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A Delicate Balancing Act: Curing Humanized Mouse Models Of Cooley'S Anemia By Reducing The Globin Chain Imbalance

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A DELICATE BALANCING ACT: CURING HUMANIZED MOUSE MODELS OF COOLEY'S ANEMIA BY REDUCING THE GLOBIN CHAIN IMBALANCE

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A DELICATE BALANCING ACT: CURING COOLEY'S ANEMIA BY REDUCING THE GLOBIN CHAIN IMBALANCE IN HUMANIZED MOUSE MODELS

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BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Cooley's Anemia (CA) is a hereditary disease which occurs when an individual inherits two null β-globin alleles. CA presents during the latter part of the first year of life due to the high levels of fetal hemoglobin (HbF) in the circulating red blood cells (RBCs) at birth. The absence of β-globin chains, to dimerize with α-globin chains in the newly formed bone marrow derived erythroblasts, results in premature destruction of the erythroid cells in the marrow and ineffective erythropoiesis. I hypothesized that increasing the amount of β-like globin chains for hemoglobin assembly in erythroblasts would lessen disease severity or even cure a humanized mouse model of CA. Employing gene editing techniques, we increased the levels of the fetal β-like globin chain, γ-globin, by introducing mutations that prevent or delay the completion of the switch from fetal to adult hemoglobin. This goal was first achieved by the introduction of a T to C promoter mutation at position -175 upstream of the γ-globin gene transcription start site (TSS). The animals generated from this experiment survived normally but were phenotypically thalassemic with enlarged spleens, extramedullary hematopoiesis and low levels of liver iron accumulation. The second approach employed germline gene editing of the Bcl11a erythroid specific enhancer (ESE) with CRISPR/Cas9. Several mutant founder lines were generated, but the mutations with the greatest effect on γ-globin upregulation extended at least 77 bp 3' of the sgRNA cut site. The 77 bp deletion resulted in a heterocellular increase in γ-globin transcription and protein chain synthesis through adulthood at levels

sufficient to prolong the survival of 84% of CA mice over 10 weeks with no RBC transfusions. The final approach was a proof-of-principle experiment in which we used CRISPR/Cas9 mediated homology directed repair (HDR) to repair the mutant β-globin gene in homozygous CA embryos. Of the mice born, two had repaired alleles and subsequent homozygotes generated demonstrated no symptoms of thalassemia.

These animals provide novel models for studying *in vivo* globin switching mechanisms, identifying new modulators of globin switching and gene editing approaches to increasing HbF, and testing new therapeutic drugs for increasing HbF levels in erythroblasts.

Key words: Cooley's Anemia, CRISPR/Cas9, gene editing, hemoglobin, mouse models

DEDICATION

This thesis is dedicated to my mother, Elizabeth Christine Glasgow, who has supported me with unconditional love and encouragement throughout this journey.

This thesis is also dedicated to the rest of my family especially my grandparents and aunts who consistently provided encouragement, called and sent messages of support.

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LIST OF ABBREVIATIONS

BMT: bone marrow transplant

CA: Cooley's Anemia

Cas9: CRISPR associated protein 9

CRISPR: clustered regulatory interspersed short palindromic repeat

dCas9: nuclease deficient Cas9

DHS: DNase I hypersensitive site

DNA: deoxyribonucleic acid

DSB: double stranded break

ESE: erythroid specific enhancer

GWAS: genome wide association study

HbA: adult hemoglobin

HbA_2: minor adult hemoglobin

HbF: fetal hemoglobin

HbS: sickle hemoglobin

HDR: homology directed repair

HLA: human leukocyte antigen

HMIP: HBS1L-MYB intergenic region

HPFH: hereditary persistence of fetal hemoglobin

HSC: hematopoietic stem cell

IVS: intervening sequence

LCR: locus control region

NHEJ: non-homologous end joining

PAM: protospacer adjacent motif

RBC: red blood cell

rHbA: repaired HbA (adult hemoglobin)

RNA: ribonucleic acid

RNP: ribonucleoprotein

RVD: repeat variable di-residue

SCA: sickle cell anemia

ssODN: single stranded oligo-deoxyribonucleotide

sgRNA: single guide RNA

TALE: transcription activator-like effector

TALEN: transcription activator-like effector nuclease

trugRNA: truncated single guide RNA

ZFN: zinc finger nuclease

ZFP: zinc finger protein

β^A: normal β-globin allele

β^0: β-thalassemia major

β^I: β-thalassemia intermedia

β^T: β-thalassemia

INTRODUCTION

Red Blood Cells and Hemoglobin Switching

When a baby is born, one of the first things which the physician or midwife does is ensure that the child is breathing. A swift slap elicits wailing that heralds the arrival of the newborn, and forces it to breath on its own. Breathing is often taken for granted, with little thought going into the exquisite process which has evolved over millions of years while diverging to adapt to specific ecosystems such as water, earth or air.[1] Most individuals go through the day completely oblivious to the small cells racing through the human body (and that of other animals), ensuring that oxygen is delivered so that the organism remains alive. But, it would do us some good to take a step back and delve into the inner working of the cells that keep us going as along as we keep breathing.

The red blood cell (RBC) is the primary constituent of what is collectively called blood and is generally the component being referred to when one mentions blood, although blood comprises multiple cell types and serum proteins. The reason is that blood gets its color from the RBCs which in turn get their color from hemoglobin which makes up over 95% of the dry weight.[2-4] Our first encounter with blood may be an unpleasant one since it is usually accompanied by pain and a metallic tinge to its smell and taste. The gravity of spilling blood is not lost on one when the amount lost is large, but the real power of these small, enucleated cells is their specific importance to our sustenance which goes unnoticed and underappreciated.

Air contains approximately 21% oxygen, the molecule essential to sustaining animal life. Oxygen is the eighth element on the periodic table (atomic number 8) which exists in nature as a covalently bonded diatomic molecule, O_2, in the gas phase at room temperature and pressure. It is transported through the body by the RBCs that are pumped to the lungs, by the heart, where the carbon dioxide (CO_2) collected from tissues is off-loaded and O_2 picked up from the alveoli. The O_2 is secured by reversibly binding to a heme moiety which is cradled in a pocket of each globin chain of tetrameric hemoglobin. The tetramer is formed of two heterodimers of α-like and β-like globin molecules that are found almost exclusively in the (RBCs) of the hematopoietic system.

RBCs are the first hematopoietic cell to develop in the growing embryo. They are manufactured in three distinct compartments as development progresses, a feat which is for the most part irreversible. About 70 % of the cells in the human body are RBCs with approximately 2.07 $x10^{11}$ new cells (~1%) entering circulation daily as aged cells undergo eryptosis.[5,6] This allotment of resources to the production of red cells gives an indication of the importance of RBCs and their function.

Hemoglobin is the molecule which facilitates the RBC's role as oxygen transporter. Each mature enucleated RBC is packed full of hemoglobin molecules that are distributed throughout the cytoplasm of these biconcave disc shaped cells. Globin holoproteins are comprised of three essential components: globin polypeptide chain, polyporphyrin ring and iron cation (together called heme). The porphyrin ring is synthesized in the developing erythroblast while Fe^{2+} is absorbed from dietary sources and recycled from destroyed RBCs that are cleared from circulation by splenic macrophages.[7-10] Heme is lodged between two alpha helices within each globular α- and β-like globin chain. Each

heme binds an O_2 molecule (four per hemoglobin tetramer) in the lungs. O_2 is transported and released to other tissues such as the muscles where myoglobin, which has a higher O_2 binding affinity than hemoglobin, binds and stores O_2.[11]

Hemoglobin molecules are tetramers made up of two heterodimers. Each heterodimers is comprised of an α-like globin and β-like globin chain translated from transcripts on two different chromosomes, 16 and 11 in humans (11 and 7 in mice) respectively (Figure 1). The human α-globin locus on chromosome 16 contains three developmentally regulated genes, the embryonic ζ-globin gene and two fetal/adult α-globin genes under the transcriptional control of an upstream enhancer sequence called R1-6 (containing HS-40).[12-14] The five genes on human chromosome 11 comprises the β-globin locus which encodes ε-, $^{G}\gamma$-, $^{A}\gamma$-, δ- and β-globin genes that are developmentally expressed from 5' to 3' (from embryonic to fetal to adult life) under the control of their own upstream enhancer called the locus control region (LCR).[15,16]

In humans, the first erythroid cells develop in the yolk sac blood islands, appear between day 16-20, and are nucleated.[17,18] These primitive nucleated cells manufacture the first hemoglobin molecules, embryonic hemoglobin: Hb Gower 1 ($\zeta_2\ \varepsilon_2$), Hb Portland ($\zeta_2\ \gamma_2$) and Hb Gower 2($\alpha_2\ \varepsilon_2$).[19,20] As the fetus grows and the site of erythropoiesis shifts from the yolk sac to the fetal liver where hematopoietic stem cells (HSCs) differentiate into definitive erythroid cells, and these cells suppress the embryonic hemoglobin in favor of fetal hemoglobin (HbF), comprised of α-globin and γ-globin ($\alpha_2\gamma_2$)[21] A few weeks later (11 week old fetus) the fetal liver HSCs begin to seed the bone marrow, the terminal site of erythropoiesis. Bone marrow becomes the primary erythropoietic organ shortly before birth and maintains that role thereafter.[22,23] This shift also results in a change in the

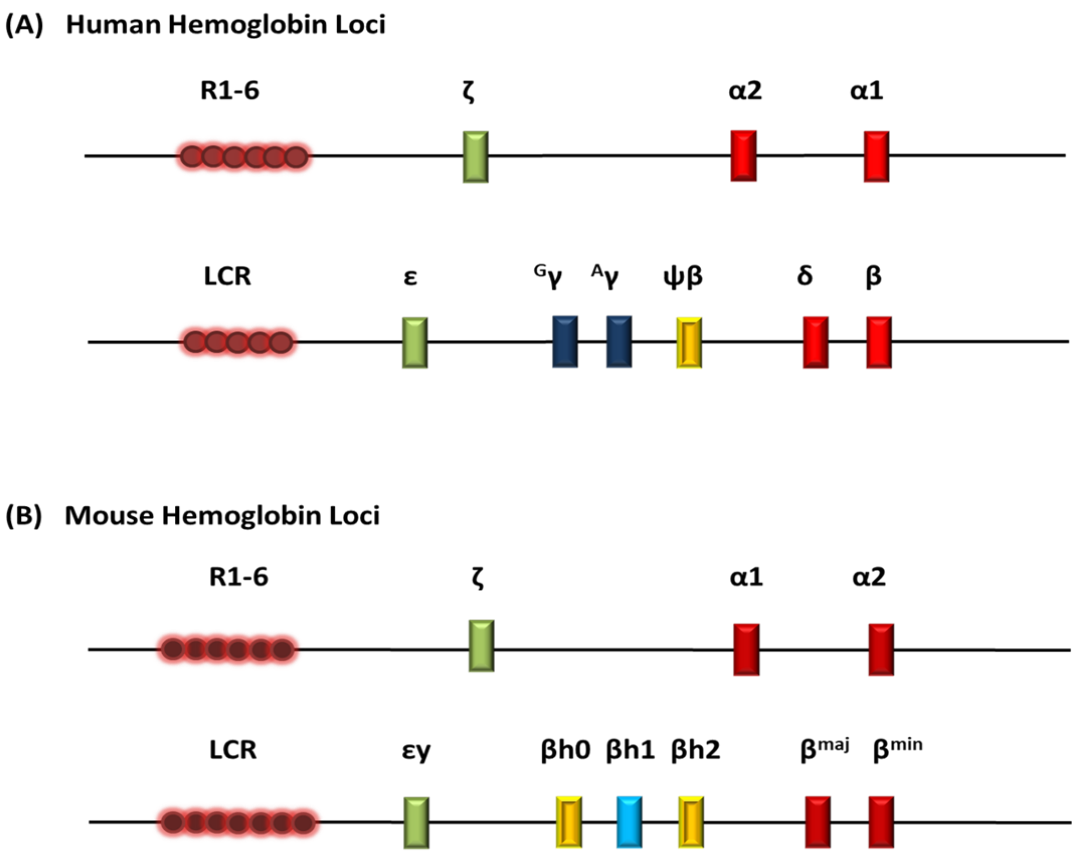

Figure 1. Human and mouse globin loci. (A) The human α-globin locus is located on chromosome 16 and contains three genes. The human β-globin locus is located on chromosome 11 and contains five expressed genes and one pseudogene (yellow). (B) The mouse α-globin locus is located on chromosome 11 and contains three genes. The mouse β-globin locus is located on chromosome 7 and contains four expressed genes and two pseudogenes (yellow).

globin chain expressed off the β-globin locus, with β-globin, and to a much smaller extent, δ-globin, being the chains expressed in adult cells.[24] This transition from fetal to adult hemoglobin (HbA: $\alpha_2\beta_2$ and HbA_2: $\alpha_2\delta_2$) is called the fetal-to-adult switch.[23,25] At birth 60-70% of the hemoglobin in circulating RBCs contain primarily HbF which steadily decreases as the newly synthesized RBCs of the rapidly growing infant are bone marrow derived erythroblasts.[26] The fetal-to-adult switch is completed within the first year of life with HbA being the primary hemoglobin molecule at about 97% with about 2.5% HbA_2, the minor adult hemoglobin. By then, HbF is usually less than 1% of the total hemoglobin, a trend which persists throughout adulthood.[27]

Hereditary Persistence of Fetal Hemoglobin

Low (0.5%) HbF levels in adult blood were thought to be the norm until a group of Swiss doctors testing army recruits happened upon some recruits with elevated HbF levels (>0.7%).[28] The benign condition coined hereditary persistence of fetal hemoglobin (HPFH) has since been identified in several population groups the world over. The persistent HbF expression can be distributed in all the RBCs (pancellular) or found in only a fraction of the cells (heterocellular). The percentage of HbF containing RBCs, termed F-cells varies widely and is dependent on the genetic mutation responsible for the phenotype. There are three broad genetic determinants of HPFH that have been described.

The first is deletional HPFH, which in most cases is due to large scale deletions in the β-globin locus. The deletions specifically encompass the β-globin gene but may extend 5’ all the way to the $^{A}\gamma$-globin gene.[24,29] Many of the deletions extend far 3’ of β-

globin, deleting more than 100kb of DNA. The common feature of these deletions is the absence of the β-globin promoter elements which recruit and interact with the LCR. With the loss of these regulatory elements and proximal enhancers, the LCR interacts preferentially with the $^{G}\gamma$-globin, and when present, the $^{A}\gamma$-globin gene promoters. Other small deletions have been identified in the γ-globin promoter.[30,31] The sequences deleted are thought to be necessary for binding factors important in silencing HbF in adulthood. These deletions result in HbF production well into adulthood, with levels varying based on deletion size and genetic background of the affected individual.

The second class of HPFH is a called non-deletional HPFH, owing to the fact that the entire globin locus is intact. Instead, the high HbF is attributed to mutations within the promoters of either of the γ-globin genes. Point mutations known to be associated with HPFH have been identified within the -100 to -202 bp region upstream of the transcription start sites of either of the γ-globin genes.[29] Identical mutations have been seen in both genes, though the effect on HbF production varies widely. The mutations have been shown either to inhibit repressor complex binding or create erythroid transcription factor binding sites that derepress the silencing of the gene proximal to the mutation.[32,33] The LCR loops to the γ-globin promoters more often when such mutations exist, increasing HbF gene transcription.

The final class of HPFH is due to transacting modulators of hemoglobin switching which are just now beginning to be identified. Mutations that affect the levels of essential erythroid protein KLF1 result in increased HbF levels.[34,35] This effect appears to be both direct (loss of KLF binding at β-globin promoter) and indirect (lower transcription of KLF1-regulated switching factors).[36]

Genome wide association studies (GWAS) have been used by researchers to identify other polymorphisms and mutations in genes that lead to HPFH independent of those in the globin locus mutations.[37] These studies have yielded several candidates that provide clues into the complexity of globin switching. One such locus is the HBS1L-MYB intergenic region (HMIP) which contains several polymorphisms associated with HPFH. This region is thought to contain enhancer sequences involved in both HBS1L and MYB expression. Transgenic mouse experiments showed reduced HBSlL and MYB expression when the HMIP sequence was disrupted, whereas embryonic globin expression was increased.[38] Similar results were obtained when shRNAs against MYB were transduced into human $CD34^+$ cells. Erythroid differentiation cultures had elevated HbF, adding evidence to support a role for MYB in globin switching, in addition to its role in erythropoiesis.

GWAS and other functional experiments have also identified BCL11A as a potential modulator of globin switching.[39-41] BCL11A is a proto-oncogene which has been identified as the master transcription factor for B-cell lineage commitment and differentiation.[42] Early studies also identified a role for this protein in neural cells, specifically axonal branching.[43] The importance of BCL11A dosage was demonstrated in individuals with loss of one allele of BCL11A. These individuals showed developmental defects, but also exhibited HPFH.[44] GWAS mapping of the location of the HPFH associated SNPs and elegant studies in human cell lines and transgenic mice identified three erythroid specific DNase I hypersensitive sites (DHSs) within the second intron of the Bcl1a gene (Figure 2).[37,45,46] Unlike global loss of the protein in exonic knockouts, mutations in the DHSs only affected protein expression in erythroid cells.[47] This provided the first muta-

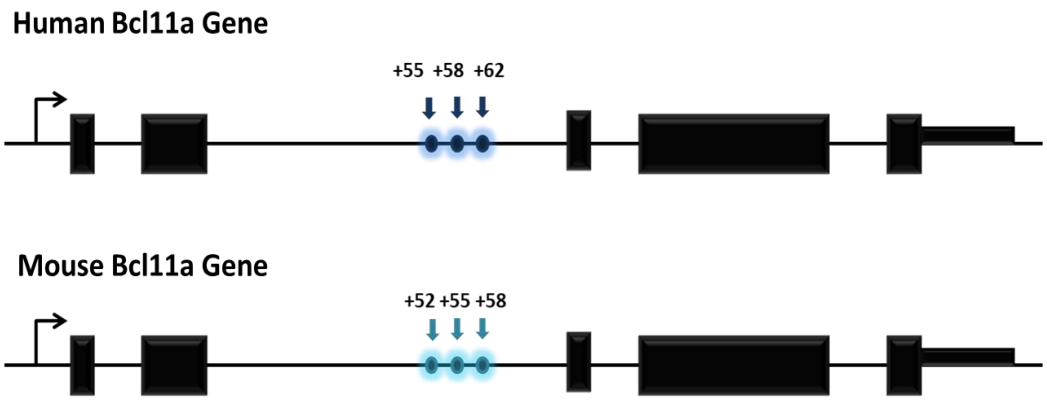

Figure 2. Human and mouse Bcl11a genes showing DNase I hypersensitive sites with erythroid specific enhancer activity. The DNase I hypersensitivity sites are numbered by their distances (in kb) from the transcription start sites in their species

ble switching transacting factor which did not affect global erythropoiesis as is seen with KLF1 and HMIP mutants.

The Thalassemias

The production of the components of the hemoglobin molecule is tightly regulated to ensure balanced translation of globin chains and availability of heme for incorporation into the globin pocket for dimerization and subsequent tetramerization. Red cell production is also tightly controlled with an equivalent number being released into and cleared from circulation daily. All this physiological efficiency and tight regulation are thrown off if even one of the many factors involved in the pathways essential to RBC production and maturation is dysregulated. RBCs have evolved their shape and deformability by having special membrane and cytoskeletal proteins as well as a critical mass of hemoglobin for functionality.[48,49] Mutations in globin chains, erythroid specific transcription factors/ regulators or cytoskeletal protein genes affecting protein synthesis, transcription or alter their functionality, can have deleterious and in some cases fatal consequences for affected individuals.

. Mutations of the α- and β-globin genes of hemoglobin are the most common gene mutations known in humans, occurring in approximately 5% of the world's population. Hemoglobinopathies, such as sickle cell anemia (SCA), are the result of single point missense mutations which affect hemoglobin function. The thalassemias, on the other hand, are diseases caused by mutations that affect globin chain production. α-thalassemia is caused by reductions in α-globin chain synthesis; whereas, β-thalassemia (β^T) is caused

by mutations that affect β-globin chain levels.[50,51] To date over 200 mutations which cause β^T have been identified.[52] The clinical manifestation and severity of the diseases depend on the alleles inherited from both parents. β^T-minor is usually a heterozygous condition where a normal β-globin gene is co-inherited with a nonfunctional β^0allele. Affected individuals can usually live normal lives with no need for clinical intervention and may be unaware of their carrier status. β^T-intermedia (β^I) is more complicated because it can arise when two low β-globin expressing alleles are co-inherited (compound heterozygous or homozygous). The clinical presentation of those affected can run the spectrum from mild (similar to β^T-minor), to severe (similar to β^T major patients) requiring periodic RBC transfusions.[53-55] Treatment regimens are patient-specific and designed with close monitoring by physicians.

With β^T-major (β^0) or Cooley's Anemia (CA), the β-globin synthesis is negligible or completely abolished. First described by Dr. Thomas Cooley in 1925, this disease arises when two mutations which abolish β-globin production are co-inherited.[56,57] The mutations may be homozygous or compound heterozygous, but the effect on the red cells remains the same: the absence of β-globin chains. CA is a microcytic, hemolytic anemia with ineffective erythropoiesis due to premature erythroblast death in the bone marrow.[58]

Pathophysiology of Cooley's Anemia

The α- and β-globin proteins have evolved obligate dimerization roles. When there is a complete loss of β-globin chains for α-globin to dimerize in adult RBCs, the α-globin chains aggregate into intracellular inclusions.[59] The insoluble inclusions associate with the cell membrane, affecting the cells' integrity.[60] The immature erythroblasts un-

dergo premature apoptosis in the bone marrow with those that escape into circulation lysing while navigating the vessels.[51] Recently, Arlet *et al*. showed that the maturational arrest and apoptosis seen in β^0-globin RBCs is also due to the sequestration of HSP70 in the cytoplasm by free α-globin chains.[61] HSP70 usually translocates to the nucleus where it binds the transcription factor GATA1 and protects it from activated Caspase3 cleavage. The cytosolic sequestration of HSP70 by α-globin leaves GATA1 exposed to Caspase-3 cleavage and unable to bind the DNA of erythroid specific target genes, leading to apoptosis of the immature RBCs. Anemia caused by apoptosis of polychromatophilic erythroblasts (polyE) in CA patients triggers the expression of the hormone erythropoietin resulting in the stimulation and expansion of erythropoiesis to meet the oxygen demands of the body.[62,63] However, this expansion of additional thalassemic erythroblasts results in ineffective erythropoiesis, which in turn leads to the expansion of the marrow cavity in attempt to generate RBCs. In addition, pathogenic extramedullary erythropoiesis in the liver, and to a larger extent the spleen (in the mouse), occurs. The spleen loses its red/white pulp architecture in favor of mostly erythroid output. The spleen becomes engorged with defective erythroid cells trapped by the reticuloendothelial system and, in many cases, splenectomies are performed.[64] The expansion of the bone marrow results in long bones that are weaker and more susceptible to fractures.[65-67] The bones of the cranium which also manufacture RBCs also expand resulting in craniofacial abnormalities. In addition to the poor endogenous red cell output, the signaling cascade causes excess iron to be absorbed from dietary sources in the gut.[8,68] Because this iron is not utilized, over time the excess iron accumulates in the organs where it is deposited leading to organ damage and

failure. One of the main organs affected by iron deposition is the heart and CA patients are at severe risk of cardiac failure.[69]

Treatments, Cures and Experimental Therapies

Allogenic Bone Marrow Transplants

The only cure currently available to CA patients is an allogenic hematopoietic stem cell HSC transplant (commonly referred to as a bone marrow transplant [BMT]) from a HLA matched donor.[70,71] Sibling matches are preferable although unrelated donors are viable options when a related donor is unavailable.[72,73] However BMTs are fraught with complications especially when performed in adults. Children transplanted before the onset of the associated disease complications, such as iron overload, have much higher rates of success. Adults transplanted face post-transplant complications, such as the immediate threat of graft rejection and graft versus host disease, either of which could subsequently develop and ultimately results in fatality.[74,75] BMT recipients may be required take immune suppressants for extended periods of time after the procedure, in some cases for the rest of their lives in order to keep their immune cells from attacking the donor cells. Immunosuppression puts them at increased risk for infections that a compromised immune system may be unable to fight; close monitoring by physicians and strict adherence to drug regimens are required.

Transfusions

The dearth of histocompatible donors and severe complications associated with HSC transplants leaves most CA patients to manage the disease with frequent blood transfusions to maintain their RBC and hemoglobin levels within the normal ranges. The frequency of the transfusions is determined by the patient and their doctor. Although establishing a transfusion therapy regimen is essential for the survival of CA patients, this therapeutic intervention comes with its own set of complications.[76] Iron overload from increased absorption in the gut in response to the anemia is compounded by exogenous iron from the transfused blood. In order to limit the deleterious effects of excess iron, transfusion therapy needs to be coupled with iron chelation therapy. There are currently three iron chelation products available in the market, with others in development. Even with strict adherence, the chelators can only do so much and iron overload is an inevitability.[77,78]

Gene Replacement Therapy

One of the avenues explored to treat CA and other hematopoietic diseases is gene replacement therapy in autologous HSCs. This involves the addition of functional genes to HSCs which can then be transplanted back into patients and differentiated into the affected cell type. The new cells derived from the transplant render the lineage functional, curing the patient of their disease. The method of delivery best thought to be capable of achieving a therapeutic goal is retroviral gene therapy. The ability of retroviruses to transduce cells and permanently insert themselves into host genomes made them excellent vectors to test this therapeutic approach. Targeting HSCs would ensure that inserted

viral genomes and their therapeutic transgenes would be passed on to all HSC derived cells providing therapeutic benefit. Unfortunately, early trials in Europe hit a snag with patients developing T-cell leukemia due to insertional mutagenesis from activated gammaretroviruses in the years following two enrollments with a total of 10 patients.[79-82] The death of one of the patients led to researchers taking a step back to assess the vectors' safety and refine the transduction protocols. Currently there are multiple lentiviral gene therapy trials underway to treat several diseases including several to treat CA (and sickle cell disease) both in the United States and in Europe.[83-86]

Butyrate and its Derivatives

Reactivation of fetal hemoglobin is an active area of therapeutic investigation. The ability to achieve such an outcome with a drug, without the need for invasive procedure such as BMTs would be a welcome development for patients. One drug which made it through two stages of clinical trials on the basis of increasing HbF in reticulocytes was butyrate and one of its derivatives.[87,88] Butyrate was administered intravenously whereas isobutyramide was an oral treatment. Some reports claimed that despite increased HbF and F-cell percentages, the levels were not high enough to warrant the administration of the drugs to CA patients.[89] It was also reported that patients developed drug tolerance because initial HbF increases reverted to background levels.[90] As with other HbF inducing drugs (hydroxyurea), results vary between patients of different genetic backgrounds within the study groups making approval difficult.[91] Conflicting reports on the efficacy of the treatments have resulted in no large scale clinical trials. A report published a decade ago showed that butyrate caused increased translation efficiency of γ-globin mRNA, though the exact mechanism continues to elude researchers.[92]

Modeling Cooley's Anemia in Mice

The study of diseases which affect humans requires study subjects under close observation, readily available for enrollment in experimental studies for delineating disease mechanisms, testing therapeutic drugs and study follow-up. Such invasions into the lives of individuals, who are in some cases dealing with life threatening conditions, cross the ethical boundaries outlined for both academic and industrial institutions. As such, modeling diseases in animals has become the mainstay of most research institutions. As many scientists will point out, lower order mammals and humans are different, but in some cases, the differences disguise the mechanistic similarities underlying physiological processes. And even when the mechanistic similarities stop short of perfectly mimicking one another, there may be small manipulations that can nudge them closer to each other.

