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THE ROLES OF KRÜPPEL-LIKE FACTOR 1 (KLF1) IN THE HUMAN FETAL ERYTHROID COMPARTMENT

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

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List of Abbreviations

5’UTR---------- 5’Untranslated Region

3’UTR---------- 3’Untranslated Region

%---------- Percent

α---------- Alpha

β---------- Beta

βh1---------- Beta-like embryonic chain (mouse hemoglobin Z)

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

APS---------- Ammonium persulfate

BCL11A---------- B-cell CLL/lymphoma 11A

BFU-E---------- Burst Forming Unit-Erythroid

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CDK---------- Cyclin Dependent Kinase

cDNA---------- complementary DNA

CFU-E---------- Colony Forming Unit-Erythroid

ChIP---------- Chromatin Immunoprecipitation

CO_2---------- Carbon Dioxide

DNA---------- Deoxyribonucleic acid

DPBS---------- Dulbecco's Phosphate Buffered Saline

E---------- Embryonic Day

FACS---------- Fluorescent Activated Cell Sorting

FBS---------- Fetal Bovine Serum

FOXM1---------- Forkhead Box M1

GFP---------- Green Fluorescent Protein

HBS---------- HEPES Buffered Saline

HEPES---------- Hydroxyethyl piperazineethanesulfonic acid

HPFH---------- Hereditary Persistence of Fetal Hemoglobin

HRP---------- Horseradish Peroxidase

HS---------- Hypersensitive Site

HSC---------- Hematopoietic Stem Cell

IgG---------- Immunoglobulin G

IMDM---------- Iscove's Modified Dulbecco's Medium

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KLF---------- Krüppel-like factor

LB agar---------- Luria Bertani agar

LCR---------- Locus Control Region

LDL---------- Low Density Lipoprotein

MNCs---------- Mononuclear Cells

Myc---------- Myelocytomatosis viral oncogene homolog

O_2---------- Oxygen

PBS---------- Phosphate Buffered Saline

PBST---------- Phosphate Buffered Saline-Tween

PCR---------- Polymerase Chain Reaction

P/S---------- Penicillin/ Streptomycin

PTHR---------- Parathyroid Hormone Receptor

PVDF---------- Polyvinylidene Fluoride

qRT-PCR---------- quantitative Real time- Polymerase Chain Reaction

RBC---------- Red Blood Cells

RT---------- Room Temperature

SDS---------- Sodium Dodecyl Sulfate

SFEM---------- Serum Free Expansion Medium

shRNA---------- short hairpin RNA

SPHK1---------- Sphingosine Kinase 1

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ABSTRACT

THE ROLES OF KRÜPPEL-LIKE FACTOR 1 (KLF1) IN THE HUMAN FETAL ERYTHROID COMPARTMENT

By Safa Fatima Mohamad, Master of Science.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2014.

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

Erythroid Krüppel-like factor (EKLF or KLF1) is a transcription factor with roles in embryonic and adult erythropoiesis. KLF1 knockout mouse embryos die due to severe anemia. Dominant human mutations in KLF1 can cause hereditary persistence of fetal hemoglobin. We show that KLF1 positively regulates β-globin and Bcl11A gene expression using KLF1 knockdown in *in vitro*-differentiated CD34+ human umbilical cord blood cells. γ-globin expression appears

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dependent on KLF1; it is increased with modest KLF1 knockdown but not in cells with low KLF1. KLF2 mRNA amounts are usually increased in KLF1 knockdown. KLF1 knockdown in CD34+ cells results in reduced colony forming ability. Interestingly, the expression of certain proliferation and cell cycle genes is reduced due to KLF1 knockout in mouse or knockdown in human erythroid cells. In conclusion, KLF1 is an important regulator of the β-globin locus and has roles in proliferation and cell cycle.

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CHAPTER ONE: Introduction

1.1 Erythropoiesis

An essential fluid in our body is circulating blood. It transports nutrients within our bodies, removes waste products, keeps our immune system healthy and our heart pumping. The blood cells that perform these functions are produced through hematopoiesis. Hematopoiesis is a carefully controlled process regulated by a complex network of transcription factors, cell signaling and chromatin remodeling (Watkins et al. 2009). Blood cells are derived from the mesoderm lineage in the developing embryo during developmental hematopoiesis (Medvinsky A et al. 2011, Wilkinson et al. 2013). The red blood cells (RBCs, erythrocytes) in particular, are formed through a process called erythropoiesis. RBCs are responsible for the normal transportation of oxygen (O_2) and carbon dioxide (CO_2) to and from the various tissues in mammals and other vertebrates. In mammals, this transportation occurs in the delivery vehicle called hemoglobin present in erythrocytes. Erythropoiesis occurs at several distinct spatiotemporal locations in the developing embryo and can be broadly divided into two stages: 1) primitive erythropoiesis and 2) definitive erythropoiesis (McGrath K, Palis J. 2008, Palis et al. 2008, Wilkinson et al. 2013).

Primitive erythropoiesis originates in the extra-embryonic mesoderm from the yolk sac, 14 to 19 days after conception in humans and persists in this organ for 9 weeks. Definitive erythropoiesis originates from the aorta-gonado-mesonephros region of the embryo proper and is detectable in the fetal liver starting at the sixth week of development and in the bone marrow beginning in the eleventh week of gestation (Figure 1.1) (Qui et al. 2008). In mouse embryos, primitive

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erythropoiesis begins as early as embryonic day 7.5 (E7.5) and definitive erythropoiesis at E11.5 (Figure 1.1) (McGrath K, Palis J. 2008, Wilkinson et al. 2013). The main difference between these two stages of erythropoiesis is that primitive erythroid cells are nucleated and megaloblastic in nature compared to their enucleated definitive counterparts. However, it is now thought that these nucleated primitive erythroblasts progressively mature and enucleate like definitive erythroid cells (Palis J. 2008). Another difference between the two is that primitive erythroid cells do not come from a true hematopoietic stem cell (HSC) as they cannot reconstitute the entire hematopoietic system of an irradiated mouse like definitive erythroid stem cells (Muller et al. 1994, Medvinsky et al. 1996, Wilkinson et al. 2013).

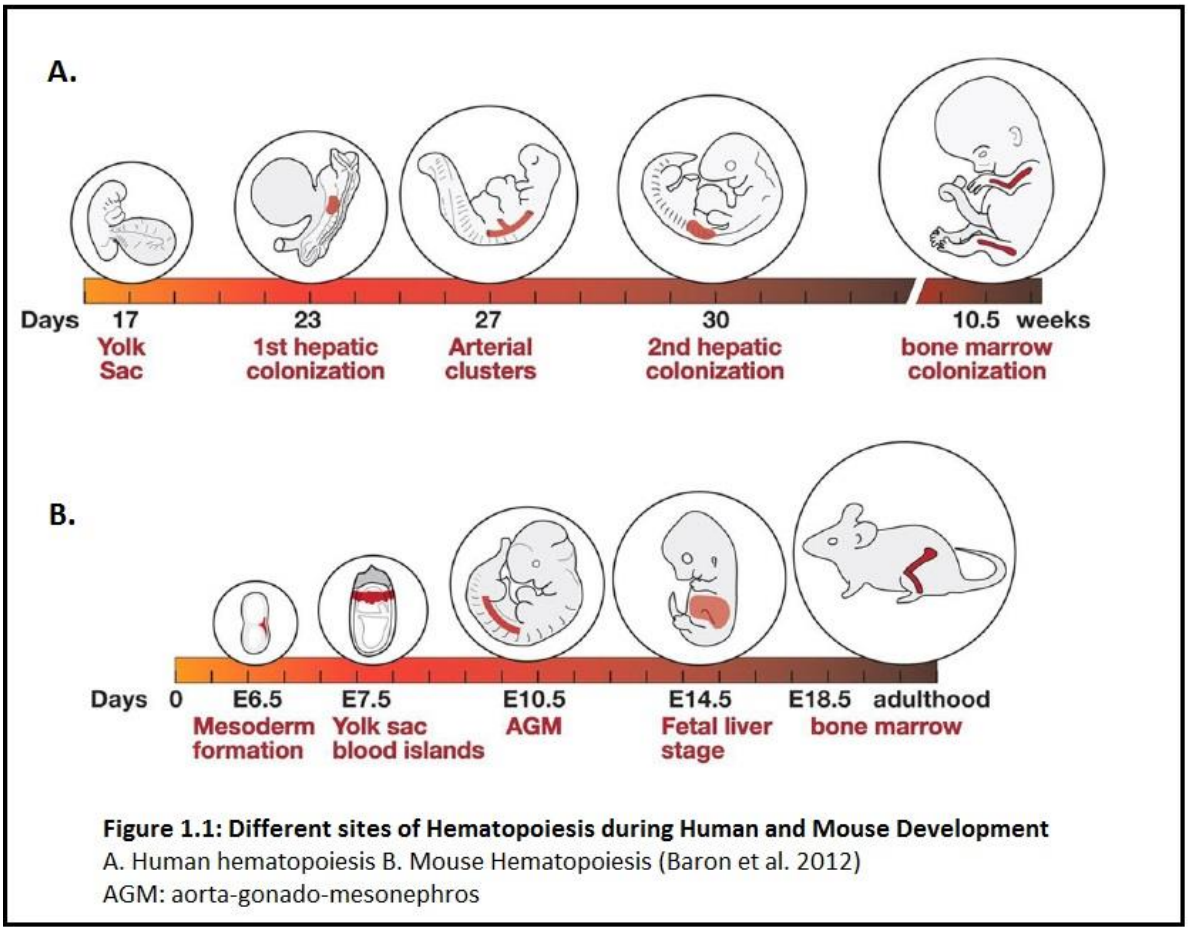

Figure 1.1: Different sites of Hematopoiesis during Human and Mouse Development
A. Human hematopoiesis B. Mouse Hematopoiesis (Baron et al. 2012)
AGM: aorta-gonado-mesonephros

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In human embryogenesis, the hemangioblast and hemogenic endothelium models have been proposed to exist for hematopoietic specification (Medvinsky et al. 2011). The hemangioblast cell-type is thought to be tri-potent and can give rise to hematopoietic, endothelial and smooth muscle cells. It forms the hemogenic endothelium which is bi-potent and has the capacity to form endothelial cell-types as well as hematopoietic stem cells (HSCs) from which all definitive blood cells arise (Lancrin et al. 2009). These HSCs first expand within the fetal liver and eventually seed the bone marrow which serves as the source of HSCs throughout postnatal life (Baron et al. 2012). HSCs usually express markers such as CD34, Sca-I and c-kit (Guo et al. 2003) which are used to isolate them to treat patients with leukemia and other bone marrow diseases. However, HSCs cannot be purified, they can only be enriched.

HSCs are the precursors that can form the erythroid, myeloid and lymphoid lineage. In the erythroid lineage, eight distinct developmental stages have been identified during erythropoiesis, as shown in Figure 1.2.A. The earliest committed progenitor that has been identified *ex vivo* is the slow proliferating burst-forming unit-erythroid (BFU-E) which further differentiates into rapidly dividing colony-forming unit-erythroid (CFU-E) (Koury et al. 2002, Tsiftsoglou et al. 2009, An et al. 2011, Hattangadi et al. 2011). BFU-Es and CFU-Es are the only two erythroid progenitors that can be functionally defined through colony forming assays. The next developmental stage, involves the formation of the earliest morphologically recognizable erythroblast called the proerythroblast. The proerythroblast differentiates to generate basophilic, polychromatic and orthochromatic erythroblasts. All of the cells up to this stage can divide and undergo mitosis. The expulsion of nuclei then leads to the formation of reticulocytes. This differentiation process involves 3-4 mitotic cycles and is commonly referred to as terminal erythroid differentiation. Throughout this differentiation several changes occur such as decrease

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in cell size, chromatin condensation, hemoglobinization and finally enucleation and expulsion of other organelles (An et al. 2011, Hattangadi et al. 2011).

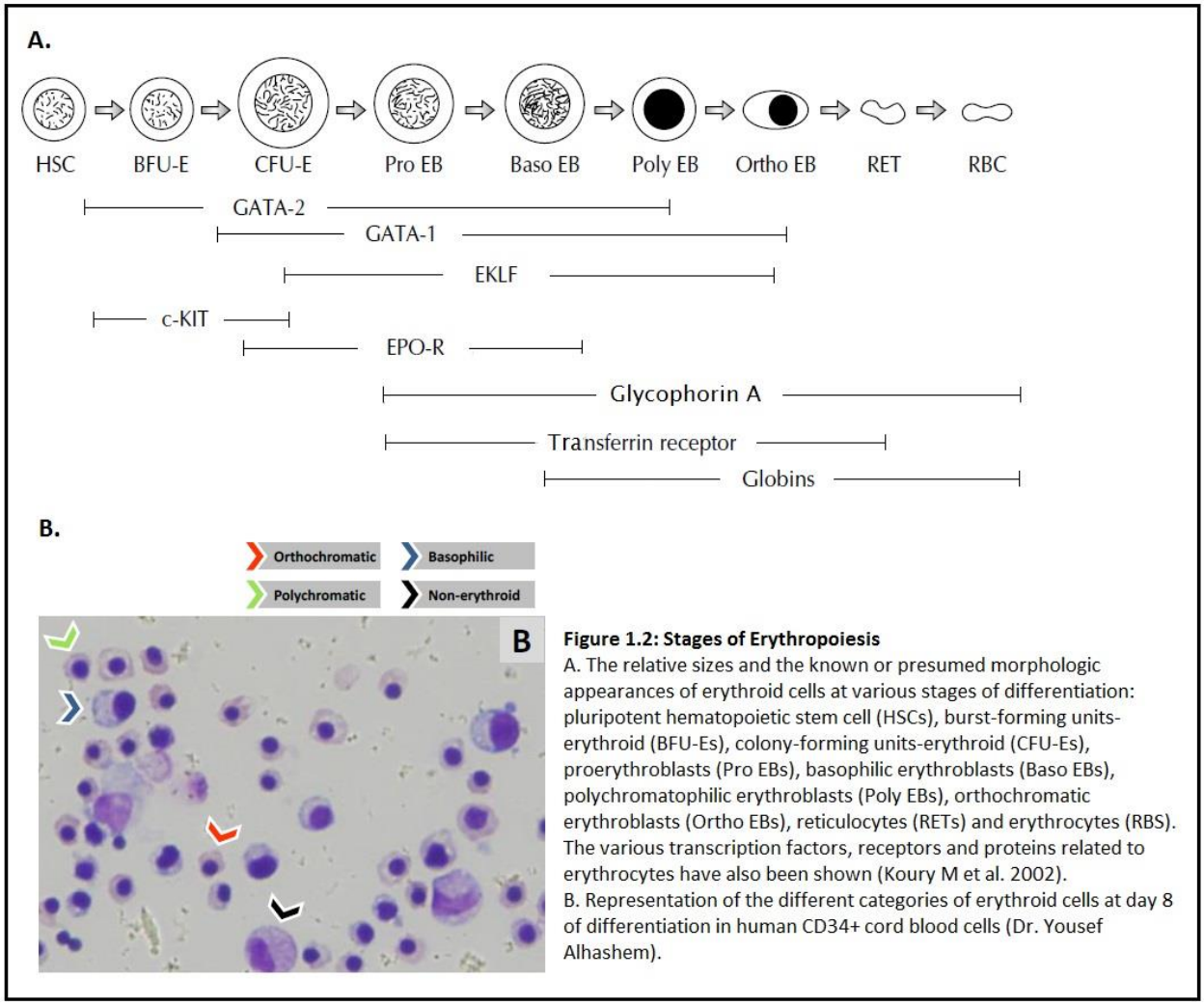

Figure 1.2: Stages of Erythropoiesis
A. The relative sizes and the known or presumed morphologic appearances of erythroid cells at various stages of differentiation: pluripotent hematopoietic stem cell (HSCs), burst-forming units-erythroid (BFU-Es), colony-forming units-erythroid (CFU-Es), proerythroblasts (Pro EBs), basophilic erythroblasts (Baso EBs), polychromatophilic erythroblasts (Poly EBs), orthochromatic erythroblasts (Ortho EBs), reticulocytes (RETs) and erythrocytes (RBS). The various transcription factors, receptors and proteins related to erythrocytes have also been shown (Koury M et al. 2002).
B. Representation of the different categories of erythroid cells at day 8 of differentiation in human CD34+ cord blood cells (Dr. Yousef Alhashem).

The work performed in our laboratory is carried out in the CD34+ human fetal cord blood model. CD34+ cells isolated from fetal cord blood are HSCs which can differentiate into the erythroid lineage. The laboratory differentiates these definitive HSCs for 8 days and equal amounts of β-globin and γ-globin mRNA are observed on this day of differentiation. Most of the erythroid cells observed on this day are polychromatic erythrocytes (60%) (Figure 1.2B). Basophilic and orthochromatic erythrocytes account for the remaining 40% (about 25% and 15% respectively)

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(Dr. David Williams and Dr. Yousef Alhashem). Common markers present on erythrocytes at this point of time are transferrin receptor (CD71) and Glycophorin A (CD235) (Figure 1.2.A) (Koury et al. 2002, Lim et al. 2011, An et al. 2011). These markers are present in different quantities at different stages of differentiation. For example, in flow cytometry there is a 100-fold increase observed for Glycophorin A on day 8 compared to day 5 of differentiation (Zeuner et al. 2011). Such markers help define the stage of differentiation that these erythroid cells have achieved.

1.2 The β-globin Locus

1.2.1 The Globin Genes

Hemoglobin is the oxygen carrying molecule present in erythrocytes. It is a tetrameric molecule composed of two α-like globin chains and two β-like globin chains (Perutz 1968, Biochemistry by Voet D and Voet J). Each of the four subunits binds a single heme group which is responsible for the characteristic red color of the blood and is the site at which each globin monomer binds one molecule of O_2 or CO_2 (Biochemistry by Voet D and Voet J). In humans, the α-like globins are encoded by four genes: ζ, α2, α1, and θ, located on chromosome 16. The β-like globins are encoded by five genes, ε, $^{A}\gamma$, $^{G}\gamma$, δ and β, located on chromosome 11 (Figure 1.3A). The genes present on both the α-globin as well as the β-globin loci are expressed during development in the order they appear on the chromosomes (Weatherall 2001). These different α- and β-globins form different types of hemoglobin at different time-points in the human embryo. During primitive yolk-sac erythropoiesis, three types of hemoglobin are expressed: Hb Gower I ($\zeta_2\epsilon_2$), Hb Gower II ($\alpha_2\epsilon_2$) and Hb Portland ($\zeta_2\gamma_2$). Definitive erythropoiesis can lead to the formation of fetal

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hemoglobin ($\alpha_2\gamma_2$) from the fetal liver and adult hemoglobin ($\alpha_2\beta_2$) and ($\alpha_2\delta_2$) synthesized in the bone marrow (Weatherall 2001, Qui et al. 2008).

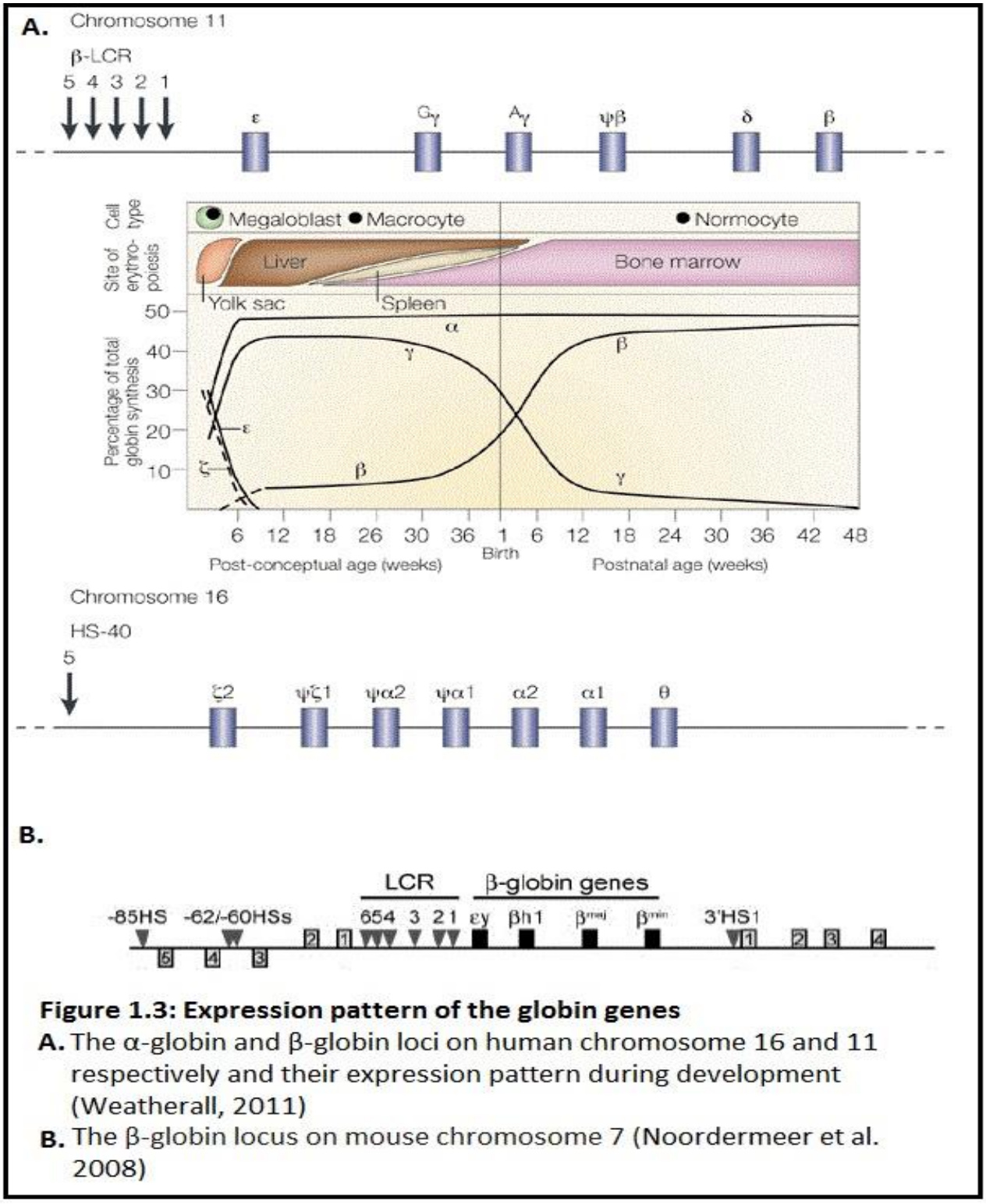

Figure 1.3: Expression pattern of the globin genes

A. The α-globin and β-globin loci on human chromosome 16 and 11 respectively and their expression pattern during development (Weatherall, 2011)

B. The β-globin locus on mouse chromosome 7 (Noordermeer et al. 2008)

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In mice, the β-globin locus is present on chromosome 7 and consists of four genes: εy, βh1, β^{maj} and β^{min} (Figure 1.3B). During primitive erythropoiesis, εy and βh1 are produced in embryonic tissues; whereas, during definitive erythropoiesis, β^{maj} and β^{min} are produced in the fetus and adult mouse (Noordermeer et al. 2008).

1.2.2 The Locus Control Region (LCR)

The locus control region (LCR) is a major distal regulatory sequence for the β-globin locus. It spans 16 kb of DNA upstream of the ε-globin gene and is composed of phylogenetically conserved five DNase I hypersensitive sites (HS): HS1, HS2, HS3, HS4 and HS5 (Labeled as 1-5 in Figure 1.3A) (Mahajan et al. 2007, Noordermeer et al. 2008, Kukreti et al. 2010). Deletion of any of these HS sites leads to abolishment of LCR activity suggesting that all of the HSs act together as a single entity or holocomplex (Cao A et al. 2002, Palstra et al. 2008). The HSs contain a number of binding sites for transcription factors such as GATA-1, NF-E2, KLF1 and Sp-1. Formation of the HSs appears to precede β-globin transcription, but the LCR needs to be linked to an active promoter to stay hypersensitive (Palstra et al. 2008). In fact, Chromosome Conformation Capture (3C) and RNA TRAP experiments, allowed the determination of the spatial organization of the LCR in the β-globin locus. These experiments proved that the LCR and the activated globin genes are in close proximity and chromatin looping occurs to bring these two sites in close contact (Noordermeer et al. 2008). Once in close proximity, the LCR colocalizes with active genes to form a structure called the active chromatin hub. The appearance and disappearance of this hub correlates with β-globin gene expression levels. This indicates that chromatin looping is necessary for β-globin regulation by LCR (Noordermeer et al. 2008).

