erythroid progenitor cells toward erythroid differentiation (Isern et al., 2010). It is possible that KLF1 is also involved in the switch from primitive to definitive erythropoiesis. It is plausible that KLF2 drives erythroid cells towards embryonic globin gene expression, opposing the role of KLF1.

Our data indicate that at E10.5, H3K9Ac and H3K4me3 are enriched at the actively transcribed globin genes, mouse Ey and βh1, and human ε and γ. Previous reports have shown
that H3K9Ac and H3K4me3 marks correlate with each other in the human β-globin locus during adult erythropoiesis (Demers et al., 2007). Our work provides the first demonstration in native embryonic erythroid cells that H3K9Ac is associated only with the actively transcribed globin genes. In contrast, in a previous study of mouse embryonic erythropoiesis, using an antibody that detects both H3K9Ac and H3K14Ac (i.e. H3Ac), H3Ac and H3K4me2 enrichment was found at both the embryonic and adult β-globin genes (Fromm and Bulger, 2009). In the human β-globin locus, H3Ac and H3K4me2 are detected within the LCR and only at the active β-globin genes (Fang et al., 2009, Fromm and Bulger, 2009). In K562, a human cell line which expresses the fetal γ- but not the adult β-globin gene, H3Ac, H3K4me2, and H3K4me3 are enriched specifically at the γ-globin gene (Kim et al., 2007a). H3K4me3 is enriched only in the actively transcribed fetal and adult β-globin genes in human and mouse tissue culture cell models, respectively (Kim et al., 2007a, Fromm et al., 2009). Our results in native embryonic erythroid cells differ in that H3K4me3 is somewhat enriched at the mouse adult βmaj-globin gene at E10.5, prior to its activation.

KLF1 binds to histone modifying proteins such as CBP, p300, and PCAF (Zhang and Bieler, 1998, SenBanerjee et al., 2004, Kim et al., 2009). Our data indicate for the first time that H3K9Ac and H3K4me3 are reduced at the mouse Ey- and βh1-, and human ε- and γ-globin genes, in primitive erythroid cells lacking KLF1. Transcription factors have previously been implicated in affecting histone modifications. The presence of GATA1 correlates with histone acetylation, H3K4me2 and H3K4me3 at the murine β-globin locus (Im et al., 2005). Histone acetylation and H3K4me3 are significantly reduced in the adult β-globin gene by the absence of KLF1 in E13.5 fetal liver cells (Bottardi et al., 2006). It is difficult to dissect cause and effect in
these cases. It is not known whether reduced transcription due to the absence of the transcription factor leads to decreased H3Ac and H3K4me3, or vice versa. However, there is a strong correlation between the presence of KLF1 and the occurrence of histone marks found in actively transcribed genes.

A simple model incorporating the available data is that KLF1 and KLF2 interchangeably (but not simultaneously) bind to the same CACCC elements in the LCR and promoters in the β-globin locus, and promote gene expression in the embryo. This model is supported by the fact that KLF1 and KLF2 have very similar DNA binding domains, there is no evidence that they can form dimers, and they are likely to be present in similar amounts in embryonic blood cells. Individually, the effect of KLF1 on globin gene regulation tends to be greater than that of KLF2, based on qRT-PCR in the mouse KO models. Interestingly, this draws a parallel with our results studying histone marks in KLF1-/- and KLF2-/- embryonic blood cells. In these experiments, KLF1 binding correlates with histone marks that are associated with transcription, while KLF2 does not appear to dictate histone modifications in the β-globin locus. Yet, both KLF1 and KLF2 positively regulate globin gene transcription through direct binding to CACCC elements in the locus. Therefore, there are distinct yet overlapping mechanistic roles for KLF1 and KLF2 in embryonic red blood cells.
CHAPTER FOUR:

Regulation of β-globin genes by KLF1 and KLF2 in the human

4.1 Introduction

Investigating the mechanism of γ-globin gene regulation in the mouse is an important venue towards understanding the mechanism underlying hemoglobin switching. However, the expression pattern of γ-globin in transgenic mice that carry the entire β-locus does not entirely mimic γ-globin expression in humans. In humans, γ-globin is expressed in the fetal liver (Weatherall, 2001, Zhang et al., 2002) while it is expressed in the yolk sac of transgenic mice (Noordermeer and de Laat, 2008). Therefore, we sought to study the regulation of the γ-globin gene in cord blood cells as a complimentary system. Human fetal liver cells would be the best cells to study γ-globin regulation. However these cells are not available for research due to ethical concerns. The best available model to study γ-globin regulation in vitro during its normal expression in humans is by using hematopoietic stem cells (CD34+) from cord blood. Therefore, we acquired cord blood units, isolated CD34+ cells from them, and then expanded and differentiated them into the erythroid lineage ex vivo. We found that γ-globin is expressed in equal amounts as β-globin which resembles the pattern of γ- and β-globin expression in newborn (Weatherall, 2001). We also found that KLF1 is expressed in increasing amounts as CD34+ cells differentiate to erythroid cells. KLF2 is also expressed but in lower amounts than KLF1. ChIP assays showed that KLF1 bind directly to the promoter of β- and γ, while KLF2 binds to epsilon and γ. To study the role of KLF1 and KLF2 in regulating γ- and β-globin genes we used a lentiviral system to knockdown either KLF1 or KLF2. KLF1 Knockdown shows a robust decrease of β-globin gene expression and insignificant change of γ-globin gene
expression. Attempts to knockdown KLF2 resulted only in 40% reduction of KLF2 which appeared to be insufficient to show any effect on γ- or β- globin genes. Histone modification and polymerase recruitment studies showed that KLF1 is important to maintain normal signature of histone modifications and polymerase II recruitment onto β- globin gene.