CA is a disorder which affects erythroblast maturation due to the inability of the cells to produce β-globin to pair with α-globin. Vertebrates use hemoglobin filled red blood cells to facilitate oxygen transport and, as such, modeling the disease in an animal, such as a mouse should have been simple. However, the differences in human and mouse physiology made such a task impossible. Mice have four functional globin genes at their β-globin locus. The two closest to the LCR, ε and βh1, are expressed in early embryos while the two more distal to the LCR, β^{maj} and β^{min} are expressed during fetal and adult life (Figure 1B and 3). Humans have five active genes on the β-locus but the timing of their expression is different (Figure 1A and 3). In humans ε-globin is expressed during the first few weeks of life but a change occurs when the fetal liver becomes the major site of erythropoiesis. The γ-globin genes are expressed for several months, as the primary β-

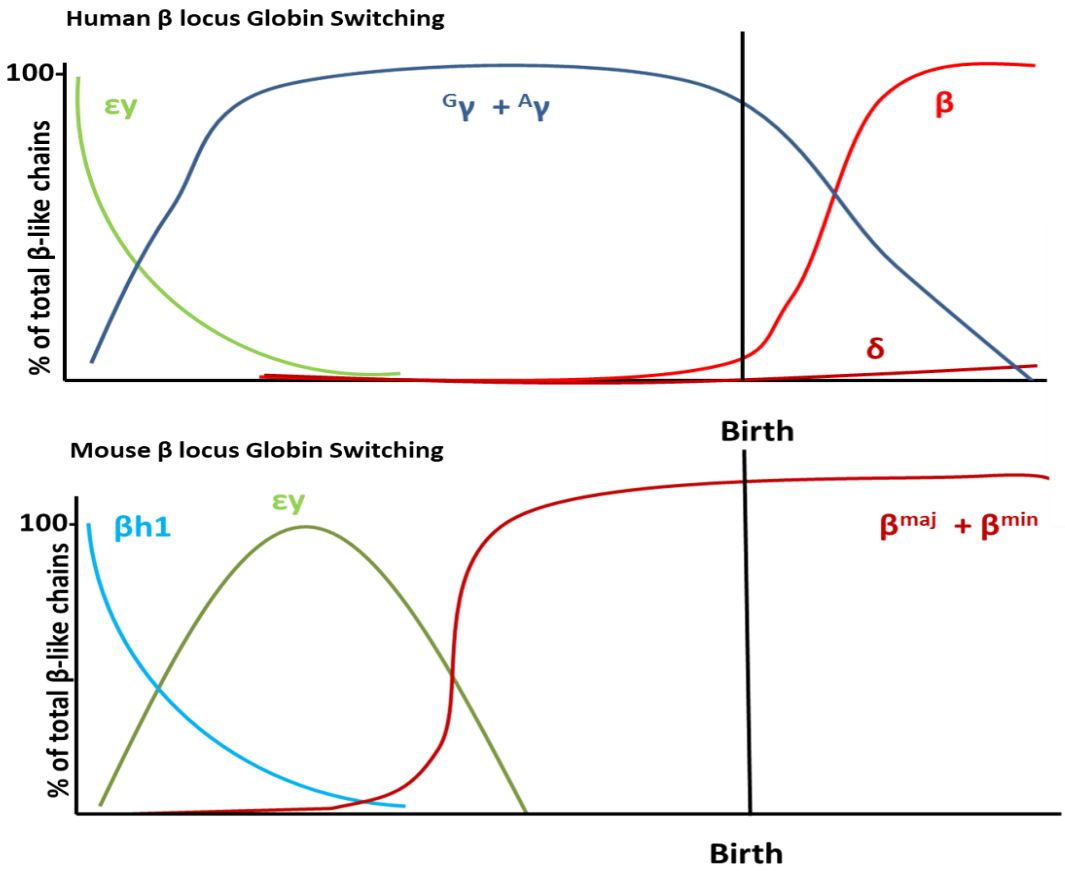

Figure 3. The difference in hemoglobin switching between humans and mice at the β-globin locus.

like chain. Expression of the adult chains, δ and β does not begin until a few weeks before birth (Figure 3). The presence of this fetal gene made all the difference in how modeling a disease affecting the adult β-globin chains would be tackled. Mice, on the other hand, do not synthesize a separate fetal hemoglobin. Rather mice begin synthesizing their adult β-globin chains early in fetal life. A knockout of both mouse adult β-globin chains results in an embryonic lethal phenotype at day 16.5 whereas humans with thalassemia major survive to birth as healthy infants due to their high HbF levels.[93]

The first description of a β-thalassemia mouse model, now referred to as the Th-1 mouse, was identified as a result of a heterozygous deletion of the mouse adult β^{maj}-globin gene. The heterozygous animals were phenotypically normal but homozygotes exhibited some of the symptoms commonly observed in thalassemia patients.[94] The animals showed growth retardation and blood smears showed cells reminiscent of thalassemia patients'. The β-globin to α-globin chain ratio was 0.75 owing to the compensatory increased expression of the β^{min}-globin gene *in cis* to the deletion. Nevertheless, this ushered in a new era in thalassemia research. We could not only use mice to understand normal cellular mechanisms in analogous systems and pathways but also to study disease conditions. Scientists now had a disease model to study disease pathology and molecular mechanisms of disease progression and to test novel therapies. The model though imperfect, opened up the avenue for further exploration and refinement.

The Th2 model, the 2nd iteration of the thalassemia model was engineered by knocking out the mouse endogenous mouse β^{maj}-globin gene by incorporating a neomycin gene into the second exon of the gene, rendering it non-functional.[95] Despite expectations that the model would mimic the Th1 mouse, this animal fared much worse. This

was explained by the newly proposed competition model of hemoglobin switching.[96] The presence of the β-globin promoter (and a neo promoter) rendered the β^{min}-globin genes less transcriptionally active and animals homozygous for the mutation died perinatally due to severe anemia.

The third attempt at a thalassemia mouse, the Th3 model, was created by deleting a 16 kb fragment encompassing both the adult β^{maj}- and β^{min}-globin genes.[93] Homozygous animals died *in utero* around day 16.5 when the mouse adult β-globin chains were the primary chains being expressed in the β-globin locus. Heterozygous animals presented as β-thalassemia intermedia as evidenced by microcytic anemia characterized by reduced hemoglobin, anisocytosis, poikilocytosis and hypochromic erythroid cells, and reticulocytosis. Heterozygotes are fertile and the phenotype is rescued when bred to transgenic animals that contain a human β-globin transgene. This model though useful, had a phenotype much more severe than that experienced by thalassemia minor patients.

Experimentally, the Ryan group determined that the human γ-globin gene is temporally expressed similar to humans when it was inserted into the mouse β-globin locus under the endogenous control of the mouse LCR. A human γ-to-β switching cassette was knocked into the mouse β-globin locus, replacing both mouse adult globins.[97] The resulting switching profile was one which mimicked human globin switching with γ-globin being the primary transcript expressed in the modified β-globin locus from E14.5 to E18.5 and decreasing steadily until complete transcriptional shutdown by three weeks after birth. The F-cells are detectable longer due to their lifespan (~60 days) but are relegated to background levels by 7 weeks after birth. A null β^0-globin switching cassette with a splice donor mutation at the first base of intron 1 (IVS1.1 G to A) was used to generate

the first CA mouse model which survived to birth solely on human HbF.[98] The mice perished within the first 24 hours but provided a template for generating subsequent CA mice that contain a human HPFH mutation in the γ-globin promoter, resulting in prolonged survival, with a median survival of 15 days, a window within which therapeutic interventions could be tested.[99] The experiments detailed in the subsequent chapters make use of the mouse lines described above, demonstrating the importance of having animal models with similar phenotypes to the patients who they are meant to represent.

Advances in Gene Editing Technology

In addition to gene replacement therapy, advances made in the last 30-40 years in the field of gene editing have made gene correction a viable alternative in the future of genomic medicine. The first breakthrough came about when DNA binding proteins were first identified and their sequence specificity recognized.[100,101] These proteins name zinc finger proteins (ZFPs), -for their metal iron cofactor- ushered in a new avenue in treating monogenic disorders such as those in renewable cell populations, including hematologic diseases. Several reports have been published using engineered zinc fingers linked *in cis* to nuclease domains (ZFNs) or activators to edit DNA or affect gene expression of target genes, respectively.[102-107] Developing ZFN technology is time consuming, expensive, and commercially available from one company, Sangamo Therapeutics (ST). While ST continues to improve the ZFN technology, such as binding specificity and obligate dimerism, other DNA binding proteins/systems have been discovered and offer cheaper alternatives to the ZFPs.

Transcription activator-like (TAL) effector proteins (activators and nucleases) splashed on to the scene in 2011 and quickly captured the imaginations of researchers across the globe. They were chimeric proteins assembled by fusing the DNA binding domain of effector proteins from bacterial genus Xanthamonas to activators and nucleases.[108,109] The effector domains demonstrated DNA binding specificity with four individual modules binding the four DNA bases. This allowed the assembly of a protein capable of specifically binding a DNA sequence from 12 to 27 base pairs long with very little constraint.[110] The minimal target requirement for TALE activity was T at position -1 of the binding sequence. Each module consisted of 33-35 amino acid repeats with the twelfth and thirteenth amino acids called repeat variable di-residue, (RVD), dictating the nucleotide specificity, as they were the only amino acids which varied within the assembled DNA binding domain. [111] The apparent simplicity of the system belied the challenge of assembling such large plasmids, especially when two were required for nuclease activity (Fok1 requires dimerization for DNA cleavage). A Golden Gate assembly method that cut down significantly on the time and number of steps required to produce the nuclease version of TALEs (TALENs) enable the use of this gene editing technology for mammalian cell use.[111] Several groups used the TALENs and activators to demonstrate DNA editing via non-homologous end joining (NHEJ) and homology directed repair (HDR) as well as transcriptional activation with VP64 conjugated proteins.[112-118]

Although researchers continue to report on TALEN driven experiments, shortly after their debut as an editing tool, another new DNA editing system emerged. Clustered regulatory interspersed short palindromic repeat (CRISPR) and CRISPR-associated (Cas) proteins demonstrated that precise genome editing could be achieved with an RNA mole-

cule complementary to a short DNA target sequence.[119] The ribonucleoprotein endonuclease complex is an archaeal and bacterial adaptive immune system which protects host microbes from phage and plasmid infections.[120,121] When bacteria or archaea are challenged by phages, the viral DNA is cleaved by host endonucleases and, short sequences called protospacers, are incorporated into the microbial genome between direct repeats in the CRISPR locus. Infection by a previously encountered phage triggers the expression of the Cas proteins and transcription of CRISPR RNAs. The RNAs are processed and complexed with endonucleases. The complex is targeted to the invading DNA, generating double stranded breaks to render the phage inactive. The microbial system made use of two RNA molecules for activity but optimization by Jinek *et al.* defined the necessary sequences within the two RNAs and engineered a chimeric single guide RNA (sgRNA) which worked efficiently. The Cas9 endonuclease, the only protein required for targeting, was codon optimized for eukaryotic cell applications. Since those first ground breaking publications reporting on the role of CRISPR locus in bacteria and its gene editing application, numerous groups have sought to optimize further the system for high on-target efficiency and low off-target mutational load, all with mixed results. Initial reports put the sgRNAs target at 20 nucleotides with a trinucleotide, NGG, protospacer adjacent motif (PAM) at the 3' end of the target sequence (23 bp total) but subsequent work has shown that longer RNAs are processed down to 20 nucleotides.[122] This discovery is of particular significance due to the other constraint of the RNA transcription mechanism. Guide RNAs transcribed off expression plasmids in mammalian cells are typically transcribed from the U6 promoter by RNA polymerase III which requires a 5' G to begin transcription. Not all target sequences are able to meet both requirements (5'GN_{19}NGG) and the

need for a longer RNA molecule may arise. Despite high on target specificity, sgRNAs were shown to be tolerable to mutations especially those which occurred outside the 12 bp seed sequence proximal to the PAM. This short seed sequence could result in off target cleavage of DNA sequences which had mismatches to the sgRNAs outside this seed sequence. To reduce off-target effects, shorter truncated sgRNAs (trugRNAs) were tested and showed lower tolerance to mismatches throughout the target.[123,124] Another effort aimed at increasing on target cleavage and enhancing HDR was the inactivation of one of the nuclease domains to generate a mutant Cas9 which only nicked one strand of DNA.[122] This strategy enables the use of two sgRNAs within 20 bases of each other to generate a double stranded break. This mechanism is similar to one employed in the used of ZFNs and TALENs where the Fok1 protein has been modified for obligate heterodimerism, thereby cleaving occurs only when the heterodimer pair meets, ruling out cleavage by homodimers.[125,126] Similarly, the nickases would only generate double stranded breaks when bound to sgRNAs on opposite strands within close proximity to one another. The strong DNA base pairing at longer distances were not favorable to "unzipping".

Since CRISPR/Cas9 was made available for widespread use, the editing system has been used to model diseases in cell lines, repair diseased alleles in cell lines, cure animal models of muscular dystrophy, screen cell lines for novel enhancer (and insulator) regions, generate new models of disease, generate disease resistant animals and plants.[46,127-132] Researchers continue to find new and innovative ways to harness the unique properties of the ribonucleoprotein complex.

The advent of the CRISPR/Cas9 endonuclease system enabled the goals of this project to be achieved by providing a fast and efficient way to target DNA.

Thesis Overview

Our basic understanding of CA and other hemoglobinopathies has greatly increased over the last several decades. Despite this increase in knowledge of the mechanisms underlying the diseases, treatment options and curative therapeutics continue to lag. CA requires interventions at multiple levels including managing iron absorption, improved iron chelation drugs, improving the safety of hematopoietic stem cell transplantation and therapeutic interventions aimed at endogenously reducing the globin chain imbalance, the cause of the pathology of the disease. **I hypothesized that reducing the globin chain imbalance in RBCs, the cause of CA, will cure the mice of their disease.** In the next chapters, I will present evidence of three gene editing approaches that address the issues of the chain imbalance in CA and offers improved outcomes for the CA mice.

In Chapter 1, the therapeutic utility of the incorporation of a single point mutation in the human γ-globin gene promoter of a pancellular HPFH is demonstrated *in vivo* in a CA mouse model. Mice with the γ-to-β switching cassette have previously been shown to mimic the human switching patterns in mice. An introduction of the IVS1.1 G to A transition which yields no functional β-globin transcripts resulted in 20% of homozygous animals making it to birth but all die within the first 24 hours of birth. The introduction of the Black -175 HPFH, a T to C transition, resulted in complete rescue of the animals from their lethal anemia. This experiment demonstrates that increased γ-globin levels can reduce the amount of unpaired excess α-globin chains-enabling erythroblasts to survive premature apoptosis.

In Chapter 2, HPFH is induced by deletions in the erythroid specific enhancer of Bcl11a, a transacting modifier of hemoglobin switching. β-thalassemia is caused by over 200 mutations throughout the gene body, deletional and non-deletional. This complexity makes specifically repairing individual mutations cost prohibitive. It therefore made sense to target a gene or enhancer which could raise γ-globin levels in all patients regardless of the disease causing variant. Most gene editing technology to date has been shown to repair preferentially the DSB in the genome via NHEJ despite the availability of homologous DNA to facilitate HDR. It therefore made sense to attempt to increase γ-globin levels by targeting the Bcl11a gene which normally represses the expression of HbF in the adult. More importantly rather than target exonic sequences of Bcl11a that would certainly have profound effects in multiple tissues, the erythroid enhancer was specifically targeted so that Bcl1a levels would only be decreased in erythroid cells but leave the expression in other cellular compartments unchanged. Deletions in the enhancer region resulted in heterocellular increases in γ-globin with F-cell levels increasing to more than 20 % in one homozygous line. This increase in γ-globin expressing cells was coupled with a 20-fold increase in γ-globin mRNA. This mutation provided a significant increase in survival of the CA mice that were bred on to the Bcl11a ESE deletion background.

Progress in the field of gene editing has far surpassed initial projections and has made the possibility of curing lethal hereditary diseases in the germline a reality.[133] Despite ethical concerns and calls for caution from the scientific fraternity, the resources are now available and testing the efficiency and safety of such biotechnological tools is paramount. In Chapter 3, I conduct a proof-of-principle germline gene editing experiment to repair the mutant β-globin gene mutation. Homozygous CA embryos were microinjected

with Cas9, a trugRNA and a β-globin repair ssODN. Four mice were derived from the experiments, and 3 of the 9 targeted alleles used the ssODN to repair the double stranded break generated in the β-globin exon. Homozygous mice generated from breeding showed normal hematology when compared with HbA and CA mice. The silent mutations incorporated in the repair ssODN did not affect erythropoiesis. This experiment serves as a demonstration of our ability to cure the humanized CA mouse model by correcting the mutant β^0-globin gene via germline gene editing.

AN HPFH MUTATION IN THE γ-GLOBIN GENE PROMOTER OF HUMANIZED COOLEY'S ANEMIA MICE RESCUES THEM FROM PERINATAL LETHALITY AND RENDERS THEM TRANSFUSION INDEPENDENT

by

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Introduction

Hemoglobin is one of the most important proteins synthesized in the human body and a primary constituent of red blood cells (RBCs) that comprise roughly 70% of the cells in the human body. [24,134] Being the only molecule capable of physiological oxygen transport, hemoglobin is indispensable for survival. Unfortunately, the frequency of mutations found in hemoglobin occur in 5% of the world's population due to the selective advantage conferred by diseases such as malaria, resulting in an overrepresentation of genetic diseases of hemoglobin, collectively referred to as hemoglobinopathies.[135-137] Sickle cell anemia (SCA) and the thalassemias are two of the more well studied hemoglobinopathies, with millions of individuals affected worldwide.[138,139] Unlike SCA which is due to a single point mutation in the first exon of β-globin, thalassemias can be caused by a myriad of mutations, deletional and non-deletional in either hemoglobin subunits, α-globin and β-globin.[50,51] β-thalassemias alone have been shown to be the result of over 200 mutations in the β-globin locus.[51] They include but are not limited to large and small deletions in the β-globin structural gene or regulatory sequences as well as point mutations in the exons and introns that destabilize the mRNA or disrupt translation of full length globin chains.[140] In the most severe presentation of β-thalassemia, negligible or no β-globin protein is synthesized, resulting in a severe hemolytic anemia which is fatal if left untreated.[141] This form of the disease termed β-thalassemia major, or Cooley's Ane-

mia (CA), only becomes apparent postnatally when the adult β-globin chains become the dominant chains synthesized in RBCs.

During development, the hemoglobin moieties expressed go through a series of changes as the site of erythropoiesis changes.[142-145] The first blood cells to develop are primitive erythroid cells that develop in the yolk sac blood islands expressing embryonic globin chains, ζ-globin and ε-globin (the α-like and β-like chains, respectively).[18,19] When the site of erythropoiesis shifts to the fetal liver, definitive erythroid cells that express α-globin and γ-globin, (adult α-like chain and the fetal β-like globin chains respectively).[22] These fetal hemoglobin, HbF, ($\alpha_2\gamma_2$) containing cells called F-cells make up the majority of fetal blood during gestation. Right before birth, the site of definitive erythropoiesis begins to shift from the fetal liver to the bone marrow, accompanied by a fetal-to-adult globin switch at the β-globin locus.[144,146] RBCs containing mostly adult hemoglobins HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$) slowly increase until the HbF level is but a minor component of total hemoglobin, usually less than 1%. The timing of the fetal-to-adult hemoglobin switch and admixture of fetal cells with adult cells are responsible for the delayed presentation of serious anemia in CA until well into the first year of life.[26,140]

The fetal-to-adult switch at the β-locus is completed during the first year of life for most individuals. However, a subset of the population has been found to express HbF levels over 1% and up to 30% throughout adulthood.[29] This condition is usually benign with no adverse effects on most individuals. This condition termed hereditary persistence of fetal hemoglobin (HPFH) showed significant amelioration of disease symptoms in patients homozygous for deleterious CA mutations.[147,148] This discovery led to a pursuit of modulators of the fetal-to-adult switch at the β-globin locus. To date many HPFH muta-

tions, point mutations and deletions, have been characterized throughout the globin locus as well as at other regions in the human genome.[37,52,149-152] The causes of the variation and the effects of these mutations, heterocellular vs pancellular, and absolute increase in HbF are active areas of research by many groups including ours.

Studying CA and hemoglobin switching at the molecular level in human patients is difficult and fraught with ethical concerns. To circumvent these issues, the Ryan lab has generated humanized mouse models that recapitulate the developmental timing of human fetal-to-adult hemoglobin switching.[97-99] These models replace the adult mouse α- and β-globin genes with a human α-globin and a γ-to-β hemoglobin switching gene cassette, respectively. These humanized mouse models synthesize 100% human hemoglobin inside their adult RBCs. The first humanized CA model ($\gamma\beta^0$) was generated by incorporating a nonfunctional human β^0-globin gene containing a splice donor site mutation (IVS1.1 G to A) so that no functional β-globin mRNA or protein is produced. Humanized $\gamma\beta^0$ CA mice were born synthesizing 100% HbF but succumb to their anemia within hours of birth. The second generation humanized CA model replaced the adult mouse β-globin genes with a $\gamma^{HPFH117}\delta\beta^0$ gene cassette containing a γ-globin gene containing the heterocellularly distributed GREEK HPFH mutation (G to A) at position -117 bp upstream of the transcription start site (TSS) in the γ-globin promoter. Inclusion of this HPFH mutation extended the survival of the fully humanized $\gamma^{HPFH117}\delta\beta^0$ CA model to about 15 days after birth, providing a window for therapeutic intervention.[98,99] I hypothesize that utilizing a pancellularly distributed HPFH mutation, specifically the Black HPFH mutation at position -175 in the γ-globin promoter would further extend the postnatal survival of humanized CA mice.

This chapter describes the generation of a humanized CA mouse model containing the Black HPFH mutation in the γ-globin promoter.[153] A putative mechanism for the high HbF expression associated with this specific mutation was recently reported using cell lines.[32] We show for the first time that a mouse can survive into adulthood synthesizing 100% human fetal hemoglobin. Furthermore, we demonstrate that a single nucleotide change in the human γ-globin gene promoter can rescue a humanized mouse model of CA from lethal anemia.

Materials and Methods

Generation of Mutated Embryonic Stem Cells and Derivation of Mouse Lines

Mice were derived from ECS modified with a targeting construct built in pBluescript vector (Stratagene). The switching cassette in the plasmid constructs contain a 1.7 kb of DNA homology 5' of mouse β^{maj}-globin gene (HindIII fragment), 4.7 kb of human $^{A}\gamma$-globin gene (Accession# U01317: 39, 013-43, 728), 4.1 kb of β-globin gene (Accession# U01317: 61, 320-65, 426) and 7kb mouse homology 3' of the mouse β^{min}-globin gene (BamHI fragment). The Black HPFH mutation, a T to C transition was introduced at position -175 of the $^{A}\gamma$-globin gene promoter. The plasmid was linearized with NotI and electroporated into an F1 129S1vImJ-C57BL/6J mouse embryonic stem cells (mESCs). The mESCs were cultured on mouse embryonic fibroblasts in ESC media (Dulbecco's Modification of Eagle Medium, 15% fetal bovine serum (HyClone, Logan, UT), 1× nucleosides, 2 mM L-glutamine, 1× nonessential amino acids, 50 IU/mL penicillin, 50 μg/mL streptomycin, 0.1 mM β-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor) and selected with Hygromycin B (125 μg/ml) and ganciclovir (1 mM) to facilitate preferential growth of ESC clones which had incorporated the cassette. ESCs with the cassette knocked in (PCR screening) correctly were injected into 8-cell stage blastocysts from D2-C57BL/6J (The Jackson Laboratory, Bar Harbor, ME). The cells were subsequently implanted into the uteri of pseudo pregnant CD1 mice (Charles River Laboratories, Inc. Wilmington MA). Animals born from these experiments were bred to hCMV-Cre, and then humanized α2α1 globin KI mice to delete the hygromycin gene and generate mice heterozygous at both globin loci (α2α1/+ $\gamma^{HPFH}\beta^{0}$/+), respectively.[154] The heterozygotes were bred to generate homozygous animals. All procedures were approved

by University of Alabama (UAB) Institutional Animal Care and Use Committee (IACUC).

Measurement of Hemoglobin Chains

Hemoglobin chains were analyzed by high-performance liquid chromatography (HPLC). Red blood cells were collected, washed and lysed in hemolysate buffer (5 mM phosphate, 0.5 mM EDTA, pH 7.4), then NaCl was added to 1%. The samples were centrifuged to remove the membranes. The mouse and human globin chains were separated by a linear gradient of increasing acetonitrile with 0.1% trifluoroacetic acid at a 1.0 ml/min flow rate on a reverse-phase C4 column (Vydac, Hesperia, CA) on a Surveyor high-performance liquid chromatography (HPLC) instrument (Thermo Scientific, Waltham, MA).

Hematological Indices and Histopathology

Peripheral blood and tissues were collected and analyzed as previously described.[99] Peripheral blood was obtained from anesthetized mice and used to make blood smears stained with Dip Quick kit (Jorgensen Laboratories, Loveland, CO). RBC counts and red cell distribution widths were measured on a HemaVet1700 (Drew Scientific, Waterbury, CT). Packed cell volume measurements were taken using a JorVet J503 (Jorgensen Laboratories) microhematocrit centrifuge. Hemoglobin concentrations were determined after conversion to cyanmethemoglobin by lysing RBCs in Drabkin's Reagent (Sigma, St. Louis, MO), removal of insoluble RBC membranes by centrifugation, measuring the absorbance at 540 nm on a spectrophotometer, and comparison to hemoglobin standards. Reticulocytes were measured by staining with thiazole orange and analyzing

by flow cytometry. [155] Mice tissues fixed in 70% formalin and delivered to the UAB Comparative Pathology Laboratory for sectioning (embedded in paraffin) and staining (hematoxylin and eosin). Liver iron was stained by Prussian Blue. All slides were analyzed on Nikon Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). Images were taken on Nikon Coolpix E990 digital camera and processed by Adobe Photoshop CS version 8.0 imaging software (Adobe Systems, San Jose, CA).

Flow Cytometric Analysis of F-cells

Peripheral blood was collected from mouse tail tips using heparinized micro-hematocrit capillary tubes (Fisher Scientific**)** and deposited into 100 µl of DPBS with 0.05 µM EDTA. The blood was centrifuged at 500g for 3 mins at room temperature in a tabletop centrifuge. The blood was washed by resuspending in 500 µl PBS and centrifuged. The supernatant was aspirated and the blood was fixed by incubation in 500 µl 0.05 % glutaraldehyde (50 % stock solution was diluted 1:1000 in PBS) for 10 minutes at RT. The sample was centrifuged as previously described and the cells were permeabilized by incubation in 500 µL 0.1 % TritonX-100 for 10 minutes at RT. The cells were washed with 500 µL PBS. 1x 10^6 cells were diluted into 100 µL PBS and 0.25 µL of PE conjugated mouse anti-human fetal hemoglobin antibody (BD Biosciences, San Jose, CA) was added to the cell suspension, mixed and incubated at RT for 30 minutes. The cells were washed with 500 µL PBS and resuspended in 300 µL of PBS and analyzed by flow cytometry (BD FACSCalibur™).

Statistical Analysis

P values were calculated by the two tailed Student's *t* test.