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Besides the LCR, three major regulatory elements have been identified: TATA, CAAT and CACCC boxes in all globin promoters. In the γ-globin promoter, two CAAT and one CACCC boxes are present; whereas, in the β-globin promoter, two CACCC boxes and one CAAT box is present (Cao A et al. 2002). Early transgenic experiments in mice, showed that even though these regulatory elements present in the globin promoter are sufficient for expression, the expression levels were very low (Palstra et al. 2008). The LCR along with the promoter elements play a crucial role in maintaining high β-globin gene expression (Weatherall et al. 2001). Its importance is portrayed in patients suffering from β-thalassemia because of β-chain imbalance even though they were shown to carry a normal β-globin gene. This form of thalassemia was manifested due to deletions in the LCR (Weatherall 2001, Noordermeer et al. 2008).

1.2.3 Hemoglobin Switching

The tetrameric molecule hemoglobin is composed of two α-like and two β-like polypeptide subunits. In humans, the composition of these subunits varies due to two developmental switches during the production of β-like subunits in the hemoglobin molecule. The first switch occurs around three months post-fertilization wherein the embryonic Hb Gower 1 ($\zeta_2\varepsilon_2$) in primitive erythrocytes is switched to fetal Hb ($\alpha_2\gamma_2$) in definitive erythrocytes (Sankaran et al. 2010, Qiu et al. 2008, Wilber et al. 2011, Tallack M and Perkins A, 2013). This is due to the simultaneous silencing of the ε-globin gene expression in the yolk-sac and the activation of the α- and γ-globin gene expression in the fetal liver. The second switch which occurs at around birth involves the shift from fetal Hb to adult Hb ($\alpha_2\beta_2$) in the bone-marrow (Figure 1.3A) (Sankaran et al. 2010, Wilber et al. 2011, Tallack M and Perkins A, 2013). In mice, there is only one switch in hemoglobin from embryonic hemoglobin to adult hemoglobin between E11 and E12

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(Noordermeer et al. 2008). Understanding this mechanism of hemoglobin switching has become an area of intense research over the past few decades since it would help in ameliorating the symptoms of sickle cell-anemia and β-thalassemia by partially inhibiting the switch to adult β-globin gene.

In the past several years, substantial progress has been made in uncovering the molecular mechanisms that regulate the developmental switch between fetal to adult hemoglobin. Genetic linkage and genome-wide association studies have identified three loci: the β-globin locus, BCL11A gene and the intergenic region between HBS1L and MYB to account for up to 50% variance in fetal Hb expression (Wilber et al. 2011). Also, evidence suggests that hemoglobin switching is influenced by multiple cis-acting elements and transcription factors like KLF1, BCL11A, SOX6, NF-E4, MBD2 and others (Sankaran et al. 2010, Wilber et al. 2011). Krüppel-like factor 1 (KLF1)/ Erythroid KLF (EKLF) is a master regulator of erythropoiesis and known to play a major role in hemoglobin switching. In the absence of KLF1, there is concomitant loss of β-globin gene expression and an upregulation in γ-globin gene regulation (Tallack M and Perkins A, 2013). In addition to binding the β-globin gene promoter, KLF1 was found to show strong binding affinity towards the BCL11A promoter a regulator of γ-globin gene silencing (Wilber et al. 2011). Recent reports have also shown that KLF1 and MYB activate each other. KLF1 binds to a single HS site located upstream of MYB and forms a structure by DNA looping to bring intergenic enhancer regions situated between the MYB and HBS1L genes into direct proximity with the MYB promoter. MYB once activated plays a role in the regulation of globin switching (Tallack M and Perkins A, 2013). Thus, KLF1 may mediate hemoglobin switching through these three mechanisms. SOX6 is another repressor of γ-globin gene expression and interacts with BCL11A. On the other hand, NF-E4 or p22 is a fetal erythroid-specific

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transcription factor that interacts with another ubiquitous transcription factor CP2 to bind γ-globin promoter and positively regulate its expression (Sankaran et al. 2010, Wilber et al. 2011). Another transcription factor MBD2 is necessary for DNA methylation and is required to repress γ-globin gene expression during the switch (Sankaran et al. 2010).

Epigenetic mechanisms such as DNA methylation and histone acetylation are other important regulators of hemoglobin switching. Histone modifications by acetylation, methylation, phosphorylation, sumoylation, ubiquitination and DNA methylation on CpG residues are among the most important epigenetic mechanisms (Wilber et al. 2011). Such epigenetic marks cause chromatin remodeling which may facilitate binding of transcription factors. Further studies in sickle cell anemia and β-thalassemia patients showed that inhibiting methylation of CpG residues present in the region of duplicated γ-globin genes ($^{G}\gamma$ and $^{A}\gamma$) using 5-azacytidine enhanced γ-globin production and in turn increased the production of fetal hemoglobin (Charache S et al. 1983, Wilber et al. 2011).

1.2.4 β-hemoglobinopathies

A genetic defect that results in the abnormal structure or reduced quantity of one or both of the β-globin chains of the hemoglobin molecule is called as a β-hemoglobinopathy. Examples of β-hemoglobinopathies are sickle cell anemia and β-thalassemia. Another condition seen in the population due to abnormal hemoglobin switching is called as hereditary persistence of fetal hemoglobin (HPFH). However, HPFH is asymptomatic; it is the abnormal production of fetal hemoglobin after birth (Medical Genetics by Jorde L, Carey J, Bamshad M).

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Sickle Cell Anemia- Sickle Cell Anemia is a monogenic, autosomal recessive disorder caused by a single missense mutation that substitutes a hydrophobic valine residue for a hydrophilic glutamic acid residue at position 6 of the β-globin polypeptide chain (Medical Genetics by Jorde L, Carey J, Bamshad M, Biochemistry by Voet D and Voet J, Higgs and Wood, 2008). The resulting hemoglobin (HbS, $\alpha_2\beta^S_2$) has an altered structure that forms aggregates under hypoxic conditions. Normal erythrocytes can squeeze through capillaries; however sickled erythrocytes (Figure 1.4) are less flexible due to the accumulation of rigid HbS fibers that extend throughout the length of the erythrocyte. Homozygous patients are thus afflicted by hemolytic anemia together with painful and sometime fatal blood flow blockages (Biochemistry by Voet D and Voet J).

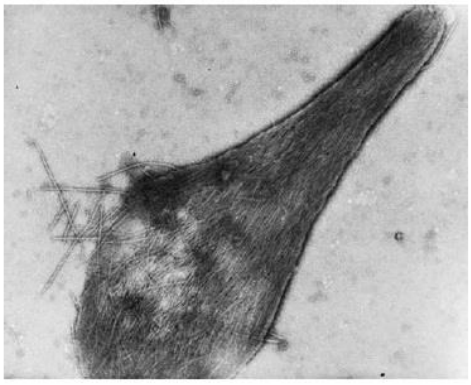

Figure 1.4: Electron micrograph of HbS fibers spilling out of a ruptured erythrocyte (Biochemistry by Voet D and Voet J).

β-thalassemia- This is also an autosomal recessive disease in which mutations result in the absence (β^0-thalassemia) or reduced quantity (β^+-, β^{++}-thalassemia) of the β-globin chain (Weatherall 2001). In β-thalassemia, homotetramers are formed due to the excess of α-globin chains present and reduction in β-globin chains. These homotetramers precipitate and damage the cell membranes of RBC precursors resulting in premature erythrocyte destruction and anemia (Medical Genetics by Jorde L, Carey J, Bamshad M, Weatherall 2001). The anemia also leads to bone marrow expansion, splenomegaly, bone deformities and infections. Over 300 different β-globin mutations have been reported which encompass all sorts of DNA and RNA mutations. In addition to deletions and mutations in the β-globin gene itself, alterations in the promoter, two

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enhancers and the LCR are also seen. Several types of splice-site mutations have also been observed (Medical Genetics by Jorde L, Carey J, Bamshad M, Weatherall 2001).

Hereditary Persistence of Fetal Hemoglobin (HPFH) - HPFH is associated with the abnormal production of HbF in RBCs throughout adult life. Generally, during birth there is a switch from HbF to adult Hb. Point mutations in the cis-acting sequences important for this switch within the γ-globin promoter can cause abnormalities in the interactions between transcription factors and the promoter leading to HPFH (Higgs and Wood, 2008, Medical Genetics by Jorde L, Carey J, Bamshad M). Besides mutations in cis-acting sequences, irregularities in trans-acting sequences such as KLF1 that regulate γ-globin gene expression are also known to cause HPFH. However, HPFH does not cause disease but it can compensate for a lack of normal adult globin in sickle cell anemia and β-thalassemia. In fact, it has been shown that increased levels of HbF in patients with β-hemoglobinopathies can alleviate symptoms and lead to a less severe phenotype due to the compensatory role of HbF (Higgs and Wood, 2008). Therefore, understanding the mechanism of hemoglobin switching from ε- to γ- to β-globin is of great clinical significance which could lead to efficient therapeutic strategies for β-hemoglobinopathies.

1.3 Transcriptional Regulation of Erythropoiesis

A complex interaction of transcription factors is required to modulate genes and signals involved in erythropoiesis. These erythroid transcription factors were recognized through their binding to specific DNA motifs present in the promoters of β-globin genes (Tsiftsoglou A et al. 2009). The function of these has already been established using gene knockout and tissue-specific conditional knockout studies in mice (Cantor A and Orkin H, 2002, Tsiftsoglou A et al. 2009,

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Hattangadi S et al. 2011). Some of the important transcription factors required for erythropoiesis are as follows:

Transcription Factors required to establish Erythroid Lineage

1. SCL (TAL1): TAL1/SCL (Stem Cell Leukemia) is a basic helix-loop-helix (bHLH) transcription factor that binds E-box (CAGGTG) DNA elements (Cantor A and Orkin H, 2002, Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009). This transcription factor is detected in early hematopoietic progenitors as well as in mature megakaryocytes, erythroid cells and mast cell (Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009). It is known to activate erythroid and repress myeloid differentiation (Perry C and Soreq H, 2002). SCL participates in a DNA-bound complex containing the transcription factors E12/E47, GATA-1, Ldb-1 and LMO2 to regulate erythropoiesis. SCL knockout mice die *in utero* at E8.5 due to total absence of primitive yolk-sac hematopoiesis. Also, conditional knockout of the SCL gene in adult mice led to failure of erythropoiesis (Cantor A and Orkin H, 2002, Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009, Hattangadi S et al. 2011).

2. Runx1: Runt-related transcription factor-1 (Runx1) also known as acute myelogenous leukemia-1 (AML-1) is a transcription factor that is constitutively expressed in all lineages of hematopoietic cells except for mature erythroid cells (Ichikawa M et al. 2013). Many lines of evidence have shown that Runx1 plays a critical role in regulating the development of hematopoiesis. Runx1 knockout mice die around E12.5 due to the lack of definitive hematopoiesis in the vascular endothelial cells and defective angiogenesis (Ichikawa M et al. 2013). However, this transcription factor is not required for the maintenance of hematopoiesis in adult mice. Deletion of Runx1 may be an important cause of human leukemia because even

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though it is not required for the maintenance of hematopoietic stem cells, it is essential for their homeostasis (Ichikawa M et al. 2013).

3. Gfi-1b: Growth factor independent 1b (Gfi-1b) is a six zinc-finger transcription factor which is required for the development of erythroid as well as megakaryocytic lineages. Gfi-1b knockout mice die at E15 due to a block in definitive erythropoiesis (Saleque S et al. 2002, Tsiftsoglou A et al. 2009). On the other hand, overexpression of Gfi-1b in hematopoietic progenitors results in a dramatic increase in expansion of erythroblasts and termination of erythroid maturation and apoptosis. Gfi-1b expression is upregulated by GATA-1 in early erythroid maturation. These expression levels decline in later stages of erythropoiesis suggesting a role for Gfi-1b in erythroid differentiation (Tsiftsoglou A et al. 2009).

Erythroid Specific/Restricted Transcription Factors

1. GATA-1: GATA-1 is a zinc finger containing transcription factor which binds to the DNA consensus sequence (A/T)GATA(A/G). It is expressed in erythroid cells, megakaryocytes, mast cells, eosinophils and dendritic cells in hematopoiesis (Cantor A and Orkin H, 2002, Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009). GATA-1 knockout mice show severe anemia and die at E11.5 due to arrested maturation and apoptosis of erythroid precursors at the proerythroblast stage (Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009). Detailed genetic analysis showed that GATA-1 null embryonic stem cells fail to differentiate beyond the proerythroblast stage due to apoptotic death in primitive as well as definitive erythropoiesis (Tsiftsoglou A et al. 2009). GATA-1 acts as an activator or repressor of hematopoietic genes. It is known to interact with several other transcription factors such as SCL, FOG1, LMO2, KLF1 and others to carry out its functions (Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009, Hattangadi S et al. 2011).

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2. GATA2: GATA-2 is also a zinc-finger containing transcription factor which binds to the same DNA consensus sequence as GATA-1. Its expression in hematopoietic stem and progenitor cells is essential to promote proliferation and block erythroid differentiation (Perry C and Soreq H, 2002). GATA-2 knockout mice are embryonic lethal and die between E10-E11 due to severe anemia during the early phase of primitive hematopoiesis. GATA-2 null embryonic stem cells proliferate poorly and undergo excessive apoptosis indicating that GATA-2 is critical for expansion and survival of hematopoietic cells (Perry C and Soreq H, 2002). GATA-1 and GATA-2 double knockouts die mid-gestation due to ablation of primitive erythropoiesis indicating overlapping roles of GATA-1 and GATA-2 at the yolk sac stage (Fujiwara Y et al. 2003).

3. NF-E2: Nuclear Factor Erythroid 2 (NF-E2) is a basic-leucine zipper heterodimer containing two subunits: p45 and p18 (Andrews NC, 1998). It is found almost exclusively in hematopoietic progenitors and is involved in γ-globin activation in association with another transcription factor CP2 (Sankaran et al. 2010, Wilber et al. 2011). Knockout models of NF-E2 do not show erythroid abnormalities, but show a marked reduction in platelet counts due to defects in megakaryocyte development (Andrews NC, 1998). This indicates that another protein is capable of substituting NF-E2 hematopoietic functions, but incapable of substituting its megakaryocytic functions (Andrews NC, 1998, Koury M et al. 2002).

4. FOG-1 (Friend of GATA): FOG-1 is a nine zinc-finger containing protein and is a close interacting partner of GATA-1 (Cantor A and Orkin H, 2002, Tsiftsoglou A et al. 2009). It is expressed in fetal liver, erythroblasts, mast cells, megakaryocytes and adult spleen (Cantor A and Orkin H, 2002, Perry C and Soreq H, 2002). FOG-1-/- mice die mid-gestation between E10.5-E11.5 due to blocked erythropoiesis similar to GATA-1-/- mice. However, unlike GATA-1-/-

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mice, they exhibit complete failure of megakaryopoiesis, indicating that FOG-1 has a role independent of GATA-1 in megakaryopoiesis (Cantor A and Orkin H, 2002, Tsiftsoglou A et al. 2009). The FOG-1/GATA-1 interaction is essential for erythroid differentiation. Ectopic expression of FOG-1 in non-erythroid hematopoietic lineages or progenitors results in the reprogramming of these cells toward the erythroid/megakaryocytic lineages. This reprogramming fails to occur when FOG-1 is expressed alone devoid of GATA-1 (Tsiftsoglou A et al. 2009).

Ubiquitous Factors

1. MBD2: Methyl- binding domain 2 (MBD2) belongs to a family of methylcytosine binding proteins that specifically recognize methylated CpG sequences and regulate the transcription of the associated gene. To carry out this function, MBD2 is known to bind to the nucleosome remodeling and deacetylation (NuRD) complex (Gnanapragasam MN et al. 2011). The MBD2-NuRD complex has been known to repress human fetal γ-globin gene expression in β-YAC transgenic mice (Rupon J et al. 2006). Also MBD2 contributes to the developmental silencing of the human ε-globin gene expression. The lack of MBD2 leads to a 15-20 fold increase in human ε-globin gene expression in adult transgenic mice (Rupon J et al. 2011).

5. BCL11A: The transcription factor B-cell lymphoma/leukemia 11A (BCL11A) is a zinc finger protein previously shown to be critical for the development of B-cell precursors (Tsiftsoglou A et al. 2009). BCL11A has two major isoforms that are expressed in primary adult human erythroid cells at the mRNA and protein levels (Sankaran V et al. 2008). It is involved in the negative regulation of γ-globin gene expression during hemoglobin switching. Bcl11A binds to sequences in the locus control region (LCR) and in the γ–δ intergenic region of the human β-globin locus in adult erythroid progenitors to regulate globin gene expression (Zhou D et al. 2010). Zhou et al in

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2010 showed that downregulation of KLF1 in human *in vitro* differentiated CD34+ peripheral blood leads to a decrease in Bc111A gene expression and in turn an upregulation in γ-globin mRNA levels. To carry out this function, it interacts with several other proteins such as NuRD, GATA-1 and FOG-1 (Sankaran V et al. 2008, Tsiftsoglou A et al. 2009). BCL11A downregulation is associated with increased HbF making it a potential therapeutic target for increasing HbF levels in patients suffering from β-hemoglobinopathies (Sankaran V et al. 2008, Tsiftsoglou A et al. 2009).

2. CREB-binding protein (CBP): CBP is a histone acetyltransferase that is known to interact with GATA-1. A simple model put forth regarding the role of CBP in erythropoiesis is that, GATA-1 recruits CBP to chromatin associated with erythroid-specific genes. Acetylation of histones around this region will facilitate erythroid gene expression (Cantor A and Orkin H, 2002). Mice that are haploinsufficient for CBP suffer from bone-marrow failure syndrome and are prone to hematologic malignancies (Kung et al. 2000).

In addition, various other transcription factors like LMO2 (LIM domain only 2) and LYL-1 (Lymphoblastic Leukemia 1) also plays a role in erythropoiesis (Cantor A and Orkin H, 2002, Perry C and Soreq H, 2002, Tsiftsoglou A et al. 2009, Hattangadi S et al. 2011). Another set of transcription factors important for erythropoiesis are Krüppel-like factor 1 (KLF1) and KLF2. KLF1 is an erythroid-specific transcription factor, whereas KLF2 is expressed in endothelial and erythroid cells. Both of them are essential for globin gene expression and are discussed in detail below.

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1.4 Krüppel-Like Factors (KLFs)

Krüppel-Like Factors (KLFs) are a family of DNA-binding transcription factors that bind to GT-rich elements including the consensus sequence CACCC present in the regulatory regions of a number of genes. They have been named after their homologous counterpart Krüppel present in *Drosophila* where it plays a critical role in embryogenesis (Turner J and Crossley M, 1999, Bieker J, 2001, Basu P et al. 2004, Pearson R et al. 2007, Limame R et al. 2013). The distinguishing feature of the KLF family is the presence of three highly conserved Cys2/His2 zinc fingers present in their C-terminus (Figure 1.5). These zinc fingers enable binding of the KLFs to DNA. The fingers are connected by a characteristic seven-amino acid spacer TGERP(Y/F)X also called as the Krüppel-link and highly conserved among family members (Pearson et al. 2007). The zinc fingers of KLF family members specifically identify the consensus sequence (N/C)CN CNC CCN, where "N" denotes any nucleotide (Turner J and Crossley M, 1999). Outside the zinc finger domain, there is relatively very little homology amongst family members allowing the various KLFs to carry out their different individual functions.

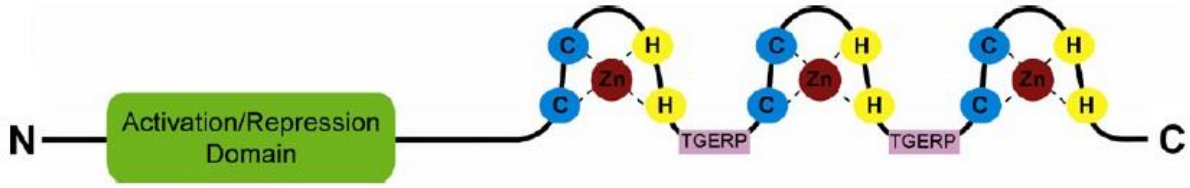

Figure 1.5: Represention of the KLF molecule
The KLF family of transcription factors share three conserved Cys2/His2 zinc fingers at the carboxyl-terminus and have an activation/repression domain at the amino-terminus. TGERP is the spacer/Krüppel-link between zinc fingers.

The first KLF to be discovered was KLF1/EKLF which is predominantly found in erythroid cells (Bieker J. 2001, Pearson et al. 2007). Thus far, seventeen mammalian KLFs have been identified

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starting from KLF1 to KLF17 (Limame R et al. 2013). Each of these proteins is expressed in different tissues and has been implicated in various functions related to differentiation, survival, proliferation and development (Turner J and Crossley M, 1999, Bieker J. 2001, Pearson et al. 2007, Limame R et al. 2013). The tissue distribution and functions of the seventeen KLFs are given in Table 1.1.

HGNC Name	Alias	Tissue Distribution	Primary activity	Knockout Phenotype	Other Functions
KLF1	EKLF	Erythroid tissues	Activator	Lethal β-thalassemia at E14.5	Erythrocyte differentiation
KLF2	LKLF	Lungs and Spleen	Activator	Lethal cardiac failure at E12.5 to E14.5	Erythroid and T-cell differentiation
KLF3	BKLF, TEF-2	Erythroid Tissues and Brain	Repressor	Myeloproliferative disorder	-----
KLF4	GKLF, EZF	Epithelial cells of epidermis, particularly in the digestive tract	Activator	Perinatal failure of skin barrier leading to lethal dehydration	Gut differentiation
KLF5	IKLF, CKLF, BTEB2	BTEB2: Widespread, IKLF: Digestive tract	Activator	Early embryonic death before E8.5	Cardiovascular development and response to injury; gut development; adipogenesis
KLF6	BCD1, COBEP, CBPB, ST12, GBF	Widespread, low levels in brain, high in muscles	Activator	Lethal failure of erythropoiesis and yolk sac vascularization at E12.5	-----
KLF7	UKLF	Widespread	Activator	Severe neurological defects lead to death within 2 d of birth	-----

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HGNC Name	Alias	Tissue Distribution	Primary activity	Knockout Phenotype	Other Functions
KLF8	BKLF3, ZNF741	Upregulated in various human cancers (prostrate, gastric, glioma, renal, breast, ovarian)	-----	Viable with a shortened life span	Promotes proliferation and tumor invasion and inhibits apoptosis.
KLF9	BTEB, BTEB1	Widespread, high in brain	Activator	Viable with impaired uterine development and defective embryo implantation	Mild behavioral defects
KLF10	TIEG, TIEG1, EGRα	Widespread, low in brain, kidneys, lungs	Activator	Viable with bone defects, skeletal abnormalities	Aged cardiac defects
KLF11	FKLF, TIEG2, MODY7	Widespread, high in muscle and pancreas	Repressor	Viable with no phenotype observed	-----
KLF12	AP2rep	Adult kidney, liver and lung	Repressor	-----	-----
KLF13	BTEB3, NSLP1, RFLAT-1	Widespread including lymphoid and cardiac	-----	Viable with defects in T cell differentiation	Heart Development
KLF14	BTEB5, SP6, EPFN	-----	-----	-----	-----
KLF15	KKLF	Widespread	-----	Viable with increased susceptibility to cardiac hypertrophy	Adipogenesis
KLF16	BTEB4, NSLP2, DRRF	-----	-----	-----	-----
KLF17	ZNF393	-----	-----	-----	-----

Table 1.1: Mammalian Krüppel-Like Transcription Factors and their Functions.