4.2 Cord blood derived CD34+ cells mimic newborn pattern of β- globin gene expression upon differentiation

In vitro differentiation of hematopoietic stem cells has emerged as an instrumental tool for understanding hemoglobin regulation. CD34 is a hematopoietic stem cell marker (Orkin, 2000). Since γ- globin is expressed at its highest amounts in the fetal liver and continue to express after birth, we sought to use cord blood to isolate CD34+ cells for in vitro expansion and differentiation. We acquired freshly collected cord blood units and isolated CD34+ cells using magnet separation techniques. The isolated cells were kept for one week in expansion media before they were allowed to differentiate to erythroid lineage by replacing the expansion media with differentiation media. During differentiation, we collected cells for RNA studies. RNA was isolated and the amounts of γ- and β- globin mRNA were measured. We found that both γ- and β- globin mRNAs increased with time as CD34+ become more erythroid (Figure 4.1). Epsilon globin mRNA was negligible at these cells and therefore it is not shown in (Figure 4.1). Starting from day six of differentiation cells show noticeable red color indicating that hemoglobin is being expressed in them. At day eight of differentiation, cells are very red and more than 90% positive for benzidine staining which stains hemoglobin. Data at later days of differentiation suggest that γ- and β- globin mRNAs continue to increase at E10 but decline at E12. The results show that the cord blood derived CD34+ cells are capable of recapitulating the status of newborn
hemoglobin if they were allowed to differentiate in vitro. Thus support the premise that cord blood CD34+ cells are a valid model to study human fetal hemoglobin regulation.

**Figure 4.1: Expression pattern of β- globin genes in cord blood derived CD34+ cells**

CD34+ cells were selected from fresh umbilical cord blood units, expanded for eight days then in-vitro differentiated for another eight days. Cells were collected every other day of differentiation for RNA extraction. (A) The amount of globin mRNAs were measured using qRT-PCR methods and expressed using the $E^{ΔCT}$ method to allow for direct comparison of the mRNAs from all differentiation time points. Cyclophilin A was used as a normalization control. N=3, Error bars: S.D. CD34+ derived erythroid cells at day 8 of Differentiation are red (B) and stain positive for hemoglobin (C, Benzidine staining).
4.3 **KLF1 is expressed in higher amounts compared to KLF2 expression in *in vitro* differentiated CD34+ cells**

KLF1 is a major transcription factor in β- globin regulation (Nuez et al., 1995, Perkins et al., 1995, Basu et al., 2007). KLF2 is important factor for embryonic and fetal globin gene regulation in the mouse. We assumed that cord blood derived CD34+ cells will express increasing amounts of KLF1 and KLF2 as they differentiate to become erythroid cells. To examine this we measured the amounts of KLF1 and KLF2 at different time points across CD34+ differentiation. The results show that the mRNA amounts of KLF1 indeed increase with differentiation (Figure 4.2). KLF2 mRNA is detectable but does not show an increasing pattern of expression with differentiation (Figure 4.2). The results suggest that KLF1 is more erythroid specific than KLF2. The ratio of KLF1 to KLF2 at day eight of differentiation is about 10:1 which is more similar to the ratio of KLF1 to KLF2 in definitive erythroid cells in the mouse (Alhashem et al., 2011).
Figure 4.2: Expression pattern of KLF1 and KLF2 in cord blood derived CD34+ cells

CD34+ cells were selected from fresh umbilical cord blood units, expanded for eight days then in-vitro differentiated for another eight days. Cells were collected every other day of differentiation for RNA extraction. The amount of KLF1 and KLF2 mRNAs were measured using qRT-PCR methods and expressed using the E^{ΔCT} method to allow for direct comparison of different mRNAs from all differentiation time points. Cyclophilin A was used as a normalization control. N=3, Error bars: S.D.
4.4 KLF1 is important for β-globin expression

To investigate the importance of γ-globin regulation by KLF1, we attempted to knockdown KLF1 using a lentiviral system that carries a short hairpin RNA (shRNA) targeted against KLF1. An independent study showed that this shRNA was able to inhibit 85% of KLF1 protein production (Bouilloux et al., 2008). Cord blood derived CD34+ cells were infected with particles containing either KLF1-shRNA or Scramble shRNA (Scr-shRNA). The infected cells should be GFP+ and they were sorted based on their ability to express GFP. Lentiviral infection with either KLF1 or scrambled shRNAs show approximately 70% GFP expressed cells. Positively infected cells were induced for differentiation for eight days. We were able to successfully knockdown KLF1 to more than 70% at day eight of differentiation compared to scr-shRNA (Figure 4.3A). At that level of knockdown the ratio of γ- and β- globin is changed in favor of γ- globin. Setting the γ-: β- globin mRNA ratio of scr-shRNA samples to 1:1, KLF1 KD changed the ratio to about 3.5:1 as shown in (Figure 4.3B). The results suggest that KLF1 either suppresses γ-globin or activates β-globin. To differentiate between the two possibilities γ- and β-globin mRNA have been normalized to the amount of cyclophilin A mRNA, a house keeping gene. The results showed that the change of γ- to β- ratio was indeed because of decrease in β-globin rather than increase of γ- globin which suggests that KLF1 functions mainly by up-regulating β-globin gene expression (Figure 4.3C). However, Data from other other studies performed on adult CD34+ cells show that KLF1 indirectly down-regulate γ-globin by up-regulating BCL11a, a zinc finger repressing transcription factor, which in turn release the suppression of γ-globin expression (Zhou et al., 2010). To investigate whether BCL11a has a role in our system we measured the amount of BCL11a in KLF1 knockdown cells. The results show that BCL11a was severely decreased when KLF1 is knocked down (Figure 4.4A).
However this decrease does not result in an increase in $\gamma$-globin regulation, as is seen in the adult system. Moreover, we noticed that as KLF1 is reduced, KLF2 mRNA show a compensatory 4-fold increase compared to Scr-shRNA (Figure 4.4A). As KLF1 goes down and KLF2 goes up to compensate for the loss of KLF1, the ratio of KLF1 to KLF2 changes from 10:1 in scr-shRNA to about 1:1.2 in KLF1-shRNA (Figure 4.4B). This compensation suggests that double knockdown of KLF1 and KLF2 may have a more dramatic effect on $\beta$-globin expression or reveal a hidden effect of KLF1 or KLF2 on $\gamma$-globin expression.
Figure 4.3: Effects of KLF1 Knockdown in CD34+ cells on the expression of γ- and β-globin genes