Results

Establishment of the $\gamma^{HPFH175}\beta^0$ KI Mouse Model

Previous work from our lab demonstrated that despite post-natal survival of humanized $\gamma\beta^0$ and $\gamma^{HPFH117}\delta\beta^0$ CA mouse models, the rapidly decreasing postnatal expression of γ-globin resulted in the early demise within hours or a couple of weeks, respectively, due to severe anemia. In this study we chose a naturally occurring HPFH mutation that was characterized as having pancellular high HbF expression, the Black HPFH. To determine the effects of this HPFH mutation, we used PCR mutagenesis to create a new targeting construct with a T to C transition at position -175 in the promoter of the γ-globin gene in the $\gamma\beta^0$ switching cassette (Figure 1A). The β-globin gene in the parent plasmid contained the IVS1.1 G to A mutation which produces aberrant transcripts incapable of producing any functional β-globin protein.[156,157] This $\gamma^{HPFH175}\beta^0$ construct was electroporated into the mouse embryonic stem (ES) cells and correctly targeted ES cell clones were identified following selection and subcloning. Chimeric animals were derived from these ES cells following their injection into C57BL/6 blastocysts. These heterozygous F1 $\gamma^{HPFH175}\beta^0$ KI mice were bred to homozygous human α2α1 KI mice to produce doubly heterozygous $\gamma^{HPFH175}\beta^0$ and α2α1 KI mice. These mice were subsequently intrabred to homozygosity to generate animals fully humanized at both globin loci ($\gamma^{HPFH175}\beta^0/\gamma^{HPFH175}\beta^0$ and α2α1/α2α1). The targeted $\gamma^{HPFH175}\beta^0$ knock-in allele was confirmed by PCR and Southern Blots using DNA isolated from F2 offspring (Figure 1A and Figure 1B.) Southern blotting and Sanger sequencing of wild type (WT), heterozygous and homozygous animals were used to confirm the genotypes at both the γ- and β-globin

genes (Figure 1B and data not shown, respectively). These fully humanized mice will be referred to as the HPFH175 model.

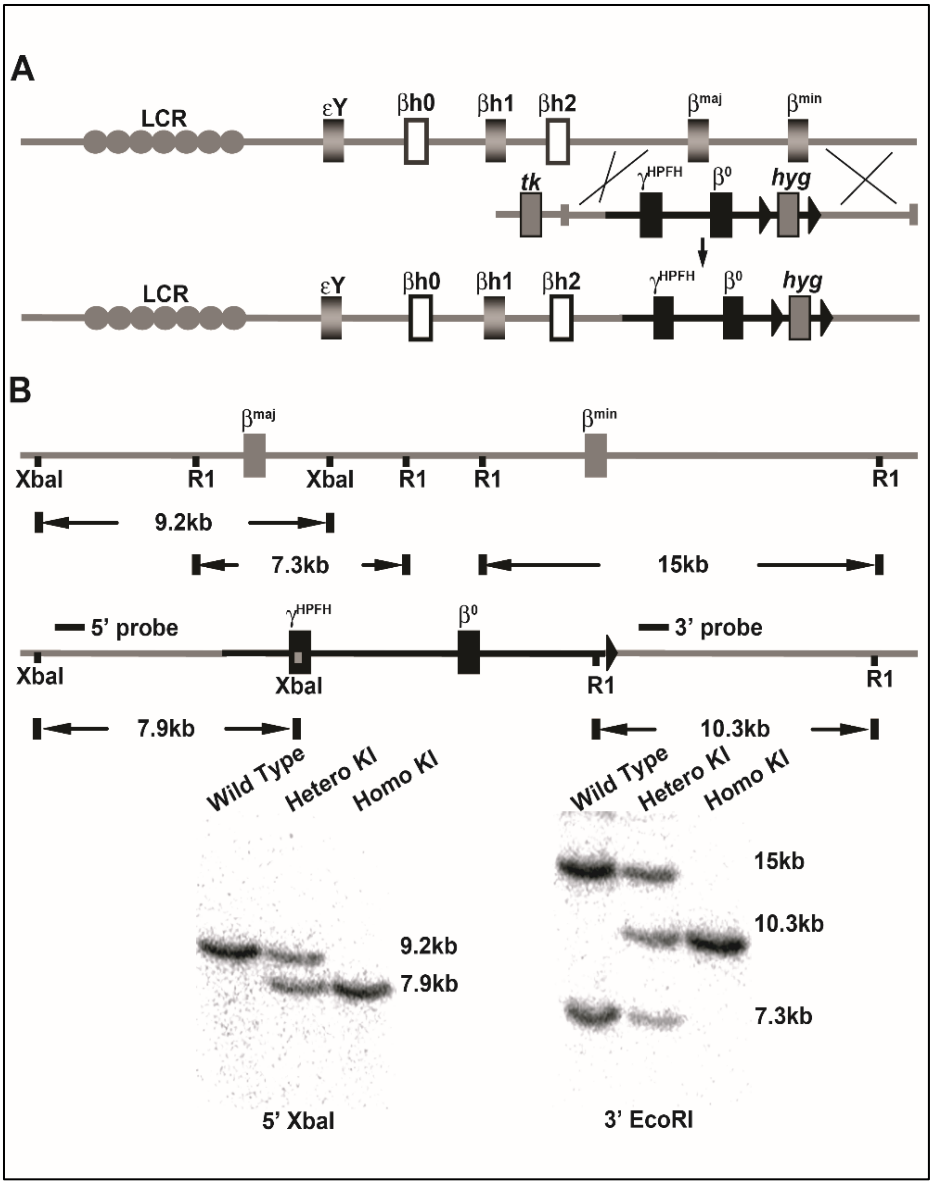
A
LCR
εY
βh0
βh1
βh2
βmaj
βmin
tk
γHPFH
β0
hyg
B
XbaI
R1
9.2kb
7.3kb
15kb
5' probe
3' probe
7.9kb
10.3kb
Wild Type
Hetero KI
Homo KI
5' XbaI
3' EcoRI

Figure 1. Humanized mice were generated from modified embryonic stem cells. C57Bl/6-129 hybrid mouse ESCs were electroporated with linearized plasmid constructs containing mouse homology and human globin constructs. (A) Mouse adult β-like globins, β^{maj} and β^{min}, were replaced with a human $\gamma^{HPFH}\beta^{0}$ cassette. The HPFH mutation is a -175 T to C mutation and the β-globin gene encoded a nullifying IVS1.1G to A transition (B) Southern blots of DNA show that homologous recombination occurred correctly. The 5' probe hybridizes to the XbaI fragment of both alleles. The mouse WT allele is 9.2 kb whereas the knock-in construct is 7.9 kb. The 3' probe hybridizes to EcoRI fragments. The WT allele has two fragments sized 15 kb and 7.3 kb whereas the knock-in allele has a 10.3 kb fragment. Lane 1: wild type control; lane 2: heterozygous knock-in; lane 3: homozygous knock-in.

HPFH175 Mice Live Normal Lifespans Surviving Solely on HbF

From the outset, the homozygous HPFH175 animals were noticeably different from humanized $\gamma\beta^0$ and $\gamma^{HPFH117}\delta\beta^0$ newborns. HPFH175 mice appeared indistinguishable from their heterozygous littermates and grew normally. Measurements of β-like globin chain ratios in compound heterozygotes ($\gamma^{HPFH175}\beta^0/\gamma\beta^A$) showed that the high (70%) γ-globin levels at birth declined to 40% of total β-like chain levels in the adults, similar to what has been observed in patients with a Black HPFH allele (Figure 2A). The animals maintained normal body weights, were fertile and had normal sized litters with expected Mendelian ratios, living as long as humanized HbA (α2α1/α2α1 $\gamma\beta^A/\gamma\beta^A$) and C57Bl/6 mice (Figure 2B). This mouse line represents the first known animal model which is able to live solely on human fetal hemoglobin. This animal makes no functional adult β-globin (neither mouse nor human) but is able to survive to adulthood, confirming that the reactivation of HbF is a viable therapeutic option for treating CA.

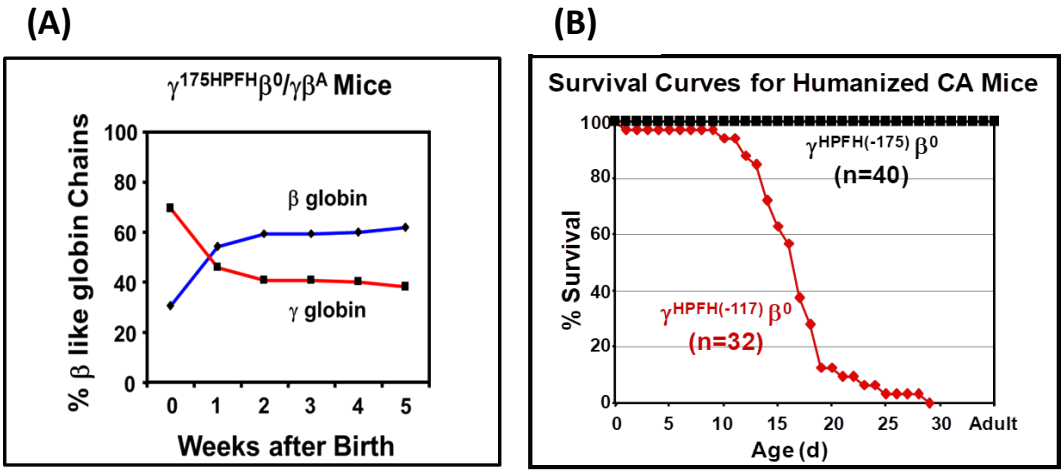

Figure 2. Hemoglobin switching in heterozygous HPFH175 mice and survival curves of homozygous mice. (A) Heterozygous mice were used to determine postnatal globin switching by HPLC analysis of hemolysates in both lines. HPFH175 heterozygote mice were born with high levels of γ-globin which decreased in the weeks after birth. The percentage of β-like chains is plotted over time. γ-globin shown by red curve; β-globin shown by blue curve. (B) Survival curves of homozygote HPFH175 mice (Black line) compared to homozygous HPFH117 mice (red line), $n \geq 32$.

HPFH175 Mice Have a Thalassemic Phenotype

The expression of hemoglobin chains is highly regulated and any genetic anomaly which impairs the production of any of the globins results in a chain imbalance affecting the integrity of the red cell and may reduce the half-life of the RBCs. As is the case in CA, the lack of β-globin chains results in excess α-globin which aggregates in the red cell forming toxic inclusions and accelerating apoptosis by sequestering HSP70 in the cytoplasm[51,61]. Despite the fact that HPFH175 mice have normal life spans, the α:γ chain ratio was not 1:1, and thus the true determinant of their health is measured by their hematological indices and tissue histology.

Blood was collected from HbA controls, heterozygous and homozygous HPFH175 mice and analyzed with a Hemavet 1700 hematology analyzer to measure the RBC indices. As can be seen in Table 1, the HPFH175 mice have hematological indices which indicate that they are indeed thalassemic. The animals have significantly reduced RBC counts, hemoglobin levels, and packed cell volumes compared to HbA controls. Both heterozygous and homozygous HPFH175 mice have increased reticulocytosis, RBC distribution widths (RDW) and splenomegaly; however the values for homozygous HPFH175 animals suggest a more severe anemia. One of the features of RBCs is their uniformity of shape and size which can be seen in the blood smear of the HbA animals (Figure 3, top left). There is significant variation in the size of the circulating red cells from the HPFH175 mice. The HPFH175 mice had an average RDW of 29.1%, a value 50% higher than the controls. The hemoglobin content of the RBCs is lower than normal and can be observed by the pallor of the cells in the blood smear (Figure 3).

The longevity of the HPFH175 mice belied their diseased state. Extramedullary erythropoiesis is a hallmark of CA and is apparent in the HPFH175 line although to a much lesser extent than untransfused $\gamma^{HPFH117}\delta\beta^{0}$ CA mice.[99] The disruption of the normal mixture of red and white pulp in the spleen to almost exclusively red pulp is a sign that the spleen is once more functioning as an erythroid organ generating red cells to help meet the oxygen demands of the tissues (Figure 3, middle left). The defect in erythropoiesis is also manifested by the enlarged spleen, (Table 1), which was packed with erythroblasts. The spleen in normal mice is less than half a percent of body weight. In comparison, the spleens of HPFH175 mice were an average of 1.7% of body weight, an almost 5-fold increase over normal. Another organ which is usually affected in CA is the liver, as evidenced by clusters of erythropoietic cells (Figure 3, middle right). The HPFH175 animal has some extramedullary erythropoiesis in the liver which is absent in the normal mouse liver (Figure 3, middle right). The liver is also known to be a repository of excess iron which can't be metabolized and excreted from the body. Consistent with the phenotype of anemia and ineffective erythropoiesis, the liver of the mice also displayed some evidence of iron loading (Figure 3, far right). Taken together, these data show that the HPFH175 mice retain some thalassemic pathology despite appearing outwardly healthy. Despite these thalassemic pathology, this new HPFH175 model has markedly improved hematological indices and histopathology compared to $\gamma^{HPFH117}\delta\beta^{0}$ CA mice accounting for their survivability.

Table 1. Hematological indices of HbA, heterozygous and HPFH175 mice. All mice were analyzed 8 to 9 weeks after birth. Values represent Mean ± SEM. Statistical significances were determined for the $\gamma^{-175HPFH}\beta^0$ globin KI mice compared to the $\gamma\beta^A/\gamma\beta^A$ control mice. *P* values were calculated by the two-tailed student *t* test. RBC represents red blood cell; HGB, hemoglobin; Hct, hematocrit; RDW, red cell distribution width; fl, femtoliter. * *p<.05*, ** *p<.001*, *** *p<.0001*, $^{n.s.}$ *not significant*

Genotype	n	RBC (10^6/μL)	HGB (g/dL)	Hct (%)	Retics (%)	RDW (%)	Spleen (%)
$\alpha_2\alpha_1/\alpha_2\alpha_1$ $\gamma\beta^A/\gamma\beta^A$	10	10.5 ± 0.1	13.7 ± 0.2	44.8 ± 0.5	2.2 ± 0.2	18 ± 0.1	0.34 ± 0.01
$\alpha_2\alpha_1/\alpha_2\alpha_1$ $\gamma^{-175}\beta^0/\gamma\beta^A$	10	10.8 ± 0.2 $^{n.s.}$	13.3 ± 0.2 $^{n.s.}$	43.9±0.6 $^{n.s.}$	3.8 ± 0.3 **	19.3 ± 0.1 ***	0.42 ± 0.03 *
$\alpha_2\alpha_1/\alpha_2\alpha_1$ $\gamma^{-175}\beta^0/\gamma^{-175}\beta^0$	5	9.4 ± 0.3 **	9.1 ± 0.4 ***	37 ± 1.7 **	27 ± 3 ***	29.1 ± 1.3 ***	2.7 ± 0.3 ***

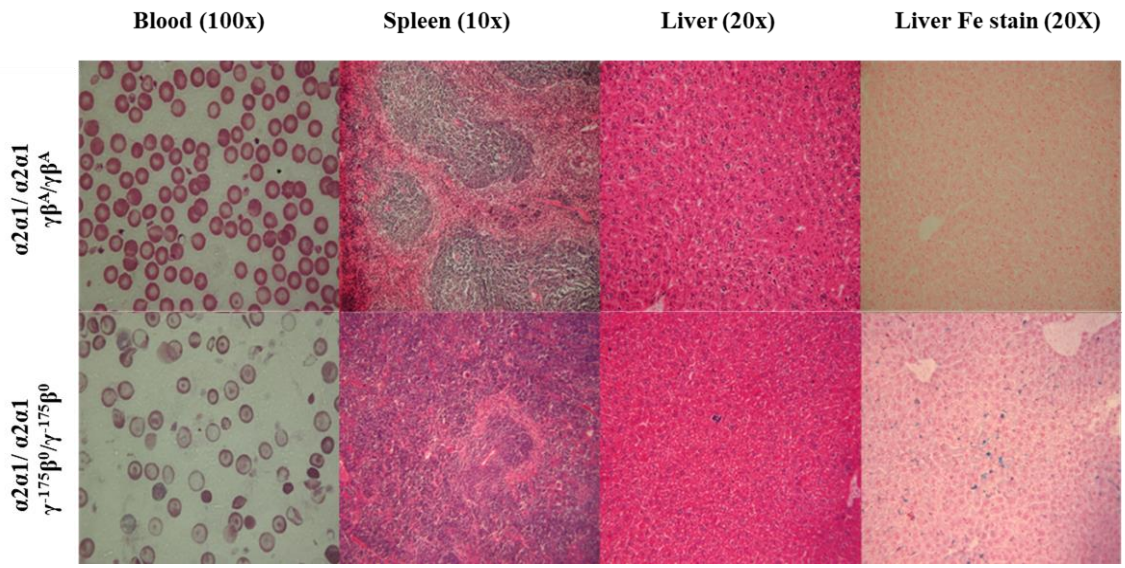

Figure 3. Histological staining of tissues comparing the HPFH175 mouse to HbA mouse. The first panel shows representative blood smears obtained from the two lines. The HbA animals have well-formed RBCs with the characteristic biconcave disc shape. There are no erythroblasts or reticulocytes visible. The spleen architecture, liver and iron staining show no signs of dysregulation. The HPFH175 animal has hypochromic RBCs along with circulating reticulocytes and erythroblasts. The spleen though enlarged and containing erythroid cells, does have observable white pulp. The liver contains almost no erythroblasts and very little, though noticeable amounts of iron in the organ.

HPFH175 is a Pancellular HPFH in Mice

The amount of γ-globin expressed from the Black HPFH allele remained constant throughout adulthood in HPFH175 mice based on analysis of compound heterozygotes, (Fig 2A). This was consistent with the amounts of HbF observed in humans carrying this particular HPFH mutation. We next sought to determine whether the distribution of the γ-globin chains within the circulating mouse RBCs would be similar to that observed in human RBCs. It has been reported that the HPFH 175 mutation causes pancellular HbF expression and results in much larger amounts of γ-globin chains.[29,153] Red blood cells were collected from adult mice, fixed, permeabilized, stained with fluorescently conjugated anti-HbF antibody and analyzed by flow cytometry. Control HbA mice showed only background levels of HbF and F cell staining, (Figure 4A). In contrast, all the HPFH175 RBCs stain positively for HbF, (Figure 4B). This model demonstrates the first pancellular expression of human HbF in an adult mouse.

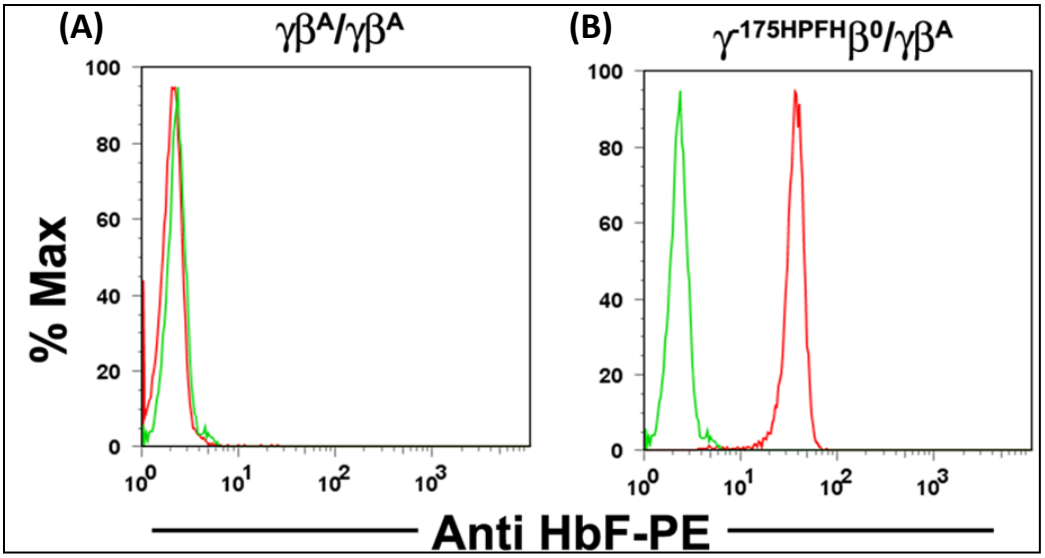

Figure 4. HPFH175 results in pancellular fetal hemoglobin distribution. Anti-HbF antibody staining of RBCs from HbA (($\gamma\beta^{A/}$ $\gamma\beta^{A}$) mice and a compound heterozygote for the $\gamma^{HPFH175}\beta^{0}$ KI allele ($\gamma^{HPFH175}\beta^{0}$/ $\gamma\beta^{A}$) shows that 100% of RBCs stain positively for HbF in the compound heterozygote.

Discussion

Thalassemias and other β-hemoglobinopathies affect millions of individuals worldwide. Despite having been studied for several decades, cures such as allogenic bone marrow transplantation remain expensive, risky and only available to patients with a suitable histocompatible donors. Researchers have characterized several naturally occurring HPFH mutations, many of which exist in the γ-globin promoter, and contribute to high HbF levels, with a potential to render patients transfusion independent for life.[52,148,158] The expression of HbF is normally reduced to <1% by two years of age but HPFH mutations prevent complete silencing thus reducing the chain imbalance responsible for CA phenotype.[26,28] Several of these naturally occurring mutations have revealed a promising direction for researchers to focus their investigations into therapeutic targets for raising HbF.[159]

In this Chapter, we show that the incorporation of the Black HPFH175 mutation in a humanized mouse model of CA is able to block the complete silencing of the fetal genes. Working from the knowledge gained from our previous HbA model demonstrating correct globin switching, we generated mice that express HbF during fetal life and switch to a non-functional β-globin gene.[97,160] CA mice carrying a wild type $^{A}\gamma$-globin gene linked *in cis* to a β^0-globin gene survive to birth but died perinatally upon completion of the fetal-to-adult hemoglobin switch at birth.[157] Similarly, the incorporation of a heterocellularly distributed Greek HPFH allele (-117) in the γ-globin gene promoter extends the postnatal survival of HPFH117 CA mice, but only for a couple of weeks after birth. Therefore, we chose to introduce a different non-deletional HPFH mutation, the Black HPFH allele (-175), into the γ-globin promoter of the $\gamma\beta^0$ hemoglobin switching cassette

to increase HbF levels and further extend the postnatal survival of humanized CA mice. Compound heterozygous $\gamma^{HPFH175}\beta^{0}/\gamma\beta^{A}$ mice express 70% HbF at birth and continue to synthesize high levels of HbF (just over 40%) in adults (Figure 2A). The mice demonstrated pancellular F-cell distribution (Figure 4B), a novel phenotype which has not been observed previously. Homozygous HPFH175 mice though thalassemic, were able to make enough HbF to survive and breed normally with no interventions.

This HPFH175 mouse model represents the first humanized murine model to survive into adulthood on solely human HbF. The reason for the pancellularity was unknown until recently published in an article by Wienert *et al.* It elucidated the molecular mechanism for this HPFH.[32] The authors showed that the T to C mutation introduces a canonical Tal1 binding site (CANNTG) on the reverse strand of the DNA. Tal1 is an important transcription factor for erythroid differentiation, along with its binding partners Lmo2, Ldb1 and Gata1.[161] The presence of this new Tal1 site enables the recruitment of the LCR to the γ-globin promoter, thereby increasing $^{A}\gamma$-globin transcription while concurrently decreasing β-globin transcription.[162] This decrease in β-globin is a direct result of the competition model of hemoglobin switching.[96] This finding leads to other questions, including why this new site is not silenced, as other promoter factor-binding sites are during globin switching.

Our HPFH175 model demonstrates that a single point mutation in the γ-globin gene promoter is sufficient to rescue CA mice from lethal anemia, suggesting a novel therapeutic target for CA patients. Current improvements in gene editing technologies may someday enable the incorporation of the Black HPFH mutation in the γ-globin gene promoters in autologous hematopoietic stem cells of CA patients. Likewise, this same

approach would be beneficial for the treatment of sickle cell anemia and many other hemoglobinopathies. These humanized CA models are perfectly poised for testing pharmacological agents and other gene editing techniques to increase further HbF levels and reduce the remaining thalassemia. The presence of human globin genes under the control of the endogenous mouse LCR and regulated by mouse erythroid transcription and epigenetic factors present a novel model of human disease and an opportunity to dissect key mechanisms involved in globin gene regulation and erythroid development and maturation.

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GERMLINE GENE EDITING OF THE BCL11A ERYTHROID ENHANCER IN HUMANIZED HEMOGLOBIN MICE RESULTS IN HETEROCELLULAR HPFH

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Introduction

Hemoglobin molecules are comprised of two different protein moieties, α-like and β-like chains, which are transcribed from α-like and β-like genes. These genes are grouped together into two globin loci located on human chromosome 16 and 11, respectively. The exact chain composition of hemoglobin changes through development. In humans, the first globins to be expressed are the ζ-globin and ε-globin chains (the embryonic α- and β-like chains) in the primitive red blood cells (RBCs) derived from the yolk sac blood islands. When the site of erythropoiesis shifts to the fetal liver, the newly derived erythroid cells express fetal hemoglobin (HbF), comprised of two α- and γ-globin chains ($\alpha_2\gamma_2$). The fetal chains are the predominant chains expressed throughout gestation until right before birth. The hematopoietic stem cells (HSCs) which seed the bone marrow begin to generate erythroid cells which express the adult β-like globin chains, forming HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$) tetramers.[24]

This switch is completed during the first year of life and, as such, the persistent but continuously waning level of HbF after birth can mask the presence of hemoglobinopathies such as sickle cell disease (SCD) and Cooley's Anemia (CA). Disease symptoms are invisible at birth and only become apparent once the falling HbF levels results in

a reduction in the steady state levels of RBCs below a threshold where the oxygen supplied to the tissues is insufficient for normal function, resulting in anemia.

In the human population there are a subset of individuals that can continue to express high levels of fetal hemoglobin well into adulthood. This completely benign condition is called hereditary persistence of fetal hemoglobin (HPFH) and the adult HbF levels can vary from 1% to over 35% of total hemoglobin in different individuals.[29,163] The expression of HbF may be restricted to a subset of circulating RBCs (heterocellular) or be present in all of the cells (pancellular). Several HPFH mutations have been mapped to the β-globin locus, mostly in the γ-globin promoter but other unmapped mutations remain to be examined.[163] HPFH has been shown to ameliorate the symptoms of both SCD and CA due to its anti-sickling properties of γ-globin while providing binding partners for the excess α-globin chains, respectively.

Recently, through two genome wide association studies (GWAS), one of a large cohort of Sardinians, single nucleotide polymorphisms (SNPs) in two genomic loci have been identified. These SNPs contribute to the incomplete silencing of the fetal globins.[37,39,152] The SNPs are located in BCL11a intron 2 and in an intergenic region between HBS1L and c-MYB. BCL11a is a zinc finger transcription factor which has emerged as a front runner in the search for a master regulator of fetal-to-adult hemoglobin switching.[37,39-41] Though an incomplete picture exists as to the role of BCL11a in globin switching, we know definitively that BCL11a binds several regions in the human β-globin locus and that erythroid specific factors regulate the expression levels of BCL11a in erythroid cells through binding to erythroid specific enhancers (ESE) in intron 2.[36,45,46,164] This ESE is delineated by three erythroid-specific DNaseI hypersensitive sites

that extend over 10 kb in intron 2 of the Bcl11a gene in both humans and mice (Figure 2, page 8). Furthermore, low BCL11A expression is correlated to high HbF expression in cell lines. In Bcl11a conditional knockout mice, embryonic-to-adult hemoglobin switching is delayed further into development.[36,40,41,45,46,164] This information has made BCL11a, and especially its ESE, a very attractive candidate target for increasing HbF levels in SCD and CA patients through gene editing, for whom increases of HbF have been shown to ameliorate disease symptoms.[45,46] Before testing gene editing technology as therapies in the clinic, exhaustive studies should be conducted in animal models and cell lines to assess their safety and efficacy. In this study, we conducted gene editing experiments in our fully humanized mouse models to assess the therapeutic value of mutating the ESE of Bcl11a.[97]

The CRISPR/Cas9 endonuclease system has recently found prominence as an inexpensive and easily programmable gene editing tool. This protein-RNA complex was first discovered in bacteria and later characterized as a bacterial adaptive immune response system.[119,120] The RNP system has since been optimized for use in eukaryotic cells, plants and animals.[165,166] The endonuclease forms a complex with an RNA molecule that possesses a programmable 17-20 nucleotide sequence complementary to the DNA target sequence of interest in the genome. There is a restrictive mechanism that requires a trinucleotide protospacer adjacent motif (PAM), NGG, at the 3' end of the target on the non-target DNA strand. It serves as a green light once Cas9 and the RNA find a complementary DNA target sequence, giving the go ahead signal for cleavage of both DNA strands using two separate endonuclease domains.[119]

Several genetic variants within the BCL11a intron have been associated with elevated HbF levels. One such variant was rs1427407, and it lies within the human +62 kb DNase1 hypersensitive site which has been characterized as an erythroid factor binding region and controls BCL11a expression levels in erythroid cells. The rs1427407 variant has been associated with elevated HbF levels in SCD patients.[45,167,168]

Experiments utilizing yeast artificial chromosome (YAC) transgenic mice that contain the entire human β-globin locus demonstrated the ability of Bcl11a to delay embryonic-to-adult globin switching of the human genes.[169,170] We identified the rs1427407 orthologous DNA segment in the Bcl11a ESE in mice as a place to start defining whether Bcl11a is a regulator of hemoglobin switching in humanized mice that contain a human fetal-to-adult hemoglobin switching cassette knocked into the endogenous mouse β-globin loci. We hypothesized that mutation of the ESE in intron 2 of Bcl11a will result in decreased expression of Bcl11a specifically in erythroid cells leading to persistent expression of HbF in humanized HbA mice.