HGNC: Human Gene Nomenclature Committee

(Turner J and Crossley M, 1999, Pearson R et al. 2007, Limame R et al. 2013, Funnell A et al. 2013)

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Several KLFs are expressed in erythroid cells and are implicated in the expression of globin genes. KLF1, KLF2, KLF3, KLF4, KLF5, KLF8, KLF11, KLF12, and KLF13 are expressed in mouse yolk-sacs, fetal liver and adult spleen at different stages of erythroid development (Zhang et al. 2005). KLF1 binds the β-globin promoter and positively regulates its gene expression. It has also been shown that KLF2 activates the human β-globin gene promoter in transiently transfected mouse fibroblasts (Anderson K et al. 1995). Using transient transfection assays in human erythroid cell-lines, it has been found that KLF3 and KLF8 bind to globin CACCC elements and represses its transcription whereas KLF2, KLF5 and KLF13 activates its transcription (Basu P et al. 2004, Zhang et al. 2005). KLF1 has also been known to activate KLF3 and KLF8 which are involved in erythroid pathways. KLF3 represses several genes that are activated by KLF1 to fine tune their expression during erythropoiesis. KLF3 null mice show reticulocytosis, increased nuclear inclusions in peripheral blood and mild anemia (Funnell A et al. 2013). One of the genes repressed by KLF3 is KLF8. A number of embryonic globin genes are derepressed in KLF3 and KLF8 null mice leading to embryonic lethality (Funnell A et al. 2013).

1.4.1 Krüppel Like Factor 1 (KLF1)

KLF1 (EKLF) is a gene located on chromosome 19 in humans and chromosome 8 in mice. It has three exons that code for a 38 kDa KLF1 protein made up of 362 amino acids. Part of exon 2 and 3 code for the zinc finger which is the characteristic feature of the KLF family of proteins. KLF1 protein expression is largely restricted to erythroid, megakaryocytic and mast cells. The mouse and human KLF1 protein share a 73% overall identity and 90% zinc finger identity. KLF1 was first identified as a gene involved in erythropoiesis using subtractive hybridization in a murine

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erythroleukemia (MEL) cell line. KLF1 has been demonstrated to physically bind DNA at a conserved CCNCNCCCN motif. The KLF1 zinc finger binds to the sequence CCACACCCT located at -90 in the β-globin promoter and the CTCCACCCA sequence in the γ-globin promoter to regulate its gene expression (Miller IJ and Bieker JJ, 1993).

In mice, KLF1 is expressed as early as E7.5 in the yolk sac even before blood begins to circulate; and its concentration increases by ~3- fold as the hemoglobin switch from primitive to definitive erythropoiesis occurs (Zhou D et al. 2006, Alhashem Y et al. 2011). KLF1+/- mice show normal development compared to wild type mice (Perkins A et al. 1995); whereas, KLF1 knockout mice are embryonic lethal and die between E14.5 and E16 due to severe anemia and β-thalassemia (Perkins A et al. 1995, Pearson R et al. 2007). These KLF1 knockout embryos appear normal during the yolk-sac stage; their anemic nature starts from E11 and progressively increases till E15. This anemic behavior is parallel to the switch from primitive to definitive erythropoiesis indicating the importance of KLF1 in adult globin gene expression (Perkins A et al. 1995). Further analysis using S1 nuclease protection assays combined with primary transcript *in situ* hybridization showed that the β-globin gene in these knockout mice failed to activate at the fetal liver stage. There was a loss of the DNase I hypersensitivity site at HS3 of the LCR and a closed chromatin structure at the β-globin promoter suggesting the involvement of KLF1 in the spatial organization of an active chromatin domain in the β-globin locus (Wijgerde M et al. 1996, Tsiftsoglou et al. 2009). Semiquantitative ChIP analysis has also shown that HA-tagged KLF1 binds to the β_{maj} globin genes in primitive and definitive erythroid cells and to HS1, HS2, HS3 and HS5 in mouse primitive erythroid cells (Zhou D et al. 2006).

KLF1 single knockout mice containing a complete human β-globin gene locus transgene have also been studied to understand the roles of KLF1 in human erythropoiesis. These knockouts

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have lower amounts of human ε- and γ-globin in E10.5 yolk sacs compared to wild type (Alhashem Y et al. 2011). However, at the fetal liver stage in KLF1 heterozygotes, there are reduced levels of human β-globin and elevated levels of human γ-globin compared to wild type mice carrying the same transgene (Wijgerde M et al. 1996, Cantor A and Orkin SH, 2002). The null mutants also show a dramatic decrease in mouse β-globin compared to wild type.

The functions of KLF1 are not restricted to globin gene regulation. Microarray studies in primitive erythroid cells as well as mRNA-seq studies in definitive erythroid cells have shown that KLF1 regulates an entire repertoire of genes involved in cell cycle, proliferation as well as apoptosis (Tallack M et al. 2012, Pang C et al. 2012). In fact, studies involving the reintroduction of KLF1 in KLF1-null erythroid cell lines showed enhanced differentiation and hemoglobinization and reduced proliferation indicating the involvement of KLF1 in the regulation of other genes involved in hemoglobin synthesis (Perry C and Soreq H, 2002). The role of KLF1 in cell cycle has also been established by Pilon et al. (2008) in KLF1-/- fetal liver and by Divya Vinjamur and Kristen Wade in KLF1-/- yolk sacs. In both cases, KLF1 proved to be essential in G1 to S phase progression. In correlation with these findings, Tallack et al. (2007, 2009) found cell cycle regulators such as E2F2, E2F4 and p18 to be directly regulated by KLF1 in definitive erythropoiesis. Evidence also suggests the association of KLF1 in inhibition of megakaryopoiesis. This function is carried out by the interaction of sumoylated KLF1 with the Mi-2β component of the NuRD repression complex (Siatecka M et al. 2007).

Several mutations in KLF1 have been found that are known to increase HbF levels. Such mutations in humans lead to haploinsufficiency for KLF1 leading to hereditary persistence of fetal hemoglobin (Satta S et al. 2011, Borg J et al. 2011). Another interesting mutation of KLF1 has been reported in heterozygous *Nan*/+ mice which gives rise to hereditary spherocytosis and

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severe hemolytic anemia. This mutation causes a reduction in expression of erythrocyte membrane skeleton proteins leading to abnormally shaped erythrocytes (Heruth D et al. 2010, Borg J et al. 2011). A missense mutation in the homologous position in humans causes congenital dyserythropoietic anemia. KLF1 mutations do not always give rise to increased HbF. Variability has been observed in the HbF levels of patients with KLF1 mutations. Some heterozygous carriers of KLF1 mutations have HbF levels in the normal range; some have extremely high levels of HbF levels alone, while others have high HbF levels accompanied by high levels of zinc protoporphyrin in their circulation (Heruth D et al. 2010, Satta S et al. 2011, Borg J et al. 2011).

1.4.2 Krüppel Like Factor 2 (KLF2)

KLF2 (Lung KLF/LKLF) is a transcription factor originally isolated from a mouse genomic library using the EKLF zinc finger region as the hybridization probe. It is located on chromosome 19 in humans and chromosome 8 in mouse. The KLF2 gene has three exons that encode a 37.7 kDa protein which contains 355 amino acids. The zinc finger domain of KLF2 (from amino acid 274 to 354 in humans) has an 89% conserved amino acid homology compared to KLF1. Overall, there is a 50% similarity between the full length protein of KLF1 and KLF2 (Anderson K et al. 1995, Basu P et al. 2005). KLF1 and KLF2 are also located close to each other on the same chromosome in mouse and human indicating divergence due to a relatively recent duplication event (Basu P et al. 2005).

Unlike KLF1, KLF2 is present in several different cell types such as lungs, blood vessels, heart kidneys, skeletal muscle, testis and the lymphoid organs (Kuo CT et al. 1997). KLF2 knockout mice die between E12.5 and E14.5 due to severe hemorrhages in the abdomen and cardiac

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outflow tract. The cause of this hemorrhage is the abnormal thinning of the tunica media and instability of the blood vessel walls (Kuo CT et al. 1997, Basu P et al. 2005). Due to this observation, the role of KLF2 in erythropoiesis was investigated. Gene expression studies showed that KLF2 has a greater role in primitive erythropoiesis compared to definitive erythropoiesis. KLF2-/- embryos at E10.5 have highly reduced expression of murine embryonic Ey- and βh1-globin but not ζ-globin and the adult β^{maj} and β^{min} globin genes compared to wild type. This developmental stage specific role is conserved in transgenic mice carrying the entire human globin locus wherein KLF2 is shown to regulate human embryonic ϵ-globin gene but not the adult β-globin gene (Basu P et al. 2005). Histological evidence has also shown abnormal morphology of erythroid cells in KLF2-/- mice. Primitive erythroid cells are irregular in shape with pseudopodia-like appendages and exhibit apoptotic features which has been confirmed using TUNEL assays (Basu P et al. 2005).

Laser capture microdissection and microarray studies have helped recognize one-hundred and ninety six genes that were significantly different in KLF2-/- embryonic erythroid cells compared to wild type. These genes were involved in varied functions in erythropoiesis such as differentiation, migration and development (Redmond LC et al. 2011). Besides having an erythroid function, KLF2 is also known to have functions in endothelial cells, T- cells and mouse embryonic stem cells. KLF2 has been demonstrated to produce induced pluripotent stem cells (iPS) in mouse embryonic fibroblasts (MEFs). Genome-wide chromatin immunoprecipitation studies have shown KLF2 to bind to several critical cell regulators such as Nanog, Sox2, Oct3/4, Myc, Tcf3 (transcription factor 3), Sall4 (sal-like 4), and Esrrb (estrogen-receptor-related receptor β).

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KLF1 and KLF2 double knockout mice are embryonically lethal and die before E11.5. This is earlier compared to KLF1 and KLF2 single knockout mice. These double mutant embryos appear anemic at E10.5 compared to KLF1-/- and KLF2-/- embryos. Also, double mutants have membrane abnormalities in erythroid and endothelial cells (Basu P et al. 2007).

1.5 KLF Model Systems

KLF1 and KLF2 single and double knockout mouse model systems may be used to study erythropoiesis *in vivo.* These models have helped elucidate the role of KLF1 and KLF2 in mouse embryonic erythropoiesis. To further this study, transgenic mouse models were created that contained a complete β-globin locus transgene. This model helped explain the role of KLF1 and KLF2 in human γ-globin gene expression. The drawback of these models is that KLF1 and KLF2 knockout mice are embryonically lethal and die before the onset of definitive erythropoiesis. Also, there is only one hemoglobin switch in mouse as compared to the two switches observed in human erythropoiesis. These models are excellent systems to study the effect of these genes on primitive erythropoiesis; however, these mouse models cannot be used to study definitive erythropoiesis due to their early death. To study definitive erythropoiesis, a human CD34+ hematopoietic stem cell *in vitro* system has been developed for KLF1 knockdown studies. These CD34+ hematopoietic stem cells are enriched from human umbilical cord blood which is collected at birth. This system cannot be used to study primitive erythropoiesis since definitive erythropoiesis has already begun at the time of collection of umbilical cord blood. Also, studying the human *in vitro* model brings us one step closer to understanding the role of KLF1 and KLF2 in β-hemoglobinopathies.

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Chapter 2: Methods

2.1 Generation of KLF1 Knockdown Erythrocytes

The *in vitro* human KLF1 knockdown model was generated to study the effect of KLF1 in human fetal erythropoiesis. The knockdown is mediated by a KLF1 sh-RNA and the whole process from enrichment of CD34+ hematopoietic stem cells from umbilical cord blood to differentiation into erythroid lineage takes 18 days (Figure 2.1B). The protocol involves the following stages (Figure 2.1A):

2.1.1 Selection and Expansion of Cord Blood CD34+ Cells

Human fetal cord blood was purchased from St. Louis Cord Blood Bank (SLCBB, St. Louis, MO). Henceforth, all steps were carried out in sterile tissue-culture conditions. The cord blood was diluted 1:1 using Dulbecco's Phosphate Buffered Saline (DPBS). Four volumes of blood was then layered over three volumes of Ficoll-Paque™ (GE Healthcare) to carry out density gradient centrifugation to isolate mononuclear cells (MNCs). The tubes were centrifuged at 400 X g for 30 mins at 24°C with zero brakes. The middle buffy layer containing MNCs was carefully removed and transferred to a new tube. This middle layer was put through two washes with at least three volumes of DPBS at 120 X g for 10 mins with zero brakes. The washed MNCs underwent CD34+ selection using the EasySep, Human CD34 selection kit (Stem Cell Technologies) according to manufacturer's instructions. The human CD34+ cells are hematopoietic stem cells that have the potential to differentiate into the erythroid and myeloid lineage. Briefly, MNCs were diluted to $5X10^8$ cells per ml with PBS containing 2% FBS and 1mM EDTA. Next, 100 µL/ml of EasySep® Positive Selection Cocktail was added to the cells

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and incubated for 15 mins at RT. 50 µL/ml of EasySep® Magnetic Nanoparticles was then added and incubated for 10 minutes at RT. The final volume of the tube was made to 2.5ml by adding PBS containing 2% FBS and 1mM EDTA and placed into the EasySep® Magnet (Stem Cell Technologies, Cat. 18000) for five minutes. Non CD34+ cells were discarded by inverting the tubes for a few seconds while it is still in the magnet. 2.5ml of PBS containing 2% FBS and 1mM EDTA was again added to repeat this magnet process another 4 times. Each sample goes through the magnet separation for a total of five times at the end of which 1-3 million CD34+ cells were obtained. These enriched cells were resuspended in the Expansion medium containing StemSpan SFEM medium (Stem Cell technologies), 10 µl/ml CC100 (Stem Cell technologies), 8 µl/ml LDL (Sigma), and 2% Penicillin/Streptomycin (P/S, Gibco) at 1-1.5 million cells per ml. The cells were expanded for one week with a change in medium every alternate day.

A hemocytometer was used for cell counting. The number of cells in one of the four large squares was counted (each large square contains 16 small squares). The cells are multiplied by the factor 10^4 to give the final cell count per ml.

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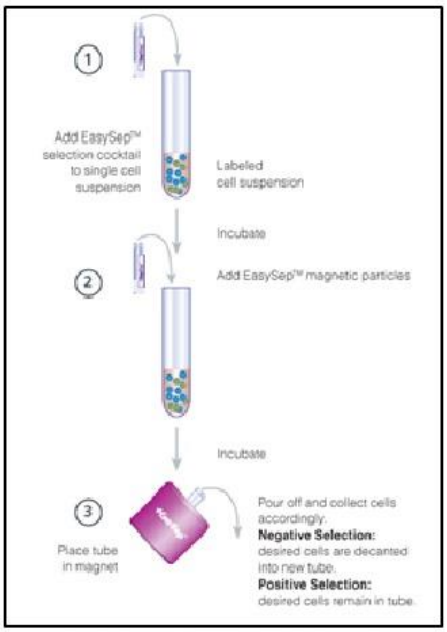
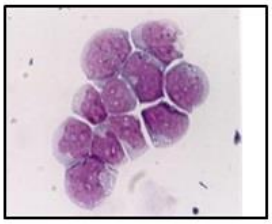
Figure 2.1 A

Umbilical Cord Blood from St.Louis Cord Blood Centre, Missouri

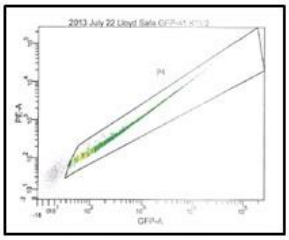


Magnetic Separation to isolate CD34+ cord blood cells

Expand CD34+ cells for 8 days



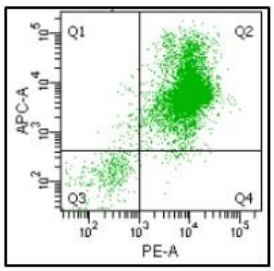
Transduction of cells with a lentivirus containing KLF1 shRNA or Scramble shRNA on day 8



Flow Cytometry for GFP positive CD34+cells on day 10

Grow and differentiate cells into erythroid lineage for 8 days

Marker sorting for glycophorin A and transferrin receptor on day 18



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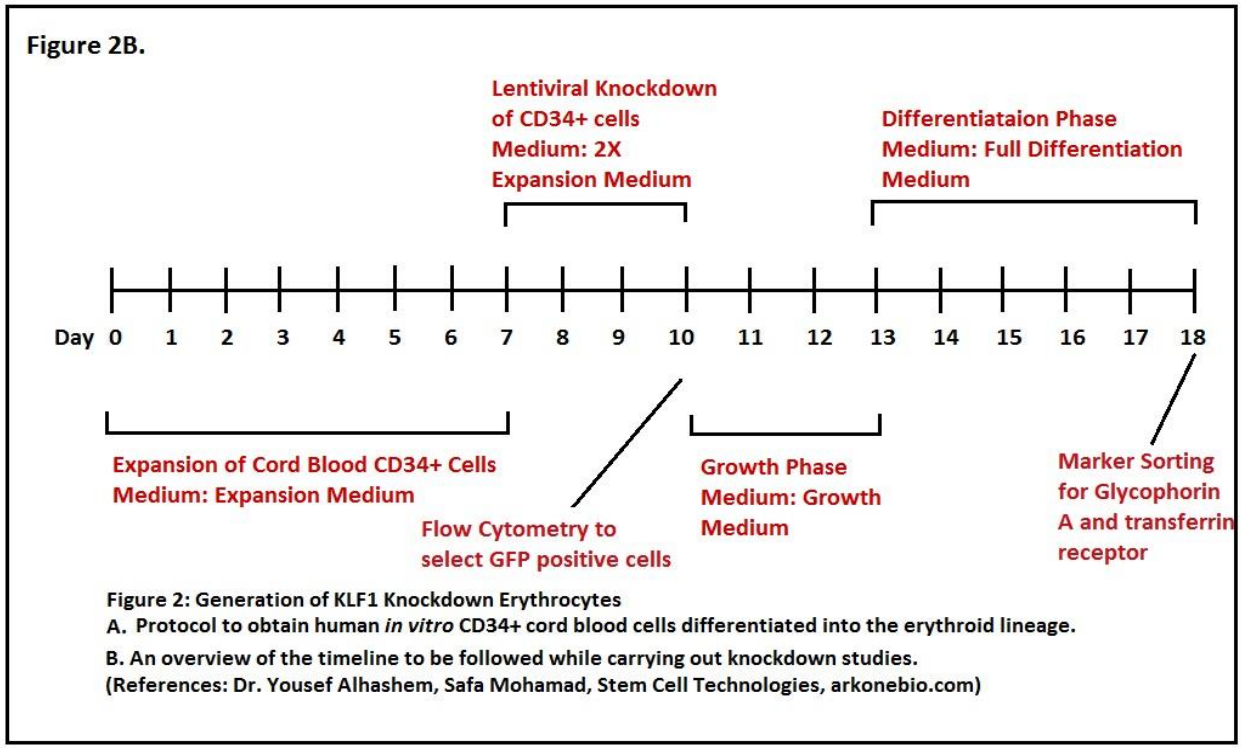

Figure 2: Generation of KLF1 Knockdown Erythrocytes
A. Protocol to obtain human *in vitro* CD34+ cord blood cells differentiated into the erythroid lineage.
B. An overview of the timeline to be followed while carrying out knockdown studies.
(References: Dr. Yousef Alhashem, Safa Mohamad, Stem Cell Technologies, arkonebio.com)

2.1.2 Cloning

The plasmids containing the human KLF1 shRNA (K1V2) and the Scramble shRNA were obtained from Dr. François Morle's Laboratory (Université de Lyon, Lyon, France). Plasmids containing new KLF1 shRNAs (K1V4, K1V5 and K1V6) were designed using RNAi Explorer (Gene Link). The difference between the KLF1 shRNAs is that K1V2 targets exon 2 whereas K1V4, K1V5 and K1V6 sh-RNA targets the 3'UTR of the KLF1 gene. To construct K1V4, K1V5 and K1V6 shRNAs, four oligonucleotides (Table 2.1) that assemble the shRNA were designed and ordered from Eurofins MWG Operon (Huntsville, AL). Oligonucleotides were kinased, ligated and annealed to form a complete shRNA (highlighted in yellow in Table 2.1) which possessed an Xho I restriction site at one end and a Mlu I site at the other. The shRNA was cloned into a precut pRRLSIN.cPPT.PGK-GFP.WPRE backbone at the Xho I and Mlu I restriction sites. Plasmids were transformed into stbl3 E.Coli (Invitrogen™) according to

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manufacturer's instructions and the transformed bacteria were plated overnight on LB agar containing 100 µg/ml Ampicillin (Thermo Fisher Scientific Inc.). Approximately five Ampicillin resistant colonies were picked from the plates for miniprep plasmid extraction (QIAprep® Spin Miniprep Kit, Qiagen) followed by plasmid sequencing at the Nucleic Acids Research Facilities, Virginia Commonwealth University. A colony once recognized with the correct shRNA sequence was then used for large scale plasmid extraction (PureLink ™ HiPure Plasmid Filter Maxiprep Kit, Invitrogen by Life Technologies).

Name	**Sequence 5'-3'**
KLF1A shRNA Version 2 (K1V2)	GATCCCGCGGCAAGAGCTACACCAATTCAAGAGATTGGTGTAGCTCTTGCCGCTTTTTGGAAA
Scramble shRNA	AGCTTTTCCAAAAAGCGGCAAGAGCTACACCAATCTCTTGAATTGGTGTAGCTCTTGCCGCGG
KLF1A shRNA Version 4 (K1V4)	ACGCGTCCCCGACTCAGTGGACACTCAGATTCAAGAGATCTGAGTGTCCACTGAGTCTTTTTGGAACTCGAG
K1V4 Probe 1	CGCGTCCCCGACTCAGTGGACACTCAGA
K1V4 Probe 2	TCTCTTGAATCTGAGTGTCCACTGAGTCGGGGA
K1V4 Probe 3	TTCAAGAGATCTGAGTGTCCACTGAGTCTTTTTGGAAC
K1V4 Probe 4	TCGAGTTCCAAAAAGACTCAGTGGACACTCAGA
KLF1A shRNA Version 5 (K1V5)	ACGCGTCCCCGGCTGATATTAACTGTCAATTTCAAGAGAATTGACAGTTAATATCAGCCTTTTTGGAACTCGAG
K1V5 Probe 1	CGCGTCCCCGGCTGATATTAACTGTCAAT
K1V5 Probe 2	TCTCTTGAAATTGACAGTTAATATCAGCCGGGGA
K1V5 Probe 3	TTCAAGAGAATTGACAGTTAATATCAGCCTTTTTGGAAC
K1V5 Probe 4	TCGAGTTCCAAAAAGGCTGATATTAACTGTCAAT
KLF1A shRNA Version 6 (K1V6)	ACGCGTCCCCGTCCAAACTGTCGTGCAAATTCAAGAGATTTGCACGACAGTTTGGACTTTTTGGAACTCGAG

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K1V6 Probe 1	CGCGTCCCCGTCCAAACTGTCGTGCAAA
K1V6 Probe 2	TCTCTTGAATTTGCACGACAGTTTGGACGGGGA
K1V6 Probe 3	TTCAAGAGATTTGCACGACAGTTTGGACTTTTTGGAAC
K1V6 Probe 4	TCGAGTTCCAAAAAGTCCAAACTGTCGTGCAAA
pRRL sequencing F	GGTACAGTGCAGGGGAAAG

Table 2.1: Sequences of shRNAs, oligonucleotides and primers used for sequencing and making new KLF1 target shRNAs.

2.1.3 Lentiviral Transduction and KLF1 Knockdown

At the end of expansion, approximately 40-70 million CD34+ cells were obtained depending upon the startup cell number. CD34+ cells were infected with lentivirus containing either KLF1 shRNA or Scramble shRNA (Control). Around 4 million cells (mock) were left untreated as another control. To make the virus, 4 million 293T cells in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% P/S were plated in 10cm tissue culture dishes and incubated overnight at 37°C, 5% CO_2. The next day, Calcium phosphate precipitation was carried out for DNA transfection into 293T cells. For each dish (each containing ~4 million 293T cells), 5.6µg of pCMVR plasmid, 2.3µg of pMD2G plasmid (Packaging plasmids obtained from Dr. Gordon Ginder's laboratory, VCU) and 7.5µg of either KLF1 shRNA pRRL plasmid or Scramble shRNA pRRL plasmid (with a GFP gene inserted in their genome) were added in a tube along with 63µl of $CaCl_2$ and 500µl of 2X HEPES Buffered Saline (HBS). The total volume was made to 1ml using tissue culture grade water. This 1ml was dropwise added to the 293T tissue culture dish so as to cover the whole dish. The dishes were incubated overnight at 37°C, 3% CO_2. The next day, the medium of each dish was changed to DMEM containing 2% FBS and

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1% P/S and the dishes were again incubated overnight at 37°C, 10% CO_2. The medium and incubation condition were inductive for virus formation. At the end of these three days, the lentivirus containing either KLF1 shRNA or Scramble shRNA is made and present in the medium. The medium containing the virus is then collected and used to infect human CD34+ cells to facilitate shRNA mediated knockdown.