CD34+ cells were selected, expanded, and differentiated as described earlier. After the end of expansion period, cells were treated with lentiviral particles carrying either scramble (Scr) or KLF1 specific shRNAs. Cells were collected for RNA studies at day 8 of differentiation. (A) The remaining amounts of KLF1 mRNA were measured using qRT-PCR and plotted after being normalized by Cyclophilin A mRNA (Cyc.A) and setting individual Scramble results as one. (B) The amounts of γ- and β-globin mRNAs were quantified and plotted as individual globin mRNA over the total globin mRNA (β- + γ). (C) The amounts of γ- and β-globin mRNAs were quantified and plotted as individual globin mRNA over Cyc.A. N=7, error bars= S.D, * = p-value <0.05 compared to the scr-shRNA.
CD34+ cells were selected, expanded, and differentiated as described earlier. Just at the end of expansion period cells were treated with lentiviral particles carrying either scramble (Scr) or KLF1 specific shRNAs. Cells were collected for RNA studies at day 8 of differentiation (A) The amounts of KLF2 and BCL11a mRNA were measured using qRT-PCR and plotted after being normalized by Cyclophilin A mRNA (Cyc.A) and setting individual Scramble results as one. (B) The relative amounts of KLF1 and KLF2 in the scr-shRNA or KLF1-shRNA treated cells were established and plotted after normalizing over Cyc.A and setting KLF1 mRNA in scr-shRNA treated cells as one and adjusting the other values accordingly. N=3, error bars= S.D, * = p-value <0.05 compared to the scr-shRNA.

Figure 4.4: Effect of KLF1 Knockdown in CD34+ cells on the expression of BCL11a and KLF2 genes
4.5 40% knockdown of KLF2 does not affect regulation of the γ- or β- globin genes

We have shown earlier that KLF2 regulates γ- globin expression in a transgenic mouse model (Alhashem et al., 2011). Here we attempted to knockdown KLF2 in CD34+ cells and observe the effect of that on γ- and β- globin gene expression. Two short hairpin (shRNA) constructs were delivered independently into CD34+ cells using a lentiviral system. One of the shRNAs, KLF2-shRNA(a), was adopted from a previously published paper showed 80% knockdown of KLF2 in endothelial cells (Dekker et al., 2005). The other, KLF2-shRNA(b), was based on the sequence of siRNA used previously to transiently knockdown KLF2 in K562 cells (Pang et al., 2012). Cord blood derived CD34+ cells were infected with particles containing KLF2-shRNA(a), KLF2-shRNA (b), or Scr-shRNA. The infected cells should GFP+ and they were sorted based on their ability to express GFP. Lentiviral infection with any of the shRNAs give approximately 70% GFP+ cells. Positively infected cells were induced for erythroid differentiation for eight days. At day eight of differentiation, RNA was isolated to measure KLF2 and other expressed genes. Unfortunately, only 40% knockdown of KLF2 was achieved using either of the two shRNAs, compared to Scr-shRNA. The poor knockdown could be explained by the low level of KLF2 mRNA in these cells initially and probably the nature of KLF2 as a GC rich mRNA which could make it more difficult for shRNA to access their target sequence on KLF2 mRNA. With 40% KLF2 knockdown no evidence of any change in ratio of γ- to β- globin mRNA (Figure 4.5B). There was also no significant change in the γ- or β- globin gene expression when normalized to cyclophilin A although data taken from KLF2-shRNA(b) suggest that reduction in KLF2 may reduce both γ- and β- globin mRNAs (Figure 4.5D). BCL11a and KLF1 mRNAs were not significantly changed by 40% KD of KLF2 (Figure 4.5C). All together, KLF2 is expressed at low levels of mRNA compared to KLF1 which could mask minor but
probably significant role of KLF2. KLF2 also failed to be knocked down optimally in the cord blood CD34+ cells using the available shRNAs.