Materials and Methods

Cas9 mRNA and In Vitro Transcription of sgRNA

Cas9 mRNA was purchased from TriLink Biotechnologies. gRNA cloning vector plasmid **(**Addgene 41824) was purchased from Addgene and modified using oligonucleotides containing two BsmBI restriction sites according to instructions. Bcl11a sgRNA1 and sgRNA2 plasmids were constructed by linearization of the modified cloning plasmid with BsmBI. A pair of complementary 23 bp oligonucleotides (Integrated DNA Technologies) corresponding to each target (with 4 bp overhang for ligation) were annealed and ligated into the vector. Bcl11a gRNAs were generated by PCR amplifying the sgRNA sequences with specific primers containing T7 promoters and common reverse primers (Table 1) (Integrated DNA Technologies). PCR templates were *in vitro* transcribed (IVT) using MEGAshortscript T7 kit (Life Technologies) according to manufacturer's instructions. IVT gRNAs were purified and resuspended in TE buffer (Tris-10 mM, 0.1 m mM EDTA), and quantified by NanoDrop (ThermoFisher Scientific).

Generation of Bcl11a Erythroid Specific Enhancer Deletion Mice

Superovulated (PMSG 5U/mouse T-72 hours and HCG 5U/mouse at T-24 hours) sexually mature female 3-week old HbA mice were mated with stud male HbA mice of overnight. Embryos were isolated from the oviducts of superovulated female mice by flushing the oviducts with culture medium. Hyaluronidase was used to disaggregate granulosa cells and release embryos into the media. The embryos were transferred to small petri dishes with KSOM (Millipore). One-cell stage fertilized embryos were identified. They were microinjected with a mixture of Cas9 RNA (100 ng/μL) and sgRNA (50

ng/μL) then, incubated overnight. Embryos that developed to the two-cell stage were injected into pseudo pregnant female CD1 mice.

DNA Isolation and Genotyping

Tail biopsies were clipped from pups and incubated in 500 μL homogenization solution (10 mM Tris-CL, 20 mM EDTA, 100 mM NaCl, 1% SDS, pH 8.0) with Proteinase K (0.6 mg/ml) (Omega Bio-tek, Norcross, GA) at 65 °C overnight. After the samples cooled, 300 μL was aliquoted into a new Eppendorf tube and 400 μL phenol- chloroform (1:1) was added. The samples were shaken and centrifuged at 13, 000 rpm for 10 minutes at 4 °C, the aqueous phase was transferred to a new tube and 400 μL chloroform was added, shaken and centrifuged at 13, 000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and two volumes of 100 % ethanol added. The tube was shaken vigorously and incubated at -20 °C (for 30 minutes or overnight). The DNA/ethanol solution was centrifuged at 13, 000 rpm for 10 minutes. The supernatant was aspirated and the DNA pellet was washed with 70 % ethanol. The DNA was resuspended in sterile 1x TE buffer. DNA samples were stored at -20 °C.

PCR reactions were performed with Bulldog BioReady rTaq according to manufacturer's protocol using primers specific to the DNA region being analyzed (Table 2). Each PCR reaction contained 0.125 μL Bulldog BioReady rTaq, 2.5 μL of 10x Reaction Buffer (with 15 mM $MgCl_2$), 0.5 μL of 10 mM dNTP (New England Biolabs), 1 μL each of primer (DHS F1 and DHS R1), 1 μL DNA and water to 25 μL. The PCR conditions used were as follows: 94 °C for 5min, 35x (94 °C for 30s, 54 °C for 30s, 72 °C for 5

mins), 72 °C for 10 mins, 4 °C forever. A small aliquot (~5 µL) of each PCR product was run on a 2% agarose gel (1x TBE).

RFLP Analysis and TA Cloning

Restriction enzyme reactions were assembled in PCR tubes follows: 16.5 µL sterile water, 2,5 µL 10 Cutsmart® Buffer, 1 µL restriction enzyme (BsaXI or MnlI) (New England Biolabs) and 5 µL PCR product and, incubated at 37 °C for at least one hour. Digestion products were analyzed on 2% agarose gel.

PCR products for individual mice were cloned using pCR2.1®TOPO-TA Cloning® kit (Invitrogen) according to manufacturer's instructions. The cloning reaction mixture was incubated at room temperature for 30 minutes and five microliters of the cloning reaction was used to transform chemically competent DH5α *E. coli* bacterial cells. The transformed cells were plated on agar plates with ampicillin (100 µg/mL) added for selection. Clones were picked and the DNA isolated was sent for sequencing.

Hematological Indices and Histopathology

Peripheral blood was obtained from anesthetized mice and used to make blood smears stained with Dip Quick kit (Jorgensen Laboratories, Loveland, CO). RBC counts and red cell distribution widths were measured on a HemaVet1700 (Drew Scientific, Waterbury, CT).

Flow Cytometry

Peripheral blood was collected from mouse tail tips using heparinized microhematocrit capillary tubes (Fisher Scientific**)** and deposited into 100 µl of DPBS with

0.05 μM EDTA. The blood was centrifuged at 500g for 3 mins at room temperature in a tabletop centrifuge. The blood was washed by resuspending in 500 μl PBS and centrifuged. The supernatant was aspirated and the blood was fixed by incubation in 500 μl 0.05 % glutaraldehyde (50 % stock solution was diluted 1:1000 in PBS) for 10 minutes at RT. The sample was centrifuged as previously described and the cells were permeabilized by incubation in 500 μL 0.1 % TritonX-100 for 10 minutes at RT. The cells were washed with 500 μL PBS. 1x 10^6 cells were diluted into 100 μL PBS and 0.25 μL of PE conjugated mouse anti-human fetal hemoglobin antibody (BD Biosciences) was added to the cell suspension, mixed and incubated at RT for 30 minutes. The cells were washed with 500 μL PBS and resuspended in 300 μL of PBS. Reticulocytes were detected by staining with Thiazole orange (0.1 μg/ml) (Sigma) at room temperature for 15 minutes. The cells were analyzed by flow cytometry (BD FACSCalibur (BD Biosciences).

Bone marrow mononuclear cells were collected and washed in DPBS. The cells were stained with the following antibodies: TER119-APC, CD71-PE B220-APC Cy7 and CD19-PE (BD Biosciences) and incubated on ice in DPBS/0.5%BSA for 30 minutes according to manufacturer's protocol. After 15 minutes of incubation, 7AAD viability staining solution (eBioscience) was added. The cells were centrifuged, supernatant discarded and washed with 1ml DPBS/0.5%BSA before sorting. Cells were sorted on BD FACSAria II (BD Biosciences).

RNA Extraction, cDNA Synthesis and qRT-PCR Analysis

Peripheral blood was collected from mouse tail tips and RNA was extracted using Trizol LS reagent (Ambion). RBCs were collected in DPBS/0.5 mM EDTA. The cells

were washed and resuspended in 50 µL DPBS and 150 µL Trizol LS was added. The tubes were shaken and incubated at room temperature for at least 10 minutes (or stored at -80 °C until it was needed). 40 µL of chloroform was added to the tubes which were shaken and incubated for a further 10 minutes at room temperature. The samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new tube and 100 µL of isopropanol was added. The sample was shaken and incubated at room temperature for 10 minutes or -20 °C overnight. The Samples were centrifuged at 13,000 rpm for 10 minutes at 4 °C, washed with 1 mL 70 % ethanol and resuspended in 20-40 µL sterile water. RNA samples were stored at -80 °C.

cDNA was synthesized using the High capacity cDNA kit (Applied Biosystems) according to manufacturer's protocol. For globin chain qRT-PCR reactions, cDNA samples were diluted 1:100 and for Bcll11a dilutions were 1:10. Each individual qRT-PCR reaction contained 3 µL diluted cDNA, 10 µL Power Up™ Sybr® Green Master Mix, 0.5 µM each primer and sterile water to 20 µL. The samples were loaded in triplicate and the plate was run on the Applied Biosystems® ViiA7 (Life Technologies) and the data was analyzed using QuantStudio ™ Real-Time PCR software (AB by ThermoFisher Scientific).

One million (1 x 10^6) erythroblasts ($TER119^+$ $CD71^+$ $CD19^-$ $B220^-$) and 1 x 10^6 B-cells ($CD19^+$ $B220^+$ $Ter119^-$) were sorted by flow cytometry. The cells were centrifuged and supernatant was aspirated. The cells were resuspended in 50 µL and 150 µL Trizol LS was added. RNA was isolated as previously described above and resuspended in 20-40 µL sterile water. RNA samples were stored at -80 °C.

Next Generation Sequencing

Amplicon library was prepared by amplifying sgRNA target sites and selected off target sites (Table 3) with Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The PCR conditions used were as follows: 94 °C for 2 minutes, 35x (94 °C for 15s, 54 °C for 30s, 68 °C for 40s), 68 °C for 10 minutes, 4 °C forever. The PCR products were purified using E.Z.N.A.® cycle pure kit (Omega Bio-tek) and Illumina adapters were ligated to the purified PCR products. A second round of PCR was performed to amplify ligation products. PCR products were first purified with E.Z.N.A.® cycle pure kit (Omega Bio-tek) and then with magnetic beads (Aline Biosciences). The DNA was submitted to UAB Genomic and Genetics Core for paired end sequencing (Illumina MiSeq System). The sequences were analyzed using CRISPResso Software (Luca Pinello) and IGV2.3 (Broad Institute of MIT and Harvard, Boston, MA).

Statistical Analysis

P values were calculated by the two tailed Student's *t* test.

Table 1. Oligonucleotides used to generate sgRNA plasmids and *in vitro* transcribed RNA.

sgRNA Name	Oligonucleotide Name	Oligonucleotide Sequence
Bcl11a sgRNA1	Bcl11a DHS gRNA1W	ACCGAGTGGCTGTTGAAAGAGGG
	Bcl11a DHS gRNA1C	AAACCCCTCTTTCAACAGCCACT
Bcl11a sgRNA2	Bcl11a DHS gRNA2W	ACCGATGGAGTGGCTGTTGAAAG
	Bcl11a DHS gRNA2C	AAACCTTTCAACAGCCACTCCAT
Bcl11a sgRNA1	T7 DHS1	TAATACGACTCACTATAGGAGTGGCTGTTGAAAGAGGG
Bcl11a sgRNA2	T7 DHS2	TAATACGACTCACTATAGGATGGAGTGGCTGTTGAAAG
	sgRNA REV	AAAAGCACCGACTCGGTGCC

Table 2. Primers for genotyping mice at Bcl11a intron 2.

Gene Name	Primer Name	Forward Primer	Reverse Primer
Bcl11a	DHS1	GTGCATGGTTGAATCACAGC	CACTCAAGGAATGCAAGCAA
Bcl11a	DHS2	TTGAATGAAGTTGGGCTTCAC	ACCACAAACAGTCCCAGTCC
Bcl11a	DHS3	AGGGCTATGCTTGGTTGTCGC	ACAAAGATGGTCCCCATCCAGC
Bcl11a	DHS4	TCAGACTTCATCACTCAAATGTGG	CCAGATAAAAAGCGGCGAGTG
Bcl11a	DHS5	TTCCATGGGAGGAAGCACTA	CATGCCCTGGGTACAACAC
Bcl11a	DHS6	ACAGGCTGAGGAGGCATGTA	CCTGTTCACCCCCAAATAGA

Table 3. Primers used for amplifying off-target sites.

Target	Genome Location (mm9)	Gene Name	Forward Primer	Reverse Primer
sgRNA1 OTS #1	chr1:172400730-172401027	Nos1ap (intron 2)	ACACAAGCGCTCACAGTCAC	TTCCTCAACTGAAGCTCCTTTC
sgRNA1OTS #2	chr1:165626036-165626328	intergenic	CAAGCAGGTGCTAGGGAAAG	TCTCTCTGTAGCAAGCCATCAA
sgRNA1OTS #3	chr12:67101073-67101347	intergenic	TTCCCTGCATCCTGATAACCC	GGCAACTTAAAGGCAGAAAGC
sgRNA1 OTS #4	chr13:110528876-110529175	Pde4d (intron 1)	TCCCTTTTTGCTTTCACACCC	CAACAGGGTAGCTGCAATATGA
sgRNA2 OTS #1	chr11:117883634-117883923	intergenic	TGTGTGTTAGCTTGGCATCAG	AGTCACACACACCCTGCAAA

Table 4. Primers used for qRT-PCR reactions.

Gene Name	Primer Name	Forward Primer	Reverse Primer
Mouse Hb-εy	m.Hb-ey EVA-2	GGTGAACTTTACTGCTGAGG	CAACAAGAAGCCTTCCCAA
Mouse Hb-βh1	m.Hb-bh1 EVA-1	GAGAAGGCAGCTATCACAAG	AAACAATCAGGAGCCTTCC
Human Hbγ	h.HbG EVA-4	CTTCAAGCTCCTGGGAAAT	CTGCAGTCACCATCTTCTG
Human Hbβ	h.HbB EVA-4	TCGGTGCCTTTAGTGATGG	CACACAGACCAGCACGTTG
Mouse Hbζ	m.HbX EVA-2	CAACCTCTCTAGTGCTTTGAC	GACAGGAGCTTGAAGTTGAC
Human Hbα	h.HbA EVA-1	CGACAAGACCAACGTCAA	ACAGGAACATCCTCTCCA
Bcl11a	Bcl11a SyF2	ACAGGAACACATAGCAGATAAA	CTGCTGGGCTCATCTTTAC
CD19	CD19	AGTGATTGTCAATGTCTCAGACC	CTCCCCACTATCCTCCACGTT
β-actin	β-actin	CCAACCGTGAAAAGATGACC	ACCAGAGGCATACAGGGACA

Results

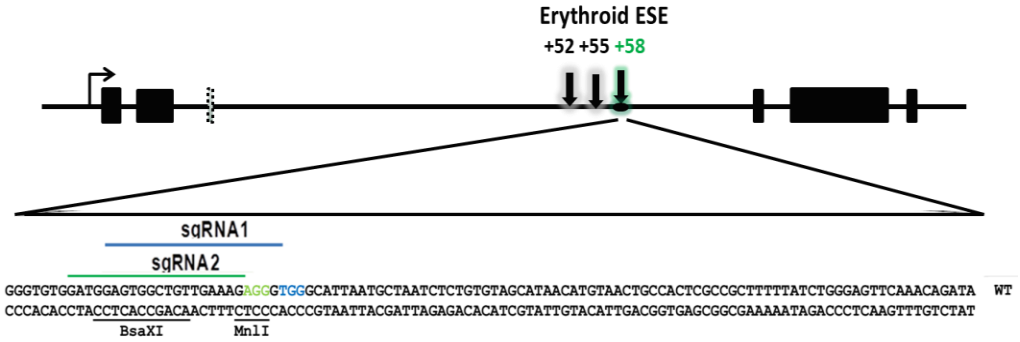
CRISPR/Cas9 Introduces Mutations into Bcl11a Intron Target

Two overlapping sgRNAs were designed to target +58 kb DNaseI hypersensitive site in the second intron of Bcl11a (Figure 2, page 8 and 1A). The target site chosen has previously been shown to contain putative binding sites for several erythroid transcription factors and is orthologous to a similar region in humans, which contains HPFH associated SNPs.[46] The chosen sgRNA DNA target sequences also overlap two restriction enzyme sites which could be used to screen animals for on-target cleavage efficiency (Figure 1A). Previous experiments evaluating the role of Bcl11a in globin switching have been largely carried out in mice carrying a transgenic human β-globin locus or transformed primary human cell lines[40,41,46,47]. In this study, a fully humanized mouse model (henceforth referred to as HbA mice), generated by our group that has the human globin genes knocked into the endogenous mouse α- and β-globin loci (α2α1/ α2α1 $\gamma\beta^A$/ $\gamma\beta^A$) was used to investigate the effect of small enhancer deletions on hemoglobin switching.[97] In these mice, all mouse adult α- and β-globin genes were replaced by human α-globin and a fetal-to-adult (γ-to-β) hemoglobin switching cassette. Humanized HbA mice synthesize high levels of HbF during fetal life and complete the switch to HbA postnatally.

Germline gene editing experiments were performed in HbA embryos in order to examine the *in vivo* effect on hemoglobin switching in the lines of HbA mice bearing specific mutations of the Bcl11a ESE. Three week old HbA female mice were superovulated and bred with HbA stud males to generate fertilized eggs. These 0.5 day old single celled HbA embryos were microinjected with one of two *in vitro* transcribed sgRNAs

(Figure 1B) and Cas 9 mRNA. The embryos were cultured overnight to the two-cell stage and transplanted into the oviducts of pseudo pregnant female mice. Of the 125 embryos transferred, 21 live born mice were obtained (16.8%) (Figure 1B). An initial screen of PCR amplified tail DNA biopsied from the 21 founder animals using a restriction fragment length polymorphism (RFLP) assay revealed that most of the animals contained on target site mutations (data not shown). Target site amplicons from each founder animal was subcloned and sequenced. Several of the 21 founders were mosaic having more than one mutated allele (Figure 1C). A summary of all mutations mapped in the founders can be found in Table 5. These data demonstrate that the CRISPR/Cas9 endonuclease system was efficient at generating wide ranging indels in the Bcl11a ESE in HbA mouse embryos.

(A)



(B)

	sgRNA 1	sgRNA 2
Eggs collected	260	
Fertilized/injected eggs	134	
2-cell stage embryos	62	63
Number of embryos transferred	47 (3)	48 (3)
Live Births	10	11

(C)

```
GGGTGTGGATGGAGTGGCTGTTGAAAGAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA     WT
CCCACACCTACCTCACCGACAACTTTCTCCCACCCGTAATTACGATTAGAGACACATCGTATTGTACATTGACGGTGAGCGGCGAAAAATAGACCCTCAAGTTTGTCTAT
                BsaXI      MnlI

TGGGTG----------------------------GGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    72-1

TGGGTGGGCATGGAGTGGCTGTTGAAAGAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    72-2

TGGGTGTGGATGGAGTGGC-------------------------------------------------------------------------------------------    73-1

TGGGTGTGGATGGAGTGGCTGT-----------GGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    73-2

TGGGTGTGGATGGAGTGGCTGTTGAAAG-GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    74

TGGGTGTGGATGGAGTGGCTGTTGAAA-------------------------------------------------------------------------------ACAGATA    76-A
                           CGAATCTTAAAATTATCTGGCATTA
TGGGTGTGGATGGAGTGGCTGTTGAAA^TGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA         76-B
                            A
TGGGTGTGGATGGAGTGGCTGTTGAAAGA^GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   77-1
                           AA
TGGGTGTGGATGGAGTGGCTGTTGAAAGA^GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   77-2

TGGGTGTGGATGGA------------------TGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    79

TGGGTGTGGAT------------------GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    80-1

TGGGTGTGGATGGAGTGGCTGTTGAA--AGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    80-2

TGGGTG----------------------------GGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    80-3

TGGGTGTGGATGGAGTGGCTGTT------GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    81-1
                             TGGCT
TGGGTGTGGATGGAGTGGCTGTTGAAAG^AGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   81-2
```

```
GGGTGTGGATGGAGTGGCTGTTGAAAGAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA     WT
CCCACACCTACCTCACCGACAACTTTCTCCCACCCGTAATTACGATTAGAGACACATCGTATTGTACATTGACGGTGAGCGGCGAAAAATAGACCCTCAAGTTTGTCTAT
                BsaXI      MnlI

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TGGGTGTGGATGGAGTGG-------------GTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    82-2

TGGGTGTGGAT------------------GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    83

TGGGTGTGGATGGAGT------------------GGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    84-1

TGGGTGTGGATGGAGTG----------GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    84-2

TGGGTGTGGATGGAGTGGCT----------GGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    84-3
                           A
TGGGTGTGGATGGAGTGGCTGTTGAAA^GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   85
                           A
TGGGTGTGGATGGAGTGGCTGTTGAAA^GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   86
                         AC
TGGGTGTGGATGGAGTGGCTGTTG^AAAGAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   87-1

TGGGTGTGGATGGAGTGGCTGTTGA--------GGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    87-2
                           A
TGGGTGTGGATGGAGTGGCTGTTGAAA^GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   88-1

TGGGT--------------------------GTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    88-2

TGGGTGTGGAT------------------GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    89-1

TGGGTGTGGATGGAGTGGCTGTTTAAAGAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    89-2
                           A
TGGGTGTGGATGGAGTGGCTGTTGAAA^GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   89-3

T---------------------------GGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA    89-4
                           A
TGGGTGTGGATGGAGTGGCTGTTGAAA^GAGGGTGGGCATTAATGCTAATCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   91

-------------------------------------------------TCTCTGTGTAGCATAACATGTAACTGCCACTCGCCGCTTTTTATCTGGGAGTTCAAACAGATA   92
```

Figure 1. CRISPR/Cas9 endonuclease system produces a diverse range of on-target indels in the Bcl11a intron. (A) Schematic representation of Bcl11a gene with exons shown in solid black boxes and targeted region at +58 kb highlighted by green arrow. Intronic sequence shows sgRNA targets with restriction enzyme sites underlined. (B) Table showing the total number of embryos isolated and injected with the individuals sgRNAs. The injected embryos were allowed to develop to the two-cell stage overnight and subsequently transferred into pseudo pregnant females. Live born mice were delivered naturally or via C-section. (C)Mutations mapped in each mouse born from the injection experiment. Mouse ID numbers I7772-81 were injected with SgRNA1 and I7782-92 were from the sgRNA2 injection. Several of the mice from both sgRNA injections have more than one mutated allele, in addition to the wild type Bcl11a allele (not shown).

Table 5. Summary table showing the individual mutations in the target region mapped in each HbA founder mouse born from the germline gene editing experiment.
(-: deletions, +: insertions)

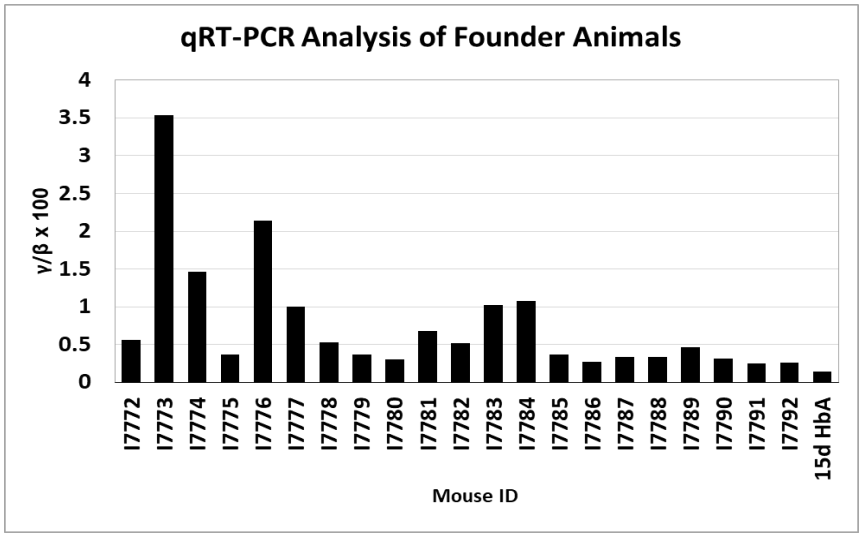
Founder ID #	Mapped on-target mutations
I7772	-28 bp, 2 SNPs
I7773	-11 bp, -702 bp
I7774	-1 bp
I7776	+25 bp, -77 bp
I7777	+1bp, +2 bp
I7779	-18bp
I7780	-2 bp, -18 bp, -28 bp
I7781	-6 bp, +5 bp
I7782	-13 bp, -1 bp
I7783	-18 bp,
I7784	-18 bp, -10 bp, -10 bp
I7785	+1 bp, -18 bp
I7786	+1 bp
I7787	+2 bp, -8 bp
I7788	+1 bp, -26 bp
I7789	SNP, -18 bp, +1 bp. -28 bp
I7791	+1 bp,
I7792	-110 bp

Mutation of the Bcl111a ESE Results in Heterocellular HbF in Gene Edited HbA Mice

Humanized HbA mice complete the fetal-to-adult hemoglobin switch after birth. By weaning age their HbF levels are less than 1% of total hemoglobin and less than 5% of their circulating RBCs are F-cells. Two week old gene edited HbA founder animals and unedited HbA controls were analyzed by qRT-PCR for the ratio of their human γ-to-β-globin mRNA levels in peripheral blood. Half the animals had elevated γ-globin transcripts when compared to the control animals (Figure 2A). At four weeks of age, when the γ-globin gene was silenced to background levels in HbA control mice, the peripheral blood of gene edited founder animals was analyzed for the presence of circulating F-cells. Fluorescently labeled anti-HbF antibody was used to stain RBCs that were then analyzed by flow cytometry. Consistent with the qRT-PCR data, most founder animals had elevated F-cell numbers (18 out of 21), with five well over 10% whereas an age matched HbA animal had only 4.5 % F-cells (Figure 2B). This suggested that the indels in the Bcl11a intron enhancer had successfully contributed to derepress the γ-globin gene, leading to prolonged HbF expression in adult mice. An F-cell analysis done at seven months of age showed that a third of the mice (7 out of 21) continued to express higher HbF amounts in their RBCs than those of control mice (Figure 2C). These data show that small deletions of the Bcl11a ESE in intron 2 are able to elevate γ-globin expression in mosaic founders derived from gene edited HbA embryos.

Interestingly, all but one of the founder animals appeared normal at birth, showed no outward phenotypes and had normal growth rates. The abnormal animal, ID I7781, was born with a unilateral defect in one eye (anopthalmia or microopthalmia), had a slower growth rate than its littermates, and died prior to weaning (data not shown).

