**The Regulation of γ-globin and LRF/ZBTB7A by Krüppel-Like Transcription Factor KLF-1 in Human Erythroid Cells**

**I. Introduction**

Sickle Cell Disease (SCD) continues to be a prominent inherited blood disorder that affects approximately 70,000-100,000 Americans per year1. A common variant of SCD, Homozygous sickle cell anemia, affects a gene located on Chromosome 11 named HBB, which encodes for the beta chain of hemoglobin and synthesizes the beta globin protein. In homozygous sickle cell anemia, an autosomal recessively inherited mutation changes the 17th nucleotide of the beta globin gene from a Thymine to an Adenine2. This results in the glutamic acid at position 6 in the beta globin gene to be replaced by Valine1,2. Due to the substitution of Valine, a hydrophobic amino acid, the point mutation causes hemoglobin to polymerize, resulting in deformed red blood cells with the marked “sickle” shape. These damaged sickle cells may block large vessels in the body, even causing areas of necrosis called infarcts, to occur in different organs.1

As sickle cell anemia continues to have deleterious effects on the body and population, scientists are actively seeking to procure a complete cure for this disease. As of now one of the major treatments of sickle cell anemia involves actively caring for symptoms and even the transfusion of red blood cells.4 However, clinical complications that arise with the latter, especially when blood transfusions are being performed regularly, involve an overload of iron.3 These high levels of iron, approximately 200 mg of iron present in 420 mL of donated blood3, continue to persist even when strict regimens are used to remove heavy metals from the body using chelating agents.3

Another complication lies in the brief timeframe in which patients with sickle cell anemia go from being healthy to beginning exhibiting harmful symptoms. Shortly after the time of birth, there is a switch of expression from HbF (fetal hemoglobin) to adult hemoglobin (HbA).4 This switch of expression is mediated by a switch in the transcription of *γ- to* β-globin. (Fig.1)

As such, humans who have inherited the mutation affecting the production of beta globin begin exhibiting symptoms around 4-5 months of age when the fetal hemoglobin (gamma globin) is replaced by the sickled hemoglobin, thus making other cells sickle as well.5 Although the fetal hemoglobin that protects the red blood cells from sickling is not present when it is switched to adult hemoglobin, a condition named hereditary persistence of fetal hemoglobin delivers promising news about the significance of γ-globin in reducing symptoms of sickle cell anemia.6 Hereditary persistence of fetal hemoglobin is categorized by the maintenance of fetal hemoglobin levels well beyond the point in which hemoglobin is switched from fetal to adult (*γ- to* β-globin). Some factors to explain this persistence include mutations in the β-globin gene cluster or changes in the *γ* gene promoter region.7 Those diagnosed with hereditary persistence of fetal hemoglobin, in addition to sickle cell disease, exhibit reduced symptoms of the disease due to the increased expression of fetal hemoglobin in the form of the γ-globin. Hereditary persistence of fetal hemoglobin can be caused by two classes of mutations: one involving deletions at the beta-globin cluster and other involving point mutations in the promoters of the gamma-globin genes. However some forms of non-deletional HPFH suggests that the promoters of gamma globin genes interact with factors that repress/silence expression so that even though gamma globin is present, it is being modulated simultaneously with the adult beta globin.

Fig 1 -The fetal-to-adult hemoglobin switch. This illustration depicts the normal timing of the developmental hemoglobin switches in humans. In the top panel, the sites and levels of various b-like globin molecules are shown with colors corresponding to the various developmental groups of genes shown below it in a model of the human b-globin locus (embryonic in blue, fetal in green, and adult in red). The bottom illustration also depicts the upstream enhancer of the b-globin locus, known as the locus control region (LCR), with its corresponding DNase I hypersensitivity sites (HSs) and a downstream HS known as the 30 HS1. (Reference 4)

As humans undergo a developmental switch from fetal γ-globin to adult β-globin expression around the time of birth, during the switch γ-globin gets silenced as the β-globin gene is expressed.4 This knowledge combined with observations of reduced SCD symptoms in patients diagnosed with hereditary persistence of fetal hemoglobin, scientists are looking towards the possible of reactivating γ-globin gene expression in adult patients.