Expanded CD34+ cells were seeded at 0.3 X 10^6 cells per 50µl for infection and added into 12 well plates at 50µl per well. 600µl of medium containing lentivirus was added to each well and incubated at 37°C, 5% CO_2 for 12 hours and then supplemented with 600µl of expansion media. Cells were kept a total of three days before they were subjected to flow cytometry to select for GFP positive cells.

Figure 2.2

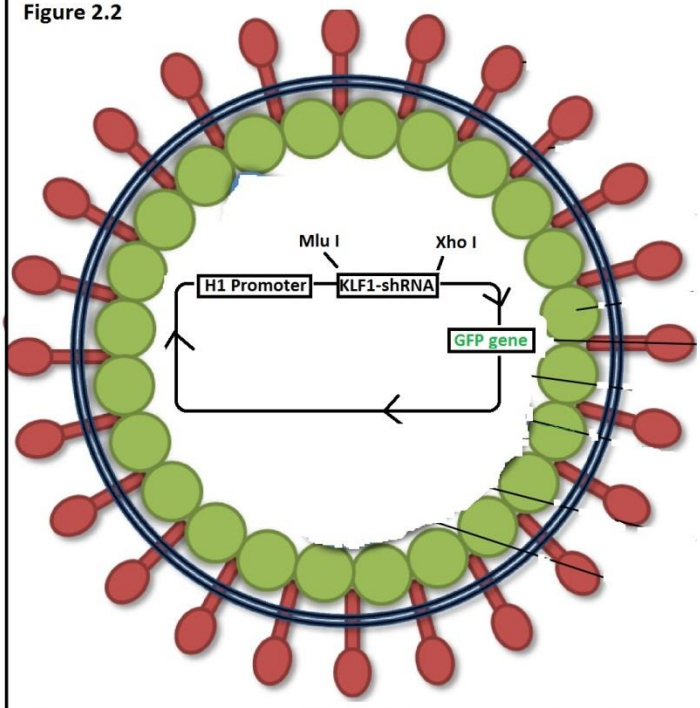

Figure 2.2: Representation of the lentivirus used to perform shRNA-mediated KLF1 knockdown.

2.1.4 Flow cytometry

Flow cytometry was carried out to sort GFP positive cells. The CD34+ cells that were infected by lentivirus would contain GFP gene in their genome (Figure 2.2) which would get translated into GFP protein. The flow sorting was done using a FACS Aria flow cytometer running FACS Diva software (BD Biosciences, San Jose, CA) at the Flow cytometry Core Facility, Virginia Commonwealth University. Usually 40-90% of cells were found to be GFP positive (Average= 65.8%, Stdev= 16.95, N= 39).

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

The next step after GFP sorting was to transfer the CD34+ cells into differentiation medium at two consecutive stages, First cells were transferred to Growth/Differentiation medium which contained IMDM, 20% FBS, 10 ng/ml SCF (Stem Cell Factor), 1 ng/ml IL-3, 1µM Dexamethasone, 1µM Estradiol, 1U/ml Erythropoietin and 2% P/S. The cells were kept in this medium for three days. The cells were then transferred to full differentiation medium for the next five days which contained IMDM, 20% FBS, 1U/ml Erythropoietin, 10 ng/ml insulin and 2% P/S. In both stages, cells were kept in 12 well plates at 1-1.5 million cells/well and medium was changed every 2-3 days.

Flow cytometry was carried out to sort cells in the erythroid lineage at day 8 of differentiation. Antibodies against CD235+ (Glycophorin A) and CD71+ (Transferrin Receptor) were used to stain cells in the erythroid lineage at day 8 of differentiation. 5µl of CD71+ (APC) and 7.5µl of 1:100 diluted CD235a+ (PE) was added to around 20 million cells. The cells were allowed to stain for 20 mins followed by two washes in PBS. The flow sorting was done using a FACS Aria flow cytometer running FACS Diva software (BD Biosciences, San Jose, CA) at the Flow cytometry Core Facility, Virginia Commonwealth University. Around 90% of the cells were found to be GFP+, CD235+ and CD71+.

2.2 Mouse Mating, Dissection and Collection of Erythroid Cells

cDNA for KLF1+/-KLF2-/-, KLF1-/-KLF2+/- and KLF1-/-KLF2-/- genotypes was prepped by Divya Vinjamur. KLF1+/- mice were mated with wild type mice to obtain KLF1 heterozygous embryos. Once plugged, pregnant mice were dissected on E10.5 after being anesthetized using isoflurane and cervical dislocation. Whole embryos were collected and placed in petri dishes

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containing PBS. One embryo was processed at a time to carefully separate the maternal tissues from the yolk sac without disturbing the embryo. Embryos with intact yolk sacs were washed in PBS and then transferred in 12 well culture plates wherein each well contained 1ml PBS. The embryos were allowed to bleed into the PBS by severing the umbilical and vitelline vessels connecting the embryo and the yolk sac. Embryonic blood once collected into the PBS was centrifuged at 12,000 g to collect the blood cells followed by RNA extraction. The embryo was used for genotyping.

2.3 Genotyping

DNA was prepared for genotyping by digesting the embryos in digestion buffer (10mMTris HCL (pH8.5), 50mM KCl, 40mM MgCl2, 0.45% Tween 20, and 0.45% NP-40) and 1µg/µl proteinase K for at least 4 hours at 55-60°C followed by alternative boiling and cooling of samples two times for 10mins each. PCR was then carried out by adding the appropriate DNA (sample DNA, control DNA or water) to each reaction tube which contained: 0.2mM dNTPs, 1.5mM MgCl2, 10X PCR buffer (Invitrogen), 0.04 U/µl *taq* polymerase (Invitrogen), 1.75 µg/µl RNase A (Invitrogen), and the KLF1 primers (FP: GGT GAA CCC GAA AGG TAC AA, RP: CTG GGA CCT CTG TCA GTT GC, product size: 170). The DNA amplification required 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. An H_2O negative control, a wild type and KLF1+/- positive controls were used in this experiment. The PCR product was run on a 2% agarose gel at 90-120V to visualize two bands for KLF1+/- and one band for wild type genotypes.

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2.4 RNA Extraction and Quantification

To extract RNA from human cord blood CD34+ cells differentiated into the erythroid lineage, TRIzol® Reagent (Ambion®, Life Technologies Corporation) was used. Approximately 0.35-0.5ml of TRIzol® Reagent was added to 2-3 million pelleted cells. These cells were incubated at Room Temperature (RT) for 5 mins for complete lysis. The cell lysate can then be stored at -80°C or used for RNA isolation. RNA isolation was carried out in 3 steps:-

Phase Separation: 0.2ml of chloroform was added per 1ml of TRIzol® Reagent. The tube was shaken vigorously by hand for 15 seconds and centrifuged at 12,000 X g speed, 4°C for 15 mins. The top aqueous layer was then separated and transferred to a new tube. Care must be taken to avoid the interface between the two layers since it is rich in DNA.

RNA precipitation: 5-10µg of RNase-free glycogen was added as a RNA carrier to the aqueous phase. Next, 0.5ml of 100% isopropanol per 1ml of TRIzol® Reagent was added to precipitate RNA. For complete precipitation, this tube was incubated at -80°C overnight. The following day, precipitated RNA was recovered by centrifuging at 12,000 X g speed, 4°C for 30 mins. A pellet should be seen at the bottom of the tube.

RNA wash: The supernatant was discarded carefully leaving the pellet. This pellet was washed using 1ml of 75% ethanol per 1ml of TRIzol® Reagent and dried for 15 mins. After the wash, the RNA pellet was dissolved in 10µl of RNase free water containing 1:20 superase enzyme.

RNA concentration was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies). The RNA kit contains chips and reagents designed for analysis of RNA

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fragments. Each chip contains interconnected microchannels which separate nucleic acid fragments electrophoretically based on size. The integrity of RNA can also be assessed since a picture of the gel is provided. Two clear bands of ribosomal RNA with minimal smearing indicate good integrity. The first step in following this kit was to filter the RNA 6000 Nano gel matrix. 65µl of filtered gel was then aliquoted into a 1.5ml microfuge tube. Simultaneously, the RNA 6000 Nano dye concentrate was allowed to equilibrate to RT for 30 mins. 1µl of this dye was added into the aliquot of filtered gel. This solution was vortexed well and centrifuged at 13,000 X g for 10 mins at RT.

Figure 2.3: RNA 6000 Nano chip in a chip priming station

A new RNA 6000 Nano chip was placed on the chip priming station (Figure 2.3). 9µl of the gel-dye mix was first loaded in the well marked G, the chip priming station was closed and the plunger was pressed until it could be held by the clip. The clip was released after 30 seconds followed by pipetting 5µl of RNA 6000 Nano marker in all sample wells and in the well marked ladder. 1µl of sample was loaded in each sample well. A ladder was provided along with the kit to separate RNA based on its size and 1µl of it was loaded in the well marked ladder. The chip was vortexed at 2400 rpm for 1 min and run in the Agilent 2100 bioanalyzer within 5mins of vortexing. Approximately 2000-3000µg of RNA is obtained from 1-1.5 million cells.

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2.5 cDNA Synthesis

The RNA isolated above needs to be treated with DNase to eliminate any traces of DNA that could have precipitated along with the RNA. A DNase I treatment kit by Invitrogen™, Life Technologies Corporation was used for this purpose. The reaction tube contained 1µg of RNA sample, 1µl 10X DNase I reaction buffer and 1µl DNase I enzyme, amplification grade, 1U/µl. The total volume was made up to 10µl. The tubes were incubated at RT for 15 mins after which the enzyme was inactivated by the addition of 1µl of 25mM EDTA followed by heat inactivation at 65°C for 10 mins.

cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). In addition to the 10µl after DNase treatment, 4µl of 5X iScript Reaction Mix and 1ul of iScript Reverse Transcriptase were added. The total volume was adjusted to 20µl using nuclease-free water. The reaction mix was incubated under the following conditions to complete cDNA synthesis: 5 min at 25°C, 30 min at 42°C, then 5 min at 85°C.

2.6 Quantitative Real Time PCR (qRT-PCR)

The expression of several human and mouse genes was quantified using qRT-PCR with SYBR green or Taqman reagents (Applied Biosystems, Foster City, CA). Human cyclophilin A and mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA were used as internal standards for normalization. For qRT-PCR using SYBR green, the following reagents were added in each reaction tube: 12.5µl of 1X Power Sybr Green master mix (Applied Biosystems®), 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 5.5µl of molecular biology grade water and 5µl of respective cDNA. For qRT-PCR using Taqman technology, the

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following reagents were added in each reaction tube: 12.5µl of 1X Taqman® Universal PCR Master Mix (Applied Biosystems®), 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl of 10µM probe, 4.5µl of molecular biology grade water and 5µl of respective cDNA. The total volume of each reaction tube for both protocols was 25µl. qRT-PCR was performed using an ABI Prism 7300 (Applied Biosystems, Foster City, CA). A dissociation step was included while quantifying samples using SYBR green chemistry to verify the presence of a single amplified product. A standard curve obtained from pooling cDNA and having set concentrations were included in each run. This was useful in measuring the quantity of unknown cDNA samples as well as calculating the efficiency of each primer set. Each unknown cDNA sample was run in quadruplet. The average quantity of the four replicates was calculated and any replicate greater/lesser than 20% of the average was excluded. Efficiency was calculated using the formula Efficiency (E) = 10^(-1/slope). Relative expression of each sample was calculated as $E^{(-1*Ct_{test\ gene})} / E^{(-1*Ct_{endogenous\ gene})}$. All comparisons were made to wild type in the mouse system which was set at 100%. In the human CD34+ system, KLF1 knockdown CD34+ cells differentiated into the erythroid lineage were compared to scramble cells which were set at 100%. Statistical significance was calculated using the Student's t-test. The list of genes quantified and their primers and probes are given below in Table 2.2.

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mRNA	Primer Sequence 5'-3'	Product Size (bp)
Human KLF1	F: GCA AGA GCT ACA CCA AGA G R: GTG TTT CCG GTA GTG GC	140
Human KLF2	F: TGC CAT CTG TGC GAT CGT R: GGC TAC ATG TGC CGT TTC ATG	71
Human Cyclophilin A	F: CCG AGG AAA ACC GTG TAC TAT TAG R: TGC TGT CTT TGG GAC CTT G	125
Human ϒ-globin	F: GTG GAA GAT GCT GGA GGA GAA A R: TGC CAT GTG CCT TGA CTT TG Probe: AGG CTC CTG GTT GTC TAC CCA TGG ACC	136
Human β-globin	F: GCA AGG TGA ACG TGG ATG AAG T R: TAA CAG CAT CAG GAG TGG ACA GA Probe: CAG GCT GCT GGT GGT CTA CCC TTG GAC CC	117
Human Bcl11A	F: AAC CCC AGC ACT TAA GCA AA R: GGA GGT CAT GAT CCC CTT CT	114
Human FOXM1	F: GCG ACA GGT TAA GGT TGA GG R: GCT GTT GAT GGC GAA TTG TA	112
Human Myc	F: TCT CTG CTC TCC TCG ACG R: CTT CCT CAT CTT CTT GTT CCT CC	118
Human CD24	F: GCC CCA AAT CCA ACT AAT GC R: ACG TTT CTT GGC CTG AGT C	126
Human Sphk1	F: AGG CTG AAA TCT CCT TCA CG R: CTC CAT GAG CCC GTT CAC	149
Human E2F2	F: CAA CTT TAA GGA GCA GAC AGT G R: GGC ACA GGT AGA CTT CGA TG	134
Human E2F4	F: CTC ACG TCC AAA TAG TCC TCA G R: GTC CTT GCT ATC AGT CCC AG	124
Human p18	F: GTA AAC GTC AAT GCA CAA AAT GG R: AGT TCG GTC TTT CAA ATC GGG	123
Mouse Foxm1	F: GGC AAA GAC AGG AGA GCT ATG R: TCT TCC AGT TCC TGC TTA ACG	136

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Mouse Sphk1	F: TGA ATG GGC TAA TGG AAC GG R: GTC TTC ATT AGT CAC CTG CTC G	137
Mouse Myc	F: TGC TGC ATG AGG AGA CA R: TCG GGA TGG AGA TGA GC	140
Mouse Cd24a	F: CTT AGC AGA TCT CCA CTT ACC G R: GTA AAT CTG CGT GGG TAG GAG	136
Mouse E2F2	F: CCC CAA AAC CCC CAA GTC T R: ACT CGC TCA GGA GGT AAA TGA ACT	92
Mouse E2F4	F: GCA GAT GCT TTG CTG GAG AT R: TCT GGT ACT TCT TCT GGC CAT TGA	105
Mouse p18	F: CGT CAA CGC TCA AAA TGG ATT R: GAC AGC AAA ACC AGT TCC ATC	130
Mouse p27	F: ACC AAA TGC CTG ACT CGT C R:GTT CTG TTG GCC CTT TTG TTT	115
Mouse Gapdh	Commercial primers (Unknown Sequence)	

Table 2.2: qRT-PCR primer sequences, probe sequences and product sizes.

2.7 Western Blot Assay

A western blot was performed to determine whether the KLF1 knockdown occurred at the translational level. The CD34+ cells obtained at Day 8 of differentiation were used for this purpose. Around 8-10million cells were centrifuged to obtain a pellet to which 4% Sodium Dodecyl Sulfate (SDS) was added for homogenization. The pellet once homogenized was sonicated for 3-5 minutes (30 seconds on/ 30 seconds off). The stickiness of the sample should be lost at the end of sonication giving the sample a fluid consistency.

Protein Estimation: The sonicated sample was diluted 1:500 then subjected to protein estimation using the Bradford Assay (BioRad Protein Assay). Bovine Serum Albumin (1.5mg/ml) was used to prepare standards for which it was diluted at 2µg/ml intervals (2-10µg/ml). To 800µl of standard/sample, 200µl of BioRad Protein estimation dye was added. The

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tubes were allowed to sit at room temperature for at least 5 minutes after which their protein was estimated using a spectrophotometer at 595nm wavelength. The machine was calibrated using distilled water to which protein estimation dye was added. A standard curve was made using which the amount of protein in unknown samples was determined. Estimation of both standards as well as sample was performed in triplicates. 4X loading dye was added to estimated samples to make 1X. These samples were boiled for 5 minutes followed by storage at -20°C.

Gel casting, loading and running: 10% SDS gels were cast using resolving gel and stacking gel. The resolving gel contained 5ml 30% Acrylamide/Bis, 6.03ml distilled water, 3.75ml 1.5M Tris-HCl (BioRad), 150µl 10% SDS, 6µl TEMED and 75µl Ammonium persulfate (APS). The resolving gel was poured and allowed to solidify between 1mm glass plates until 1 inch from the top. The stacking gel was poured for the remaining 1 inch of the plate and contained 660µl 30% Acrylamide/Bis, 3.03ml distilled water, 1.3ml 0.5M Tris-HCl (BioRad), 50µl 10%SDS, 5µl TEMED and 20µl APS. A comb was inserted between the two glass plates which later solidified to form the wells of the gel. Once the gel was completely solidified, it was placed in a gel running apparatus. Running buffer which contained 12g Tris base, 57.6g Glycine, 4g SDS in 4 liters of distilled water was used for the run.

Equal amounts of protein for different samples were loaded into the wells of the gel (50-100µg). A precision plus molecular weight marker was loaded as a ladder. The gel was run at 90-120V for 2-3 hours.

Transfer and Blocking: For transfer, sponges and blotting paper are wetted in cold transfer buffer which contains 12.12g Tris base, 57.6 Glycine and 400ml of methanol in 2.3 liters of distilled water. A transfer stack was then built which contains sponge, blotting paper,

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Polyvinylidene Fluoride (PVDF) membrane, gel, blotting paper and sponge. The PVDF membrane was activated by dipping in methanol prior to assembling the stack. The gel always faces the negative electrode in the transfer apparatus. The transfer was run at 100V for 1 hour on ice. After transfer, the membrane was blocked for 4 hours at room temperature in 5% blocking solution (Add milk powder 1:20 in Phosphate Buffer Saline + 0.05%Tween-PBST).

Antibody Staining and Development: The PVDF membrane was stained using primary antibody: polyclonal EKLF (F-20) (Santa Cruz Biotechnology) or monoclonal anti-β-actin antibody (Sigma Aldrich). The primary antibody was added 1:200 for KLF1 and 1:8000 for actin in 5% blocking solution (~10ml) and incubated with the membrane overnight in a cold room. The membrane was then put through three 10 minute washes with PBST. The membrane was incubated for 45 minutes with donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) for KLF1 (1:5000) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) for actin (1:10,000). This was followed by another two washes in PBST and one wash in PBS.

For development, SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) was used for KLF1 Western Bolt Assay and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for actin. The blots were allowed to react with the respective developing solution in the dark for 5 minutes after which they were exposed to X-ray films for 30 seconds to 5 minutes. The X-ray films were then put through the developer to observe for the required molecular weight bands. The bands were quantified using the Alpha-Innotech software.

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2.8 Colony Forming Assay

A progenitor assay was performed to determine the colony forming ability of erythroid progenitors in KLF1-shRNA knockdown cells compared to Scramble-shRNA cells. To perform this assay, CD34+ hematopoietic stem cells from human umbilical cord blood were enriched, expanded, infected with the appropriate lentivirus and subjected to flow cytometry for GFP sorting as explained in section 2.1. After sorting, the cells were plated in a viscous methylcellulose medium called MethoCult™ H4034 Optimum (Stem Cell Technologies), which is optimized for the detection and quantification of human hematopoietic progenitors in cord blood samples and has the cytokines required for erythroid differentiation.

Plating Methylcellulose: The cells were diluted in Iscove's Modified Dulbecco's Medium (IMDM) to 120,000 cells/ml. In a 5ml tube, 0.1ml of these diluted cells was added to 1.4ml of MethoCult™ medium to make a final volume of 8000 cells/ml of medium. The tube was vigorously vortexed for around 30 seconds and then allowed to sit for 15-20 mins at room temperature to allow the bubbles to surface. Using a 1ml syringe with a 16 gauge needle, 1ml of this medium with cells was plated into a 35x10mm culture dish. These dishes were incubated at 37°C, 5% CO_2 for 14 days.

Benzidine Staining: On day 14 of incubation, plates were stained with benzidine and counted for blue colonies. To make the stain, 3µl of 50% hydrogen peroxide was added to 1ml of benzidine stain stock (0.2% benzidine dihydrochloride in 0.5M acetic acid). 0.5ml of this stain was immediately layered onto the methylcellulose dish. The benzidine/peroxide solution stains hemoglobinized erythroid colonies a dark blue-brown color which is evident after 5-10 minutes of incubation at room temperature (Figure 2.4A).

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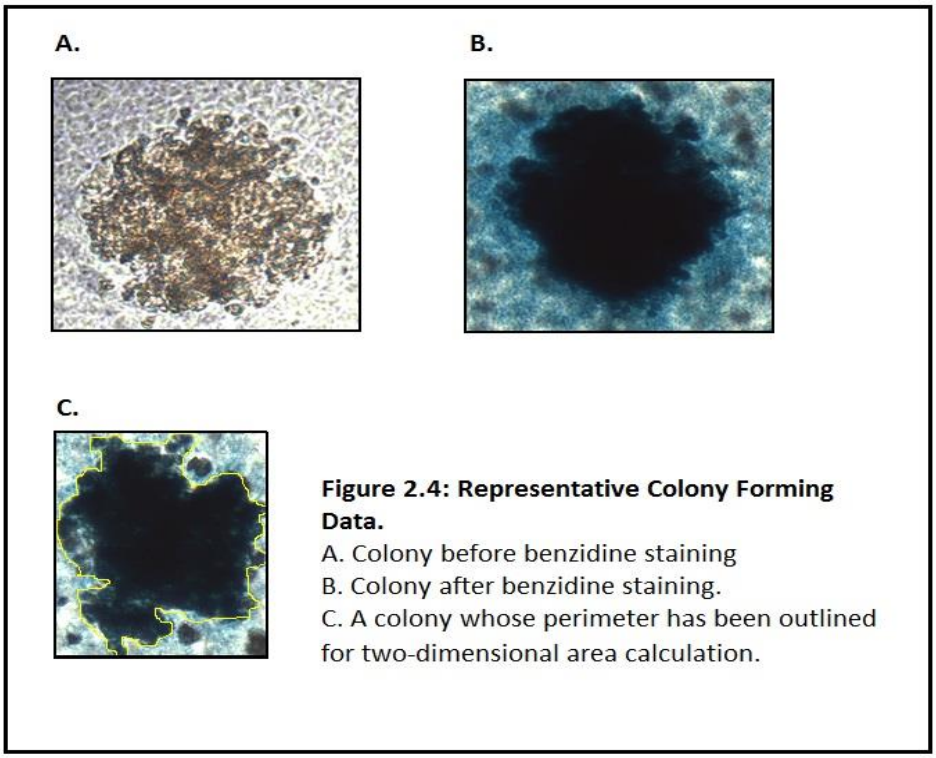

Figure 2.4: Representative Colony Forming Data.
A. Colony before benzidine staining
B. Colony after benzidine staining.
C. A colony whose perimeter has been outlined for two-dimensional area calculation.

Colony images were captured before and after staining using an Olympus IX70 microscope and Q-Color 3 camera (Olympus America). All images were taken at 100X magnification. The colonies once stained were counted using the same microscope (N=3). Colonies close to each other and connected by a trail of stained cells were counted as one. ImageJ software was used to calculate the two-dimensional surface area of the colonies in μm^2. An outline was made around the colonies to calculate the area (Figure 2.4C). The area of ~60 colonies was measured for each scramble and KLF1 knockdown colonies. The area of colonies that had already burst was not calculated. Statistical significance was calculated using the Student's t-test.

2.9 Chromatin Immunoprecipitation (CHIP)

Mouse as well as human CHIP samples were prepped by Dr. Yousef Alhashem using an anti-KLF1 antibody (Abcam), an anti-KLF2 antibody (Prepared by Dr. Yousef Alhashem; method

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published in Alhashem et al. 2011) and an anti-IgG antibody (Abcam). The samples were subjected to qPCR as described above using the primers given in Table 2.3. Primers were designed using software provided by Integrated DNA Technologies and Invitrogen. BLAST was used to make sure that the primers give a single PCR product. A dissociation curve was also plotted during qRT-PCR to ensure the same. Fold enrichment was calculated as $2^{(Ct_{input} - Ct_{test})}$ and expressed relative to a IgG control or a pre-immune control.