(A)


qRT-PCR Analysis of Founder Animals
γ/β x 100
4
3.5
3
2.5
2
1.5
1
0.5
0
I7772
I7773
I7774
I7775
I7776
I7777
I7778
I7779
I7780
I7781
I7782
I7783
I7784
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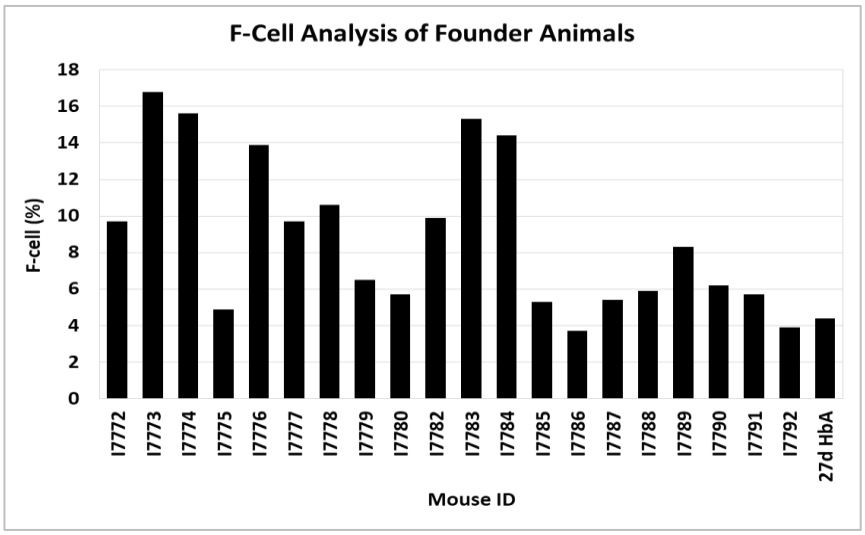
I7787
I7788
I7789
I7790
I7791
I7792
15d HbA
Mouse ID

(B)


F-Cell Analysis of Founder Animals
F-cell (%)
18
16
14
12
10
8
6
4
2
0
I7772
I7773
I7774
I7775
I7776
I7777
I7778
I7779
I7780
I7782
I7783
I7784
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I7787
I7788
I7789
I7790
I7791
I7792
27d HbA
Mouse ID

(C)

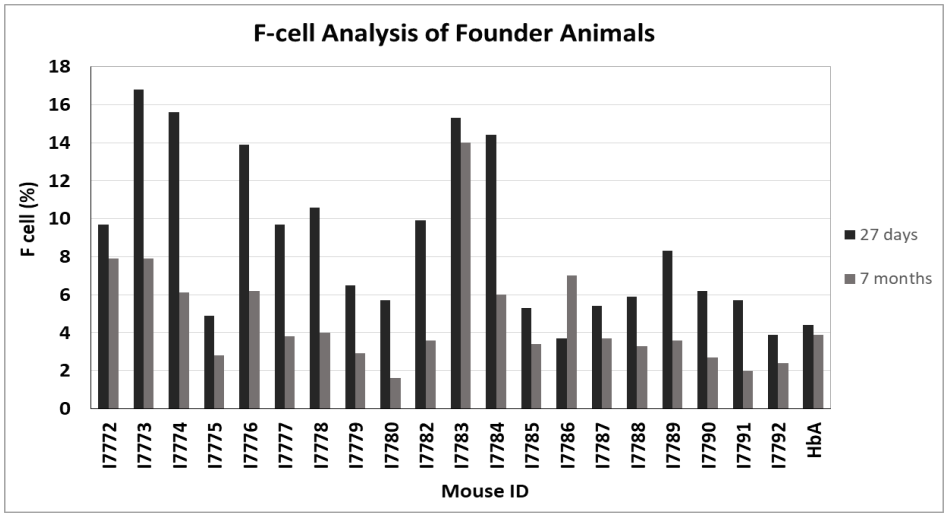
F-cell Analysis of Founder Animals
F cell (%)
18
16
14
12
10
8
6
4
2
0
I7772
I7773
I7774
I7775
I7776
I7777
I7778
I7779
I7780
I7782
I7783
I7784
I7785
I7786
I7787
I7788
I7789
I7790
I7791
I7792
HbA
Mouse ID
27 days
7 months

Figure 2. Germline gene editing results in persistent heterocellular HbF expression in adult mice. (A) RNA prepared from peripheral blood of 15 day old gene edited founder mice was analyzed by qRT-PCR for the ratio of human β-like globin gene expression. By day 15 HbA control mice have γ-globin transcripts below 0.5% of β-globin. All of the founder animals of the same age have higher γ-globin transcript levels. Each assay was conducted in triplicate and the results were averaged. (B) Peripheral blood was collected from gene edited HbA founder mice on day 27 and stained with fluorescently conjugated anti-HbF antibody. Cells were then analyzed via flow cytometry and gated using a C57Bl/6 mouse (no HbF) for the negative stained control. (I7781 is not listed because it was euthanized before this time point; n=4 for HbA control) (C) A comparison of F-cell levels at 27 days and 7 months. An adult HbA mouse (9 weeks old) was used as control.

Mutations Generated by Germline Gene Editing were Transmitted to Offspring and Showed Heterocellular HPFH Phenotype

Since most of the gene edited founders were mosaics demonstrated by the occurrence of both wild type and multiple different target site mutations of the Bcl11a ESE, we bred those founder mice with the highest HbF levels to HbA mice to assess the germline transmission of the individual mutations and measure the contribution of the specific ESE mutations to HbF elevation. All of the founders which were bred were able to successfully generate first generation offspring (F1), demonstrating that the embryo injections and germline editing did not affect their fertility. F1 animals harboring individual ESE indels were brother-sister mated to generate second generation (F2) animals with homozygous ESE mutations. Several of the F2 lines of homozygous animals (8 out of 14) had persistent expression of γ-globin well into adulthood (Figure 3 and Figure 4). Two of the founder animals (I7773 and I7776) produced homozygous F2 lines with far greater levels of HbF and F-cell percentages. Founder I7773 transmitted a large 702 bp deletion and I7776 passed on a 77 bp deletion, both of which extended beyond the 3' end of the sgRNA target site (Figure 1C). Animals homozygous for the 702 bp deletion had F-cell percentages over 30%. The 77 bp deletion (henceforth called $HbA^{\Delta 77/\Delta 77}$) had an average F-cell percentage close to 25% at one month of age. The 702 bp deletion was only recently characterized and will not be discussed further. The $HbA^{\Delta 77/\Delta 77}$ had F-cell amounts that were a 5-fold increase over adult HbA mice which have about 4.5 % F-cells at one month of age (Figure 3).

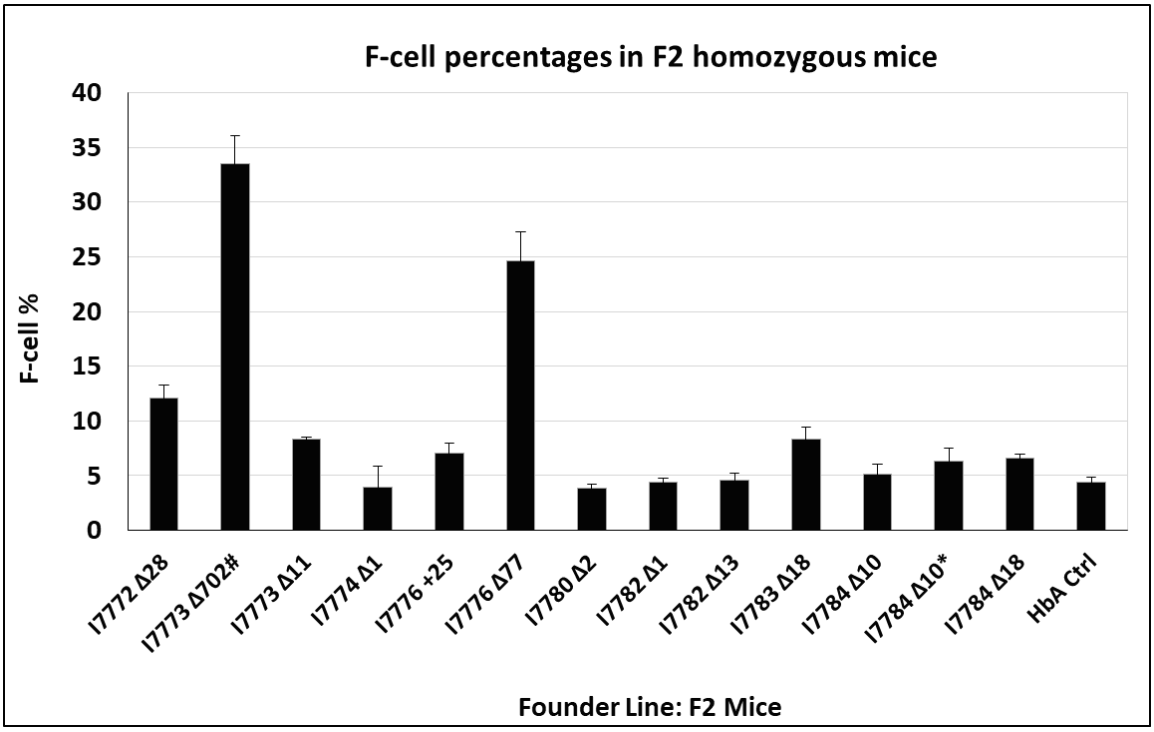

Figure 3. HbA mice with homozygous Bcl11a ESE mutations had heterocellular distribution of HbF. Founder mice with elevated F-cell numbers were bred in order to segregate mutant alleles to generate F2 animals with specific homozygous mutations. Blood was collected from F2 homozygous mutants (n≥4) and stained with anti-HbF antibody and analyzed by flow cytometry. The two lines with the highest F-cell numbers had the largest deletions mapped to date. Mouse were bled after weaning age (between d25 and d44; all mice within groups are born no more than 6 days apart). Results are plotted as averages ± SD.

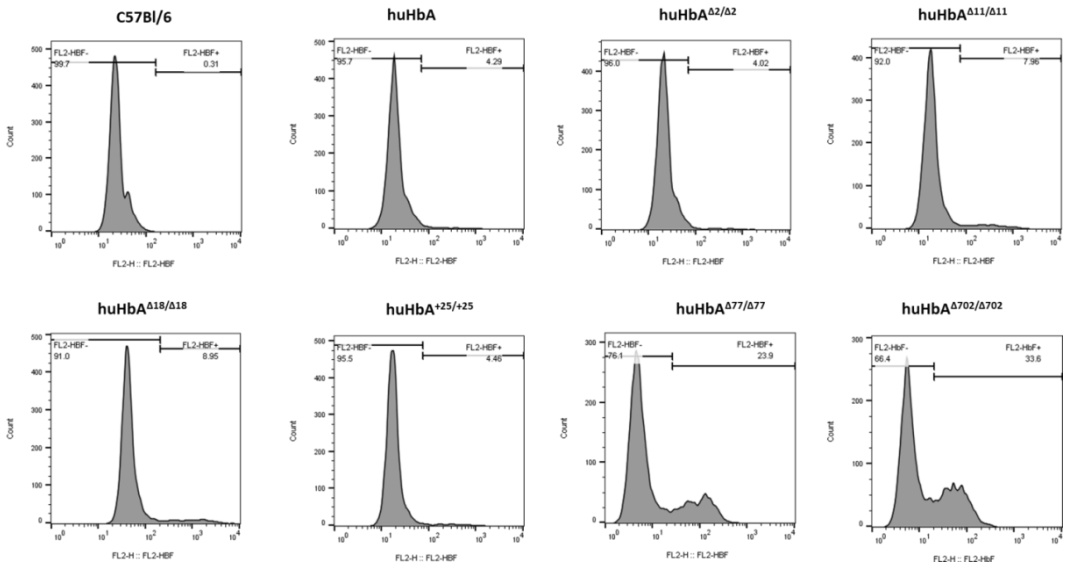

Figure 4. Representative FACS plots for some of the F2 homozygotes. Peripheral blood was stained with anti-HbF antibody and analyzed by flow cytometry. Mouse genotypes are labeled. C57Bl/6 which has no human globins is a negative control. HbA is negative control which has low level HbF expression from switching cassette.

Bcl11a Enhancer Deletions are Erythroid Specific

Bcl11a is a transcription factor involved in several tissue specific functions of neurological and hematopoietic importance.[36,42,171,172] Deletions, translocations and over-expression of certain isoforms have been implicated in several malignancies.[44,164,173-177] In order to assess the erythroid specificity of the Bcl11a deletion, two month old $HbA^{\Delta 77/\Delta 77}$ mice were analyzed and compared to HbA mice. Peripheral blood from both lines were analyzed for reticulocytes and F-cells while sorted bone marrow erythroblasts and B-cells were analyzed by qRT-PCR for globins, Bcl11a, and Cd19 gene expression. The reticulocyte percentages were normal for both groups, with no statistical difference between them (Figure 5A). The F-cell staining showed significant difference in the number of γ-globin expressing cells with the HbA mice at 1.54 % ±0.48 and the $HbA^{\Delta 77/\Delta 77}$ mice having 15.5 % ± 1.08 ($p<0.001$). The F-cell percentages in the mice were lower than had been previously observed and will be addressed later. The relative level of Bcl11a transcription in the erythroid compartment ($Ter119^{+}$ $Cd71^{+}$) was significantly decreased in the $HbA^{\Delta 77/\ \Delta 77}$ mice when compared to the control animals, 0.76 ± 0.32 to 0.35 ± 0.093, a 2-fold decrease (normalized to β-actin) ($p<0.05$) (Figure 5C). The erythroid cells were also analyzed for changes in the expression level of all the globin genes. The γ- as a percentage of β-globin transcripts in the $HbA^{\Delta 77/\ \Delta 77}$ mice was 1.34% ± 0.13 compared to 0.075 % ±0.007 of the wild type HbA mouse, an almost 18-fold increase ($p<0.001$). Surprisingly, we also saw a significant difference in the expression of the embryonic α-like globin, ζ-globin, 0.37 % ± 0.058 (Δ77) vs 0.096 % ± 0.028 (wild type), an almost 4-fold increase in transcripts (Figure 5E). It has previously been shown that Bcl11a binds within the α-globin locus in human erythroid cells but this is the first report of Bcl11a knockdown increasing the ζ-globin transcription level in mice.

Knockout mutations of Bcl11a in mice are perinatal lethal and Bcl11a is required for B-cell development.[42,169] As such, we sought to ensure that the $HbA^{\Delta 77/\Delta 77}$ animal had no hematopoietic dysregulation in their B-cells affecting the erythroid compartment through some unknown mechanism. Bone marrow cells were stained with anti-Ter119, anti-Cd71, anti-B220 and anti-Cd19 antibodies. Anti-B220 and anti-Cd19 double positive B-cells cells were sorted, and the level of Bcl11a and Cd19 transcripts in each population was analyzed for the control HbA and $HbA^{\Delta 77/\Delta 77}$ mice. There were no significant expression differences observed between these two populations for Bcl11a and Cd19 expression (Figures 5F and 5G). These data demonstrate that the intronic enhancer deletion of the Bcl11a gene in the $HbA^{\Delta 77/\Delta 77}$ is erythroid specific.

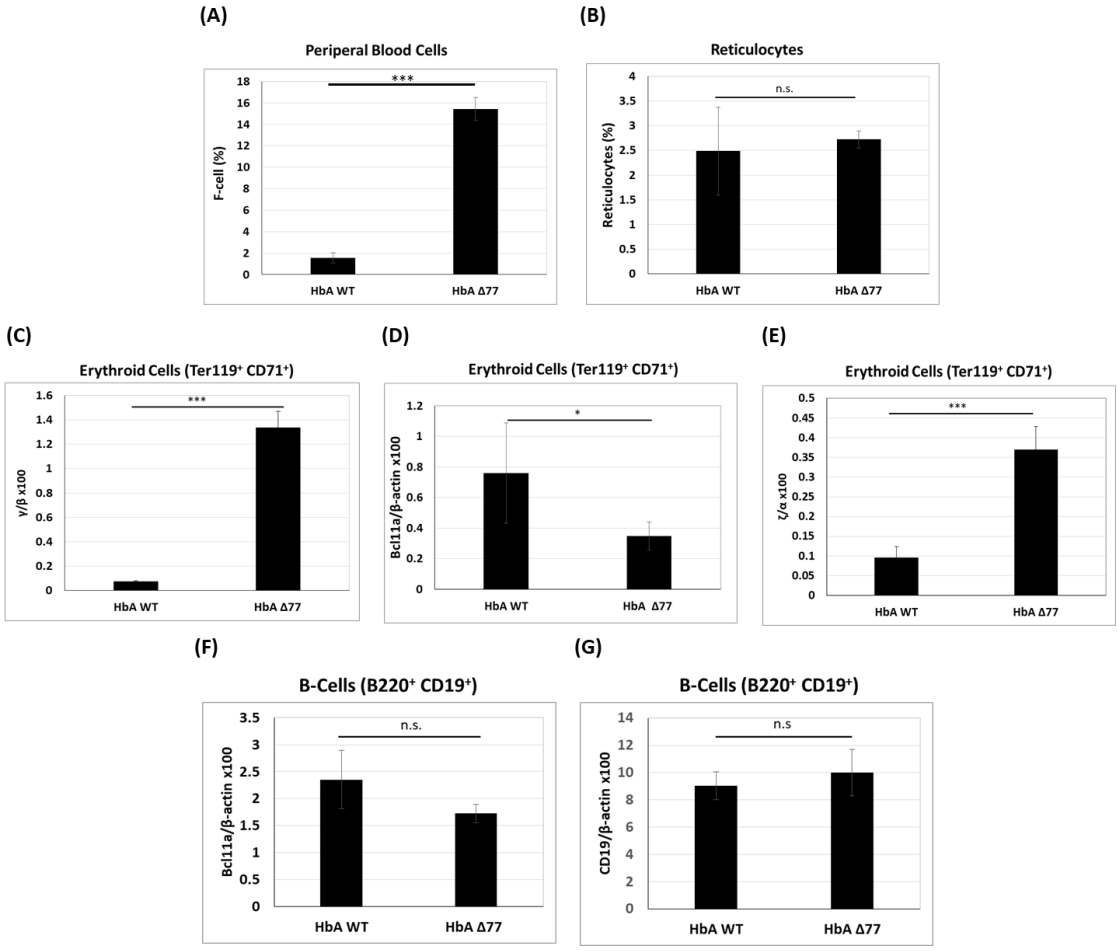

Figure 5. Enhancer deletions in the Bcl11a intron specifically affect erythroid cells. (A-B) Peripheral blood cells (PBCs) were collected from HbA and $HbA^{\Delta 77/\Delta 77}$ mice (n=4 in each cohort) and stained with anti-HbF antibody to measure F-cell percentages. PBCs were also analyzed for circulating reticulocytes by thiazole orange staining. (C-E) qRT-PCR analyses were performed on bone marrow erythroblasts ($Ter119^{+}$, $CD71^{+}$) from HbA and $HbA^{\Delta 77/\Delta 77}$ mice. (F-G) qRT-PCR analyses were performed on bone marrow B-cells from HbA and $HbA^{\Delta 77/\Delta 77}$ mice. Results were plotted as Mean ± SD. (n.s-not significant, *$p<0.05$., ***$p<0.0001$.

Percentage of F-cells in HbA$^{\Delta 77/\Delta 77}$ Mice Decrease with Age

HbA mice usually complete their hemoglobin switch by 8 weeks of age, reducing their circulating F-cell levels to around 2% for the remainder of their lives (Figure 5A). We expected the same to be true for the HbA$^{\Delta 77/\Delta 77}$ line, but were surprised to observe a different trend. RBCs collected from older mice had a significantly lower percentage of F-cells compared to younger mice. The number of F-cells in HbA$^{\Delta 77/\Delta 77}$ mice is highest at birth, and then gradually wanes with age before stabilizing at 21-weeks of age (Figure 6). This observation points to a previously unreported mechanism, by which reduced levels of Bcl11a expression can lead to a long delay in the fetal-to-adult hemoglobin switch in erythroblasts in the adult bone marrow.

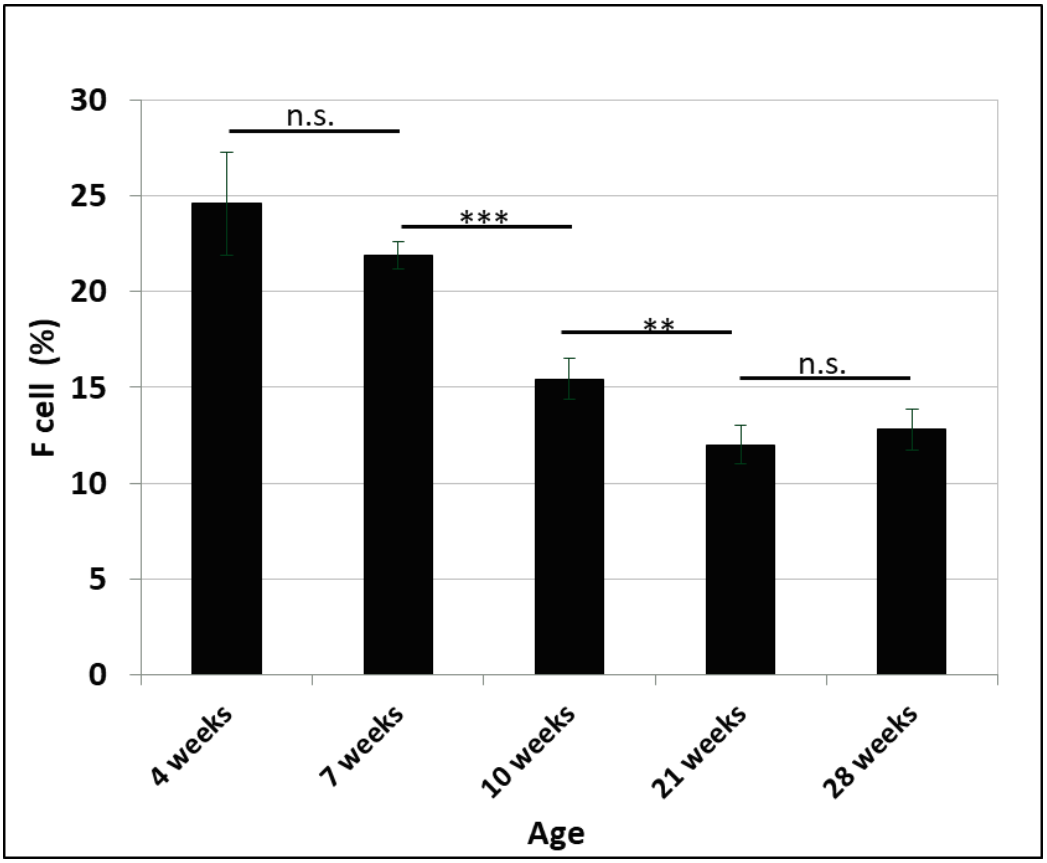

Figure 6. Percentage of F-cells in HbA$^{\Delta 77/\Delta 77}$ decreased with age. F-cell analysis performed on peripheral blood of four HbA$^{\Delta 77/\Delta 77}$ mice shows a significant decline in F-cell percentages with age before stabilizing between 10 weeks and 21 weeks of age. (Error bars represent SD. P-values were calculated using two tailed Student's t test. n.s.: not significant, **: $p<0.01$, ***: $p<0.001$)

Bcl11a Enhancer Deletion Prolongs Survival of Humanized CA Mice

Humanized $\gamma^{HPFH117}\beta^0$ CA mice recapitulate the onset of severe anemia as is seen in human CA patients. These CA mice have a median survival of two weeks, surviving solely on 100% human HbF generated from a Greek HPFH allele, a G to A mutation at position -117 bp upstream of the transcription start site in the human γ-globin promoter. They require RBC transfusions for survival into adulthood.[99] I sought to determine the effect of the Bcl11a 77bp ESE deletion on the lifespan of CA mice by interbreeding the $HbA^{\Delta 77/\Delta 77}$ and $\gamma^{HPFH117}\beta^0$ CA mice to generate mice homozygous for both the null β^0-globin gene and the 77 bp Bcl11a ESE deletion. A male homozygous CA mouse which had been transfused to sexual maturity and had fathered other litters previously, was bred to a $HbA^{\Delta 77/\Delta 77}$ to generate compound heterozygous F1 offspring ($\gamma^{HPFH117}\beta^0/\gamma\beta^A$ Δ77/+). Homozygous mice were then generated by breeding heterozygous mice together and genotyping offspring for the desired mutations at the β-locus and Bcl11a intron. Since the homozygous mice have two null β^0-globin genes, their survival is solely due to the expression of HbF. Doubly homozygous CA mice with the 77 bp ESE deletion (henceforth referred to as $CA^{\Delta 77/\Delta 77}$) appeared paler than $HbA^{\Delta 77/\Delta 77}$ and heterozygous ($\gamma\beta^A/\gamma^{-117}\beta^0$) littermates at birth and their growth rate lagged slightly behind. The animals were kept under close observation due to the early death observed in the CA mice. Surprisingly, all $CA^{\Delta 77/\Delta 77}$ mice survived beyond 4 weeks of age (Figure 7). At 10 weeks, there was 84% survival; whereas, untransfused CA mice seldom survive beyond 4 weeks of age. Peripheral blood smears from $CA^{\Delta 77/\Delta 77}$ mice show that the animals are becoming more anemic with time (Figure 8). However, the cells of a 13 day old mouse appear much

healthier than a CA mouse at the same age, possibly due to the increase in the number of cells making it past the maturation block responsible for ineffective erythropoiesis.

This prolonged survival could only be attributed to the heterocellular increase in expression of γ-globin chains and F-cells that were being generated due to the Bcll11a enhancer deletion. Because the levels of γ-globin expression and F-cell numbers continue to decrease after 10 weeks of age in $HbA^{\Delta 77/\Delta 77}$ mice, if a similar decline is observed in the $CA^{\Delta 77/\Delta 77}$ mice, then we could expect to observe an increase in mortality beyond 10 weeks. Indeed, out of the first 25 $CA^{\Delta 77/\Delta 77}$ mice born, 56% were dead by 13 weeks. This implies that the F-erythroblasts are not making sufficient γ-globin transcripts to generate sufficient HbF within those cells. The nature of heterocellular HbF is that it varies from one individual to another and we've had two $CA^{\Delta 77/\Delta 77}$ mice live up to 21 weeks. The data demonstrate that the Bcl11a ESE deletion results in a further increase of γ-globin chains which prolongs the survival of CA mice.

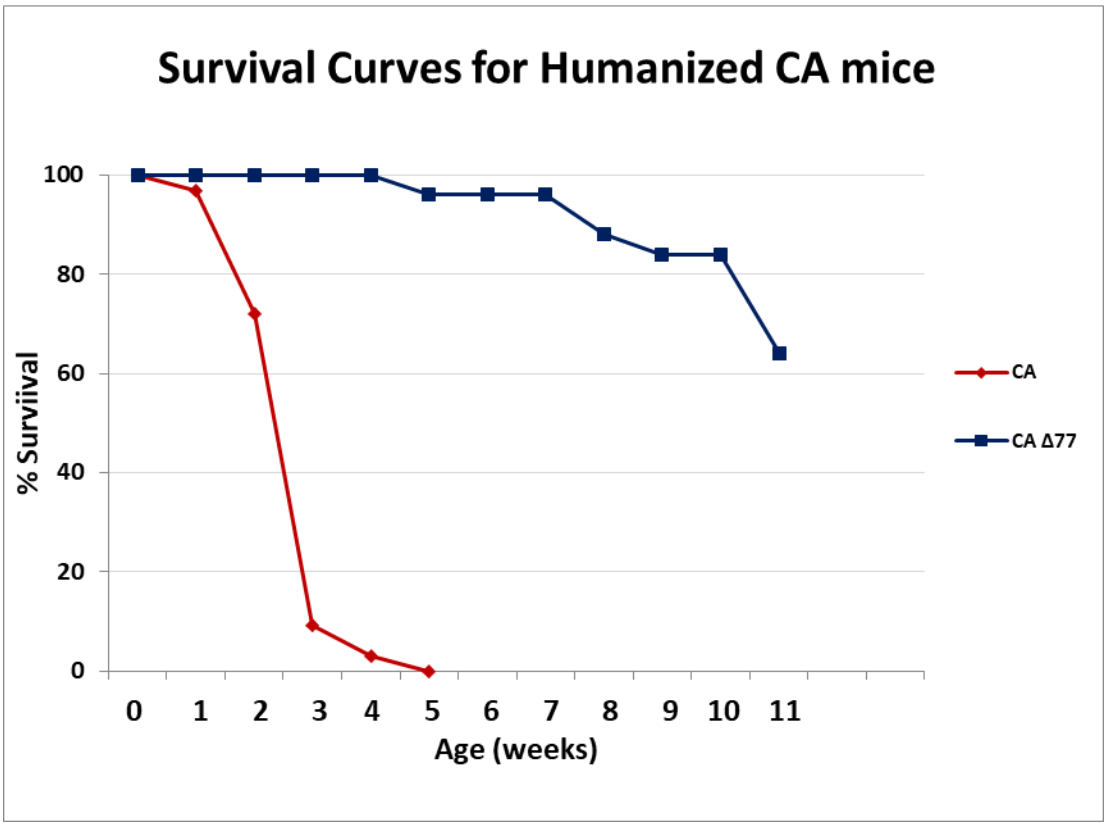

Figure 7. Bcl11a intronic deletion prolongs survival of humanized CA mice. Survival curves of humanized homozygous CA ($\gamma^{HPFH117}\beta^{0}$ / $\gamma^{HPFH117}\beta^{0}$) mice and $CA^{\Delta 77/\Delta 77}$ ($\gamma^{HPFH117}\beta^{0}$ / $\gamma^{HPFH117}\beta^{0}$ Δ77/Δ77). 97% of the CA mice were dead by 4 weeks whereas, all of the $CA^{\Delta 77/\Delta 77}$ mice were alive at that time point. 84 % of $CA^{\Delta 77/\Delta 77}$ mice were alive at 10 weeks.