In order to achieve this reactivation, it is necessary to identify factors that silence γ-globin. Two known γ-globin silencing factors are BCL11A & LRF/ZBTB7A which are transcription factors that mediate silencing of γ-globin by binding in various locations.8

BCL11A, a far more studied transcription factor, works to repress gamma globin by binding to the upstream locus control region (LCR) and intergenic regions between the fetal (γ) and adult (δ and β) globin genes.9 LRF/ZBTB7A however, is a transcription factor that has not been studied as heavily and has been known to work independently from BCL11A in silencing γ-globin.10 LRF/ZBTB7A has been known to do this by binding to DNA and recruiting a special NuRD repressor complex through its N terminal BTB domain.11 This NuRD complex causes chromatin to be tightly wound which results in less transcription of γ-globin occurring.

Regulating these silencing transcription factors, especially the lesser known LRF/ZBTB7A can provide an outlet to useful therapies by manipulating the levels of expression of these factors in erythroid cells. The key to one form of regulating the expression of known repressors of γ-globin may lie with a Krüppel-Like Transcription Factor named KLF1 which increases γ-globin expression as its function is decreased. In Norton et al (2017)14, the scientists looked towards determining whether KLF1 drives the expression of LRF/ZBTB7A in erythroid cells. They were able to accomplish this with the use of the experimental technique, *chIP-Sequencing (chIP-Seq)*,

*Fig. 2 - KLF1 enrichment peaks across the gene body of ZBTB7A, with a high number of peaks at the ZBTB7A promoter, a result of chIP-Sequencing which indicates that there is some binding of KLF1 to the ZBTB7A promoter.*

in order to determine whether KLF1 binds directly to the ZBTB7A promoter. In chIP Sequencing, Chromatin Immunoprecipitation is conducted by the fixation of chromatin with formaldehyde and subsequent fragmentation of DNA. Then, DNA-protein complexes present with protein-specific antibodies are isolated through immunoprecipitation. After this DNA obtained has been amplified by Polymerase Chain Reaction in order to produce enough to conduct an analysis. It is then sequenced using Next Generation Sequencing (chIP-Seq) and the reads produced are mapped to the genome, allowing researchers to possibly identify transcription factors through the observation of the presence of isolated DNA. In Norton et al (2017), the researchers first observed the initial levels of LRF/ZBTB7A in *KLF1-/-* murine fetal liver cells and noticed a reduction of LRF/ZBTB7A expression compared to the wild-type controls. This could account for a possibility that LRF/ZBTB7A in downregulated in the absence of KLF1. Norton et al (2017) then began to conduct chIP-Seq experiments on both mouse and human cell lines in order to determine whether KLF1 binds directly to the ZBTB7A promoter.

According to the results displayed by the chIP-Seq *(Fig. 2)14,* it was found that the ZBTB7A promoter was one of the highly enriched KLF1 peaks. Furthermore, a large number of KLF1 peaks were present within introns and enrichment of peaks were also observed in the promoter region and intergenic region. Due to the experiment conducted by Norton et al (2017), we can observe that KLF1 binding is highly enriched at the promoter of LRF/ZBTB7A in both murine and human cell lines and can further hypothesize that KLF1 can act in upregulating LRF/ZBTB7A.

If we are able to control the amount of KLF1 that is being expressed, then it is possible to then regulate the activity of LRF/ZBTB7A in order to increase γ-globin gene expression.

Relating to the findings presented by Norton et al (2017), a hypothesis presented central to this proposal would be that LRF/ZBTB7A levels would be decreased in an absence of KLF1. This may in turn increase the levels of *γ-globin* since LRF/ZBTB7A is a known *γ-globin* repressor. However, one issue with the absence of KLF1 is that knocking *out* KLF1 altogether wouldn’t serve a purpose in obtaining results since KLF1 is an erythroid regulator that positively regulates hemoglobin12. So, proceeding with a *knockdown* of KLF1 may be beneficial to observe levels of LRF/ZBTB7A and *γ-globin* while still producing hemoglobin.