Figure 4.5: Effects of KLF2 Knockdown in CD34+ cells on the expression of γ- and β-globin genes
CD34+ cells were selected, expanded, and differentiated as described earlier. After the expansion period, cells were treated with lentiviral particles carrying either scramble (Scr) or KLF2 specific shRNAs. Two different KLF2 sequences were used. Cells were collected for RNA studies at day 8 of differentiation. (A) The remaining amounts of KLF2 mRNA were measured using qRT-PCR and plotted after being normalized by Cyclophilin A mRNA (Cyc.A) and setting individual Scramble results as one. (B) The amounts of γ- and β- globin mRNAs were quantified and plotted as individual globin mRNA over the total globin mRNA (β- + γ). (C) The amounts of KLF1 (K1) and BCL11a (BC) mRNA were measured using qRT-PCR and plotted after being normalized by Cyclophilin A mRNA (Cyc.A) and setting individual Scramble results as one. (D) The amounts of γ- and β- globin mRNAs were quantified and plotted as individual globin mRNA over Cyc.A. N=2 for KLF2-shRNA (a) and N=3 for KLF2-shRNA (b), error bars= S.D, * = p-value <0.05 compared to the scr-shRNA.
4.6 Erythroid maturation

We have shown that KLF1 is critical for β-globin gene expression. To address that the decrease of β-globin mRNA is not due to a delay in erythroid maturation, slides of scr- or KLF1-shRNA treated erythroid cells were prepared at day eight and twelve of differentiation. Slides submitted anonymously to a hematologist for hematological evaluation (Dr. David Williams, Pathology, VCU-MCV). Erythroid cells change from basophilic to polychromatic to orthochromatic upon differentiation (Zhang et al., 2003, Fraser et al., 2007). Basophilic erythroid cells have blue cytoplasm and large uncondensed nuclei. Orthochromatic erythroid cells have pink shade of cytoplasm and more condensed nuclei and they are smaller in size. Polychromatic erythroid cells fall in between the two categories (Figure 4.6B). At day eight of differentiation erythroid cells are mostly polychromatic erythrocytes (about 60%) (Figure 4.6A). Basophilic and Orthochromatic erythrocytes account for the remaining part of erythroid cells (about 25%, 15% respectively). At day eight of differentiation (the same day at which the mRNA analyses were done) the status of erythroid maturation in cells treated with KLF1-shRNA is almost identical to the status of erythroid cells treated with scr-shRNA (Figure 4.6A). The results confirm that the effect of KLF1 on β-globin mRNA is direct and not due to changes is cell differentiation.

Another report obtained from experiments performed on bone marrow derived CD34+ show that erythroid maturation was not affected by KLF1 knockdown (Zhou et al., 2010). At day 12 of differentiation, erythroid cells are more heterogeneous between samples compared to day eight (compare error bars of Figure 4.6A at day 8 vs 12). With the greater heterogeneity, it seems that KLF1 knockdown causes delay of erythroid maturation. Orthochromatic erythrocytes look more abundant, but not significantly different, in the KLF1-shRNA treated cells when compared to Scr-shRNA (Figure 4.6A).
Figure 4.6: Effect of KLF1 knockdown on erythroid maturation

CD34+ cells were selected and expanded as described earlier. Just at the end of expansion period cells were treated with lentiviral particles carrying either scramble (Scr) or KLF1 specific shRNAs. (A) At day 8 or 12 of differentiation, cytospin slides prepared and level of erythroid maturation was scored. 100 cells from each slide were scored based on their morphology and called as basophilic, polychromatic or orthochromatic erythroid cells. N= 5, error bars = S.E.M. Student’s T-test was done but there was no significant differences in number of cells at each category when compared to its relevant control. (B) Representation of the different categories of erythroid cells at day eight of differentiation.
4.7 Generation and characterization of the custom KLF2 antibody

ChIP grade KLF2 antibodies were not available commercially. ChIP assays for KLF2 has been reported using mouse ES and a custom made antibody targeted against mouse KLF2 (Jiang et al., 2008). We received a sample of the antibody and the construct that was used to generate the antibody. The amount of anti-KLF2 antibody was enough to ChIP KLF2 in primitive mouse erythroid cells (Alhashem et al., 2011, Pang et al., 2012). Therefore, we sought to generate more of the antibody to use in CD34+ cells. The cDNA encoding the amino acids 90-255 of the mouse KLF2 was cloned into the pET42b plasmid. The plasmid encodes a protein fused to GST and 6-His Tags at the C-terminus. The plasmid was then transferred into BL21 bacteria and induced for protein expression by adding IPTG. The soluble lysate of bacterial protein was proceeded to run on a GST column and then a nickel column for purification (using molecular biology core facility, VCU). Four different preparations of the purified protein (two were gel extracted 43.6 kD bands and two were whole purified protein) were sent to a custom antibody production facility (Cocalico Biologicals, Inc, Reamstown, PA) to immunize rabbits. Pre-immune serum was saved from each rabbit used for the antibody production. Test antisera were collected after 35 and 56 days of immunization. Immunized rabbits were given booster doses of the antigen (mouse GST-His-KLF2_{90-255} protein) after 14, 20, and 49 days of first immunization. Production serum was collected 98 days after the first immunization and used to check the binding of KLF2 onto known binding sites (i.e Nanog Promoter and enhancer, Fbxo15 promoter). ChIP assays show that preparation (B) of the KLF2 antibody gives the highest fold enrichment at the tested sites compared to pre-immune serum (Figure 4.7A). Lysate from IPTG induced GST-His-KLF2_{90-255} bacteria shows that prep (A) was specific to the induced protein (Figure 4.7B). Lysates from K562 transfected with an over-expression construct for KLF2 were used to test whether the
antibody preparations can detect KLF2. Western blotting shows that Anti KLF2 (D) has the most specific KLF2 detection (Figure 4.7C). The results suggest that Anti-KLF2 (B) is good for ChIP and anti-KLF2 (D) is good for Western blotting.
Figure 4.7: Characterization of custom KLF2 antibodies

Four preparations of KLF2 antibody obtained by immunizing rabbits with purified GST-His-KLF2^{90-255} proteins (Anti-KLF2 (A-D)). (A) Chromatin from mESCs was precipitated with pre-immune serum or one of the four antibody preps. Precipitated chromatin was purified and quantified using qPCR with primers specific to Nanog enhancer, Nanog promoter, Fbxo15 promoter or Necdin promoter. N= 2, error bars = S.E.M. (B) and (C) represents western blot to test each one of the KLF2 preps on IPTG induced bacteria that express GST-His-KLF2^{90-255} protein (B) or K562 with KLF2 overexpression construct (C).
4.8 **KLF1 and KLF2 occupy distinct sites in the β-globin locus**