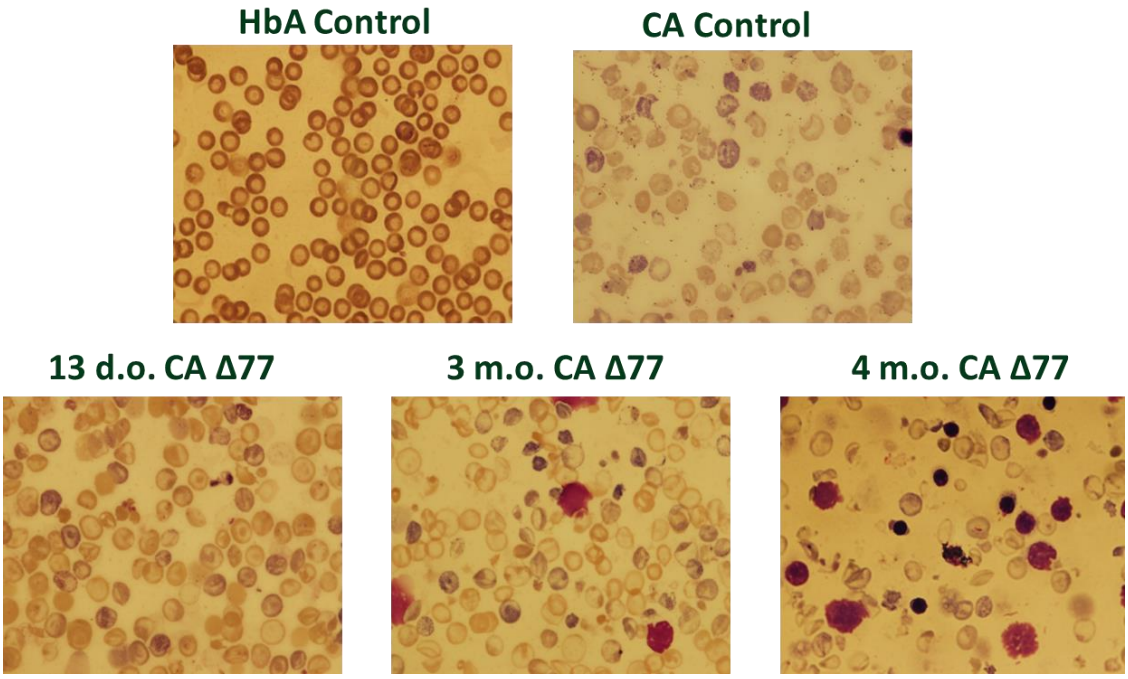

Figure 8. Bcl11a intronic deletion ameliorates RBC pathology in humanized CA mice. Peripheral blood smears from HbA, CA, and $CA^{\Delta 77/\Delta 77}$ mice at different ages were stained with JorVet Dip Quick Stain for an analysis of RBC morphology. Humanized CA mice had a severe anemia and median survival of just 15 days, but all $CA^{\Delta 77/\Delta 77}$ mice survived beyond this time point and showed improved RBC morphology. However, by 3 months of age, $CA^{\Delta 77/\Delta 77}$ mice also exhibited a severe thalassemic phenotype (Images are shown at 100x magnification)

Germline Gene Editing Introduced Off-Target Site Mutations in Founder Mice.

One of the primary concerns associated with gene editing is the possibility of introducing off-target site (OTS) mutations which could cause harm to the organism. As researchers discovered, retroviral vectors can cause insertional mutagenesis and even improved lentiviral vectors can still result in unwanted side effects.[83,178] Gene editing with endonucleases also faces a similar challenge because much of the genome is degenerate and thus prone to off target site cleavage.

Using ZiFit Targeter software available online for predicting OTS that closely match the sgRNA target, I chose the four top hits for sgRNA1 and one for sgRNA2 (Table 6).[179,180] These OTS sites were amplified from founder mice and select offspring, and sequenced via next generation sequencing (NGS). Several of the sites chosen had indels in multiple founders (Table 7). This is just a small sample of all the potential OTS sites that are present in the genome. Other groups have shown that there exist genomic 'hot spots' which are prone to mutation even in the absence of sgRNAs.[181] In addition, several F1 to F3 mice derived from the gene edited founder animals were identified with congenital defects (Figure 9). The defects observed in these mice have not been identified previously or since in other animals in our colony derived from non-microinjected mouse lines. In order to determine the full extent of OTS mutagenesis and the cause of the congenital defects, whole genome sequencing would have to be carried out on each of the lines, a task which is laborious and resource intensive.

Table 6. Genome locations with sequence similarity (1-2 nucleotides) to each sgRNA analyzed for off-target mutagenesis. Nucleotide mismatches are shown in red, with 'x' denoting a deletion**.**

Target	Genome Location (mm9)	Gene Name	Target Sequence
sgRNA1	chr:11:24036267 - 24036289	Bcl11a (intron 2)	GAGTGGCTGTTGAAAGAGGG TGG
OTS #1	chr1:172400854 - 172400876	Nos1ap (intron 2)	GAaTGGCTGTTGAAAGAGGG aGG
OTS #2	chr1:165626192 - 165626213	intergenic	GAxTGGCTGTTGAAAGAaGG aGG
OTS #3	chr12:67101226 - 67101247	intergenic	GAGTGcCTGxTGAAAGAGGG aGG
OTS #4	chr13:110529015 - 110529037	Pde4d (intron 1)	GAGTGGCTGTTGAgAGAGGG aGG
sgRNA2	chr11:24036263-24036285	Bcl11a (intron 2)	GATGGAGTGGCTGTTGAAAG AGG
OTS #1	chr11:117883634-117883923	intergenic	GAgGGAGxGGCTGTTGAAAG GGG

Table 7. Off-target site mutations mapped in founder mice.

Founder ID #	OTS #1	OTS #2	OTS #3	OTS #4
I7772	-	-117	-	-
I7773	-	-	-	SNP, -11, -7
I7774	-	-	-6	-23
I7775	-	-	-	-
I7776	-75, -1	-	-	-
I7777	-	-	-	-
I7778	-	-	-	-
I7779	-	-	-	-1
I7780	-	-	-	-
I7781	-	-	-	-

Founder ID #	OTS
I7782	-
I7783	+1
I7784	+1, +1
I7785	-
I7786	-
I7787	-
I7788	-6,+1, SNPS
I7789	-
I7791	-
I7792	-

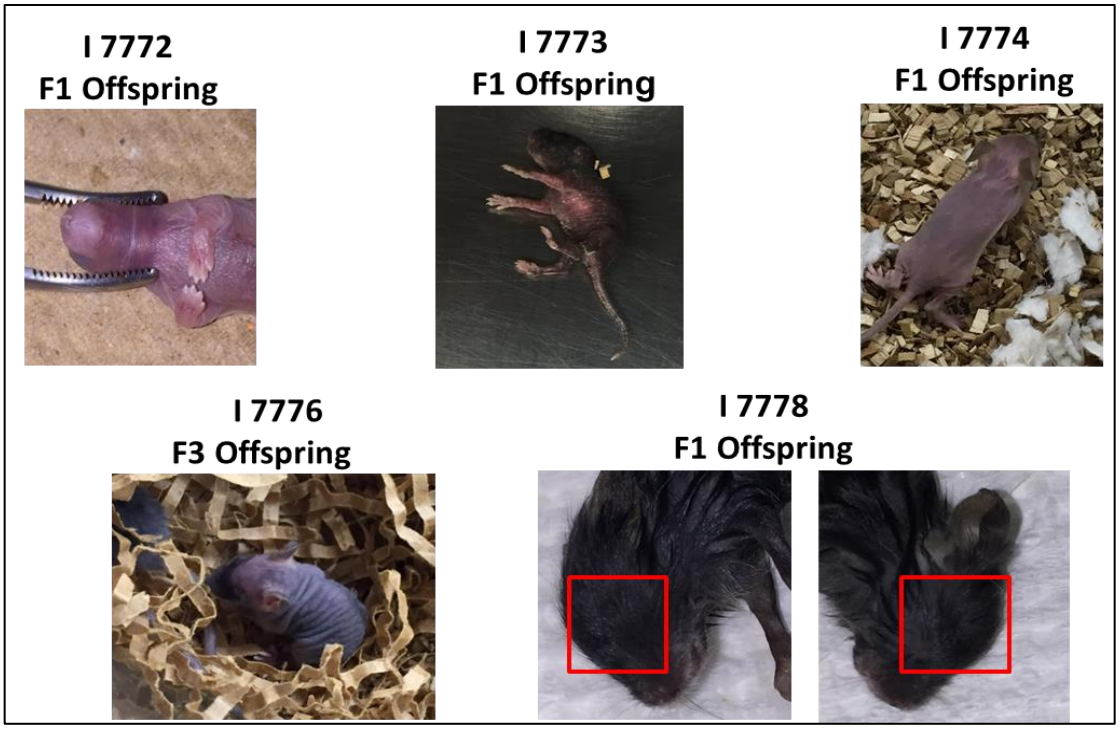

Figure 9. Birth defects were observed in first generation founder offspring and subsequent generations. Offspring from germline gene edited founders and some from subsequent generations demonstrated gross phenotypic abnormalities which had not previously been observed in the colony.

Discussion

Despite the diversity of the mutations that cause CA, ranging from large deletions to point mutations, HbF reactivation is a viable treatment option for all patients regardless of the underlying genetic mutation.[28] The identification of non-globin locus proteins, such as Bcl11a, which modify globin switching and definitive linkages of SNPs within those genes associated with HPFH phenotypes made such a goal attainable.[39,182] SNPs in the second intron provided a starting point to identify the role of Bcl11a in globin switching. Manipulation of this gene can achieve elevated HbF in RBCs.[45]

Using CRISPR/Cas9 endonuclease system, 21 germline gene edited HbA founder mice were generated with mutations in the second intron of Bcl11a, in the +58 kb (from the TSS) DNaseI hypersensitive region which demonstrated erythroid specific enhancer activity (Figure 1 and Table 5). The earliest analysis of the mice showed that their HbF transcripts were elevated above that of HbA controls (Figure 2A). This observation confirmed that the region targeted was linked to the expression of the hemoglobin genes, particularly the switching at the β-globin locus. Further analysis demonstrated that the F-cell percentages in the mice were reflective of the γ-globin expression previously seen (Figure 2B). The mosaicism of the founders required breeding to segregate the alleles and determine which one of the mutations was responsible for the high HbF levels. While most of the mutations were small and resulted in no or small increases in F-cell percentages, two deletions, 702 bp and 77 bp, when made homozygous resulted in over 25% F-cells at 4 weeks of age (Figure 3). The recent characterization of the 702 bp deletion made its further study for this thesis unfeasible. However, the 77 bp deletion had been identified early in the study and the exciting result led us to focus on this line. The mutation extended 3’

of the Cas9 cut site into a region which was shown to contain potential binding sites for erythroid factors, including Gata1 and Tal1.[46]

Experiments were undertaken to compare HbA and HbA$^{\Delta 77/\Delta 77}$ mice to delineate the cause of the differences identified in mice. It was determined that elevated HbF expression was not attributed to stress erythropoiesis and premature release of erythroblasts and reticulocytes from the bone marrow but instead was due to the decrease in Bcl11a expression in mutant erythroblasts (Figure 5). Expression levels of Bcl11a in lymphoid lineage cells (B-cells) confirmed that Bcl11a transcript levels were not statistically different between the two mouse lines. This confirmed, in our model, that the deleted region contained an enhancer for Bcl11a expression in erythroid cells but not in other lineages, as has been reported.[47] qRT-PCR analysis of transcripts from the α-globin locus also showed elevation of the embryonic chain ζ-globin in the HbA$^{\Delta 77/\Delta 77}$ cells. This study is the first to report that Bcl11a is involved in globin switching at the α-globin locus.

Continued monitoring of the HbA$^{\Delta 77/\Delta 77}$ mice as they aged revealed a distinctive trend. F-cell numbers were decreasing with age (Figure 6). F-cell numbers were cut in half from 25% at the first time point at 4 weeks down to 12.5% by 21 weeks of age. This is quite distinct from HbA mice that attain their low steady state F-cell levels of less than 2% by 7 weeks of age. The prolonged decline in F-cell numbers in HbA$^{\Delta 77/\Delta 77}$ mice suggests that the overall level of Bcl11a not only dictates the final steady state F-cell levels achieved, but also the duration required to complete the fetal-to-adult switch. A major finding of these studies is that mutation of the ESE of Bcl11a results in increased levels of γ-globin gene expression, but this increased in HbF is restricted to only a portion of total cells; that is, ESE deletion results in heterocellular increases in HbF.

The increase in γ-globin gene transcripts and number of F-cells in $HbA^{\Delta 77/\Delta 77}$ mice enabled us to examine the *in vivo* efficacy of mutation of the Bcl11a ESE in our preclinical humanized CA mouse model which normally succumbs to their disease within the first month of life. In the absence of β-globin chains in CA, even small increases in γ-globin can have a dramatic effect. Therefore, even though deletion of the Bcl11a ESE resulted in a limited heterocellular effect, these were sufficient to extend the lifespan of $CA^{\Delta 77/\Delta 77}$ mice from a couple of weeks to 3 or 4 months. Amazingly one $CA^{\Delta 77/\Delta 77}$ animal continues to survive without transfusion out to 31 weeks. This mouse is the longest surviving $CA^{\Delta 77/\Delta 77}$ animal with the HPFH117 and no β-globin chains.

The effective increase of γ-globin in $CA^{\Delta 77/\Delta 77}$ mice may be due to two concurrent phenomena. The decrease of Bcl11a transcripts in the ESE deletion mutant makes less protein available for repressor complex assembly, driving a further increase in γ-globin transcripts and protein. The perplexing question remains as to why the Greek HPFH and homozygous ESE mutants are heterocellular, a mystery which will need to be addressed if Bcl11a downregulation is to be pursued as a clinical treatment in CA patients.

The data presented in this chapter are both cause for encouragement and caution. The Bcl11a ESE deletion mice showed for the first time, that Bcl11a regulates human γ-globin expression in mice when γ-globin is under the control of the mouse LCR. This makes a case for the contextual dependence of phenotypic differences seen in our model and transgenic mice. In addition, the rise in HbF and F-cells resulted in prolonged survival of $CA^{\Delta 77/\Delta 77}$ mice. These new models will provide important insights into the regulation of globin switching by Bcl11a and its binding partners in humanized mice that undergo correct globin switching at the β-globin locus. However, it will be important to

tread carefully as the fidelity of CRISPR/Cas9 cannot be fully guaranteed. As seen in our mice, the mutations which arose at the on-target site vary from 1 bp insertions to 702 bp deletions. As with other gene editing tools, we cannot define the exact size of the deletions and have to screen mutations at the end of the experiment. This underscores the lack of control and uncertainty associated with using such technology in human patients.

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HUMANIZED COOLEY'S ANEMIA MICE CURED BY GERMLINE GENE EDITING

by

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HUMANIZED COOLEY'S ANEMIA MICE CURED BY GERMLINE GENE EDITING

Introduction

β-thalassemia is a disorder caused by loss-of-function mutations in the adult β-globin gene. Over 200 thalassemia causing mutations have been identified throughout the gene body resulting in diverse phenotypic consequences.[140,183] The severity of the disease varies depending on the causal mutation.[184] β-thalassemia major, Cooley's Anemia (CA), the most severe form of the disease, is caused by negligible or no β-globin chain synthesis. Cooley's Anemia is fatal within the first decade of life if left untreated.[141] The medical therapy most widely available to most CA patients is lifelong blood transfusions coupled with iron chelation therapy.[76,77,185-190] Hemochromatosis is one of the primary challenges facing both patients and their physicians, as humans have no effective iron clearance pathways leading to iron deposition in organs such as the liver and heart.[69,188] Despite the success of chelation monotherapy, clinicians are also experimenting with combination chelation therapies in the hope of better outcomes for patients.[191,192]

The only cure for the CA is a bone marrow transplant (BMT) from an unaffected HLA matched donor, preferably from a sibling, but most affected individuals do not have sibling matches and unrelated matched donors are rare.[193] An alternative to allogenic BMT is to deliver a normal gene to the patient's own hematopoietic stem cells (HSCs) via viral transduction *ex vivo* followed by transplanting the transduced autologous HSC

population back into the patient. In both the allogenic and autologous BMT transplantation, harsh myeloablative conditioning of the patient is employed to remove the diseased marrow. The first autologous transplants were done in France using gammaretroviral vectors to cure X-linked SCID but those trials were halted when malignancies associated with insertional mutagenesis arose in several of the test subjects.[80-82,194] Learning from the lessons from of this early trial, safer lentiviral vectors have been developed for gene delivery and are currently in trials for sickle cell anemia (SCA) and CA in Europe and the United States.[83,84,195] These ongoing studies have reported some positive, yet variable, patient outcomes. Scientists are seeking to do away with viral based gene therapy entirely, in favor of more direct gene editing based approaches.

Recent advances in gene editing technology have paved the way for researchers to explore the correction of diseases causing mutations directly in patient's autologous HSCs to cure monogenic diseases such as CA and SCA, for which there are few complication-free treatment options. The CRISPR/Cas9 endonuclease system functions as a bacterial adaptive immune system capable of specifically targeting plasmid and phage DNA which had been previously encountered by the bacteria.[119,120] The endonuclease complexes with an RNA molecule with 5' nucleotides (17-20 bases) complementary to a specific DNA target. The sole requisite for DNA targeting is the presence of a trinucleotide protospacer adjacent motif (PAM) of the sequence NGG immediately 3' of the target. This simplistic requirement makes it possible to target just about any accessible chromosomal location in the human genome. Since the initial reports, the field has moved at lightning speed to push the boundaries of DNA editing using of CRISPR/Cas9 to generate knock-out animals, correct diseased animal models, and most controversially, to ge-

netically modify human embryos.[127-129,132,165,166,196-199] The push to edit human embryos and mend mutated genes is bringing to the forefront ethical implications associated with generating germline edited individuals and has prompted the release of a guiding text by a committee of leading experts in the field.[200] Before gene correction can be used to treat human patients either in germ cells or adult stem cells, such as hematopoietic stem cells (HSCs), due diligence must be followed to ensure safety and efficacy.

HSCs are the adult stem cells responsible for generating all the differentiated cell types that make up the body's hematopoietic system. This pool of cells is constantly undergoing self-renewal as well as differentiation. In addition, there are many lineages derived from HSCs that are affected by specific mutations in lineage specific genes. Thus the HSC is one of the few cell lines which can be replaced, and/or edited to cure a hematologic disease. HSCs are notoriously difficult to culture *ex vivo* while maintaining their long-term repopulating potential, a process required to introduce editing reagents into them. HSC transfection is also a challenging procedure and mouse HSCs are even more difficult to transfect than human HSCs.

In this study I employ a humanized mouse model of CA to conduct a proof-of-principle gene editing experiment to cure CA by directly correcting the mutant β-globin gene in order to test the safety and efficacy of the procedure. I opted to use CA mouse embryos as a surrogate for HSCs to determine the extent of HDR vs NHEJ which might take place at the β-globin gene. The humanized $\gamma^{HPFH117}\beta^{0}$ CA mouse model was previously generated in the Ryan laboratory and contains human α-globin genes in place of the adult mouse α-globins and a human fetal-to-adult hemoglobin switching cassette ($\gamma^{HPFH117}\beta^{0}$) in place of the mouse adult β-globin genes.[99,157] A Greek HPFH, G to A at

position -117 in the γ-globin promoter and a non-functional β^0-globin gene, bearing a splice donor site mutation (IVS1.1 G to A) results in animals that survive on 100% human fetal hemoglobin at birth; however they soon succumb to lethal anemia prior to weaning after switching expression from the fetal $\gamma^{HPFH117}$-globin allele to their nonfunctional adult β^0-globin gene.[99] This humanized CA model is ideal for testing new gene editing reagents because similar to patients, CA mice have a single human β-globin gene target inserted into each mouse β-globin locus which is correctly developmentally regulated. This humanized CA mouse is a prime candidate for the preclinical testing of novel therapies before making the leap into human patients.

Herein we use the Cas9 endonuclease, a 17 nucleotide truncated sgRNA (trugRNA) that targets the first exon of the human β-globin gene, and a 100 bp single stranded oligodeoxyribonucleotide (ssODN) donor homology to repair the β-globin gene mutation via HDR in homozygous CA mouse embryos. [124] The ssODN was designed to incorporate the IVS1.1 correction and silent mutations that are not complementary to the trugRNA and would thus be less tolerable to re-cleavage once the repaired DNA was incorporated. The three other silent mutations also provide us with the ability to track repaired alleles with one of the incorporated nucleotides creating a novel restriction enzyme site.

Materials and Methods

Cas9 mRNA and In Vitro Transcription of sgRNA

GeneArt Platinum Cas9 nuclease (protein) was purchased from ThermoFisher Scientific. gRNA cloning vector plasmid **(**Addgene 41824**)** was purchased from Addgene and modified using oligonucleotides containing two BsmBI restriction sites according to instructions. The β-globin trugRNA plasmid was constructed by linearization of the modified cloning plasmid with BsmBI. A pair of complementary 20 nt oligonucleotides (Integrated DNA Technologies) corresponding to the exonic target (with 4 nt overhang for ligation) was annealed and ligated into the vector. β-globin trugRNA was generated by PCR amplifying the sgRNA sequence with a specific primer containing T7 promoter and common reverse primer (Table 1) (Integrated DNA Technologies). The PCR template was *in vitro* transcribed (IVT) using MEGAshortscript T7 kit (Life Technologies) according to manufacturer's instructions. IVT trugRNA was purified and resuspended in TE buffer (Tris-10 mM, 0.1 m mM EDTA), and quantified by NanoDrop (ThermoFisher Scientific).

Generation of Mice

Superovulated (PMSG 5U/mouse T-72 hours and HCG 5U/mouse at T-24 hours) sexually mature female 5-6 week old CA mice were mated with stud male CA mice of overnight. Embryos were isolated from the oviducts of superovulated female mice by flushing the oviducts with culture medium. Hyaluronidase was used to disaggregate granulosa cells and release embryos into the media. The embryos were transferred to small petri dishes with KSOM (Millipore). One-cell stage fertilized embryos were identi-

fied, microinjected with a mixture of Cas9 protein (300 ng/μL), sgRNA (50 ng/μL) and ssODN (200 ng/μL). Embryos were injected into pseudo pregnant female CD1 mice on the same day.

DNA Isolation and Genotyping

Tail biopsies were clipped from pups and incubated in 500 μL homogenization solution (10 mM Tris-CL, 20 mM EDTA, 100 mM NaCl, 1% SDS, pH 8.0) with Proteinase K (0.6 mg/ml) (Omega Bio-tek, Norcross, GA) at 65 °C overnight. After the samples cooled, 300 μL aliquots were distributed into new Eppendorf tubes and 400 μL phenol-chloroform (1:1) were added. The samples were shaken and centrifuged at 13, 000 rpm for 10 minutes at 4 °C, the aqueous phase was transferred to a new tube and 400 μL chloroform was added, shaken and centrifuged at 13, 000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and two volumes of 100 % ethanol added. The tube was shaken vigorously and incubated at -20 °C (for 30 minutes or overnight). The DNA/ethanol solution was centrifuged at 13, 000 rpm for 10 minutes. The supernatant was aspirated and the DNA pellet was washed with 70 % ethanol. The DNA was resuspended in sterile 1x TE buffer. DNA samples were stored at -20 °C.

PCR reactions were performed with Bulldog BioReady rTaq according to manufacturer's protocol using primers specific to the DNA region being analyzed (Table 2). Each PCR reaction contained 0.125 μL Bulldog BioReady rTaq, 2.5 μL of 10x Reaction Buffer (with 15 mM $MgCl_2$), 0.5 μL of 10mM dNTP (New England Biolabs), 1 μL each of primer (DHS F1 and DHS R1), 1 μL DNA and water to 25 μL. The PCR conditions used were as follows: 94 °C for 5min, 35x (94 °C for 30s, 54 °C for 30s, 72 °C for 5

mins), 72 °C for 10 mins, 4 °C forever. A small aliquot (~5 μL) of each PCR product was run on a 2% agarose gel (1x TBE).

RFLP Analysis and TA cloning

Restriction enzyme reactions were assembled in PCR tubes follows: 16.5 μL sterile water, 2,5 μL 10 Cutsmart® Buffer, 1 μL restriction enzyme (BsaBI or AciI) (New England Biolabs) and 5 μL PCR product and, incubated at 65°C and 37 °C, respectively, for at least one hour. Digestion products were analyzed on 2% agarose gel.

PCR products for individual mice were cloned using pCR2.1®TOPO-TA Cloning® kit (Invitrogen) according to manufacturer's instructions. The cloning reaction mixture was incubated at room temperature for 30 minutes and five microliters of the cloning reaction was used to transform chemically competent DH5α *E. coli* bacterial cells. The transformed cells were plated on agar plates with ampicillin (100 μg/mL) added for selection. Clones were picked and the DNA isolated was sent for sequencing.

RNA Extraction, cDNA Synthesis and qRT-PCR Analysis

Peripheral blood was collected from mouse tail tips and RNA was extracted using Trizol LS reagent (Ambion). RBCs were collected in DPBS/0.5 mM EDTA. The cells were washed and resuspended in 50 μL DPBS and 150 μL Trizol LS was added. The tubes were shaken and incubated at room temperature for at least 10 minutes (or stored at -80 °C until it was needed). 40 μL of chloroform were added to the tubes that were shaken and incubated for a further 10 minutes at room temperature. The samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new tube and 100 μL of isopropanol was added. The sample was shaken and incubated at

room temperature for 10 minutes or -20 °C overnight. The Samples were centrifuged at 13,000 rpm for 10 minutes at 4 °C, washed with 1 mL 70 % ethanol and resuspended in 20-40 µL sterile water. RNA samples were stored at -80 °C.

cDNA was synthesized using the High capacity cDNA kit (Applied Biosystems) according to manufacturer's protocol. For globin chain qRT-PCR reactions, cDNA samples were diluted 1:100. Each individual qRT-PCR reaction contained 3 µL diluted cDNA, 10 µL Power Up™ Sybr® Green Master Mix, 0.5 µM each primer (Integrated DNA Technologies) and sterile water to 20 µL (Table 3). The samples were loaded in triplicate and the plate was run on the Applied Biosystems® ViiA7 (Life Technologies) and the data was analyzed using QuantStudio ™ Real-Time PCR software (AB by ThermoFisher Scientific).

Flow Cytometry

Peripheral blood was collected from mouse tail tips using heparinized microhematocrit capillary tubes (Fisher Scientific**)** and deposited into 100 µl of DPBS with 0.05 µM EDTA. The blood was centrifuged at 500g for 3 mins at room temperature in a tabletop centrifuge. The blood was washed by resuspending in 500 µl PBS and centrifuged. The supernatant was aspirated and the blood was fixed by incubation in 500 µl 0.05 % glutaraldehyde (50 % stock solution was diluted 1:1000 in PBS) for 10 minutes at RT. The sample was centrifuged as previously described and the cells were permeabilized by incubation in 500 µL 0.1 % TritonX-100 for 10 minutes at RT. The cells were washed with 500 µL PBS. 1x 10^6 cells were diluted into 100 µL PBS and 0.25 µL of PE conjugated mouse anti-human fetal hemoglobin antibody (BD Biosciences) was added to

the cell suspension, mixed and incubated at RT for 30 minutes. The cells were washed with 500 μL PBS and resuspended in 300 μL of PBS. Reticulocytes were detected by staining with Thiazole orange (0.1 μg/ml) (Sigma) at room temperature for 15 minutes. The cells were analyzed by flow cytometry (BD FACSCalibur (BD Biosciences).