Gene	Primer Sequence 5'-3'	Product Size (bp)
mFoxM1	FP: CACGTAACCGCAAGTCTAGG RP: ACTCGGTTACCCCTGGG	92
mNecdin	F: TTCGTCCAGCAGAATTACCTGAAG R: GGACCCCCAGAAGAACTCGTA	78
hFOXM1 (1)	F: AGCCGAGCTTTGAAAAGGG R: GAGCCGAGGGAGAGTTTG	145
hFOXM1 (2)	F: AACTCGTGACCTCAAGTGATC R: AAATAAACAAATGTGGGCTGGG	94
hE2F2 (1)	F: GCAGCTTGGGAGAGTAGAAG R: TCTGAAAGGCTGGGTAACTTG	139
hE2F2 (2)	F: TCACCCCTCTGCCATTAAAG R: CAGGCCCTTTTGTTAGTTTGC	112
hE2F2 (3)	F: CTCTGCACTGTGAATCCCG R: GATTGCAACACTAGCTTCGC	130
hE2F4 (1)	F: CTGTGGTCCCAGCTACTTG R: AGATCAATGCCGTCTCAGC	74
hE2F4 (2)	F: AAGACGAGCGAAAGATGAGG R: ACCCCATCTATTCCTATCCCTC	109
hE2F4 (3)	F: GCAGATTGGCAAGGAAAGTTC R: AATGTCCAGCCTCCGAATAAG	143
hInsulin	F: GGCAGATGGCTGGGGGCTGA R: CCCACAGACCCAGCACCAGG	

Table 2.3: Primer sequences used in CHIP.

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Chapter 3: The Role of KLF1 in Human Globin Gene Regulation.

3.1 Introduction

Haploinsufficiency of KLF1 leads to hereditary persistence of fetal hemoglobin (HPFH) (Satta S et al. 2011, Borg J et al. 2011). Patients with a single copy of KLF1 have varying amounts of increased γ-globin gene expression. On the other hand, KLF1 knockout mice containing a complete human β-globin gene locus transgene have lower amounts of human ε- and γ-globin in E10.5 yolk sacs compared to wild type (Alhashem Y et al. 2011). However, at the fetal liver stage in KLF1 heterozygotes, there are reduced levels of human β-globin and elevated levels of human γ-globin compared to wild type mice carrying the same transgene (Wijgerde M et al. 1996, Cantor A and Orkin SH, 2002). This suggests that KLF1 both positively as well as negatively regulates γ-globin gene expression. The null mutants also show a dramatic decrease in mouse β-globin compared to wild type. To understand the complicated regulation of γ-globin by KLF1 we moved to an *in vitro* human model by using CD34+ hematopoietic stem cells enriched from umbilical cord blood. Therefore, we obtained umbilical cord blood, enriched CD34+ cells from this blood, expanded and then differentiated them into the erythroid lineage. This entire model was developed and initially characterized by Dr. Yousef Alhashem, a former PhD student in our laboratory. A lentiviral system coupled with a shRNA against KLF1 was used to knockdown KLF1 in these CD34+ hematopoietic stem cells.

We already had a good working shRNA against KLF1 for knockdown studies. However, to reconfirm our results, we designed other shRNAs against KLF1 (K1V4, K1V5 and K1V6). Knockdown was demonstrated at the transcriptional as well as translational level. Once KLF1

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was knocked down, we established its role in γ-globin, β-globin, Bcl11A and KLF2 gene expression in erythropoiesis.

3.2 The identification of a third new shRNA against KLF1.

To investigate the complex regulation of γ-globin by KLF1, we established a knockdown model using a lentiviral system that carries a short hairpin RNA against KLF1. A previous independent study by Bouilloux et al. 2008 had used two shRNAs against KLF1. The first KLF1 shRNA (K1V1) was capable of decreasing KLF1 mRNA by approximately 50%. The second shRNA (K1V2) knocked down KLF1 by approximately 80%. This shRNA was mainly used for all our analysis. Using these two shRNAs, Dr. Yousef Alhashem started the studies of KLF1 knockdown. However, to confirm our results obtained using K1V2 there was the need to identify a third shRNA capable of causing a greater than 50% KLF1 knockdown. Hence, we designed three new shRNAs (K1V4, K1V5 and K1V6). CD34+ hematopoietic stem cells were infected with K1V2/K1V4/K1V5/K1V6 or scramble-shRNA. The lentivirus contains the gene for GFP adjacent to the shRNA. Infected cells were flow sorted on the basis of their ability to express GFP. Lentiviral infection with any shRNA showed an average of 65% infection efficiency with a standard deviation of 17% (N=39) (Figure 3.1A, B, C). The cells once sorted were differentiated into the erythroid lineage for eight days. Erythroid marker analysis was performed on day 8 of differentiation. Cells were stained using antibodies against Glycophorin A and Transferrin receptor. The cells were sorted on the basis of their ability to express GFP, Glycophorin A and Transferrin receptor. Cells in the Q2 quadrant in Figure 3.1.E were considered to be erythroid in nature at the erythroblast stage of differentiation. 93.5% of Scramble-shRNA infected GFP positive cells were positive for both the erythroid markers (average=93.5, stdev= 1.3, n=3).

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Similarly, 93.9% of KLF1-shRNA infected GFP positive cells were positive for both the erythroid markers (average=93.9, stdev= 2.5, n=3).

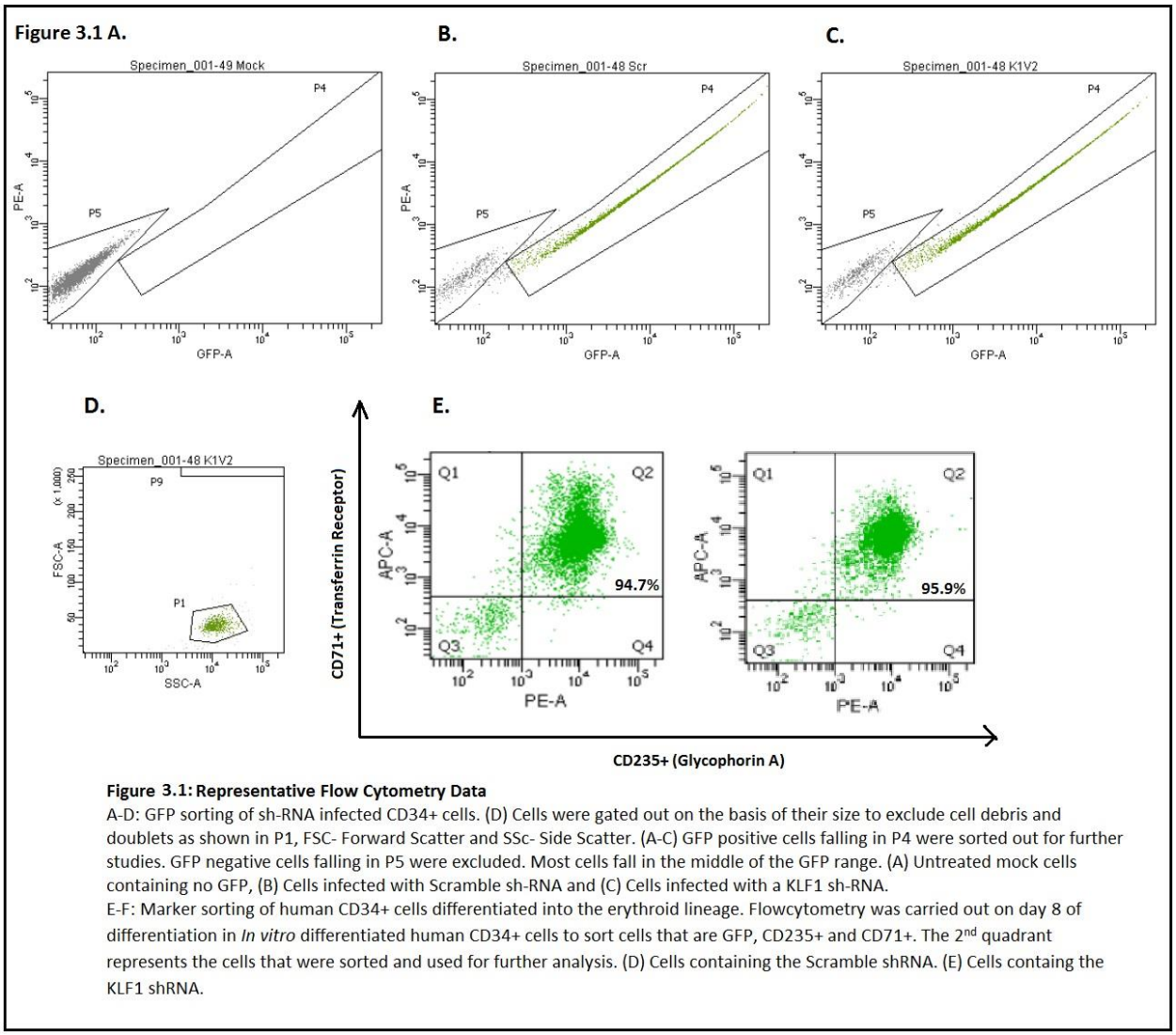

Figure 3.1: Representative Flow Cytometry Data
A-D: GFP sorting of sh-RNA infected CD34+ cells. (D) Cells were gated out on the basis of their size to exclude cell debris and doublets as shown in P1, FSC- Forward Scatter and SSc- Side Scatter. (A-C) GFP positive cells falling in P4 were sorted out for further studies. GFP negative cells falling in P5 were excluded. Most cells fall in the middle of the GFP range. (A) Untreated mock cells containing no GFP, (B) Cells infected with Scramble sh-RNA and (C) Cells infected with a KLF1 sh-RNA.
E-F: Marker sorting of human CD34+ cells differentiated into the erythroid lineage. Flowcytometry was carried out on day 8 of differentiation in *In vitro* differentiated human CD34+ cells to sort cells that are GFP, CD235+ and CD71+. The 2nd quadrant represents the cells that were sorted and used for further analysis. (D) Cells containing the Scramble shRNA. (E) Cells containg the KLF1 shRNA.

We were able to successfully knockdown about 46% of KLF1 (stdev= 8%, n=3) using K1V1. K1V2 proved to be more successful with approximately 80% knockdown (stdev=12.29%, n=18). Amongst the newly designed shRNAs, K1V6 was incapable of causing any knockdown (average=106.7%, stdev=29%, n=2) and was not used in further analysis. K1V5 caused around 58% knockdown (n=1). The most successful newly designed shRNA was K1V4 with an average

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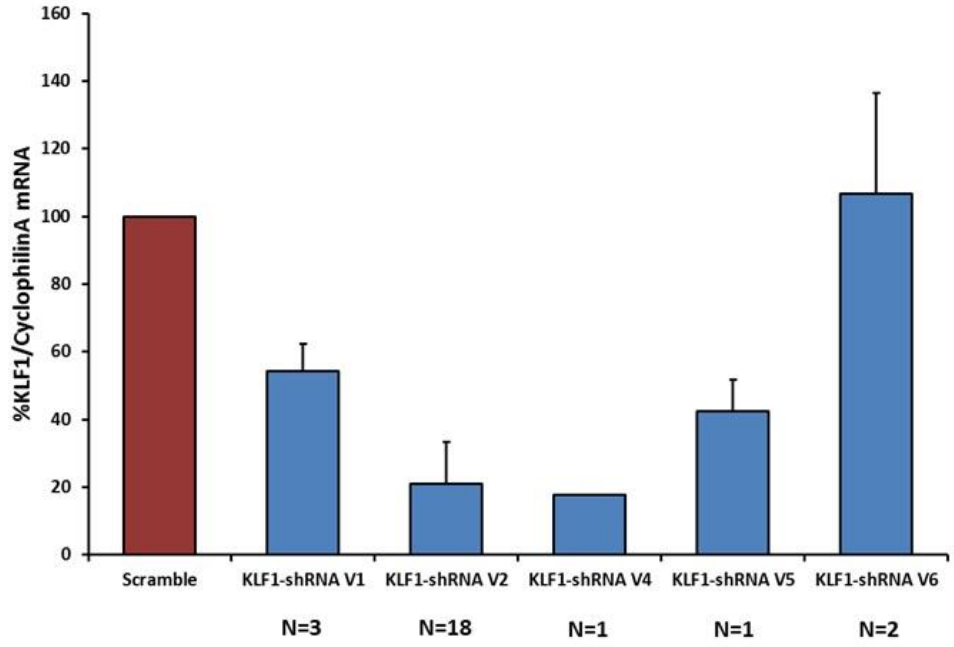
knockdown of 82% (n=1) (Figure 3.2). The results suggest that out of the three KLF1 shRNAs newly designed, K1V4 might be the most promising.

Figure 3.2



3.2: Percentage knockdown of KLF1 using various shRNAs.
qRT-PCR was performed to determine the amount of KLF1 knockdown at the mRNA level using different shRNAs compared to scramble. Cyclophillin A was used as an internal standard. Scramble was taken as 100%. Error bars indicate standard deviation.

3.3 Lentiviral knockdown using a KLF1-shRNA causes a reduction in KLF1 at the transcriptional as well as translational level.

Although KLF1 knockdown has been shown transcriptionally through qRT-PCR, it is important to prove the knockdown at the translational level. KLF1 knockdown CD34+ cells and scramble CD34+ cells were collected on day 8 of differentiation and sonicated to lyse the cells. A western blot was performed to measure the KLF1 protein levels in these lysed cells. Actin was used as a

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loading control. The sample used for western blot analysis had a 90% KLF1 knockdown at the mRNA level (Figure 3.3A). However, at the protein level, even though a band cannot be clearly seen on the blot under KLF1 shRNA; only ~60% KLF1 knockdown was observed. This result indicates that even a small amount of KLF1 mRNA is capable of translating approximately 40% of KLF1 protein. KLF1 protein stability might be one of the factors responsible for the increased protein levels. Also, this is only an n=1. There is a possibility that there was an error while quantifying the protein levels or that the suggested band is not KLF1. More samples need to be tested and quantified to determine whether the assumed KLF1 band has different knockdown levels at the transcription and translational level.

Figure 3.3

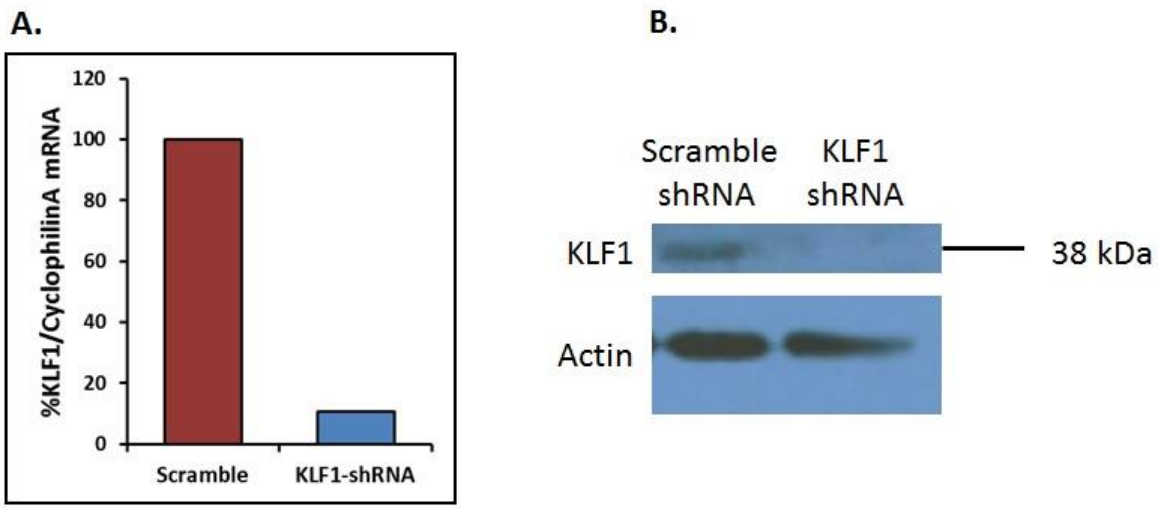

Figure 3.3: KLF1 knockdown at transcriptional and translational level.
CD34+ hematopoietic stem cells were transfected using lentivirus which contained either a KLF1-shRNA or a Scramble-shRNA. Once infected, the cells were differentiated into the erythroid lineage. On day 8 of differentiation cells were collected for either qRT-PCR analysis or Western Blot assays. (A) qRT-PCR was used to determine the mRNA levels of KLF1 knockdown CD34+ blood cells compared to scramble. (B) A western blot was carried out on the same sample to prove knockdown at the translational level.

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3.4 KLF1 positively regulates β-globin and Bcl11A gene expression but has a complex role in the regulation of γ-globin and KLF2 gene expression.

To investigate the regulation of β-globin, Bcl11A, γ-globin and KLF2, we used the KLF1 knockdown model previously described in section 3.2. Three samples were prepped by Dr. Yousef Alhashem using K1V1. Eleven samples were prepped by Dr. Yousef Alhashem and Divya Vinjamur using K1V2. Whereas, I prepped seven samples using K1V2 and all of the samples using K1V4 and K1V5. qRT-PCR was performed to assess the role of KLF1 in gene regulation. We observed a strong positive correlation between β-globin and KLF1 (r^2=0.79, $p<0.0001$) (Figure 3.4A) indicating that KLF1 regulates β-globin gene expression in human CD34+ cord blood cells.

Next we investigated γ-globin gene regulation by KLF1. At about 50% KLF1 knockdown we observed approximately 2-fold increase in γ-globin mRNA levels. At about 80% KLF1 knockdown, no change in γ-globin mRNA levels was observed. Also, at extremely low levels of KLF1 (~6%) we observed a decrease in γ-globin gene expression. To determine the relationship between γ-globin and KLF1, we tried plotting a regression correlation. However, no significant correlation was observed. Hence, we plotted a smooth curve to explain γ-globin gene regulation by KLF1 (Figure 3.4B). The smooth curve suggested that γ-globin has almost a linear relationship with KLF1 up to 50% KLF1, but at more than 50%, γ-globin mRNA levels seemed to have started reducing with increasing KLF1. This result indicated that the relationship between γ-globin and KLF1 is complicated and may need more research.

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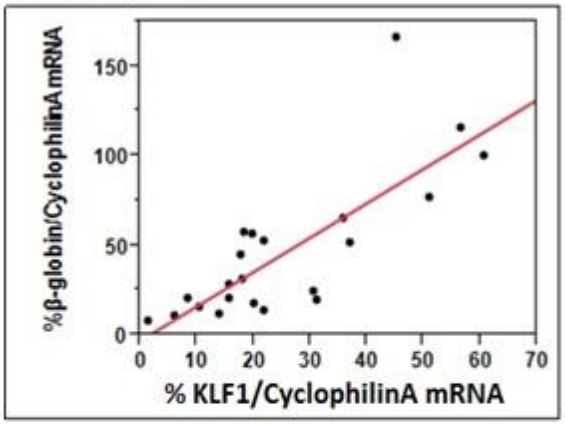
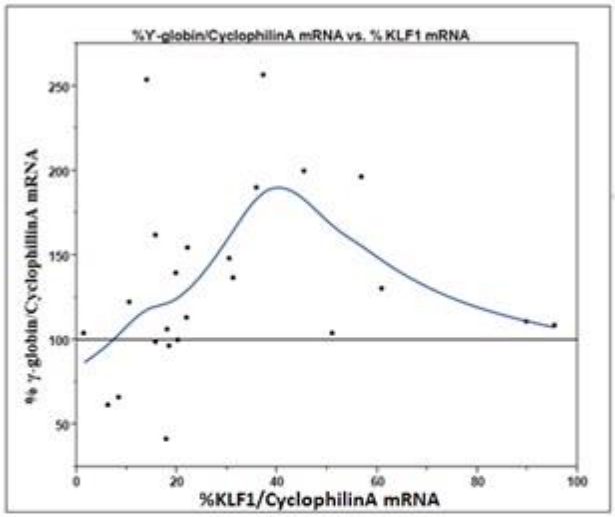
Figure 3.4. A.



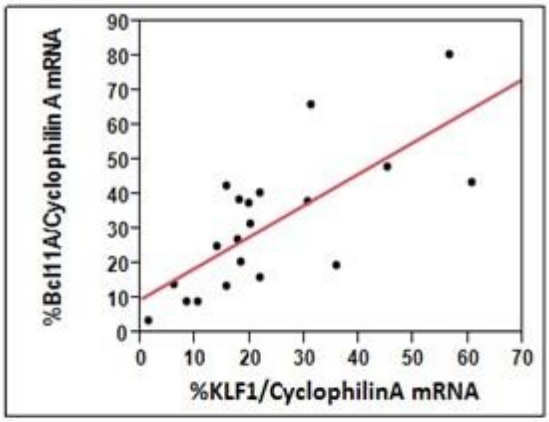
Correlation: 0.79
p- value <0.0001
N=22

B.



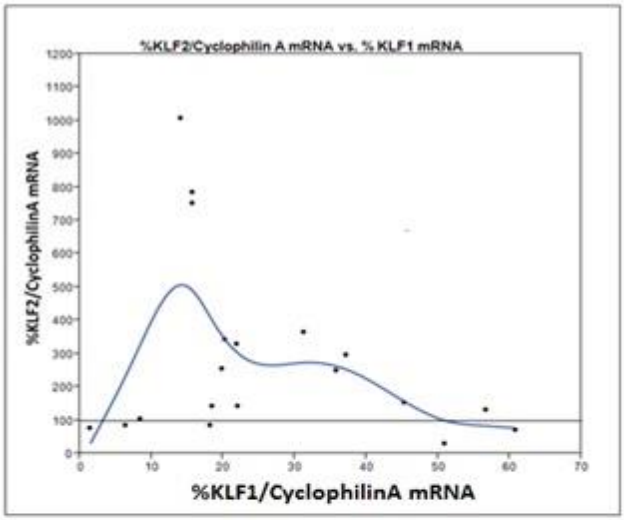
N=24

C.



Correlation: 0.73
p- value=0.0002
N= 22

D.



N=21

Figure 3.5: Analysis of β-globin, Bcl11A, Y-globin and KLF2 gene expression in KLF1 knockdown *in vitro* differentiated CD34+ human cord blood cells.

CD34+ hematopoietic stem cells were transfected using lentivirus which contained either a KLF1-shRNA or a Scramble-shRNA. Once infected, the cells were differentiated into the erythroid lineage. On day 8 of differentiation cells were collected for qRT-PCR analysis. The amounts of β-globin, Bcl11A, Y-globin and KLF2 mRNA was quantified. A regression correlation was plotted for (A) β-globin gene expression and (C) Bcl11A gene expression. No regression correlation was observed for Y-globin and KLF2 mRNA. Therefore, smooth curves were plotted for (B) Y-globin gene expression and (D) KLF2 gene expression. For all graphs, cyclophillinA was the internal standard and Scramble was set at 100%.

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Another gene important for globin regulation is Bcl11A. KLF1 has been found to show strong binding affinity towards the BCL11A promoter which is a regulator of γ-globin gene silencing (Wilber et al. 2011). To determine the role of KLF1 in Bcl11A gene regulation, a regression correlation was plotted. A strong correlation was observed between KLF1 and Bcl11A mRNA (r^2=0.73, p<0.0004) (Figure 3.4C) which indicates that KLF1 positively regulates Bcl11A. Because Bcl11A is also involved in γ-globin gene regulation, it along with KLF1 and possibly other factors regulated by KLF1 may be responsible for the complicated γ-globin gene regulation.

Finally, we noticed that KLF2 mRNA also acts differently during KLF1 knockdown (Figure 3.4D). It increased several-fold between ~ 60-80% KLF1 knockdown, but at lower or higher % knockdown, it remained unchanged. This complex regulation of KLF2 gene expression could not be explained using a regression correlation or a smooth curve. The unusual regulation of KLF2 in KLF1 knockdowns is hard to explain and needs to be studied in much greater detail to understand its entire mechanism.