**II. Experiment**

This experiment will aim to determine if a knockdown of KLF1 will alter the levels of LRF/ZBTB7A and γ-globin expression in erythroid cells. Specifically, the experiment will serve as a determinant as to whether KLF1 is able to regulate the expression of LRF/ZBTB7A and to what extent. In order to perform this experiment, erythroid cells will have to be infected by a lentivirus in order to knockdown KLF1, followed by using qRT-PCR to measure expression levels of LRF/ZBTB7A and γ-globin after successful knockdown of KLF1. Hypothetical results obtained from this experiment could suggest that knocking down KLF1 can decrease the repressive activity of LRF/ZBTB7A which can then increase γ-globin gene expression in red blood cells, thus proving to be a viable direction to explore when searching for alternates to increase γ-globin to help alleviate symptoms of sickle cell anemia.

Knockdown of KLF1

In order to be able to measure the expression of γ-globin, it is imperative to not completely knock out the amount of KLF1 in the erythroid cells since doing so would halt the production of γ-globin. In order to be able to observe and measure LRF/ZBTB7A and subsequently γ-globin levels, KLF1 will proceed to be knocked down through a lentivirus infection.



Lentiviral Infection

Infection via a lentiviral vector has posed as a vital tool in aiding nucleic acid delivery. Specifically, a lentiviral infection allows for a stable integration of a specific nucleic acid sequence into the genome of the target cell. Further utilizing this method of nucleic acid delivery by a lentiviral vector can lead to the expression of a gene construct, especially one which has the man-made shRNA inside. A key aspect that sets lentiviral infection apart from say transfection of nucleic acids is that while the transfection may result in only transient expression of the transgene, the infection system

**Fig. 3 -** Making Lentiviral Vectors.

Lentiviral vectors are created by cotransfection of a packaging cell line (293T/293FT) with the cDNA/shRNA expressing transfer plasmid along with two helper/packaging plasmids which encode the structural and envelope (typically VSV-G) proteins. The packaging cells produce infectious particles, whose genome only encodes sequences from the transfer plasmid, which can be used to transduce the target cells. The transfer plasmid alone is transfected into a packaging cell line (Phoenix) that already contains the helper constructs. (Ref. 11)

of the lentivirus allows for the transgene to be inherited and then *continuously* expressed over repeated cell divisions. Another specific facet of the lentiviral vector is that it produces “self-inactivating” particles. These particles, true to their name allow for the delivery of the desired sequence into the target cells but does not allow continued viral replication. In the case of this experiment, having self-inactivating particles can prove to be useful, since continuing viral replication would lead KLF1 down a path of being knocked out completely. As functional as the lentiviral infection sounds, there are multiple components in order to transfect the target cell and successfully harvest the lentivirus to deliver the desired sequence (Fig 3). There are currently three generations or methods to create lentiviral plasmids, however due to wanting a higher viral yield, the method employed in this proposal will be the 2nd generation of creating lentiviral plasmids. This method, in Figure 3, uses three plasmids that make up the lentiviral system. These three plasmids include the:

1. Transfer Plasmid: The transfer plasmid is used to transfer the gene of interest into the target cells. In the transfer plasmid, there is a deletion of the U3 region and transcriptionally active sequences in the 3’ LTR, which results in the LTR being self-activating. The 5’ LTR then drives the expression of the packaged genomic RNA.
2. The Packaging Plasmid: The Packaging Plasmid contains viral proteins *Gag, Pol, Rev,* and *Tat*. Gag and Pol are viral proteins that are needed for the development and maturation of the virus. Rev and Tat are needed to positively regulate transcriptional activity and export the genomic RNA from the nucleus.
3. The Envelope Plasmid: The Envelope Plasmid is comprised of envelope proteins that determine the tropism (the orientation or turning movement of an organism in response to an environmental stimulus) of the virus that is subsequently harvested.

Transfer plasmids which carried a KLF1 targeted shRNA and a scrambled shRNA coding sequences separately was obtained from Dr. Francois Morle of Universite Claude Bernard Lyon1, (Lyon France) and Dr. Fawsia Louache of INSERM (Villejuif, France). The use of a lentiviral infection in order to knock down KLF1 has been exhibited and performed before in Vinjamur et al (2016)12 In this experiment, Vinjamur et al sought to knockdown KLF1 by first conducting a calcium phosphate transfection of the 293T Cell line. Three subcategories were divided evenly for the calcium phosphate transfection—scramble, K1V1, and K2V2. All three categories were separately transfected, with scramble shRNA added to scramble category and K1V1 mock and K1V2 knockdown added respectively. Due to a calcium phosphate formation which caused cells to die after 18 hours, the medium containing the viral particles was filtered, thus making the lentivirus ready for infection of (in their experiment), an artificial cell line named HUDEP-2 cells.