Next Generation Sequencing

Amplicon library was prepared by amplifying sgRNA target sites and selected off target sites (Table 2) with Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The PCR conditions used were as follows: 94 °C for 2 minutes, 35x (94 °C for 30s, 60 °C for 30s, 68 °C for 40s), 68 °C for 10 minutes, 4 °C forever. The PCR products were purified using E.Z.N.A.® cycle pure kit (Omega Bio-tek) and Illumina adapters were ligated to the purified PCR products. A second round of PCR was performed to amplify ligation products. PCR products were first purified with E.Z.N.A.® cycle pure kit (Omega Bio-tek) and then with magnetic beads (Aline Biosciences). The DNA was submitted to UAB Genomic and Genetics Core for paired end sequencing (Illumina MiSeq System). The sequences were analyzed using CRISPResso Software (Luca Pinello) and IGV2.3 (Broad Institute of MIT and Harvard, Boston, MA).

Statistical Analysis

P-values were calculated by the two tailed Student's *t* test.

Table 1. Oligonucleotides used for trugRNA plasmid construction, IVT reaction, and β-globin ssODN. (Silent mutations incorporated in the ssODN are shown in blue and wild type β-globin IVS1.1 nucleotide highlighted).

gRNA Name	Oligonucleotide Name	Oligonucleotide Sequence
β-globin trugRNA1	Beta tGRNA 1F	ACCGTTGGTGGTGAGGCCCT
	Beta tGRNA 1R	AAACAGGGCCTCACCACCAAC
	T7 trugRNA	TAATACGACTCACTATAGGTTGGTGGTGAGGCCCT
	sgRNA REV	AAAAGCACCGACTCGGTGCC
β-globin ssODN	β-globin ssODN	CCTGTGGGGCAAGGTGAACGTGGATGAAGTCGGCGGTGAGG CTCTGGGCAG**G**TTGGTATCAAGGTTACAAGACAGGTTTAAG GAGACCAATAGAAACTGG

Table 2. Primers for genotyping founder animals and amplification of β-globin gene target and off-target sites for sequencing.

Gene Name	Genome Location (mm9)	Primer Name	Forward Primer	Reverse Primer
β-globin	chr 7 (β-globin knock-in)	Beta 1	GAGAAGTCTGCCGTTACTGCC	TGCAGCTTGTCACAGTGCAG
β-globin	chr 7 (β-globin knock-in)	Beta 3	GACAGGTACGGCTGTCATCA	TCATTCGTCTGTTTCCCATTC
Target	**Genome Location (mm9)**	**Gene Name**	**Forward Primer**	**Reverse Primer**
β-globin	chr 7 (β-globin knock-in)	β-globin	CACAACTGTGTTCACTAGCAACC	GGTAGACCACCAGCAGCCTA
OTS #1	chr5:129726316-129726607	intergenic	CGGTGCCTGTGATTATCCTAA	AAGCATCCTTCAGTGGTTCG
OTS #2	chr8:97075192-97075466	Copine-2 (intron 3)	CAGTGCCCAGTTGGATGAG	GTGCCAGACTCTCTGGGCTA
OTS #3	chrX:80324307-80324595	Dystrophin (intron 1)	CACCTACGCACCTACCCACT	AGTGTTGCAGGATTAGCAAGG
OTS #4	chr10:54569534-54569815	intergenic	AAGCAGAGTGGGAATGCAAC	GCCCCTCCACAAACTCTCTA
OTS #5	chr18:50642888-50643183	intergenic	GACAATCGCTCCTAACCAAA	TGTGAAAAGTTAGGTTCTGCATTC

Table 3. Primers for qRT-PCR analysis of globin transcripts.

Gene Name	Primer Name	Forward Primer	Reverse Primer
Mouse Hb-εy	m.Hb-ey EVA-2	GGTGAACTTTACTGCTGAGG	CAACAAGAAGCCTTCCCAA
Mouse Hb-βh1	m.Hb-bh1 EVA-1	GAGAAGGCAGCTATCACAAG	AAACAATCAGGAGCCTTCC
Human Hbγ	h.HbG EVA-4	CTTCAAGCTCCTGGGAAAT	CTGCAGTCACCATCTTCTG
Mouse Hbζ	m.HbX EVA-2	CAACCTCTCTAGTGCTTTGAC	GACAGGAGCTTGAAGTTGAC
Human Hbβ	h.HbB EVA-4	TCGGTGCCTTTAGTGATGG	CACACAGACCAGCACGTTG
Human Hbα	h.HbA EVA-1	CGACAAGACCAACGTCAA	ACAGGAACATCCTCTCCA
β-actin	β-actin	CCAACCGTGAAAAGATGACC	ACCAGAGGCATACAGGGACA

Results

β-globin Gene was Successfully Repaired by CRISPR/Cas9 Editing

Germline gene editing in fertilized embryos was used to modify directly the mutant human β^0-globin gene in humanized CA mice. Male and female CA mice containing the IVS1.1 G to A splice donor site mutation were transfused weekly to extend their survival to sexual maturity so they could breed to generate CA embryos for this study. The fertilized one-cell stage embryos were microinjected with ribonucleoprotein (RNP) complex consisting of Cas9 protein and *in vitro* transcribed trugRNA, along with a 100 nt donor ssODN. The ssODN contained four mismatched nucleotides compared to the human β-globin gene sequence. The first mismatch corrects the IVS1.1 mutation returning the mutant A back to a G, thereby repairing the splice donor site defect. Three additional mismatches were included to repress retargeting of corrected alleles by Cas9 as these mismatches occur within the trugRNA target sequence. Additionally, these mismatches incorporate synonymous mutations in exon 1 of β-globin and generate a new *AciI* restriction enzyme site allowing easy confirmation of corrected alleles. (Figure 1A). Injected embryos were transferred to the oviducts of pseudo pregnant CD1 mice and four pups were delivered by cesarean section 20 days later. Tail DNA was PCR amplified using primers that flank the β-globin target site. The 607 bp amplicon was digested with two restriction enzymes to determine whether any editing had occurred. The *BsaBI* enzyme digests the original CA IVS1.1 mutant allele, while *AciI* cuts targeted β-globin alleles that have been repaired by the donor ssODN. As can be seen in the agarose gel image in Figure 1B, three out of the four founder DNA samples were cut by *BsaBI* and two were

cut by *AciI*. It is also readily apparent from the gel image that in two of the samples (F0-2 and F0-4) the presence of two bands in the uncut samples suggests the existence of a deletion in at least one of the target sites; one of which (F0-4) appears to be quite large (Figure 1B). RNA was extracted from blood obtained from the mice at the time of genotyping and the β-goblin transcript was amplified. The two mice with apparently repaired alleles (F0-1 and F0-2) both had wild type β-globin transcript in the blood, similar to the HbA control (Figure 1C). Mouse F0-2 also had an aberrant transcript, as did the other two mice which had no *AciI* digestion products.

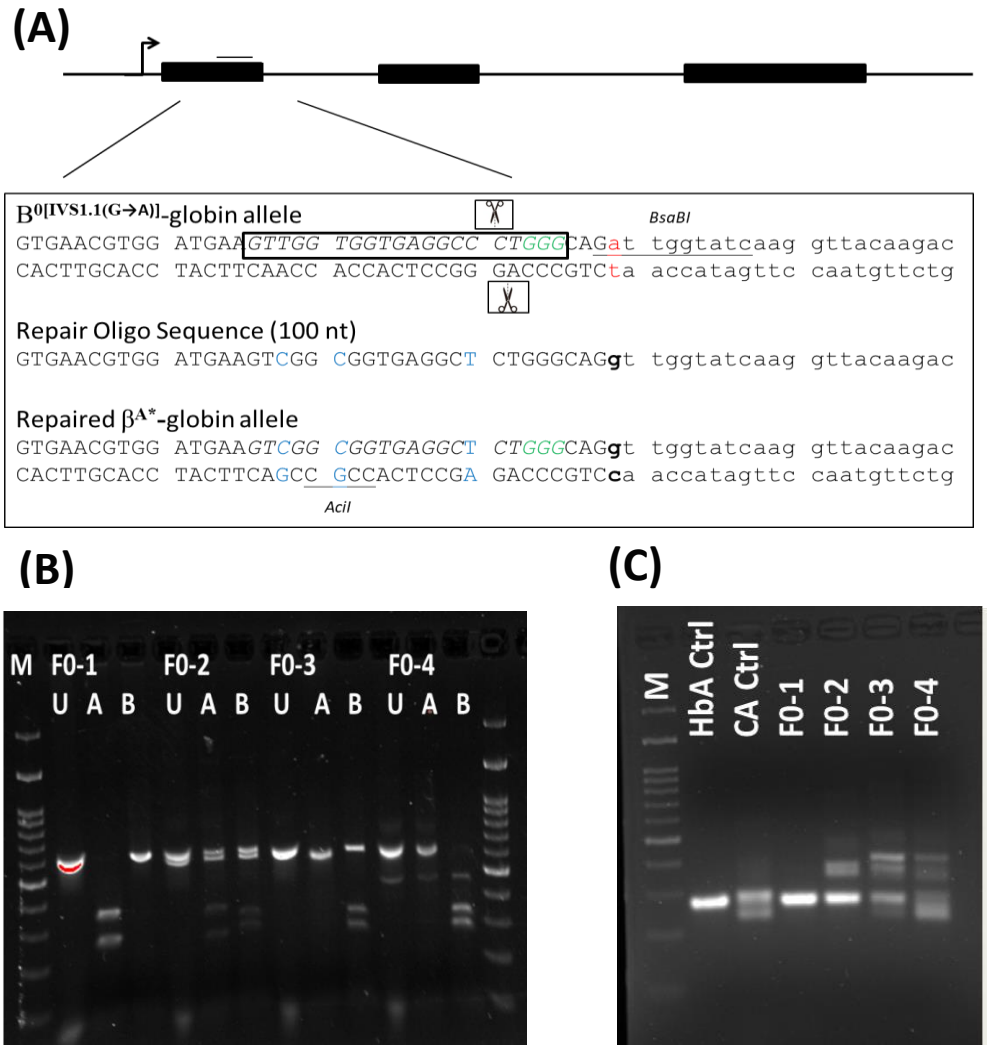

Figure 1. Mutant β-globin gene targeted by CRISPR/Cas9 reagents. (A) Representation of β-globin gene with transcription start site (arrow), exons (black bars), untranslated sequences-UTRs and introns (black lines) and *BsaBI* restriction site (underlined). The targeted region in exon one is expanded to show trugRNA target sequence (boxed), PAM (green), CA mutation at IVS1.1 (red). The repair ssODN donor is shown in part (60/100 bases) with silent mutations (blue) and wild type base at IVS1.1 (black, bold). The double stranded repaired allele is shown with incorporated changes and new restriction site *AciI* (underlined). (B) Gel image of PCR products from β-globin gene amplified from each animal. M- 100 bp DNA marker, U-uncut DNA products, A-*AciI* digests, B-*BsaBI* digests.

(C) Gel image showing β-globin cDNA amplified from reverse transcribed RNA transcripts from each animal, an HbA control and a CA control.

DNA from all four mice was amplified, TA cloned, and sequenced to determine the number of alleles (modified and unmodified) in each founder animal. From the DNA gels, we were unsurprised to find that F0-1 had a homozygous correction with all of the silent mutations incorporated into the alleles (Figure 1B, 1C and 2B). F0-2, the other mouse is mosaic; it showed some evidence of HDR by partial cutting with *AciI,* had a corrected allele, a non-targeted mutant allele, and a 31 bp deletion encompassing the Cas9 cleavage site (Figure 2A). The existence of this 31 bp deletion can account for the extra band observed in the uncut amplicon in Figure 1B and some of the aberrant transcripts observed on the cDNA gel in (Figure 1C). F0-3 was targeted on both alleles with a deletion on one and indels on the other. Despite what appears to be a correction at IVS1.1 on one allele, the other silent mutations were not incorporated and a 2 bp insertion at the cut site resulted in a frameshift rendering the β-globin allele non-functional (Figure 2A). Thus this mouse has two new null β-globin alleles, both of which came about from NHEJ. The final founder, F0-4 was mosaic with non-targeted mutant allele and three new targeted mutations. This mouse possessed the original CA allele, a 7 bp deletion (same as F0-3), a single SNP which is present in the repair oligo, but none of the other silent mutations or IVS1.1 correction is present, and a large 154 bp deletion which extends all the way into exon 2 deleting the entire IVS1.

In summary, all four of the mice (100%) generated in this experiment had evidence of gene editing. Half of the mice demonstrated mosaicism with more than two alleles being obtained per animal. Out of the 9 targeted alleles discovered in the four founders, 5 alleles were repaired by NHEJ while 4 alleles had evidence of HDR. Three

of the HDR alleles repaired the disease causing mutation and incorporated all of the mismatches present in the repair oligo representing a 33% correction efficiency of all targeted alleles.

(A)

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CA:      GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGattggtatcaaggttacaagacaggtttaaggagaccaatag
OLIGO:   GCAAGGTGAACGTGGATGAAGTCGGCGGTGAGGCTCTGGGCAGgttggtatcaaggttacaagacaggtttaaggagaccaatag

F0-1:    GCAAGGTGAACGTGGATGAAGTCGGCGGTGAGGCTCTGGGCAGgttggtatcaaggttacaagacaggtttaaggagaccaatag

F0-2.1: GCAAGGTGAACGTGGATGAAGTCGGCGGTGAGGCTCTGGGCAGgttggtatcaaggttacaagacaggtttaaggagaccaatag
F0-2.2: GCAAGGTGAACGTGGATGAAGTTGGT------------------------------tacaagacaggtttaaggagaccaatag
F0-2.3: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGattggtatcaaggttacaagacaggtttaaggagaccaatag

F0-3.1: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGC-------AGattggtatcaaggttataagacaggtttaaggagaccaatag
                                              AA
F0-3.2: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGC^CTGTGCAGgttggtatcaaggttacaagacaggtttaaggagaccaatag

F0-4.1: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGC-------AGattggtatcaaggttacaagacaggtttaaggagaccaatag
F0-4.2: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCTCTGGGCAGattggtatcaaggttacaagacaggtttaaggagaccaatag
F0-4.3: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGattggtatcaaggttacaagacaggtttaaggagaccaatag
F0-4.4: GCAAGGTGAACGTGGATGAAGTTGGTGGTGAG--------//---TACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATC
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(B)

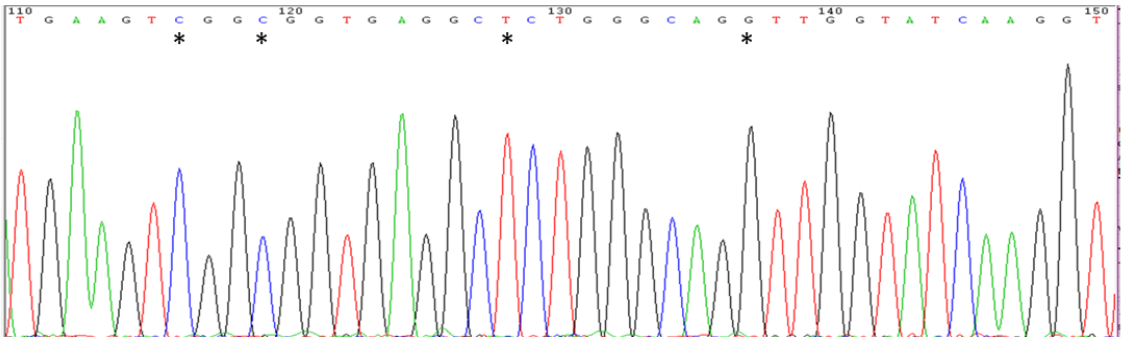

Figure 2. Sequence analysis of founder animals derived from CA germline gene editing experiment. (A) All of the animals born contained alleles which had been modified by on-target DNA cleavage. Both HDR, NHEJ, and the original non-targeted mutant sequences were observed. (B) Chromatogram of F0-1 PCR sequencing result of the β-globin gene. The trugRNA target is underlined with the PAM in green and β-globin mutation in red (the uppercase letters are in the exon and lowercase letters are in the intron).

The silent mutations incorporated are blue, the β-globin correction highlighted in yellow and indels and SNPs in red. Asterisk denotes ssODN donor silent nucleotide changes.

β-globin Correction Rescues Thalassemic Phenotype

The driver of the pathophysiology in CA is a severe globin chain imbalance resulting in the early destruction of RBCs in the peripheral blood and developing erythroblasts in the bone marrow leading to anemia.[201] Thalassemia major individuals and humanized CA mice, are unable to make enough functional hemoglobin in their RBCs to meet the everyday oxygen demands required for life. In order to compensate for their increasing anemia, a vast bone marrow and extramedullary expansion of ineffective erythropoiesis occurs. Evidence of this expansion can be observed in the blood of our CA mice that contains abnormally high numbers of immature RBCs termed reticulocytes and nucleated erythroblasts. Peripheral blood smears made from each of the founder mice during their first week of life showed that F0-1 and F0-2 mice that had repaired β-globin alleles, had improved RBC pathology compare to the other two animals (Figure 3). F0-2 was mosaic so only some of the erythroid cells produced contain corrected alleles, but compared to F0-3 and F0-4, there was no doubt how much the correction aided in the production of mature RBCs.

Despite the homozygous correction at the β-globin allele, F0-1 had to be euthanized due to trauma sustained during the cesarean delivery. Second generation homozygous mice with the same genotype as F0-1 were generated from F0-2 offspring. This provided an opportunity to assess the hematological indices, F-cell percentages, and γ-globin-to-β-globin transcript ratios. Humanized mice that were homozygous for two HDR repaired alleles, termed rHbA mice, no longer had any signs of CA. Their RBC counts, hemoglobin levels, and packed cell volumes were all normalized compared to wild type HbA mice (Table 4). The reticulocyte levels that were over 70% in CA mice re-

turned to normal (less than 3%) in rHbA mice. Likewise, the RBC distribution width indicates that rHbA mice were not producing thalassemic RBCs and the absence of splenomegaly in rHbA mice meant that there was no ineffective erythropoiesis or extramedullary erythropoiesis.

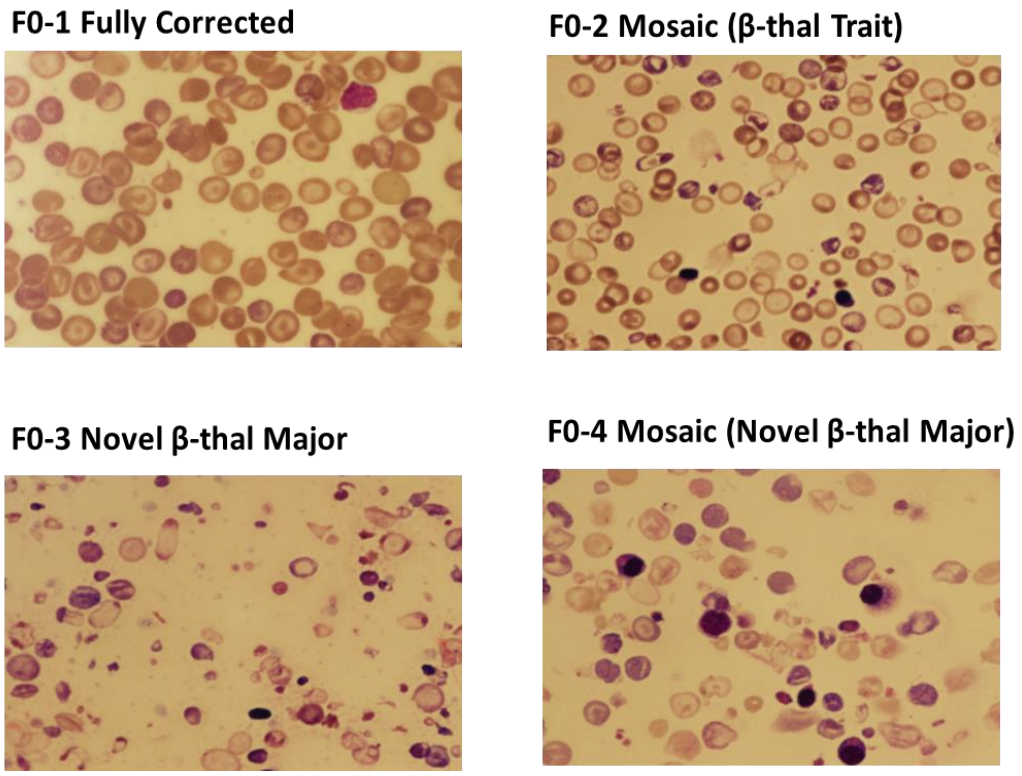

Figure 3. RBC morphology in peripheral blood smears correlates with founder animals' β-globin genotype. The two mice (F0-1 and F0-2) that had repaired alleles had significant improvements in RBC morphology. The two animals (F0-3 and F0-4) that had only new mutant and original CA alleles had smears which contained no RBCs with normal pathology. Nucleated erythroid cells, and hypochromic, anisopoikilocytotic, and fragmented RBCs predominated in these two thalassemia major founder animals.

Table 4. Hematological indices of homozygous HbA, CA, and rHbA mice. HbA and rHbA mice were analyzed beyond 8 weeks of age. CA mice were analyzed when they were euthanized. Values represent Mean ± SEM. Statistical significances are determined for the rHbA ($\gamma^{HPFH117}\beta^{A}/\gamma^{HPFH117}\beta^{A}$) globin mice compared to the CA ($\gamma^{HPFH117}\beta^{0}/\gamma^{HPFH117}\beta^{0}$) control mice. *P* values were calculated by the two-tailed student *t* test. (HbA hematological indices included as normal control). RBC represents red blood cell; HGB, hemoglobin; Hct, hematocrit; RDW, red cell distribution width; fl, femtoliter. ***p<.05*, **** p<.0001*

Genotype	N	RBC (M/uL)	HB (g/dL)	HCT (%)	Retic (%)	RDW (%)	Spleen/BW Ratio (%)
$\gamma\beta^{A}/\gamma\beta^{A}$	5	10.6 ± 0.2	13.4 ± 0.2	44. ± 0.7	2.2 ± 0.3	17.9 ± 0.2	0.33 ± 0.03
$\gamma^{HPFH117}\beta^{0}/\gamma^{HPFH117}\beta^{0}$	5	2.8 ±0.7	2.6 ± 0.5	15.0 ± 0.03	57.8 ± 2.1	39.7 ± 2.2	4.1 ± 1.0
$\gamma^{HPFH117}\beta^{A}/\gamma^{HPFH117}\beta^{A}$	4	9.9 ± 0.4***	12.3 ± 0.4***	35.6 ± 1.2***	1.8 ± 0.2***	19.5 ± 0.3***	0.35 ± 0.03**

Homozygous rHbA Mice Demonstrate Heterocellular HbF Expression

The CA embryos used in this experiment had the Greek HPFH (G to A at -117) mutation in the $^{A}\gamma$-globin gene promoter. In this model, the null β^0-globin gene linked *in cis* results in only HbF being expressed from each allele after birth. Previous work by our lab showed that compound heterozygous adult mice ($\gamma^{HPFH117}\beta^0/\gamma\beta A$) expressed HbF heterocellularly in 36.4% of their cells (Huo *et al*. in revision). However, only 10% of total β-like chains were γ-globin chains. When this $\gamma^{HPFH117}$ β^0 allele is homozygous in CA mice, the level of HbF produced was sufficient for mice to survive for an average of 15 days without transfusions.

The homozygous rHbA mice generated from germline gene editing correction by HDR in this study have the Greek HPFH117 allele linked *in cis* to the functional repaired β-globin gene. These animals provided a novel opportunity to evaluate persistent γ-globin expression when linked to a functional β-globin gene. RBCs from homozygous and compound heterozygous rHbA animals were stained with fluorescently labeled anti-HbF antibody and analyzed via flow cytometry. Compound heterozygous ($\gamma^{HPFH117}\beta^A/\gamma\beta^A$) mice had an average of 20.0% F-cells while homozygotes ($\gamma^{HPFH117}\beta^A/\ \gamma^{HPFH117}\beta^A$) mice had 30.7% F-cells. The presence of one or both corrected $\gamma^{HPFH117}\beta^A$ alleles results in a significant increase in F-cell levels over control $\gamma\beta^A$ alleles in HbA mice (Figure 4A). The difference in F-cells observed between compound heterozygous CA and rHbA animals (36.4% versus 20% F-cells, respectively) can be explained by the increased survival advantage of F-cells in the thalassemic CA compound heterozygote. The additional γ-globin chains present in F-cells in the compound heterozygous CA mice are able to pair with the otherwise toxic excess α-globin chains. Thus, the heterocellular fraction of

erythroblasts that continue to synthesize γ-globin chains are able to remain in the circulation longer than more thalassemic cells that have no persistent γ-globin chains and only express β-globin off one allele. The difference in the F-cell percentages when one allele has an HPFH mutation versus two is not lost on us. The alleles express independently of each other so it is conceivable that while some cells only have one allele 'on' a fraction of the cells can have both HPFH alleles turned on, and though some of those cells overlap, a subset of them do not. In homozygous rHbA mice the F-cells offer no survival advantage to the cells since both β-globin genes are functional and the RBCs are not thalassemic.

Peripheral blood RNA was used to analyze the γ-to-β globin expression ratios of homozygous rHbA ($\gamma^{HPFH117}\beta^{A}/\gamma^{HPFH117}\beta^{A}$) adult mice by qRT-PCR. The γ-globin transcripts averaged over 4% of the β-globin transcripts levels in rHbA blood compared to 0.055% in control HbA mice (Figure 4B). This is a 70-fold increase over the transcript ratio levels of the control HbA mice. Of course, this increased γ-globin expression is restricted to a subset of the cells, the F-cells; therefore, the true ratio of γ-to-β-globin expression in F-cells is presumably even higher**.** This new rHbA model provides us an opportunity to study *in vivo* globin switching in an HPFH mouse model which is not thalassemic.