3.5 Discussion:

No previous reports have been published on the role of KLF1 in globin gene regulation in CD34+ cord blood derived erythroid cells. Bouilloux et al. 2008 had designed two shRNAs against KLF1 to determine its role in megakaryocytic differentiation using CD34+ cord blood cells. However, one of the shRNAs did not cause a greater than 50% KLF1 knockdown; whereas the other caused ~80% knockdown. We designed three new shRNAs out of which one successfully caused ~80% KLF1 knockdown (K1V4). We confirmed the knockdowns at the transcriptional level using qRT-PCR for all of the shRNAs (K1V1, K1V2, K1V4, K1V5, and

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K1V6). To confirm the knockdowns at the protein level, we performed western blot assays and observed a band at 38 kDa which we suggest is KLF1. We observed a 60% KLF1 knockdown (n=1) at the protein level in a sample which had 10% KLF1 mRNA remaining after knockdown. The difference in KLF1 at the mRNA and protein level might be sample specific and the disparate results might not be observed once we quantify more samples. Also, KLF1 is a transcription factor present in very low quantities at the mRNA and protein level making it difficult to quantify. The half-life of KLF1 protein is 9 hrs in mouse fetal liver cells. Quadrini K and Bieker J, 2006 have shown that this half-life can be increased to 23hrs in the presence of 26S proteasome inhibitors. Lentiviral knockdown may affect the regulation of proteasome inhibitors in turn increasing the half-life of KLF1. More will be known after testing and quantifying the KLF1 protein levels of other samples.

Earlier work in our laboratory showed that KLF1 can positively as well as negatively regulate γ-globin gene expression. Alhashem et al. in 2011 demonstrated a positive regulation of γ-globin gene expression in KLF1-/- transgenic mice (primitive erythropoiesis). This is different from the reports suggested by various other groups that KLF1 only represses γ-globin gene expression (Zhou et al. 2010, Tallack M and Perkins A, 2013). Our data indicates a complicated regulation of γ-globin by KLF1. γ-globin has an almost linear relationship with KLF1 up to 50% KLF1; however, at more than 50% KLF1, γ-globin mRNA levels are reduced to normal, indicating the importance of KLF1 in hemoglobin switching. We also confirmed the well-known role of KLF1 in human β-globin gene regulation (Perkins et al., 1995, Zhou et al., 2010). Our large sample size with over 20 biological replicates (umbilical cord blood samples) and a range of KLF1 knockdowns is an excellent model to demonstrate the roles of KLF1 in globin gene regulation.

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Another gene deemed important for globin regulation is Bcl11A. This gene acts like a repressor to γ-globin thus playing a significant role in hemoglobin switching. BCL11A binds to sequences in the locus control region (LCR) and in the γ–δ intergenic region of the human β-globin locus in adult erythroid progenitors to regulate globin gene expression (Zhou D et al. 2010). Also, KLF1 has a strong binding affinity towards the BCL11A promoter (Wilber et al. 2011). Zhou et al. in 2010 showed that downregulation of KLF1 in human *in vitro* differentiated CD34+ peripheral blood leads to a decrease in BCL11A gene expression and in turn an upregulation in γ-globin mRNA levels. The importance of KLF1 in BCL11A gene regulation has been shown through our work. We observed a strong positive correlation between KLF1 and BCL11A expression. Because BCL11A is also involved in γ-globin gene regulation, it along with KLF1 and possibly other factors regulated by KLF1 may be responsible for the complicated γ-globin gene regulation. Finally, we investigated the role of KLF1 in KLF2 gene regulation. Dr. Yousef Alhashem has shown that KLF2 binds to the γ-globin promoter in human definitive cord blood erythroid cells similar to its binding in transgenic mouse primitive erythroid cells. We observed a complex regulation of KLF2 by KLF1 in KLF1 knockdown human CD34+ differentiated erythroid cells. The relationship between the two could not be explained through a regression correlation; however, using a smooth curve we usually observed an increase in KLF2 mRNA levels in KLF1 knockdown cells compared to scramble. We suggest future KLF2 knockdown studies which would help us elucidate its role in γ-globin gene regulation.

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Chapter 4: The Effect of KLF1 and KLF2 on Cell-Cycle and Proliferation in Erythropoiesis.

4.1 Introduction

KLF1 regulates Ey- and βh1-globin in mouse primitive erythropoiesis and human γ-globin and adult mouse and human β-globin gene expression in definitive erythropoiesis. KLF2 regulates Ey- and βh1-globin in mouse primitive erythropoiesis, with an unknown role in adult globin gene expression (Wijgerde M et al. 1996, Cantor A and Orkin SH, 2002, Basu P et al. 2005). Previous laboratory results have analyzed the complementary role of KLF1 and KLF2 in primitive erythropoiesis. It was observed that KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos have a reduced numbers of blood cells and extremely pale phenotypes compared to wild type at E10.5. KLF1-/-KLF2+/- embryos also have reduced numbers of blood cells compared to wild type; however, their phenotype was not pale or anemic at E10.5. The globin content of KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 embryos was significantly different compared to wild type, but not significantly different from each other (Basu P et al. 2007, Divya Vinjamur). In an attempt to explain this phenotype the proliferative effects of KLF1 and KLF2 was assessed through colony forming assays. KLF1 single knockout colonies showed a reduction in size, whereas KLF2 single knockout colonies showed a reduction in number compared to wild type (Divya Vinjamur, PhD thesis). A reduction in size of colonies may be due to reduced proliferation or apoptosis of cells within colonies and a reduction in number of colonies may be attributed to reduced colony forming ability or increased apoptosis.

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Microarray as well as mRNA-seq studies have shown that KLF1 regulates an entire repertoire of genes involved in cell cycle, proliferation, differentiation and apoptosis (Tallack M et al. 2012, Pang C et al. 2012). In fact, studies involving the reintroduction of KLF1 in KLF1-null erythroid cell lines showed enhanced differentiation and hemoglobinization and reduced proliferation indicating the involvement of KLF1 in the regulation of other genes involved in hemoglobin synthesis (Perry C and Soreq H, 2002). Redmond LC et al. (2011) performed microarray studies that showed that KLF2 regulates several genes involved in cell migration, differentiation and development. In this project, we have investigated the regulation of proliferation and cell-cycle genes by KLF1 and KLF2 and their role in the above mentioned mouse phenotypes. Following this, we studied the erythroid colony forming potential of human CD34+ hematopoietic stem cells upon KLF1 knockdown. It was determined that unlike KLF1-/- colonies, KLF1 knockdown colonies exhibit a reduction in number and perhaps in density, but not size of colonies. This led us to follow up on the same cell-cycle and proliferation genes affected by KLF1 and KLF2 in mice and define their regulation by KLF1 in a human *in vitro* model.

4.2 KLF1 and KLF2 modulate the expression of mouse proliferation and cell-cycle genes.

Progressively fewer blood cells are observed in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 embryos compared to wild type with no significant difference in the number of blood cells between KLF1+/-KLF2-/- and KLF1-/-KLF2-/-. Also, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos looked pale and anemic compared to wild type and KLF1-/-KLF2+/-. The globin content of these three genotypes was significantly different compared to wild type; however, they were not significantly different from each other (Divya Vinjamur, unpublished data). Based on previous microarray analyses, it was demonstrated that the expression of five

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proliferation-associated genes, Foxm1 (Forkhead Box Protein M1), Cd24a, Myc, Sphk1 (Sphingosine Kinase 1) and Pthr (Parathyroid Hormone 1 Receptor), is progressively reduced in wild-type, KLF1-/- and KLF1-/-KLF2-/- E10.5 blood cells (Pang C et al. 2012). In order to validate the microarray results, qRT-PCR was performed to assess the expression of these genes in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- compared to wild type and KLF1+/- blood cells. The objective of this experiment was to determine if there were trends in expression of these genes that were similar to the trend observed in the number of blood cells. The amounts of all five mRNAs are significantly reduced in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- blood cells compared to wild-type, verifying the microarray analyses (Figure 4.1A). We also wanted to determine whether haploinsufficiency of KLF1 may change regulation of these genes. Gene expression in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/- KLF2-/- blood cells was also significantly reduced compared to KLF1+/- blood cells for all of the mRNAs with the exception of Pthr. Pthr mRNA in KLF1-/- was not significantly different even from wild type due to its high standard deviation. On the other hand, Cd24a KLF1+/- mRNA proved to be significantly different from wild type indicating the importance of haploinsufficiency in the regulation of this gene. Foxm1 mRNA is the only mRNA that is significantly reduced in KLF1+/-KLF2-/- and KLF1-/-KLF2-/- compared to KLF1-/-KLF2+/- (Figure 4.1A), specifically correlating with the anemia phenotype and reduced peripheral blood cell number. Sphk1 mRNA amounts are significantly lower in KLF1-/-KLF2-/- than in KLF1-/-KLF2+/-, but not in KLF1+/-KLF2-/- compared to KLF1-/-KLF2+/- (Figure 4.1A). Myc mRNA amounts are not significantly different between KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/-. Pthr and Cd24a mRNA amounts are reduced in KLF1-/-KLF2+/- compared to KLF1+/-KLF2-/-, negatively correlating with anemia phenotype (Figure 4.1A).

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Reduced expression of any or all of these 5 genes may contribute to the decreased number of erythroid cells in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- embryos compared to wild-type. However, FoxM1 is the only gene that correlates with the E10.5 anemia phenotype, having lower expression in KLF1+/-KLF2-/- and KLF1-/-KLF2-/- than in KLF1-/-KLF2+/- blood cells. We next followed up on these results by performing ChIP analysis to determine whether KLF1 and KLF2 might directly regulate FoxM1. Three consensus binding sites for KLF1 and KLF2 were detected in the region 1000 bp upstream of the FoxM1 start site (Figure 4.1B). Primers were designed encompassing two of these sites and within 300 bp of the third site. The site at -309 bp matched all nine nucleotides of the consensus sequence. However, the site at -359 bp matched only six and the site at -626 bp matched seven of the nine nucleotides in the KLF binding consensus sequence. On carrying out ChIP, we observed a 2.5 fold increase in the binding of KLF1 to the FoxM1 promoter compared to IgG (control); however, we were unable to prove the binding of KLF2 to FoxM1 (Figure 4.1C). The binding of KLF2 to FoxM1 was significant at -309 bp and -359 bp; however, these results were not considered relevant due to a higher binding of KLF2 to necdin which was a negative control to which KLF1 and KLF2 presumably does not bind. KLF2 might be binding to the consensus site 626bp upstream of the start site which our primers may or may not have covered.

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Figure 4.1.A

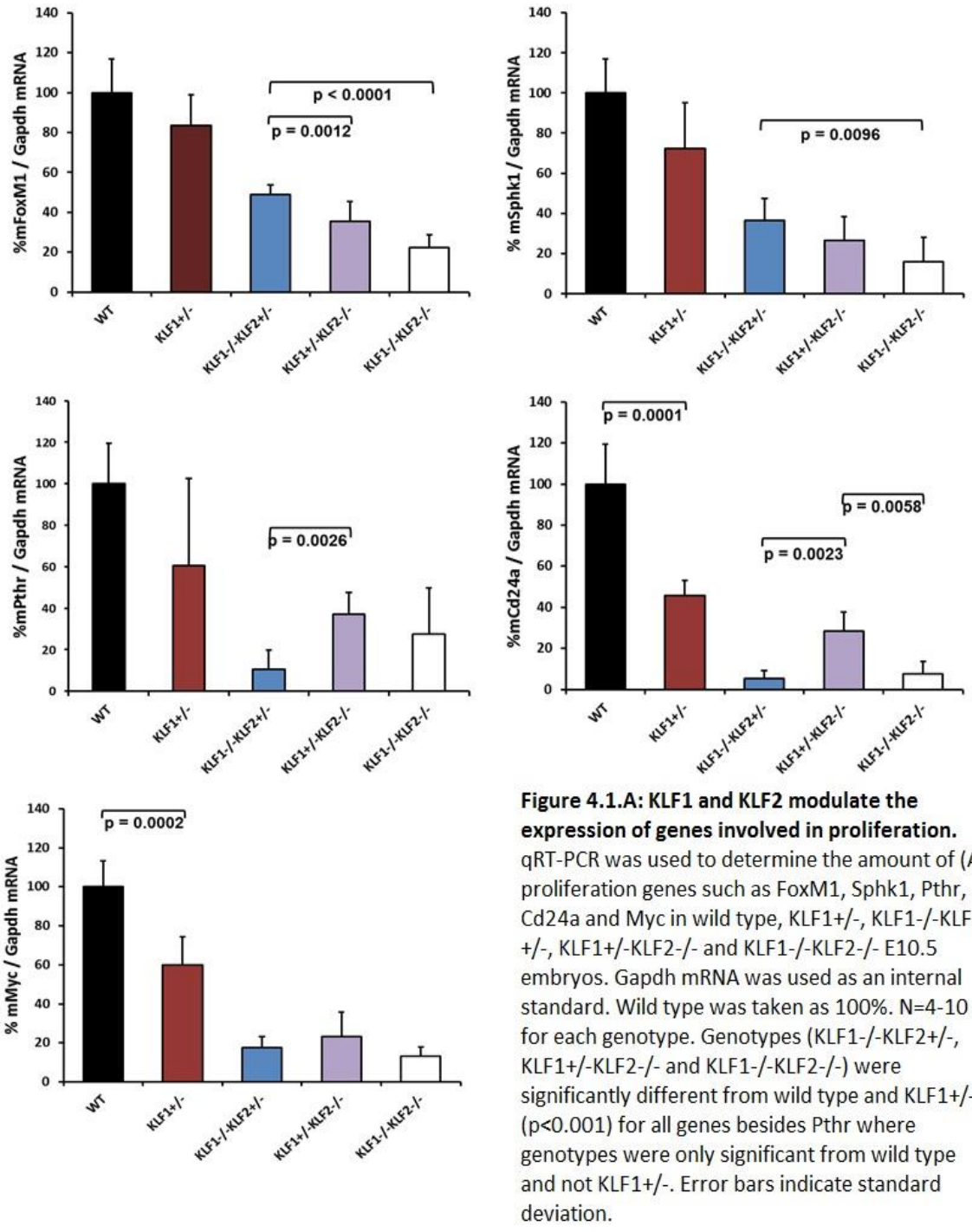

Figure 4.1.A: KLF1 and KLF2 modulate the expression of genes involved in proliferation. qRT-PCR was used to determine the amount of (A) proliferation genes such as FoxM1, Sphk1, Pthr, Cd24a and Myc in wild type, KLF1+/-, KLF1-/-KLF2 +/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 embryos. Gapdh mRNA was used as an internal standard. Wild type was taken as 100%. N=4-10 for each genotype. Genotypes (KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/-) were significantly different from wild type and KLF1+/- ($p<0.001$) for all genes besides Pthr where genotypes were only significant from wild type and not KLF1+/-. Error bars indicate standard deviation.

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Figure 4.1.B

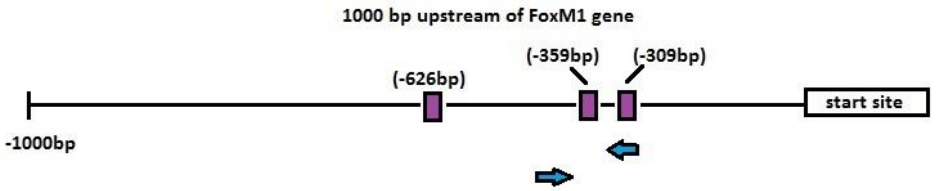

Figure 4.1.C

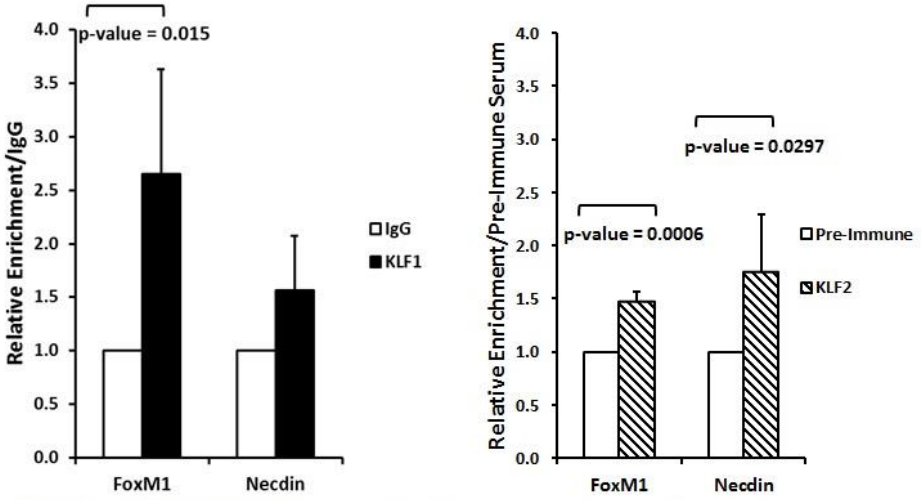

Figure 4.1 (B and C): KLF1 binds the mouse FoxM1 loci in primitive erythroid cells.
ChIP assays were performed on primitive erythroid cells of wild type mouse using polyclonal antibodies against KLF1, KLF2 and non-specific IgG (control). (A) A representation of the regulatory region 1000bp upstream of the FoxM1 start site. Three KLF1 and KLF2 binding consensus sites (CGAGTGGGT at -309bp, AGGCACCCC at -359bp and GGCCACCCA at -626bp) were observed within this 1000bp domain and are marked in purple. The primers specific to FoxM1 are also shown using arrows. (B) Graphical representation of the KLF1 and KLF2 ChIP data. The Y-axis represents the relative fold enrichment. The mean IgG enrichment was set at 1.0 and the enrichment of KLF1 and KLF2 was scaled appropriately. Primers specific to Necdin were used as a negative control. N=4 for the KLF1 CHIP and N=3 for the KLF2 CHIP. Error bars indicate standard deviation.

We also investigated the expression patterns of certain cell cycle regulators such as E2F2, E2F4, p18 and p27 to determine whether phenotypic differences between embryos are associated with differential regulation of cell cycle-related genes. The amount of E2F2, E2F4 and p18 mRNA was significantly reduced in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- compared to wild type as well as KLF1+/-. There were no significant differences in p27 mRNA expression

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between the four genotypes (Figure 4.2). However, none of these genes show significantly different expression between KLF1+/-KLF2-/- and KLF1-/-KLF2+/- blood cells, so there is no correlation with the relatively reduced number of blood cells in KLF1+/-KLF2-/- and KLF1-/-KLF2-/-. Another interesting observation was that E2F4 showed modest yet significant reduction in KLF1+/- compared to wild type. This shows that haploinsufficiency also causes changes in regulation of this cell-cycle gene.

Figure 4.2

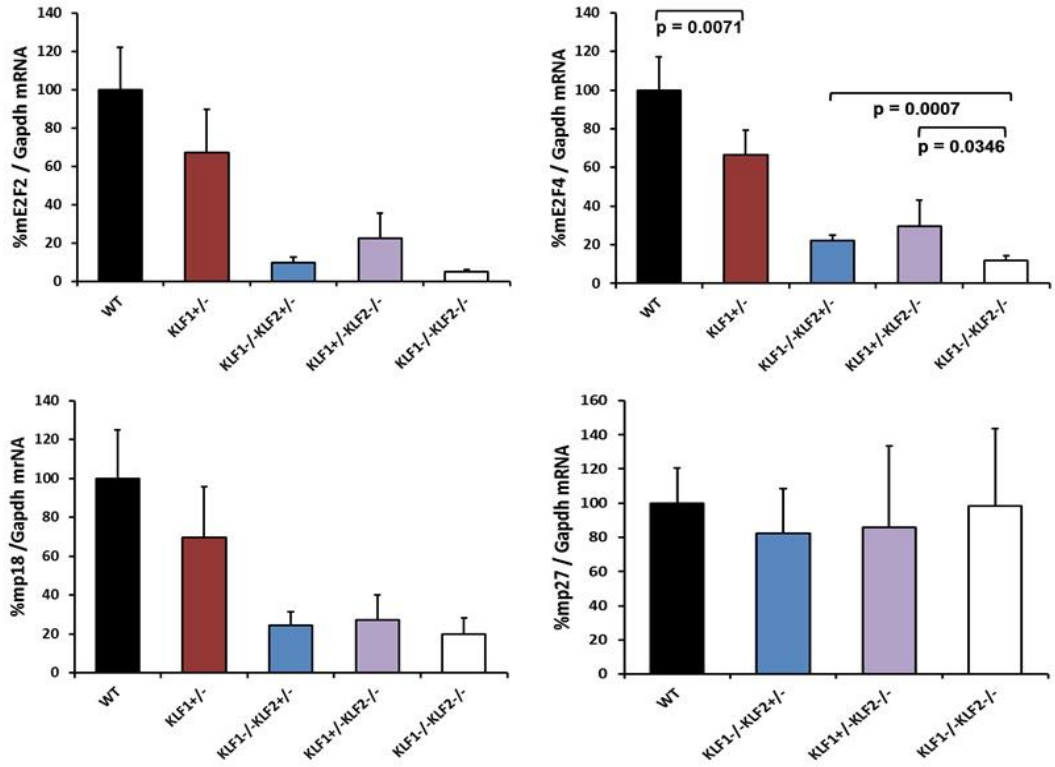

Figure 4.2: KLF1 and KLF2 modulate the expression of genes involved in cell cycle.
qRT-PCR was used to determine the amount of cell cycle genes such as E2F2, E2F4, p18 and p27 in wild type, KLF1+/-, KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 embryos. Gapdh mRNA was used as an internal standard. Wild type was taken as 100%. N=4-10 for each genotype. Genotypes (KLF1-/-KLF2 +/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/-) were significantly different from wild type and KLF1+/- ($p<0.001$) for all cell cycle genes. Error bars indicate standard deviation.

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4.3 KLF1 knockdown human CD34+ cells express reduced potential of colony formation.

To investigate the importance of human KLF1 in colony forming potential, we performed a colony forming assay in KLF1 knockdown CD34+ cells. Cord blood derived CD34+ cells were infected with lentivirus containing either KLF1-shRNA or Scramble-shRNA. The infected cells were flow sorted to retrieve those that express the GFP gene, which is adjacent to the shRNA in the lentiviral genome. A constant number of cells containing either KLF1-shRNA or Scramble-shRNA were then plated in a semisolid methylcellulose medium containing the appropriate cytokines for the formation of erythroid colonies. Approximately 1-2 million cells were differentiated for eight days in a liquid medium, followed by qRT-PCR to determine the amount of KLF1 knockdown (Figure 4.4B).

Burst Forming Unit-Erythroid (BFU-Es) colonies were observed and counted on day 14 of growth. Representative colonies are presented in Figure 4.3. A within patient analysis showed a reduction in the number of colonies in KLF1 knockdown CD34+ cells compared to Scramble CD34+ cells (Figure 4.4A and 4.4C). Also, the mock control had a greater number of colonies compared to scramble; however, this did not prove to be statistically significant (p-value=0.1116) (Figure 4.4A). These results are different compared to the colony forming assays carried out in mice using KLF1-/- cells. A reduction in the size but not number of colonies was observed in mouse KLF1-/- compared to wild type. To determine whether these KLF1 knockdown colonies exhibit a difference in size, we calculated the area of KLF1 knockdown colonies compared to scramble colonies. No significant reduction in size was observed as shown in Figure 4.5. However, the KLF1 knockdown colonies generally looked less stained compared to scramble colonies suggesting that they may contain less cells in their colonies or less hemoglobinized cells or less hemoglobin per cell in comparison to scramble (Figure 4.6).

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These results indicate a role for KLF1 in erythroid colony forming potential. Loss of KLF1 may lead to increased apoptosis of cells present in the colonies due to which fewer colonies are observed on day 14. Another possibility is that loss of KLF1 might lead to the inability of CD34+ hematopoietic stem cells to proliferate.