KLF1 regulates the expression of human and mouse β-globin genes in transgenic mice and in human cells (Nuez et al., 1995, Perkins et al., 1995, Basu et al., 2007, Alhashem et al., 2011). KLF2 regulates the expression of epsilon and γ-globin genes in the mouse system (Basu et al., 2005, Alhashem et al., 2011). Next, we asked whether KLF1 and KLF2 bind the promoters of β-globin genes, suggesting the regulation is direct. We carried out chromatin immunoprecipitation assays on CD34+ cells derived from cord blood and differentiated to erythroid cells for eight days. The results showed that KLF1 binds directly to the β-globin locus control regions 5’HS-3 and 5’HS-2 in addition to the γ- and β- globin promoters (Figure 4.8A). The binding of KLF1 is apparently more prominent on the promoter of the β-globin than the γ-globin gene, which correlates with the role of KLF1 on β-globin gene expression. For KLF2 ChIP custom KLF2 antisera was generated as described above. ChIP assays also showed that KLF2 binds to the promoter of γ-globin gene and 5’HS-3 (Figure 4.8B). Binding of KLF2 to the γ-promoter strongly suggests that KLF2 has more important roles in γ-regulation than KLF1. There was no evidence of KLF2 binding to the promoter of β-globin genes or to the 5’HS-2.
Figure 4.8: Binding of KLF1 or KLF2 to the β- globin locus

CD34+ cells were selected, expanded, and differentiated as described earlier. At day 8 of differentiation, cells prepared for ChIP assays. Chromatin from cells was incubated with either KLF1 (A) or KLF2 antibodies. Nonspecific antibodies were used as negative control. Precipitated chromatin was subjected to qPCR to quantify the amount of DNA precipitated by KLF1 (A) or KLF2 (B) at the different sites shown. N= 2, error bars = S.E.M, * = p-value <0.05 compared to nonspecific antibodies.
4.9 Histone modifications and RNA polymerase II recruitment are altered in the absence of KLF1.

Our data shows that histone modifications indicating open chromatin and active transcription are attenuated at the β-globin locus in the absence of KLF1 in transgenic mice (Alhashem et al., 2011). Here we sought to examine the status of chromatin when KLF1 is knocked down in erythroid differentiated cord blood derived CD34+ cells. We chose two histone marks, H3K4me3H3K9Ac, which indicate open chromatin and active transcription, respectively (Kiefer et al., 2008, Hosey et al., 2010). We also chose to test the abundance of RNA polymerase II (Pol II) and its active form which indicates active elongation of RNA transcripts, Pol II phospho-serine 2 (Pol II S2P) as a measure of transcription and chromatin status (Bottardi et al., 2006). Since Histone modifications and RNA Pol II recruitment could be different in the promoter and downstream sequences, we tested both sites at the γ- and β-globin genes.

ChIP for H3K4me3 at day eight of CD34+ differentiation showed that: (1) H3K4me3 marks were enriched on all sites tested in both scr-shRNA and KLF1-shRNA samples when compared to the enrichment of non-specific IgG (Figure 4.9A); (2) enrichment is greater at genes, including β- Actin, when compared to the enrichment at the LCR, assuming primer efficiencies are similar at all sites (Figure 4.9A); (3) H3K4me3 marks are reduced in KLF1-shRNA samples at the β- globin but not other globin genes when compared to scr-shRNA (Figure 4.11).

ChIP for H3K9Ac at day eight of CD34+ differentiation showed that: (1) H3K9Ac marks were enriched on all sites tested except β- Actin in both scr-shRNA and KLF1-shRNA samples when compared to the enrichment of non-specific IgG (Figure 4.9B); (2) similar to H3K4me3, more H3K9Ac was observed at the γ- and β- globin genes compared to the LCR (Figure 4.9B);
(3) H3K9Ac marks are reduced at the γ-globin promoter but not at the downstream gene sequence while reduced at both the β- globin promoter and downstream gene when KLF1 is knocked down (Figure 4.11).

Pol II ChIP assays indicate that the RNA polymerase recruitment at the β- globin gene is reduced in the KLF1-shRNA samples indicating that KLF1 is important in recruiting Pol II to the β- globin gene (Figure 4.10A, Figure 4.11). Recruitment of Pol II is not decreased at the γ-globin gene which correlates with no regulation of γ- by KLF1 at the mRNA level (Figure 4.10A, Figure 4.11). The antibody that detects only the phosphorylated form of RNA pol II show similar pattern of recruitment as the total Pol II indicating that KLF1 effect is on the recruitment of Pol II rather than activation of or elongation by Pol II (Figure 4.10B, Figure 4.11).
Figure 4.9: Effect of KLF1 Knockdown on histone modifications

CD34+ cells were selected, expanded, and differentiated as described earlier. At day 8 of differentiation, cells prepared for ChIP assays. Chromatin from cells was incubated with either H3K4me3 (A) or H3K9Ac antibodies. Nonspecific antibodies were used as negative control. Precipitated chromatin was subjected to qPCR to quantify the amount of DNA precipitated by H3K4me3 (A) or H3K9Ac (B) at the different sites shown.