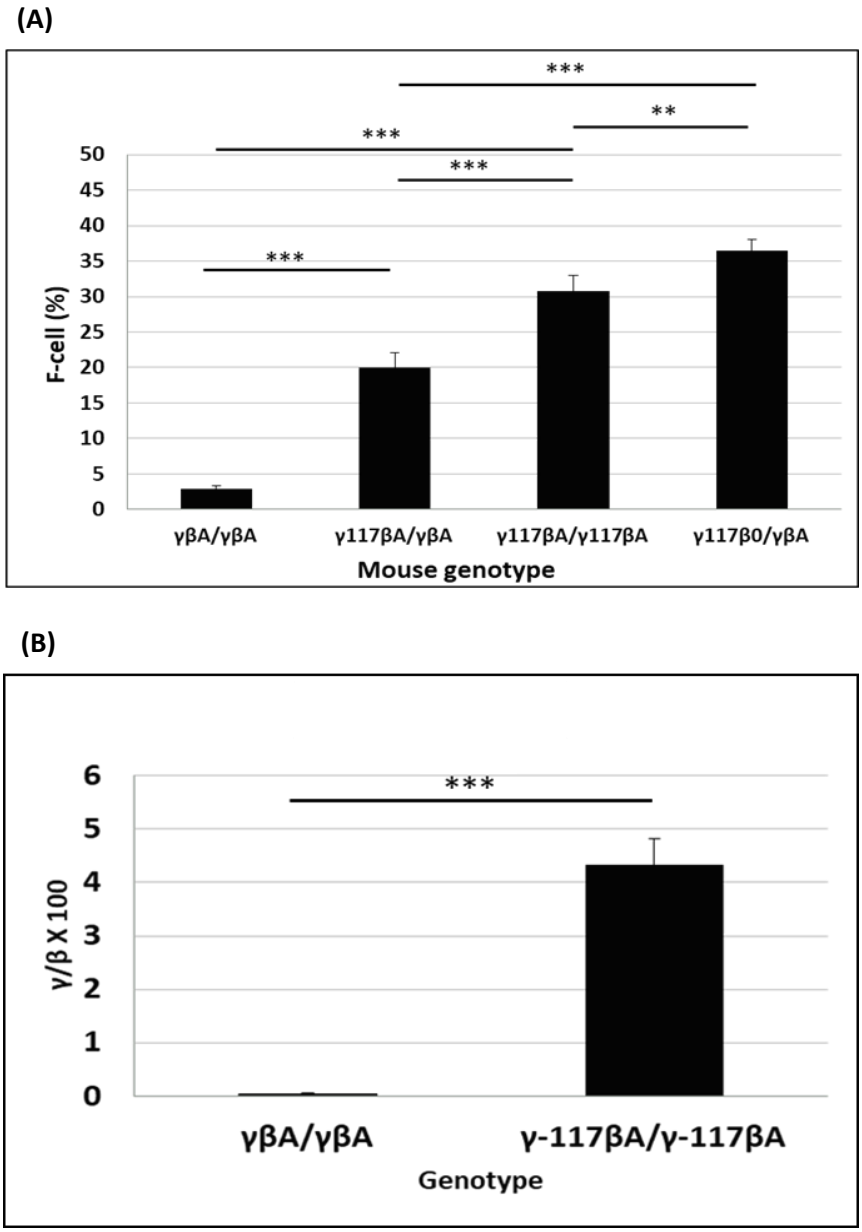

Figure 4. HPFH117 mutation results in heterocellular HPFH in heterozygous and homozygous rHbA mice. (A) F-cell analysis of RBCs by flow cytometry show that significant percentages of cells in both heterozygous and homozygous mice contain HbF (n≥3 in each cohort). (b) qRT-PCR analysis of PBCs to assess level of γ-globin transcription in WT HbA and rHbA mice. (Error bars represent SD; **p<0.05 ***-p<0.001)

Off-Target Site Mutations are Detected in Germline Corrected Mice

One of the concerns associated with using gene editing technology to modify embryos, even to repair deleterious mutations, is the possibility of causing mutations in genes and regulatory sequences that bear close sequence homology to the target sequence. To determine the level of off-target site (OTS) mutagenesis present in our germline edited mice, we specifically amplified the top five OTS with sequence similarity to the trugRNA target site (Table 5). The amplified DNA was sequenced via next generation sequencing (NGS). Of the five sites amplified, four of them had mutations in at least one animal (Table 6). The homozygous corrected mouse which was euthanized had mutations at three of those sites. It is unclear whether any of those mutations, two of which were located within the introns of genes, contributed to any *in utero* trauma or perinatal cesarean seen in the newborn. The mosaic F0-2 founder that was bred to generate rHbA homozygotes had two mutated alleles, one of which was germline transmitted to offspring (Table 7). To date there have been no congenital defects or abnormalities detected in these animals due to the mutation. It is of note that the only site which harbored no off-target mutations had a single base pair mismatch three base pairs upstream of the PAM. The other OTSs had their mismatches at least 8 bp from the PAM.

Table 5. Genomic locations with sequence similarity to β-globin trugRNA target sequence. Mismatches are highlighted in red.

Target	Genome Location	Gene Name	OTS Sequence
β-globin	Chr 7 (β-globin knock-in)	β-globin (exon 1)	GTTGGTGGTGAGGCCCT GGG
OTS #1	chr5:129726468 - 129726487	intergenic	GTTGGTGGTGAGGCtCT aGG
OTS #2	chr8:97075283 - 97075302	Copine-2 (intron 3)	GTTGaTGGTGAGGCCCT tGG
OTS #3	chrX:80324381 - 80324400	Dystrophin (intron 1)	GTTGGTGGaGAGGCCCT tGG
OTS #4	chr10:54569662 - 54569681	intergenic	GTTGGTGGgGAGGCCCT tGG
OTS #5	chr18:50643010 - 50643029	intergenic	tTTGGTGGTGAGGCCCT tGG

Table 6. CRISPR/Cas9 targeting introduced mutations at off-target sites analyzed in founder mice.

Mouse ID	Target site mutations	OTS #1 (C:5)	OTS #2 (C:8)	OTS #3 (C:X)	OTS #4 (C:10)	OTS #5 (C:18)
F0-1	HDR	-	Δ12	Δ8	Δ7, SNP; SNP	-
F0-2	Δ31; HDR	-	Δ9	-	-	Δ1
F0-3	Δ7; +1,SNP	-	Δ8	Δ1	-	SNP
F0-4	Δ7; Δ154; SNP	-	-	Δ1	-	-

Table 7. First and second generation animals analyzed had germline transmitted off-target site mutations

Mouse ID	Target site mutations	OTS #2 (C:8)
F0-2	Δ31, HDR	Δ9
F0-2 Offspring	**Target site mutation**	**OTS #2 (C:8)**
F0-2.1	HDR	Δ9
F0-2.2	HDR	-
F0-2.3	HDR	-
F0-2.1.1	HDR	Δ9
F0-2.1.2	HDR	-
F0-2.1.3	HDR	-

Discussion

Thalassemias are hemoglobinopathies caused by globin chain imbalances.[201] The most severe form of either α-thalassemia or β-thalassemia is lethal without intervention. The former leads to *in utero* fatality. β-thalassemia major, or CA, is more forgiving because the child is born with high levels of fetal hemoglobin which declines within the first year leading to the appearance of disease symptoms.[145] The most common CA treatment available right now is transfusion therapy coupled with iron chelation. Allogenic BMT is available to those with an HLA matched donor to cure the disease but researchers and clinicians are pursuing autologous transplantation of modified HSCs to circumvent the need for donors and harsh conditioning and post-transplant regimens.

The loss of β-globin chains can be partially compensated by elevated γ-globin expression which has been shown to ameliorate disease severity.[202] However, to date, no HPFH mutation has conferred the full reversal of the globin switch; the highest observed is almost 40% γ-globin chains in heterozygotes and pancellular γ-globin expression. [52] The best way to substitute the need for elevated HbF is to repair mutant β-globin genes in affected individuals.

Herein I describe the generation of mice cured of lethal β-thalassemia with germline gene repair of a null β^0-globin gene. The embryos were produced by breeding homozygous CA mice which had to be transfused to sexual maturity.[99] The fertilized one-cell stage embryos were then injected with CRISPR/Cas9 RNP complex and a ssODN carrying silent mutations as well as the correct nucleotide, guanine, at IVS1.1 to facilitate correct splicing of the mRNA and translation of functional β-globin chains.

Four founder animals were born in this experiment, of which one had homozygous correction of both alleles and one mosaic founder had one of three alleles corrected. Homozygous rHbA animals that were generated, containing both β^0-globin genes repaired, were no longer anemic and all their RBC indices were normalized. Unlike our humanized pancellular HPFH175 CA model that still had thalassemic pathology, rHbA mice show no sign of reticulocytosis nor splenomegaly (Table 1, page 42 and Table 4, page 120). This is the first report of a cured mouse model of CA by HDR correction of the β^0-globin by gene editing. In all, 33% of the targeted β^0-globin alleles were corrected in the four founder animals. If this same efficiency could be achieved editing adult HSCs, then CA animals transplanted with the corrected population of HSCs would surely be rescued. This is because there would be a great survival advantage of the erythroblasts derived from corrected HSCs. We have shown in transplantation studies that as little as 1% normal HSCs transferred into CA recipients can rescue the CA mice from lethal anemia (Huo et al., submitted). However, achieving any level of consistent editing of murine HSCs will require the development more efficient transfection methods for delivery of gene editing reagents into the HSCs.

The initial gene therapy studies were predicated on the introduction of foreign DNA into HSCs to deliver functional copies of mutant genes.[194] Unforeseen complications forced researchers to re-evaluate the vectors, transduction protocols and follow-up of patients such that only a few trials are in progress right now.[81] However the diligence of those researchers may indeed yield fruit as gene editing technology such as zinc finger proteins, CRISPR/Cas9 and derivatives are refined towards the goal of human gene editing. This study employs germline gene editing to achieve the goal of repairing the mutant

gene but the more likely use of such technology will be editing adult stem cells for HSC transplantation. Such therapy would need to be tested stringently and employed under strict GMP conditions to make it past the first stages of clinical trials. Some argue that humans are the best test subjects for human targeted therapy but we risk exacerbating disease symptoms or causing new morbidities that may be far worse than the initial condition.

The risks associated with germline gene editing as it currently stands far outweigh the benefits. The efficiency of target site editing will need to be much higher, while off-target editing will need to be greatly reduced. Our modest effort to identify off-target editing at a handful of predicted sites demonstrated the presence of indels in 4 out of 5 sites analyzed in at least one of the founders (Table 6, page 125). Furthermore, because these mutations are in the germline, the will be passed on to successive generations (Table 7, page 126). There is no doubt that whole genome sequencing would reveal many more unwanted mutations throughout the genome at sites that share less sequence similarity for the simple fact that the genome is vast.

However, patients need not lose hope as the field progresses at breakneck speed toward optimizing Cas9, testing transfection, and transduction and injection protocols to minimize deleterious effects. The mice generated from this experiment have demonstrated no adverse effects, breed normally and have normal hematological indices. These corrected rHbA animals will be useful for studying the mechanisms regulating the heterocellular expression of human γ-globin from the HPFH allele because human fetal-to-adult globin switching is occurring correctly under the control of endogenous mouse LCR and transcription factors, most of which have human orthologues with identical functions.

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CONCLUSIONS

CA was first described close to 100 years ago and is one of the most prevalent monogenic diseases affecting individuals across the globe.[56,57,138] Researchers and clinicians all over the world have worked tirelessly to raise the age of mortality from childhood to adulthood, but there remains much work to be done.[64] Current treatment options though successful in prolonging life, impact the quality of those lives.[188] Allogenic BMTs cure the disease but research shows that those transplanted during childhood fare better than adults.[203] Blood transfusions are able to suppress endogenous erythropoiesis but iron overloading causes organ failure, usually in the fourth decade of life; it is the leading cause of death for CA patients.[141] Chelation therapy is necessary for transfusion dependent CA but areas of improvement remain to be addressed for the drugs currently available and in development.[190,191] Drugs developed to raise HbF have marginal success for some patients and no effect for others leading to the termination of clinical trials due to the strict standards required to merit further investigation.

The successes seen in allogenic BMT were marred by high morbidity and mortality rates of the treatment and forced researchers to search for alternatives, such as the development of autologous transplantation therapy.[83,193,204] Though challenges have arisen, researchers remain dogged in their pursuit of cures and the field continues to push forward in the hopes of making autologous BMTs safe and effective for patients who can af-

ford them.[80-83,86] The curative potential of patients own cells holds promise for patients of CA and other diseases of the hematologic system.

Our ability to study this disease has been greatly enhanced with the generation of humanized hemoglobin mouse models.[97,99,157,160] The ability of the mice to switch correctly from embryonic to fetal to adult globins provides a unique opportunity to test novel therapeutic treatment options in a laboratory setting before going into the clinic.[97] Increasing HbF levels has become a primary focus for treating CA but the issues of specific amounts of HbF necessary, and best way to achieve clinically relevant HbF threshold are still being investigated.[159,205]

In this dissertation, I show that the human Black HPFH175 mutation is sufficient to rescue a humanized mouse model of CA from perinatal lethality. HbF levels also rose significantly in HbA mice with unmodified γ-globin promoters and normal β-globin genes when the ESE of Bcl11a was deleted using CRISPR/Cas9 mediated NHEJ. The *in vivo* efficacy of this approach for treating CA was also demonstrated in $CA^{\Delta 77/\Delta 77}$ mice that had an extension of their average survival from 15 days to several months. The successful germline repair of the null β^0-globin gene was achieved by gene editing via HDR in humanized CA embryos.

Initial attempts to generate mouse models of CA which closely mimicked the human disease were met with challenges due to the differences in the temporal control of globin gene regulation between the two species. Mice have no true fetal gene and begin expressing their adult β-globin early in gestation in the fetal liver. Simply deleting the mouse adult β-globin genes resulted in *in utero* death.[93] Similarly, providing large BAC

transgenes containing the entire human β-globin locus were also unsuccessful at generating a true fetal hemoglobin because in the context of the whole human globin locus the human fetal genes were temporally expressed as mouse embryonic genes, the expression of which was restricted to the primitive yolk sac derived erythroblasts of the early embryo.[41,97] It was only after our group inserted a human γ-to-β^0 globin switching cassette into the mouse locus, replacing both adult β-globins (in mouse ESCs), was the first CA mouse model which survived to birth solely of human fetal hemoglobin created. [98] Despite the perinatal lethality of this first humanized CA model, it suggested that if human HbF levels could be raised, then there existed the possibility that a more useful model could be generated.

We hypothesized that incorporating human HPFH mutations in the γ-globin gene promoter in the null β^0-globin background could raise fetal hemoglobin levels to those observed in patients and prolong the survival of the mice. Two HPFH mutations, a Greek -117 G to A transition and a Black -175 T to C transition, were selected and separate constructs and used to knock the switching cassettes into mouse ES cells that were later used to generate mice (Figure 1A, page 36).[99] The homozygous HPFH175 mice which were generated appeared normal at birth, and survived beyond the median 2 weeks of life seen in the HPFH117 mice. The HPFH175 animals lived as long as HbA mice. Analysis of these animals' erythroblasts, tissues and hematological indices held the answer to the differences observed. HPFH175 mice showed pancellular HbF expression (Figure 4, page 45), a phenotype observed in humans who carry the mutation heterozygously. HPFH175 mice were born with 60-70% γ-globin chains which decreased to and plateaued at 40% of total β-like chains throughout adulthood (Figure 2A, page 39). Despite thalassemic hema-

tological indices when compared to HbA mice, the histology of homozygous HPFH175 animals clearly shows how these animals were able to survive normally. Compared to the more severe HPFH117 CA model their hemoglobin, hematocrit, and RBC numbers were increased; their reticulocyte counts are down, their spleens are a smaller fraction of total body weight, all characteristics of a less severe anemia (Table 1, page 42). The splenic architecture is more similar to HbA mice and iron loading is significantly lower than what was observed in HPFH117 animals. This experiment successfully demonstrated that (1) mice can survive normally solely on human fetal hemoglobin, and (2) a single point mutation in the γ-globin promoter is sufficient to raise HbF to clinically relevant levels.

Considering such a great outcome, we sought other ways to increase HbF in our HbA mice without needing to manipulate the globin gene locus by introducing promoter mutations. The introduction of the HPFH alleles into the mouse chromosome was achieved via homologous recombination in ES cells requiring the use of large targeting constructs containing long homology arms and positive selection for antibiotic marker genes. The efficiency of gene targeting in ES cells was only about 1 in 10^6 cells. New developments in the field of gene editing have provided the opportunity to make and test new mouse models much more quickly and efficiently. CRISPR/Cas9 endonuclease system, which was modified for eukaryotic cell editing, is an affordable, highly efficient way to target the entire mouse genome.[119,166]

Two groups had discovered that mutations in genes and intergenic regions outside of the β-globin locus resulted in HPFH in humans.[37,39] Among them was Bcl11a which encodes a transcription factor shown to bind within the β-globin locus. The Bcl11a intron 2 ESE regions provided potential targets that could be mutated to reduce Bcl11a expres-

sion only in erythroid cells without affecting its expression in other tissues. In contrast, mutations of other trans-activating proteins and loci, such as KLF1 and HMIP, had deleterious side effects, including anemia.[35,38] KLF is a master regulator in erythropoiesis and regulates multiple erythroid-specific proteins, not just the globins.[206,207] KLF1 mutations which decrease the levels of the protein or inactivate one copy affect global erythroid differentiation even though HbF transcript levels increase. HMIP mutations in humans were shown to also affect erythroid cell differentiation leading to mild anemia; thus, neither gene was seen as a viable target to raise HbF. [38,208,209] Therefore, the ESE in the Bcl11a intron 2 was selected as the best target to test for achieving high HbF while keeping expression levels in other tissues unchanged.[45,47]

Two overlapping sgRNAs were designed to target a region 58 kb 3'of the Bcl11a TSS. The orthologous region in humans had been shown to contain SNPs associated with high HbF levels.[37,39,168,210] The sgRNAs were injected independently along with Cas9 mRNA into fertilized one-cell stage embryos derived from the HbA line due to the ease of breeding these animals and the near complete silencing of HbF observed in the adult animals. Mice born from both experiments were screened for γ-globin mRNA and several of the founder animals showed transcript levels higher than the age matched control (Figure 2A, page 72). F-cells analysis with anti-HbF antibody revealed several founders with HbF expression above the HbA animal of the same age. RFLP analysis of target site DNA amplified by PCR showed that most of the founders had mutations at the target site. Segregation of the mutations and breeding them to homozygosity in F2 mice revealed that the largest F-cells increases were in mouse lines with 702 bp and 77 bp deletions extending 3' of the target (Figures 3, page 75 and Figure 4, page 76). The 702 bp deletion

line continues to be analyzed but a more comprehensive analysis of the mice from the 77 bp deletion have confirmed that the increase in F-cells was due to a reduction in the Bcl11a transcript level in erythroblasts. The reduction was not seen in B-cells, demonstrating its specificity. HbA mice usually complete their fetal-to-adult hemoglobin switch by three weeks, with F-cells at background levels by seven weeks. However, F-cell levels in $HbA^{\Delta 77/\Delta 77}$ mice do not reach their background levels until sometime between 10 and 21 weeks. Importantly, the HPFH observed in the $HbA^{\Delta 77/\Delta 77}$ animals is heterocellular and the γ-globin transcript levels were relatively low. qRT-PCR measurements put the γ-globin transcript levels at 1.5% of the β-globin transcript level in $HbA^{\Delta 77/\Delta 77}$ mice which is about 20 to 40-fold higher than what is normally found in our HbA mice. An interesting observation which has not yet been reported is the increase in ζ-globin transcripts seen in our $HbA^{\Delta 77/\Delta 77}$ mice when compared to HbA controls. This novel role of Bcl11a as a regulator of the α-globin locus has not been previously reported.

In addition to confirming that Bcl11a ESE mutations play a role in globin gene switching, the CRISPR/Cas9 edited founder mice also had off-target mutations. Next generation sequencing performed on the founder animals revealed that several of them harbored mutations at the sites chosen for analysis based on sequence similarity to the target. In addition, these OTS mutations were transmitted to progeny. There were also several F0, F1 and F2 animals from multiple lines born with birth defects including anophthalmia, delayed hair growth, dry scaly skin, and one pup born with astomia, the congenital absence of a mouth (Figure 9, page 89). These congenital abnormalities may not be a direct effect of CRISPR/Cas9 off-target effects but it seems likely. The only animals which have been observed with the defects to date are from the experimental co-

horts. Despite the protestations of investigators who use CRISPR/Cas9 and defend its fidelity, the wide variation in on-target mutations alone should be sufficient to give one pause before proposing to take this technology from the lab bench to the bedside.

The final experiment described in this thesis demonstrates the ability of CRISPR/Cas9 to repair a mutant β-globin gene in humanized CA embryos when provided with single stranded donor DNA homology. The homologous DNA provided contained silent mutations making the trugRNA less tolerable to mismatches unable to recut a previously repaired DNA target gene. Of the four founder mice born from the experiment, three of them showed evidence of HDR, two of the three founders had repaired the β-globin allele, and one of these had a biallelic correction. Three of the mice showed evidence of NHEJ as well as the original mutant β^0-globin allele. The mosaicism observed in the HDR experiment was similar to what was observed in the Bcl11a ESE deletion experiments. One of the mice with a corrected β-globin allele was bred to obtain homozygous animals which were analyzed for HbF, hematological indices, and transcript stability since the silent mutations could have affected the RNA. The data show convincingly that these rHbA animals are cured of their lethal anemia. The HPFH117 allele contributes about 30% F-cells when homozygous in our founder animals. This low percentage of F-cells may be due to the lack of a survival advantage required by the RBCs, unlike the thalassemia trait mice which have 36% F-cells as heterozygotes. NGS analysis also showed that in our founder animals there were a few off-target mutations that were also germline transmitted. However, the developmental defects observed in the previous Bcl11a editing experiments have not been seen in this cohort. The presence of these mutations stresses

the need for stringent testing of sgRNAs, continued optimization of Cas9 proteins and, long term follow up and analysis of animals derived from such experiments.

Impact of This Work and Future Directions

Hemoglobin, in its various iterations, is one of the most well studied molecules. It was one of the first molecules to have its crystal structure solved, resulting in a Nobel Prize.[211] Despite our extensive knowledge of the protein's structure and function, many questions remain about the internal and external cellular factors that are responsible for the switch from fetal to adult globin. Erythroblasts made in the fetal liver manufacture HbF while the erythroblasts in the bone marrow make adult hemoglobin.[22,144] The conditions that support the expression of the two distinct genes are not well understood. The fact that hemoglobinopathies that are caused solely by mutations of the adult β-globin gene can be cured by reactivating the previously silenced γ-globin gene linked *in cis* at the locus has driven this work. The humanized hemoglobin switching animals used in this work and the new models generated over the course of this project could provide the key to unlock the secrets of the erythroblasts.

The introduction of the Black HPFH mutation rescued a CA mouse from perinatal death, pinpointing a single molecular change in the genome, a T to C transition, at position -175 bp in the γ-globin promoter capable of curing patients. The survival of the CA model was due specifically to the upregulation of γ-globin transcription and protein chain synthesis. The ability of the mouse to be cured by a single biallelic point mutation in a promote region makes this site a possible target for exogenous activators. As was de-

scribed by Wienert *et al*. the mutation creates a canonical binding site for an erythroid transcription factor, TAL1, which is involved in normal globin gene expression and switching.[32] Perhaps the simultaneous incorporation of both the Greek and Black HPFH mutations *in cis* in the same γ-globin promoter will raise HbF levels even higher thereby eliminating any residual thalassemia. Unfortunately, efficient gene editing requires the generation of a double strand break (DSB) in the DNA to stimulate recombination and repair. This fact provides challenges to the introduction of the HPFH mutations at the γ-globin gene promoters because there are four identical γ-globin alleles in the human genome. Generating multiple DSBs in the β-globin locus *in cis* and *in trans* could lead to gene deletions or chromosomal inversions or translocations. Other groups report difficulty in specifically targeting an individual γ-globin gene promoter since there were four identical targets in the human cell lines.[31] The mixture of outcomes, not to mention potential off-target mutations, could provide an insurmountable challenge for researchers, clinicians and patients.

However, based on the results of this experiment and knowledge of Tal1 interactions at the globin locus, HbA mice can be used to investigate the effect of delivering a transgene encoding Tal1 linked to a DNA binding domain with specificity to the γ-globin promoters into HSCs.[212] This can be done with lentiviral vectors containing a γ-globin specific sgRNA and nuclease deficient Cas9 (dcas9) (or a smaller orthologue such as *Cj*Cas9) fused to Tal1 that are expressed using erythroid specific promoters and enhancers.[213] The correct folding and activity of Tal1 could be assayed by its ability to activate β-globin in human K562 cells. Once this has been established, HSCs could be isolated from humanized HbA mouse bone marrow, transduced and transplanted into recipient

HbA mice to determine whether the erythroid expression of Tal1-dCas9 fusion protein will specifically activate γ-globin. The effect of the presence of a naturally occurring mutation may differ greatly from the forced looping by a large chimeric fusion protein which would be required in this case. If successful, such an approach could be tested for application in human HSCs for autologous transplantation of the activators with only the need for incorporation of the transgene in the genome of a fraction of cells, which is yet to be definitively determined.

The deletion of the Bcl11a ESE has been previously reported to increase γ-globin expression but none of the groups used a model with γ-globin under the control of the mouse LCR.[41,47,169] The human βYAC transgene is regulated differently in mice than in the endogenous context in humans. In those transgenic mice, γ-globin expression is confined to primitive cells. In the model presented in Chapter 2, γ-globin is expressed as a fetal gene which switches off after birth. Our experiment demonstrates that switching is delayed further into adulthood when the ESE deletion is 77 bp or larger. The expression is heterocellular which is yet unexplained since all of the HSCs and erythroblasts have the same mutation. This mouse line, along with the rHbA mice, may play a pivotal role in uncovering globin switching mechanisms and kinetics of Bcl11a DNA binding at the globin loci. Comparisons of F-cell percentages and γ-globin transcripts between the mouse models from birth to about 25 weeks would define the stage at which HbF expression plateaus. Analysis of RNA from bone marrow erythroblasts at multiple time points will help elucidate the pathways and mechanisms causing the delayed switch in the Bcl11a ESE deletion mice. The differentiation stage at which erythroblasts commit to a particular globin moiety (γ- or β-globin) expressed at the β-globin locus is unknown.

With the heterocellularity of HbF expression in these models and a large number of cells available for studying erythroblasts at various maturational stages could be sorted and analyzed for globin transcripts. High HbF and low HbF erythroblasts can be analyzed for the expression of Bcl11a and other erythroid switching factors (RNA-seq or qRT-PCR) to determine whether a difference in expression profiles exists for the different populations. In our current F-cell assay, the cells are fixed for sorting thus making further analysis of those cells impossible. With the use of single cell analysis, the cell populations will be sorted for stage specificity, RNAs analyzed and then grouped accordingly (HbF^{hi} vs HbF^{low}) for further analyses. This analysis may lead to the discovery of unknown partners within the complexes, which currently remain undefined.

The delayed switching and decline over time in F-cells observed in the Bcl11a ESE deletion mice ($HbA^{\Delta 77/\Delta 77}$) can be interrogated by analyzing cell populations isolated at multiple time points after the 7 week mark to determine exactly when the decline stops. Right now, we know that there is no change between 21 weeks and 28 weeks so the F-cell levels plateau between 10 weeks and 21 weeks, at a yet unknown time point. Analysis of erythroblasts isolated before and after steady state γ-globin levels are attained could provide cues to the mechanism of delayed silencing in the $HbA^{\Delta 77/\Delta 77}$ model. Importantly, this decline is likely the reason why the $CA^{\Delta 77/\Delta 77}$ mice begin to die off after the 10 weeks of age.

The most successful of the experiments carried out with respect to restoring globin chain balance and reversing the CA phenotype was the repair of the β-globin gene. Three of the nine targeted alleles from the four mice were corrected. The homozygous rHbA mice generated showed no developmental abnormalities or symptoms of thalasse-

mia. RBCs appear as uniform biconcave discs with no abnormal morphology. Hematological indices are all within the normal ranges. This mouse model provided us the novel opportunity to quantify F-cell percentages when the HPFH117 mutation is linked *in cis* to β-globin. The incorporated silent mutations appear not to have affected β-globin transcription, as no aberrant transcription was observed in the biallelic corrected animal. The β-globin correction restored globin chain balance to what is required by RBCs to function optimally. In an ideal world where gene editing worked perfectly, gene correction for diseases such as SCD and non-deletional CA would be the default medical prescription. But it is not an ideal world. One small consolation derived from the generation of the new null β^0-globin mutations in the targeted alleles repaired by NHEJ is the knowledge that these small mutations did not make the mice any worse than they have been without the experimental treatment. One side benefit is that each new null mutant allele generated is potentially the beginning of new humanized CA model for study. However, the presence of OTS mutations in the HDR and Bcl11a ESE deletion experiment are cause for concern and caution due to the randomness and unpredictability of the OTS mutations, varying from cell to cell in mosaic animas and between generations after breeding. Modifying HSCs for autologous therapy carries with it the great risk of triggering a malignancy in any one of the many hematopoietic cell lineages derived from the "mother cell'. The ability to correct the disease causing mutations doesn't necessarily mean that the benefit outweighs the associated risks.

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APPENDIX

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 15-Jun-2017

TO: Ryan, Thomas M

FROM: 

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal
Care and Use Committee (IACUC) on 15-Jun-2017.

Protocol PI:	Ryan, Thomas M
Title:	Humanized Hemoglobin Mouse Models of Anemia
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-20954

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 14-Jun-2020.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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