Figure 4.3

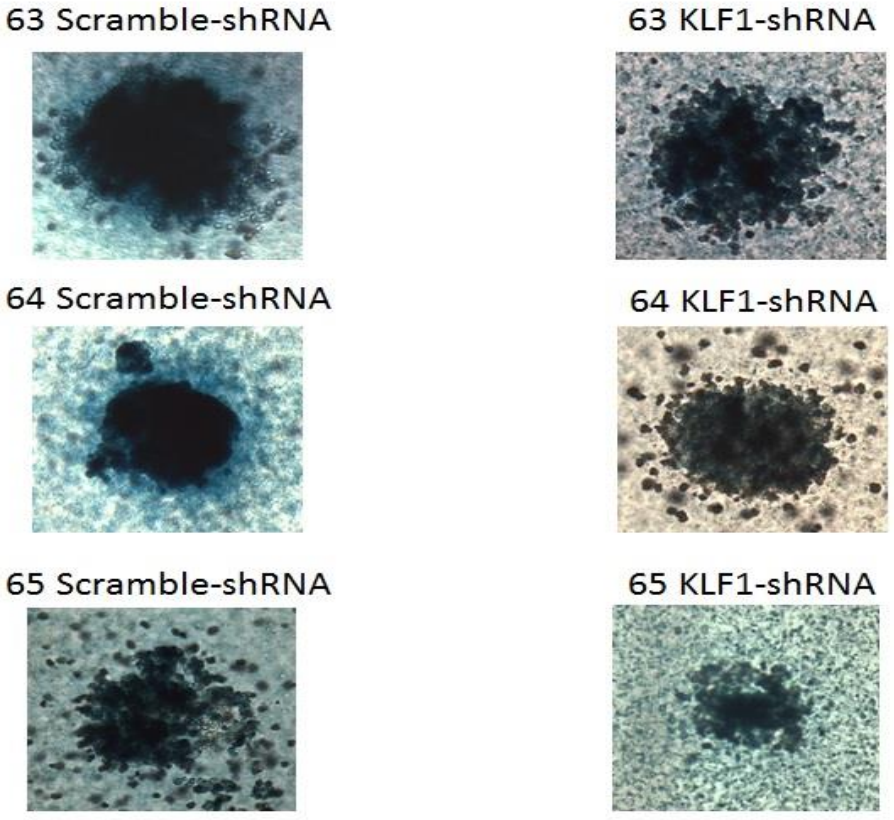

Fig 4.3: Representative Colonies Observed in the Colony forming Assay. Human CD34+ hematopoietic stem cells were infected with lentivirus containing either KLF1-shRNA or Scramble-shRNA. The infected cells were flow sorted depending upon their GFP intensity. The cells were then plated in a semisolid methycellulose medium. Colonies were observed on day 14 of growth. This figure represents the colonies observed in the three KLF1 knockdown samples and their representative scramble controls.

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Figure 4.4

A. Number of Colonies

Sample ID	Mock	Scramble-shRNA	KLF1-shRNA	% Number of colonies in KLF1 knockdown
63	190	119	63	53
64	267	225	141	63
65	239	98	48	49

B. Amount of KLF1 knockdown in sample

Sample ID	Scramble-shRNA	KLF1-shRNA
63	100	78
64	100	82
65	100	69

C.

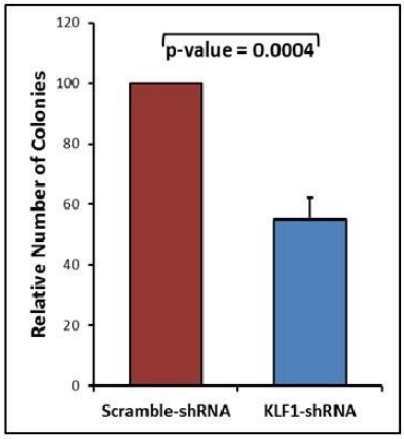

Figure 4.4: Colony number pattern on plating cord blood derived CD34+ cells.
Burst Forming Unit-Erythroid (BFU-E's) colonies were observed and counted on day 14 of growth. A within patient analysis showed a reduction in the number of colonies in KLF1 knockdown CD34+ cells compared to Scramble CD34+ cells. (A) Raw data for the number of colonies observed in the three KLF1 knockdown samples compared to their respective scramble controls. (B) Amount of KLF1 knockdown observed. (C) Graphical representation of the number of colonies in KLF1 knockdown compared to Scramble. Scramble was taken as 100 colonies. N=3. Error bars indicate standard deviation.

Figure 4.5

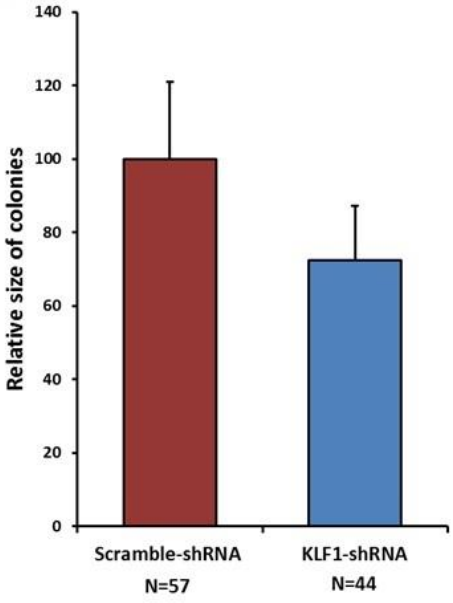

Figure 4.5: Colony size pattern on plating cord blood derived CD34+ cells.
The area of 57 Scramble colonies and 44 KLF1 knockdown colonies was measured using ImageJ software. This figure is a graphical representation of the size of various KLF1 colonies compared to scramble. The area of scramble colonies was taken as 100. Error bars indicate standard deviation. There was no statistically significant difference in size.
Average size of KLF1 knockdown colonies= 19,980µm2
Average size of Scramble colonies= 27,800µm2

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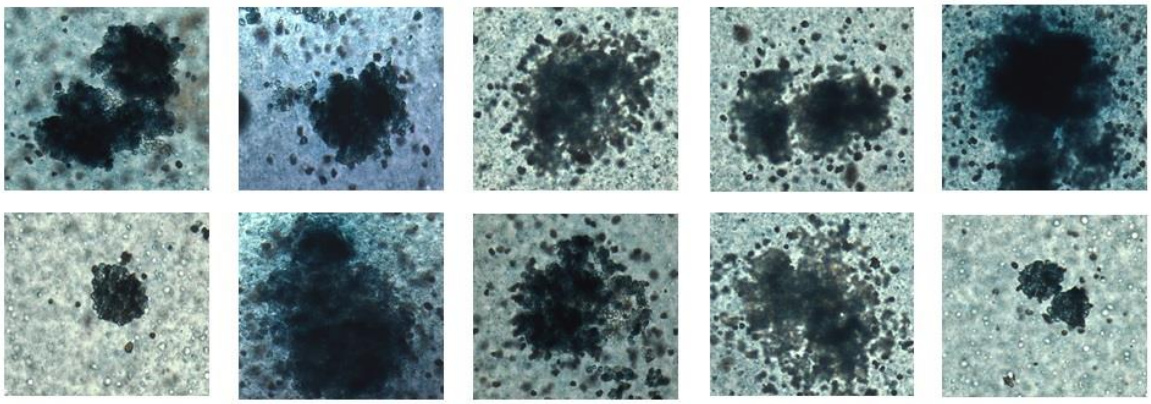
Figure 4.6

A. Scramble Colonies



B. KLF1 Knockdown Colonies

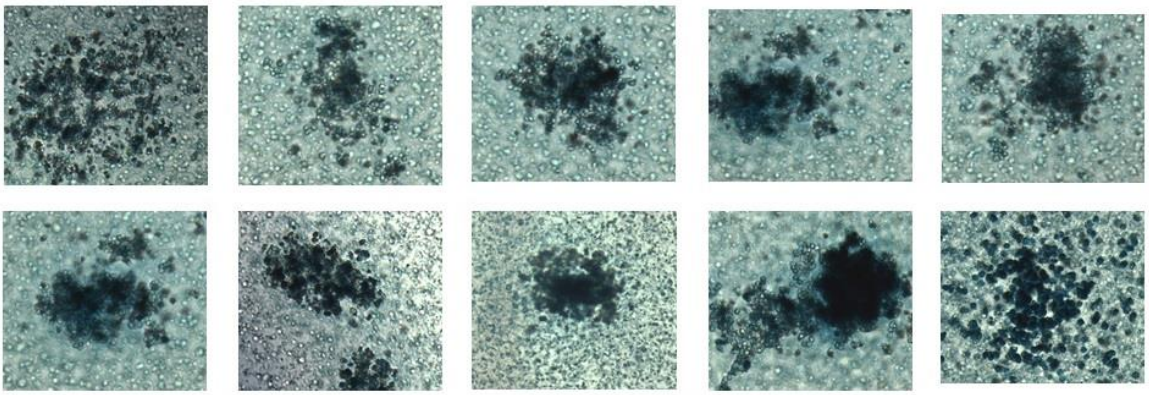


Figure 4.6: KLF1 knockdown colonies are less compact compared to scramble colonies.
The compactness of the colonies was visually observed between KLF1 knockdown and scramble. It was observed that KLF1 colonies seem less dense/compact compared to scramble. (A) Ten random pictures of Scramble colonies. (B) Ten random pictures of KLF1 knockdown colonies. The pictures were taken at 100 X magnification and are in the same scale.

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4.4 KLF1 regulates the expression of human proliferation and cell-cycle genes.

The human colony forming assay demonstrated a reduction in the number of colonies in KLF1 knockdown CD34+ cord blood cells which may be due to reduced proliferation. The mouse microarray analyses (Pang C et al.2012) led us to determine the expression patterns of proliferation genes such as FOXM1, SPHK1, Myc, CD24 and PTHR in KLF1 knockdown human CD34+ cord blood cells. These microarray assays were performed on E10.5 primitive mouse blood cells with complete knockout of KLF1; whereas, the CD34+ blood cells are definitive in nature and have around 20% KLF1 remaining after knockdown. Even then we expected to observe some evidence of KLF1 in regulation of these genes. qRT-PCR was performed to assess the expression patterns of these genes in KLF1 knockdown cells compared to scramble. The mRNA levels of FOXM1 were significantly downregulated by ~2 fold in KLF1 knockdown cells compared to scramble. On the other hand, unlike the mouse data SPHK1 was significantly upregulated by 2-fold in KLF1 knockdown cells compared to scramble. No change was observed in the expression levels of CD24 and Myc mRNA (Figure 4.7A). The mRNA levels of Pthr were not quantified in this human *in vitro* model because this gene is expressed at a low level, and there was not enough cDNA available for qRT-PCR.

The expression patterns of cell cycle regulators such as E2F2, E2F4 and p18 were also investigated. We observed a 4-fold reduction in E2F2 and a 2-fold reduction in E2F4 mRNA amounts in KLF1 knockdown CD34+ cord blood cells compared to scramble. No change was observed in p18 mRNA amounts (Figure 4.7B). KLF1 through its regulation of proliferation genes such as FOXM1 and cell cycle regulators such as E2F2 and E2F4 may be responsible for reduced proliferation in erythropoiesis. This could be one of the reasons that a reduction in the number of colonies in KLF1 knockdown cells compared to scramble is observed.

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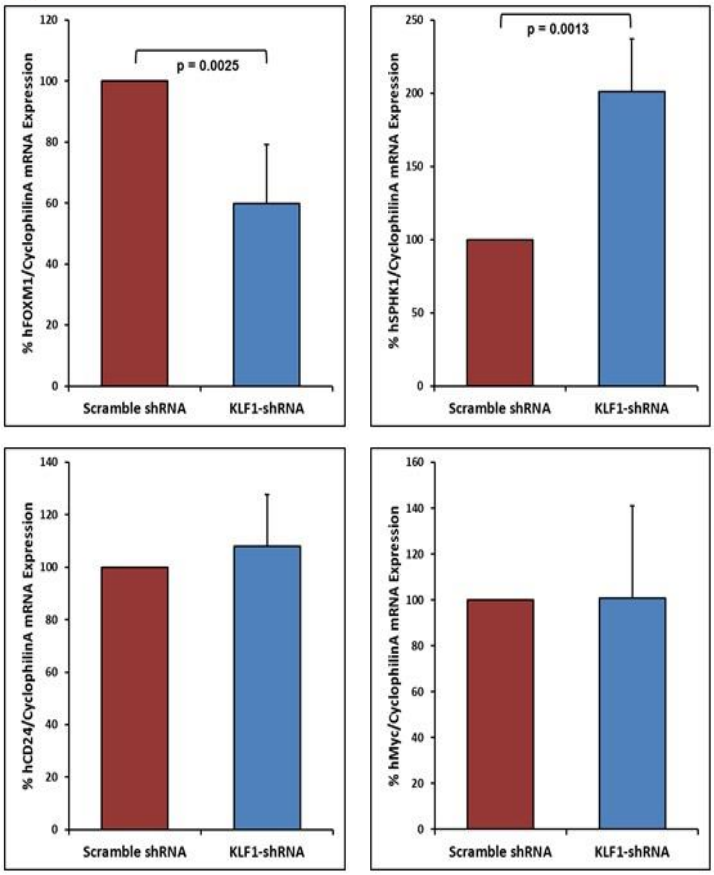
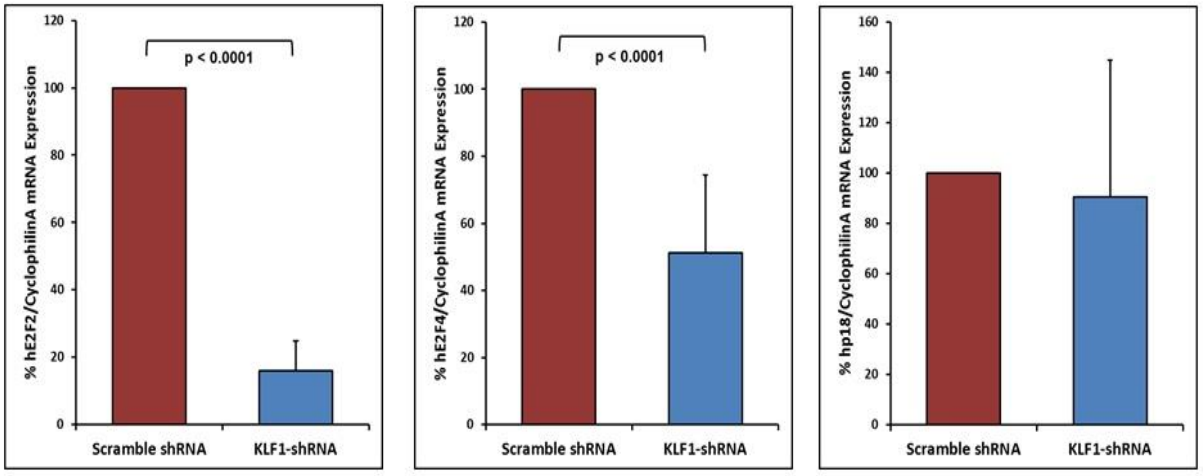
Figure 4.7 A.



4.7: KLF1 regulates human proliferation and cell cycle genes. qRT-PCR was performed to determine the role of KLF1 in regulation of proliferation and cell cycle genes. (A) The percentage mRNA levels of four proliferation genes: FOXM1, SPHK1, Myc, CD24 and (B) three cell-cycle genes: E2F2, E2F4 and p18 was quantified in KLF1 knockdown cells compared to scramble. Cyclophillin A was used as an internal standard. Scramble was taken as 100%. N=4-5. Error bars indicate standard deviation.

B.



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These results were followed by performing ChIP analysis to determine whether KLF1 binds to FOXM1, E2F2 and E2F4. Two consensus binding sites for KLF1 were detected in the region 1000 bp upstream of the FOXM1 start site; four were observed 1000 bp upstream of the E2F2 start site; and four were observed 1000 bp upstream of the E2F4 start site (Figure 4.8). The sequences of these sites are given in Table 4.1. Primers were designed encompassing these sites. On carrying out ChIP for FOXM1, we observed a 3-fold enrichment in the binding of KLF1 compared to IgG to the FOXM1 promoter using Primer Set 1 (FOXM1 (1) in Figure 4.9). The second primer set for FOXM1 showed a 5-fold enrichment in the binding of KLF1; however, the large standard deviation did not allow a significant result even though the range of binding for the three samples was from 3-fold to 7-fold (FOXM1 (2) in Figure 4.9). ChIP analysis for E2F2 showed ~4-fold enrichment in binding of KLF1 to the E2F2 promoter at two primer sets (E2F2 (1) and E2F2 (2) in Figure 4.9). On performing ChIP analysis for E2F4, an ~2.5-fold enrichment in binding of KLF1 to the E2F4 promoter was observed (E2F4 (2) in Figure 4.9). Some of the qRT-PCR assays used for these ChIP analyses gave high cycle numbers. High cycle numbers might indicate a possibility of errors within the data set. A range of cycle numbers for the various primer sets is provided in Table 4.2. These results show that KLF1 binds FOXM1, E2F2 and E2F4.

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Gene	Sequence	Position and Primer Set no.	Match to the KLF1 Consensus Sequence
FOXM1	GCCCACCCA	-215 (P2)	9/9
	ATCCACCCG	-801 (P1)	7/9
E2F2	3’ GAGGTGGGG 5’	-128 (P3)	8/9
	AACCACCCT	-274 (P3)	8/9
	CCTCACCCC	-528 (P2)	7/9
	3’ GCAGTGGGT 5’	-862 (P1)	8/9
E2F4	AGACACCCA	-93 (P2)	7/9
	AGTCACCCG	-214 (P2)	6/9
	3’ CCAGTGGGG 5’	-461 (P1)	7/9
	3’ AAGGTGGGT 5’	-536 (P1)	8/9

Table 4.1: KLF1 binding sites observed in FOXM1, E2F2 and E2F4.

	Input (Cycle Range)	IgG (Cycle Range)	KLF1 (Cycle Range)
FOXM1 (1)	16-17	25-28	24-27
FOXM1 (2)	23-25	**33-37**	30-35
E2F2 (1)	22-24	31-34	29-32
E2F2 (3)	21-23	31-34	27-33
E2F2 (2)	21-23	30-32	28-31
E2F4 (1)	23-25	31-32	30-32
E2F4 (2)	18-19	27-28	26-27

Table 4.2: Range of cycle numbers in qRT-PCR for the various primer sets.

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Figure 4.8 A

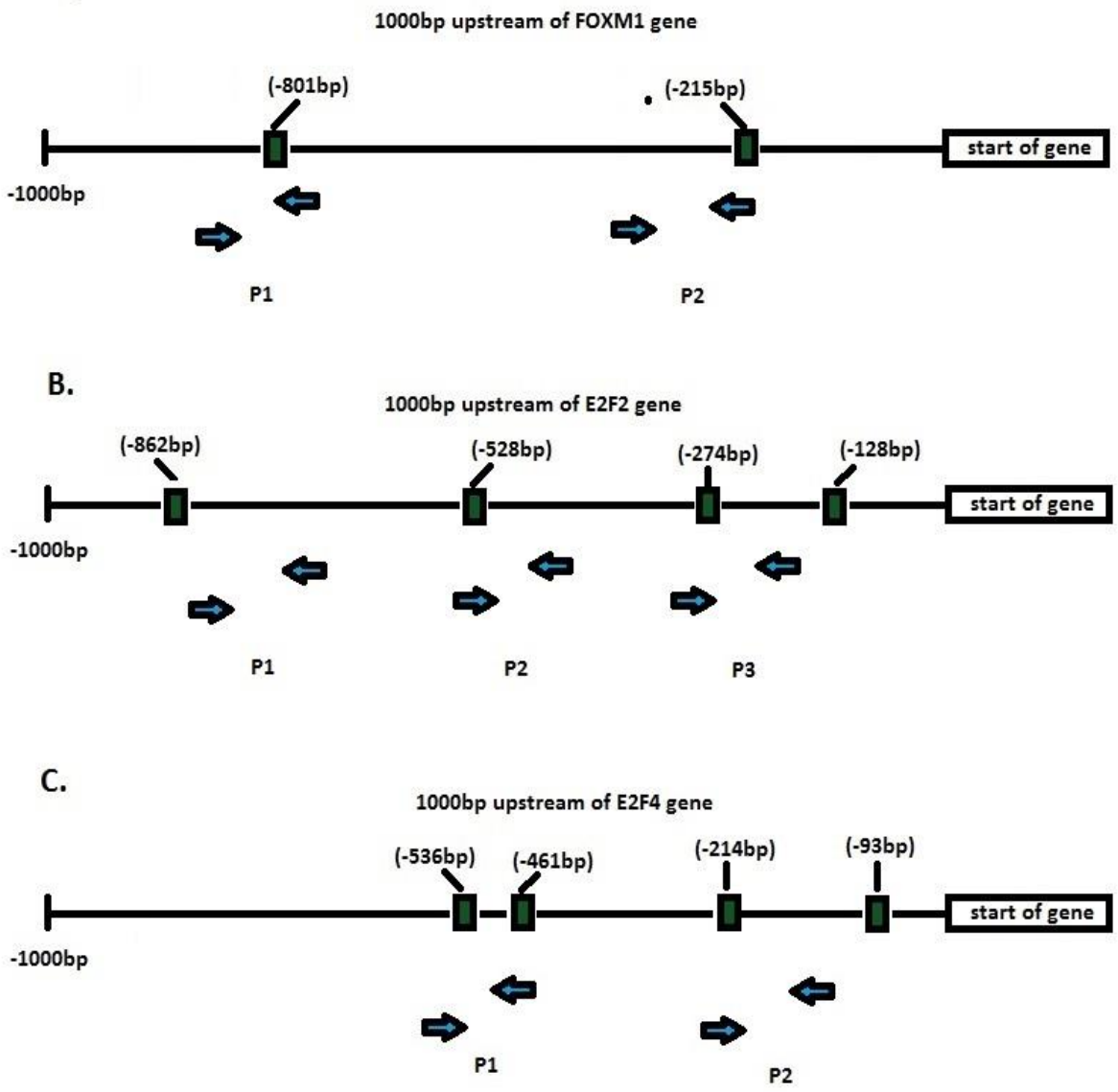

Figure 4.8: Promoters of human FOXM1, E2F2 and E2F4 genes.
ChIP assays were performed on CD34+ cord blood derived erythroid cells using a polyclonal antibody against KLF1 and non-specific IgG (control). A representation of the regulatory region 1000bp upstream of the (A) FOXM1 (B) E2F2 and (C) E2F4 start site has been shown. The KLF1 binding consensus sites were observed within this 1000bp domain and are marked in green. Primers sets to specific regions are also shown using arrows.

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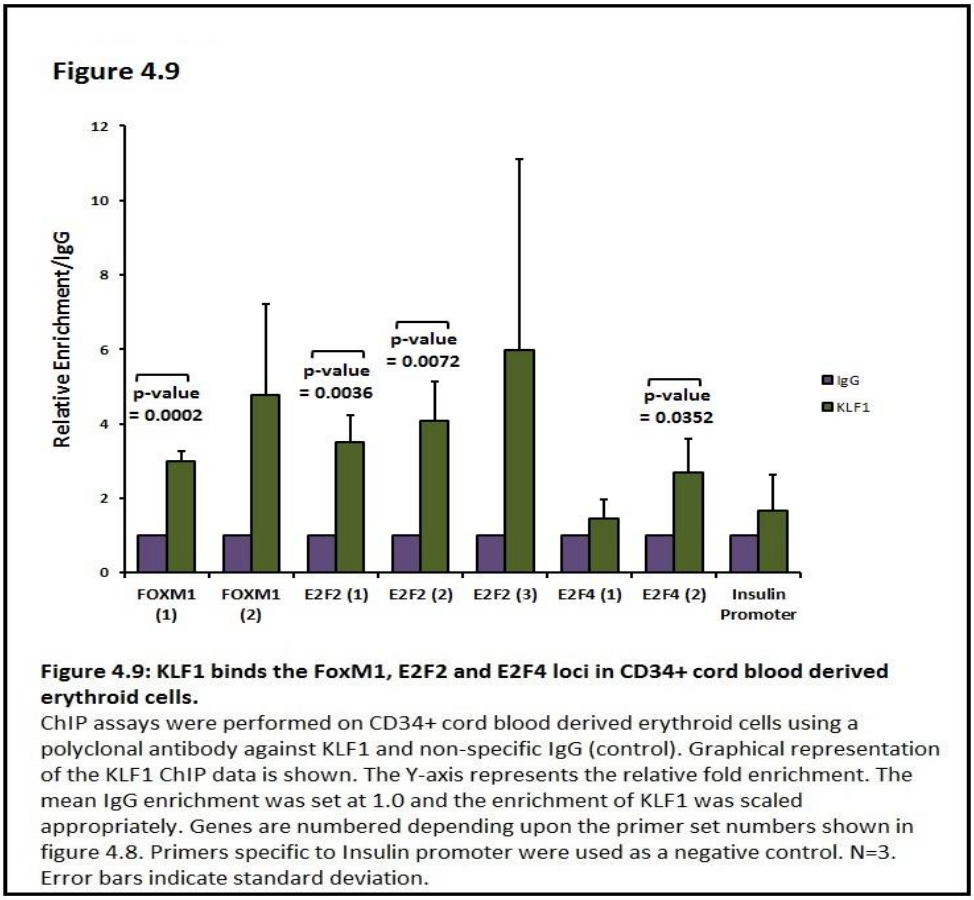

Figure 4.9: KLF1 binds the FoxM1, E2F2 and E2F4 loci in CD34+ cord blood derived erythroid cells.
ChIP assays were performed on CD34+ cord blood derived erythroid cells using a polyclonal antibody against KLF1 and non-specific IgG (control). Graphical representation of the KLF1 ChIP data is shown. The Y-axis represents the relative fold enrichment. The mean IgG enrichment was set at 1.0 and the enrichment of KLF1 was scaled appropriately. Genes are numbered depending upon the primer set numbers shown in figure 4.8. Primers specific to Insulin promoter were used as a negative control. N=3. Error bars indicate standard deviation.