N= 4, error bars = S.E.M. * = p-value <0.05 comapred to Scr-shRNA
Figure 4.10: Effect of KLF1 Knockdown on RNA polymerase II recruitment

CD34+ cells were selected, expanded, and differentiated as described earlier. At day 8 of differentiation, cells prepared for ChIP assays. Chromatin from cells was incubated with either total Pol II (A) or activated pol II (Pol II Ser2P) antibodies. Nonspecific antibodies were used as negative control. Precipitated chromatin was subjected to qPCR to quantify the amount of DNA precipitated by H3K4me3 (A) or H3K9Ac (B) at the different sites shown. N= 4, error bars = S.E.M. * = p-value <0.05 compared to Scr-shRNA
Results from Figures 5.7 and 5.8 were compiled together after setting individual signal from Scr-shRNA treated cells as one and adjusting the signal from KLF1-shRNA treated cells appropriately. N=4, error bars = S.E.M. * = p-value <0.05 compared to Scr-shRNA

4.10 Discussion

Our work showed that cord blood CD34+ cells supports the idea that γ- globin regulation can be studied in human cord blood hematopoietic stem cells as a complementary model besides the transgenic mouse models. Erythroid cells derived from ex-vivo differentiation of cord blood CD34+ cells show similar pattern of γ- and β- globin gene expression compared to blood of newborn babies. The expression of the γ- globin gene in transgenic mice is slightly different that its expression in humans. Most human globin regulation studies were performed on cell lines (e.g K562 cells) or adult hematopoietic stem cells (e.g. derived from peripheral adult blood). Fetal liver cells would be a better model for studying γ- globin regulation if available. In newborn
humans, γ- counts for about 50% of the expressed β- globin genes (Weatherall, 2001, Zhang et al., 2002). Therefore, cord blood cells, which are rich in hematopoietic stem cells and are commercially available for research, are a useful model for γ- globin regulation. Our data obtained from ex vivo differentiated cord blood hematopoietic stem cells showed that γ- and β- globin genes are expressed in equal amounts making them similar to newborn humans. Recent study on peripheral blood CD34+ cells showed that γ- and β- globin switch as cells differentiate in culture (Li et al., 2012). Differences in culture conditions and source of CD34+ cells may account for the different results in γ- and β- globin mRNA in our experiment vs Li et.al. We also showed that high expression of KLF1 is concomitant with high expression of both γ- and β- globin mRNAs. This is the first hint that KLF1 may not repress γ- globin expression as suggested by other reports (Borg et al., 2010, Zhou et al., 2010). KLF2 mRNA is detectable in cord blood derived erythroid cells although not as high as KLF1. Since, Cord blood is derived from the fetal liver, we expect to see more KLF1 comapred to KLF2 as was seen in the mouse (Alhashem et al., 2011).

There were no previous reports on the role of KLF1 and KLF2 in cord blood derived erythroid cells. Knockdown of KLF1 and KLF2 in the ex vivo differentiated cord blood CD34+ cells was attempted to investigate the roles of these two transcription factors on γ- and β- globin gene expression. Knockdown of KLF1 clearly confirmed the well-known role of KLF1 on adult β- globin regulation in the mouse and human (Perkins et al., 1995, Zhou et al., 2010). However, regulation of γ- globin by KLF1 is not clear. We showed, like others, that the ratio of Γ- over γ- and β- (G/G+B) is increased when KLF1 is knocked down (Borg et al., 2010, Zhou et al., 2010). The caveat of this calculation is it does not differentiate between the increase in γ- or decrease in β. In our case, we normalized the expression of γ- over cyclophilin A. We showed that γ- is not
up-regulated by KLF1 knockdown. Reports have shown that an increase in γ- used a normalization control that was reported as a target of KLF1 (e.g β- or α- globins). BCL11a is a repressor that is thought to mediate KLF1 repression of γ- globin (i.e KLF1 up regulates BCL11a, then BCL11a down-regulates γ- globin) (Borg et al., 2010, Sankaran et al., 2010, Zhou et al., 2010). As KLF1 does not regulate γ- in cord blood CD34+ derived erythroid cells, we asked whether KLF1 regulates BCL11a. Our data showed that KLF1 does regulate the expression of BCL11a as shown by severe reduction of BCL11a mRNA when KLF1 is knocked down. Our data suggest that in addition to BCL11a, there must be other factors that are more important repressors of γ- globin in the cord blood erythroid cells. These repressors presumably are not regulated by KLF1. Recent reports based on ChIP sequencing and RNA sequencing show that KLF1 is involved in all functions important for erythropoiesis. This make it difficult to identify these targets (Tallack and Perkins, 2010, Tallack et al., 2010, Pilon et al., 2011, Tallack et al., 2012). Knockdown of KLF2 in cord blood derived erythroid cells was not successful. We obtained a 40% reduction in KLF2 which is not enough to show any significant change in γ- or β- globin expression. Thus, regulation of γ- globin by KLF2 as inferred from mouse studies cannot be ruled out. Since KLF2 knockdown seems to be difficult, one could approach the role of γ- globin regulation in primary erythroid cells by KLF2 over-expression followed by measurements of β- globin genes.

KLF1 regulates the expression of β- globin gene in cord blood erythroid cells and regulation of γ- globin by KLF2 cannot be ruled out. Therefore, we asked whether KLF1 and KLF2 bind directly to the promoters of β- globin genes and the LCR. Commercially available ChIP grade anti-KLF1 and our generated anti-KLF2 antibodies were used in ChIP assays to answer the question. KLF1 was found to bind to the β- globin promoter and to the LCR. KLF1
binding to the γ-globin promoter was observed to a lesser extent. The binding of KLF2 to the γ-globin promoter in the human cord blood primary erythroid cells is similar to its binding in mouse primitive erythroid cells and both suggests that KLF2 is γ-specific. The CACCC element, KLF binding site, in γ-globin promoter is not identical to the one in β-globin promoter and the differential binding of KLF1 and KLF2 to γ- and β- suggest that KLF2 prefer the γ-globin CACCC element or KLF1 does not compete with KLF2 on the γ-globin promoter.