Measuring levels of Expression (of LRF/ZBTB7A and γ-globin)

Once KLF1 had been successfully knocked down by lentiviral infection, the levels of LRF/ZBTB7A and γ-globin had to be measured in order to determine if KLF1 had an effect on the regulation of either and if it did, to what extent? For this experiment, qRT-PCR would be used as a method to determine and measure changes in gene expression of both LRF/ZBTB7A and γ-globin.

Quantitative Reverse Transcriptase-PCR (qRT-PCR)

Quantitative Reverse Transcriptase-PCR (qRT-PCR) is a technique that is employed to measure the amount of a specific RNA. The standard steps of a PCR are still included as a qRT-PCR is being performed, which is comprised of three steps13(Fig. 4):

1. DNA Denaturation: Denaturation causes the DNA to unzip and separate, exposing nucleotides.
2. Primer Annealing: the primers pair up or anneal with the single-stranded template sequence of DNA that needs to be copied. The polymerase then attaches and starts copying the template.
3. Extension: DNA building blocks that are complementary to the template are then coupled to the primer, making a double stranded DNA molecule.

However, qRT-PCR takes it a few steps further and determines he progress of the PCR as it occurs (in real time), and quantitative data is collected after each cycle in the PCR process, rather than only at the end of PCR (similar to what may be achieved through end-point PCR). In doing this, as the technique is true to its name, qRT-PCR is most helpful in determining how much of a specific DNA or gene is present in a given sample. In order to detect and quantify the product from the PCR, two common methods are used:

1) fluorescent dyes that serve as intercalating agents and wedge into the double-stranded DNA.

**Fig. 4 –** a schematic detailing the steps in a Polymerase Chain Reaction conducted (PCR).

2) DNA probes that consists of labeled reports which are sequence specific. In the scope of this experiment, qRT-PCR is being used to measure a number of different gene expressions, but specifically measuring β-globin mRNA, KLF1 mRNA, and γ-globin mRNA. However, since the results produced from the qRT-PCR are quantitative, in order to provide a better visual by graphing the trends (if there were any present), it would be effective to provide a control with which to measure the levels of expression against, in this case, cyclophilin A mRNA.

**III. Discussion**

If the experiment proposal is followed as such, then hypothetical results I should expect to see are that when KLF1 is knocked down, the activity of LRF/ZBTB7A is reduced and thus γ-globin gene expression levels increase. In order to provide a better visual or schematic of this trend, it may be of note to conduct a statistical analysis, such as a Pearson’s correlation plot. An example from Vinjamur et al (2016) is provided below to show an example of on such trend that could occur. However, one more unwelcome result that I could possibly receive when conducting this experiment is that the trend between the amount of LRF/ZBTB7A and the ending result of gamma globin may not be as significant as the trend compared to a more prominent repressor, such as BCL11A. Although it is worth noting if LRF/ZBTB7A may act in the same fashion as a gamma globin repressor as BCL11A, but most likely not to the greater effect that BCL11A may have compared to LRF/ZBTB7A.

Some pitfalls I may encounter rely mostly on the lentivirus preparation. Instead of knocking down KLF1 with a direct form of genome editing (perhaps from a CRISPR/Cas9 protocol), the use of an outside virus that requires many steps beforehand to prepare for infection can leave room for error, especially since there is a use of three plasmids. A limitation in this experiment is that even if the hypothesis surrounding the underlying proposal proves itself to be true, there are still far many steps to implement the ongoing observations of this mechanisms in order to alleviate symptoms of sickle cell anemia. Although the regulation of this mechanism doesn’t provide a steadfast answer to curing sickle cell disease, it does provide scientists and medical professionals to focus on gamma globin and possibly increasing it through the manipulation of many different repressors, notably LRF/ZBTB7A.

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