4.5 Discussion

Our work has demonstrated that erythroid colonies derived from definitive CD34+ hematopoietic stem cells are reduced in number in KLF1 knockdown compared to scramble. However, these KLF1 knockdown colonies look less stained suggesting that they might contain fewer cells or fewer hemoglobinized cells or less hemoglobin per cell compared to scramble. Previous studies in our laboratory of primitive erythroid progenitors produced colonies of the same frequency but less hemoglobinized and of a smaller size from E8.5 KLF1-/- embryos compared to wild type (Divya Vinjamur, unpublished data). These disparate results may be due to the difference in origin of the colony precursors. The colony progenitors in the mouse model were primitive in nature and true progenitors. The colony precursors in the human CD34+ model were definitive and further differentiated from the progenitor stage. Also, EryP-CFC's were counted in the

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mouse colony forming assay (Palis J, 2008); whereas BFU-Es were counted in the human assay. The reason for this change is that CFU-Es are a rarity in the human colony forming assay; whereas BFU-Es are seen in abundance. Even though the human KLF1 knockdown colonies did not show a significant change in size, they seemed to contain fewer cells compared to scramble which would be similar to the results observed in the mouse model. The number of cells per colony in the human KLF1 knockdown colonies could be counted and compared to scramble in the future to test this hypothesis. Pilon et. al (2008) previously performed colony forming assays using mouse E13.5 KLF1-/- and wild type fetal livers. They counted the number of BFU-E colonies on day 10-12 postplating and observed no significant difference between the number of KLF1-/- colonies compared to wild type. On the other hand, an increase in the number of CFU-Es in KLF1-/- was observed compared to wild type embryos, which was attributed to a block in erythropoiesis. In addition, the BFU-E and CFU-E colonies from KLF1-/- embryos contained less hemoglobin and required 28-48 hours longer to reach the same size as wild-type BFU-Es and CFU-Es, suggesting a defect in proliferation (Pilon et al. 2008) similar to our findings for mouse embryonic progenitors. This suggests that KLF1 plays a role in proliferation in both mouse primitive as well as definitive erythropoiesis. However, in human definitive erythropoiesis, KLF1 may have a role in limiting the colony forming ability of CD34+ cells through reduced proliferation and may also be reducing the number of colonies through apoptosis. Tallack et al. (2012) have shown some evidence of a modest increase in apoptosis of KLF1-/- definitive erythroid cells. Another interesting project for the future would be to determine the effect of KLF2 on the colony forming ability of human CD34+ hematopoietic stem cells. Mouse primitive E8.5 KLF2-/- have reduced number of EryP-CFC colonies compared to

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wild type. We are currently establishing a model for KLF2 knockdown studies in our laboratory and hope to soon perform human colony forming assays using KLF2 knockdown CD34+ cells.

The importance of mouse and human KLF1 in the regulation of several proliferation and cell cycle genes has also been demonstrated. The transcription factor FoxM1 is known to stimulate proliferation by promoting S-phase and M-phase entry in the cell cycle. It is also known to activate all five Cdk/Cyclin complexes showing its importance in cell cycle progression. Previous work by other laboratories has shown that Foxm1 complete knockout mice are embryonically lethal and die between E13.5 and E16.5 (Inken Wierstra 2013). The gene expression results in E10.5 mouse embryos shows that FoxM1 mRNA is present in lower amounts in KLF1+/-KLF2-/- than in KLF1-/-KLF2+/- embryos, suggesting that it is more responsive to KLF2 than KLF1. This trend correlates with the anemia phenotype observed in KLF1+/-KLF2-/- embryos compared to wild type and KLF1-/-KLF2+/- embryos at E10.5. ChIP assays also demonstrated the direct binding of KLF1 to the FoxM1 promoter; however, we could not demonstrate the binding of KLF2. Along with FoxM1, several other proliferation genes such as Pthr, Cd24a, Sphk1 and myc were also reduced in KLF1-/-KLF2+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- genotypes; however, they did not correlate with the anemia phenotype. We speculate that the small changes in FoxM1 gene expression could have large effects in combination with the other proliferation genes such as Cd24a, Pthr, Sphk1 and Myc which are dysregulated by KLF1. These proliferation genes were also tested in the human KLF1 knockdown model. We observed a downregulation in the FOXM1 mRNA levels and an upregulation in SPHK1 mRNA levels in KLF1 knockdown cells compared to scramble. Myc and CD24 were not significantly regulated by KLF1 in this system. Also, we demonstrated direct binding of KLF1 to the FOXM1 promoter. A direct comparison between the two systems cannot

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be made because the mouse model is primitive in nature; whereas, the human model uses definitive erythroid cells. Also, the mouse model has one copy of KLF1 (50% knockout) or no KLF1 (100% knockout). On the other hand; the human CD34+ model has varying amounts of KLF1 with an average of 20% remaining after knockdown.

KLF1 affects G1 to S-phase cell-cycle progression during primitive and definitive erythropoiesis (Tallack M et al. 2007 and 2009). The same group has also proved the direct regulation of E2F2, E2F4 and p18 by KLF1. To determine whether both KLF1 and KLF2 are important for cell cycle progression, our laboratory performed cell cycle profiles of E9.5 erythroblasts in KLF1-/-KLF2-/-, KLF1-/-, KLF2-/- and wild type littermates. KLF1-/- and KLF1-/-KLF2-/- erythroblasts displayed aberrant G1- to S-phase cell cycle progression; whereas, KLF2-/- cell cycle profiles were similar to wild type (Kristen Wade, Divya Vinjamur, unpublished data). The data suggested that KLF1 has a unique role in cell-cycle progression which is not shared by KLF2. Furthermore, to determine whether an aberrant cell cycle may be responsible for the gross phenotype in KLF1+/-KLF2-/- and KLF1-/-KLF2-/- E10.5 embryos compared to KLF1-/-KLF2+/- and wild type, we investigated the expression of certain genes important for cell cycle regulation. Downregulation of E2F2, E2F4 and p18 was observed in KLF1-/-KLF+/-, KLF1+/-KLF2-/- and KLF1-/-KLF2-/- compared to wild type. However, their expression amounts did not correlate with the gross phenotypic differences in the embryos. The expression amounts of E2F2, E2F4 and p18 mRNA were also determined in the human CD34+ cord blood model. We observed direct regulation by KLF1 of E2F2 and E2F4. p18 was not significantly regulated by KLF1 in KLF1 knockdown cells compared to scramble. These three genes are all downregulated in E14.5 KLF1-/- mouse fetal liver compared to wild type (Tallack M et al. 2007 and 2009). A 100% knockdown of KLF1 might regulate p18 in a similar manner to KLF1-/- mouse fetal liver.

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CHAPTER FIVE: DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion

Erythropoiesis is a complex process that occurs at several distinct spatiotemporal locations in the developing embryo and can be broadly divided into two stages: 1) primitive erythropoiesis which originates from the yolk sac and 2) definitive erythropoiesis that occurs first in the fetal liver, then spleen and finally bone marrow (McGrath K, Palis J. 2008, Palis et al. 2008, Wilkinson et al. 2013). A complex network of transcription factors is required to regulate the genes and signals involved in erythropoiesis. These erythroid transcription factors were mainly recognized through their binding to specific DNA motifs present in the promoters, LCR and enhancers of β-globin genes (Tsiftsoglou A et al. 2009). One of the important transcription factors that recognize the promoters of β-globin genes is Krüppel-like factor 1 (KLF1). KLF1 is an erythroid-specific transcription factor that belongs to a family of 17 proteins (KLFs) whose main function is gene regulation by binding to DNA elements in the promoters of various genes.

Our previous work on KLF1 in the mouse model and our current work in the human CD34+ cord blood model illuminate the similarities and differences between the two systems. We have defined the role of KLF1 in regulation of important genes which are necessary for erythropoiesis in the human CD34+ *in vitro* differentiated erythroid cells. Before comparing the two models we need to understand the basic differences between the two. Our laboratory work in the mouse model was entirely based on primitive erythropoiesis. However, our work in the human CD34+ model was based on definitive erythropoiesis. Another difference between the two models is the stage at which KLF1 was knocked out/down. KLF1 is completely knocked out in the mouse

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model from the one-cell stage of development. On the other hand, we induce KLF1 knockdown in CD34+ cells which are already committed hematopoietic precursors and cannot differentiate into other lineages. The knockdown achieved through our KLF1 shRNA-mediated knockdown is on an average 80% as opposed to the complete knockout of KLF1 in the mouse model. Based on our results and previous work performed by our laboratory and various other research groups, we summarize the role of KLF1 in mouse and human erythropoiesis (Figure 5.1).

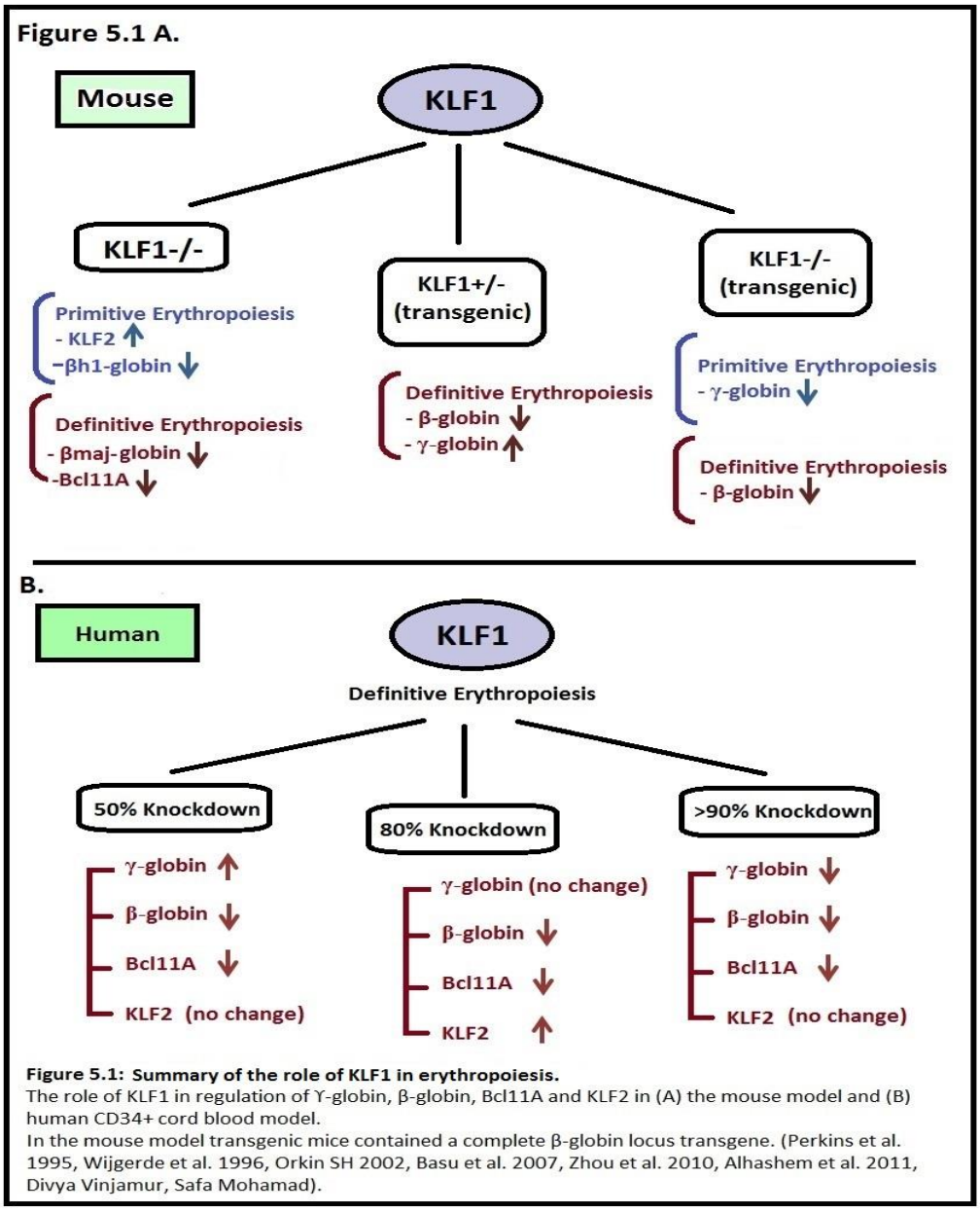

Figure 5.1: Summary of the role of KLF1 in erythropoiesis.
The role of KLF1 in regulation of γ-globin, β-globin, Bcl11A and KLF2 in (A) the mouse model and (B) human CD34+ cord blood model.
In the mouse model transgenic mice contained a complete β-globin locus transgene. (Perkins et al. 1995, Wijgerde et al. 1996, Orkin SH 2002, Basu et al. 2007, Zhou et al. 2010, Alhashem et al. 2011, Divya Vinjamur, Safa Mohamad).

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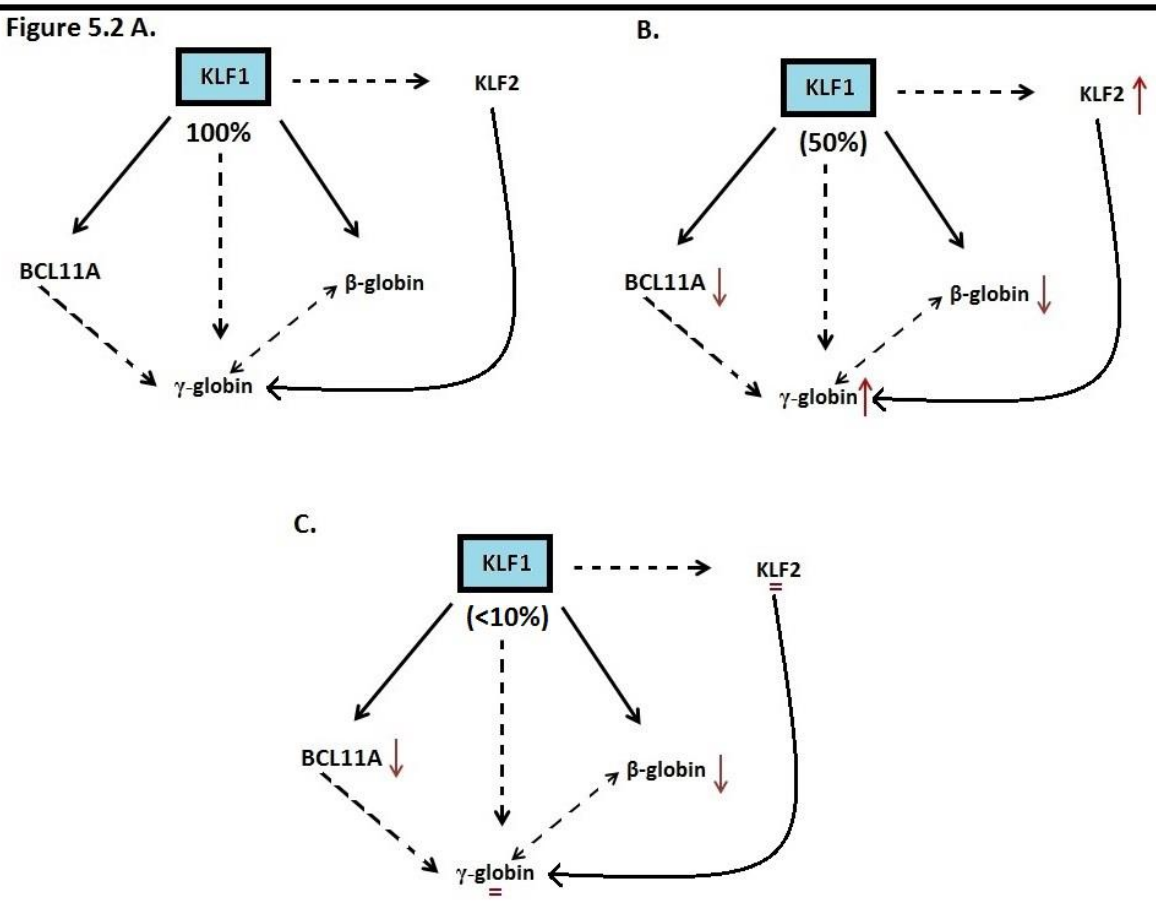

Figure 5.2 Models depicting the role of KLF1 in erythropoiesis.
A model was designed representing the role of KLF1 based on our knockdown studies in the human CD34+ model. The role of KLF1 was determined when it did not undergo knockdown (100% KLF1 mRNA was present (A), underwent ~50% knockdown (B) and > 90% knockdown leaving only <10% KLF1 mRNA (C). The increase (↑), decrease (↓) or no change (=) in the expression levels of various genes altered in erythropoiesis due to direct or indirect regulation by KLF1 have been shown.

Data from other groups of researchers as well as our data consistently shows that KLF1 positively regulates β-globin gene expression in mouse primitive and definitive erythropoiesis as well as human fetal erythropoiesis (Figure 5.1A and B, Figure 5.2B and C). On the other hand, previous data of γ-globin gene regulation by KLF1 is not consistent. Our data with KLF1 knockout mice that contain a complete β-globin locus transgene indicate that KLF1 positively regulates the γ-globin gene. This is different from the reports suggested by various other groups that KLF1 only represses γ-globin gene expression (Figure 5.1A) (Zhou et al. 2010, Tallack M and Perkins A, 2013). Our data in the human CD34+ model pointed out a complicated regulation

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of γ-globin by KLF1. γ-globin had almost a linear relationship with KLF1 up to 50% KLF1 (Figure 5.2B and C); however, at more than 50% KLF1, γ-globin mRNA levels started reducing (Figure 5.1B). The human data is similar to the data obtained by our laboratory in mouse primitive erythropoiesis, but different from the data observed by other groups. One of the main reasons for this difference may be the range of KLF1 knockdowns that we have obtained due to our large sample size which helps us perceive various situations of globin gene regulation compared to just KLF1+/- or KLF-/- mice.

BCL11A is another gene that is thought to regulate γ-globin gene expression. Zhou et al. in 2010 showed that downregulation of KLF1 in human *in vitro* differentiated CD34+ peripheral blood as well as in KLF1-/- transgenic mouse models (definitive erythropoiesis) leads to a decrease in BCL11A gene expression and in turn an upregulation in γ-globin mRNA levels. We have shown that KLF1 positively regulates BCL11A in our human CD34+ cord blood model (Figure 5.2B and C); however, our γ-globin gene expression data does not decrease parallel to an increase in BCL11A gene expression. This indicates that Bcl11A alone does not regulate γ-globin gene expression. Many other factors such as MBD2, NF-E2 and KLF2 may be involved in this complicated regulation and BCL11A alone cannot repress γ-globin (Sankaran et al. 2010, Divya Vinjamur unpublished data).

Finally, our investigation regarding the role of KLF1 in KLF2 gene regulation mostly showed an increase in KLF2 mRNA levels (Figure 5.2B) similar to our data in KLF1 knockout mouse primitive erythropoiesis (Divya Vinjamur, unpublished data). Based on all our data in the human CD34+ model, we designed models (Figure 5.2) representing the role of KLF1 in erythropoiesis. KLF1 directly positively regulates β-globin gene expression. Also, it indirectly regulates γ-globin gene expression through other factors one of them being BCL11A. KLF1 also regulates

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KLF2 which may directly or indirectly be involved in γ-globin gene regulation. β-globin and γ-globin expression levels might also be interdependent due to competition which is represented by the two-pointed arrow in Figure 5.2.

The similarities and differences between the two systems could also be seen in our colony forming assays. Divya Vinjamur saw a reduction in the size and not number of colonies in KLF1-/- genotype compared to wild type E10.5 embryos. On the other hand, the human assay showed a reduction in the number of KLF1 knockdown colonies compared to scramble colonies. This disparity in the results might have been caused due to the differences in the models listed above. However, we did observe a reduction in the staining of these KLF1 knockdown colonies suggesting fewer cells within colonies. To verify this hypothesis, cells within individual colonies could be counted in the future in KLF1 knockdown colonies compared to scramble.

We investigated the role of KLF1 in proliferation and cell cycle to extend these colony forming results. In these studies, not all of the genes analyzed in the human CD34+ model correlated with our results in the mouse model. Genes such as CD24, Myc and p18 were not regulated by human KLF1 compared to mouse KLF1. The differences in the range of KLF1 knockdowns compared to complete knockouts might be one of the main reasons for these disparities. Studies by Coghill et al. 2001 involving the reintroduction of KLF1 in KLF1-null erythroid cell lines showed reduced proliferation. However, the reintroduction of KLF1 was at a later stage of erythropoiesis due to which it could not rescue the cell line's proliferative ability. Contradicting this study, microarray studies by Pang et al. 2012 in mouse primitive erythroid cells and mRNA-seq studies by Tallack et al.2012 in mouse definitive erythroid cells showed a number of proliferative genes which were downregulated in KLF1-/- genotypes. In the human KLF1 knockdown model, we saw a reduction in FOXM1 mRNA levels and an increase in the mRNA levels of SPHK1. The

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transcription factor FoxM1 is known to stimulate proliferation by promoting S-phase and M-phase entry in the cell cycle. It is also known to activate all five Cdk/Cyclin complexes showing its importance in cell cycle progression. Interestingly, KLF1 binds to the FOXM1 promoter and thereby FOXM1 may be directly regulated by KLF1 in mouse primitive as well as human definitive erythropoiesis. This may indicate a common role of KLF1 in proliferation and cell cycle in both mouse and human systems. To determine whether the proliferative ability is decreased or increased due to KLF1 knockdowns, proliferation assays could be performed in human KLF1 knockdown CD34+ *in vitro* differentiated erythroid cells.

Understanding the effect of KLF1 and KLF2 is important for therapeutics. Our research established a unique role of KLF1 in γ-globin gene regulation. Increased γ-globin can help alleviate some of the symptoms in β-hemoglobinopathies. Also, it is essential to determine the role of KLF1 and KLF2 in regulation of genes other than globin. Our results demonstrate that KLF1 has an important role in proliferation and cell cycle in mouse and human erythropoiesis. Reducing KLF1 expression levels in patients as a therapeutic strategy might have adverse effects on proliferation and cell cycle. Therefore, we need to extensively study alternate factors involved in globin gene regulation that do not affect proliferation and cell cycle. Overall our research could facilitate effective therapeutic strategies for hemoglobinopathies in the future.

5.2 Future Directions

This work focuses on the role of KLF1 in human fetal erythropoiesis and its importance in β-globin regulation, cell cycle and proliferation. To expand this work, we need to study the role of KLF2 in human fetal erythropoiesis since KLF2 is also thought to regulate globin gene

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expression. Our laboratory has already started the preliminary work to fulfill this goal. Divya Vinjamur a current PhD student in our laboratory has successfully established human KLF2 knockdown CD34+ *in vitro* differentiated erythroid cells. Our next aim should be to establish the role of KLF2 in β-globin regulation, cell cycle and proliferation. We have previously shown through colony forming assays in mouse that KLF2 knockout mice show a reduced number of colonies compared to wild type. It would be interesting to determine whether human KLF2 knockdown results in a reduced number of colonies compared to scramble. Also, previous work by Basu et al. 2007 and Alhashem et al.2012 demonstrates the combined role of KLF1 and KLF2 in globin gene expression in mouse primitive erythropoiesis. Our future goals should include KLF1 and KLF2 double knockdown studies which would help us establish the dual combined role of these two transcription factors in human fetal erythropoiesis.

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