We have shown that KLF1 alters the epigenetics signature of the β-globin locus in mouse primitive erythroid cells by decreasing the histone acetylation and tri-methylation of specific amino acids in the histone tails that correlate with open chromatin and active transcription (Alhashem et al., 2011). Here we also show that KLF1 has the same capability of decreasing these histone marks, H3K9Ac and H3K4me3, only at the β-globin gene. These finding are supported by other reports the KLF1 can bind to some histone modifying enzymes such as CBP, p300, and PCAF (Zhang and Bieker, 1998, SenBanerjee et al., 2004, Kim et al., 2009). Moreover, our data show that recruitment of RNA Pol II and its active form, Pol II S2P, to the β-globin gene correlates with the activation of β-globin gene by KLF1. The alteration of recruitment and activation of Pol II at the β-globin gene in KLF1 knockdown cells suggest that KLF1 is important in assembling the transcription pre-initiation complex (PIC) at the β-globin gene rather than only affecting the transcription of β-globin gene. The pre-initiation complex contains other subunits beside RNA polymerase such as transcription factor IIB (TFIIB) (Ross et al., 2009). The notion that KLF1 binds to the LCR more than to the promoters correlates with others’ findings that PIC is assembled onto the LCR then transferred by means of looping to the βt globin promoters (Johnson et al., 2001, Ross et al., 2009). We did not find any change in the recruitment of RNA Pol II and RNA Pol II S2P between the promoter and the downstream
region which indicate that total polymerase is decreased from to begin with when KLF1 is knocked down. This is in contradiction with the role of KLF1 in the mouse fetal liver cells, in which KLF1 knockout lead to a reduction in the activated Pol II but not in the total Pol II (Ross et al., 2009). This indicate that the mouse KLF1 is required for transcription elongation rather than transcription initiation, while in human cells KLF1 is required for transcription initiation as depicted by reduction in total RNA Pol II.
5.1 Discussion

Erythropoiesis is a complicated process that takes place in different tissues at different developmental stages. Blood islands in the yolk sac are considered the initial site of hematopoiesis. From these blood islands, the first blood vessels arise and blood circulates by means of forces originating from the beating of the heart in the embryo. In mice, erythropoiesis starts at day E7.5 and blood begins to circulate at E8.25 (Huber et al., 2004, Palis, 2008). Primitive erythropoiesis occurs first in the yolk sac and starts by producing large nucleated erythroblasts which undergo maturational process while circulating in the blood stream to end up with small enucleated mature erythrocytes. Definitive erythropoiesis occurs later in the fetal liver, then the spleen and finally in the bone marrow.

Transcription factors are being extensively studied to elucidate their roles in erythropoiesis and in genetic disorders. Krüppel like factors (KLFs) are a family of 17 proteins whose main function is gene regulation by binding to DNA elements in the promoters of various genes. KLF transcription factors recognize the CACCC-elements and act as activators or repressors of the promoters of genes to which they bind. Among the 17 family members, KLF1, and KLF2 share high homology. KLF1 is the founding member of the family and is erythroid-specific protein. KLF2 is expressed in erythroid, endothelial, and many other cells.

KLF1 and KLF2 have a high degree of homology in their zinc finger domains and can partially compensate for each other in embryonic erythroid cells, probably because they regulate
common target genes (Basu et al., 2007). Functional overlap between family members of transcription factors is common. GATA1 and GATA2, for example, can compensate, fully or partially, for each other during erythropoiesis (Takahashi et al., 2000, Fujiwara et al., 2004). Primitive erythroid cells are present in GATA1 knockout (KO) embryos. GATA2 KO embryos show a modest reduction in the number of primitive erythroid precursors. However when both GATA1 and GATA2 are ablated, no primitive erythroid cells are detected (Fujiwara et al., 2004).

When expressed under the control of the GATA1 hematopoietic regulatory domain, GATA2 can rescue the GATA1 KO phenotype (Hosoya-Ohmura et al., 2006). Moreover, cross-regulation can occur between transcription factor family members. KLF2, for example, positively regulates KLF1 in primitive erythroid cells (Basu et al., 2007). KLF8 is upregulated by KLF1 and downregulated by KLF3 (Eaton et al., 2008). KLF1 also can activate KLF3 by directly binding to its promoter (Funnell et al., 2007). GATA1 negatively regulates GATA2 in definitive erythroid cells and megakaryocytes (Weiss et al., 1994, Grass et al., 2003, Muntean and Crispino, 2005). Proteins related to Pax and Hox transcription factor families have functional overlap during vertebral column and limb development (Peters et al., 1999, Boulet and Capecchi, 2004, Basu et al., 2007). Pax1 and Pax9 are important transcription factors for vertebral development. Whereas a deficiency in Pax9 could be fully compensated by Pax1, Pax9 can compensate only partially for the Pax1 deficiency. Mutating Pax1 and Pax9 simultaneously results in the absence of vertebral bodies and the intervertebral discs (Peters et al., 1999).

Our work in the mouse and human erythroid cells shed some light to the similarities and differences between the two models. We have shown that the cord blood CD34+ derived erythroid cells are similar to the fetal liver cells when comparing the expression pattern of KLF1 and KLF2. However they are different when comparing γ- and β- globin gene expressions. KLF1
and KLF2 in the mouse fetal liver and the human cord blood erythroid cells have a ratio of 10:1. However the expression of \( \gamma \)-globin is observed mainly in the yolk sac then quickly declined in the fetal liver cells of transgenic mice.

Although KLF2 expression is less than KLF1 in the human cord blood erythroid cells, the ChIP results show that KLF2 binding to the \( \gamma \)-promoter is greater than the binding of KLF1. In mouse primitive erythroid cells where KLF1 and KLF2 are expressed equally and \( \gamma \)-globin is expressed in the yolk sac, KLF1 binding to the \( \gamma \)-globin promoter is greater than KLF2 binding. These results suggest that the human KLF1 and KLF2 may behave differently compared to the mouse KLF1 and KLF2 due to possible differences in their trans-activation domains. We know that KLF1 and KLF2 have >90% similarity in the zinc finger domains between mouse and human but outside the zinc finger domains they are less similar.

The roles of KLF1 on histone modifications are also different between mice and human. While almost all tested sites in the mouse and the human \( \beta \)-loci in transgenic mice have decreased H3K4me3 and H3K9Ac, only histone marks at the adult \( \beta \)-globin gene are decreased. This suggests that the human \( \beta \)-locus in primitive erythroid cells may have different chromatin structure than in the definitive erythroid cells.

Our results support the notion that KLF1 is important in hemoglobin switching although argue against the combined role of KLF1 and BCL11a in \( \gamma \)-globin regulation. In our system, cord blood CD34+ cells, we show that BCL11a Knockdown as a result of KLF1 knockdown does not play important role in \( \gamma \)-globin reactivation. This insignificant role could be due to the limited starting amount of BCL11a in the cord blood \textit{ex vivo} differentiated erythroid cells. In addition, our results suggest that the ratio of KLF1 to KLF2 could be an important factor that drives
hemoglobin switching in erythroid cells. In mouse primitive cells, the ratio of KLF1 and KLF2 is near to one and at that level the expression of γ-globin is favored over β-globin. More experiments need to be performed to investigate KLF1 and KLF2 expression at different ratios to prove that the KLF1:KLF2 ration is in fact a hemoglobin switching factor.

Because KLF1 is important for many aspects in erythropoiesis other than β-globin regulation, targeting it might not be the best therapeutic strategy to reverse hemoglobin switching. KLF2 is a similar transcription factor that seems to preferentially regulate γ-globin expression and is not required for as many genes in erythroid cells as KLF1. Therefore targeting KLF2 as a therapy for sickle cell disease and β-thalassemia could be less risky than targeting KLF1.

5.2 Future Directions

This work focuses on the roles of KLF1 and KLF2 in β-globin regulation during adult and fetal erythropoiesis. To expand this work, the role of KLF1 and KLF2 could be studies in the context erythropoiesis in general. Our lab has generated genome wide expression profiles of primitive mouse erythroid cells in KLF1−/−, KLF2−/−, and double knockouts KLF1−/−KLF2−/− (Redmond et al., 2008, Redmond et al., 2011, Pang et al., 2012). Harnessing the power of these profiles to discover part of the erythropoiesis network that is governed by KLF1 or KLF2, or both in human erythroid cells from cord blood is of great value.

We have successfully knocked down KLF1 in cord blood derived erythroid cells. KLF2 knockdown was not enough to confirm its role as hypothesized, but using different shRNA
constructs could enhance the knockdown efficiency. However, if for any reason, KLF2 knockdown cannot be achieved, KLF2 overexpression could be a complimentary approach.

Our results suggest that KLF1 knockdown results in compensatory increase in KLF2 and KLF2 binds to γ- globin promoter suggesting that a positive regulation of γ- globin by KLF2 may be hidden by KLF1. Therefore, double knockdown of KLF1 and KLF2 may reveal a synergistic effect of KLF1 and KLF2 as seen in the mouse embryonic globin genes (Basu et al., 2007). Lentiviral transduction of CD34+ cells using two different shRNAs requires two different selection markers to be sure that transduced cells picked up both shRNAs. Our lentiviral system uses GFP as a selection marker for cell sorting. Red Fluorescent Protein can easily replace the GFP gene in our plasmid making it useful and easy to use in double knockdown studies.

As we let cord blood CD34+ cells to differentiate to erythroid cells _ex vivo_, we noticed that the percentage of non-erythroid cells in each sample varies. However, our result showed that KLF1 does not play a role in erythroid differentiation in our system. Therefore, selecting only the erythroid population of differentiated CD34+ cells based on their CD71 (transferrin receptor) for RNA studies could enhance the experiment specificity. Nonetheless, KLF1 roles was described as a major player in all aspects of mouse definitive erythropoiesis, including differentiation and proliferation(Tallack and Perkins, 2010, Tallack et al., 2010, Pilon et al., 2011, Tallack et al., 2012). Therefore, studying the erythroid maturation and proliferation using colony forming assays and flowcytometry is worthy.

Finally, our work confirms the important role of KLF1 in primitive and fetal erythropoiesis. In addition it establishes KLF2 as a positive regulator of γ-globin gene expression.
in transgenic mice and highly suggests that human KLF2 has a similar role in the fetal human erythroid cells.
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VITA

Yousef Alhashem received his Bachelor of Science degree in Clinical Laboratory Sciences from King Saud University, Riyadh, Saudi Arabia in 1999. He worked in King Abdulaziz Medical City, Riyadh, Saudi Arabia as a medical technologist in the department of Pathology and Laboratory Medicine from 2000 to 2006. He joined Virginia Commonwealth University in 2007 from where he earned his Master’s degree of Science in Human and Molecular Genetics in 2009 then earned his PhD degree in 2012 from the same university.