HEMOGLOBIN SWITCHING, THALASSEMIA AND SICKLE CELL DISEASE IN HUMANIZED KNOCKIN MICE

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2011
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ABSTRACT

In this dissertation we describe knockin mouse models for the study of human hemoglobin disorders. These knockin human globin genes, in contrast to transgenes, replace the adult mouse globin genes, remain under control of endogenous mouse globin enhancer sequences and are inherited in a manner identical to globin alleles in man. Starting with various knockin alleles composed of unique human α, β and γ globin gene sequences, we evaluate several allele combinations towards our goal of generating improved models of thalassemia and sickle cells disease. We show that humanized knockin mice complete a fetal to adult hemoglobin switch during postnatal development, similar to humans, allowing investigation of switching mechanisms. We demonstrate targeted repair of a β thalassemia allele in mouse stem cells, with introduced human β globin gene expression correcting their anemia. In fully humanized mice a single inactivated β globin gene results in β thalassemia minor, unlike previous mouse models but comparable to human trait patients. Within the context of sickle cell disease in humanized knockin mice we also investigate the role of α thalassemia to improve the hematological phenotype and increase survival independent of the role of fetal hemoglobin. In conclusion, humanized knockin mice faithfully model essential aspects of human hemoglobin disorders, advancing our fundamental understanding of their pathology and providing a platform to test novel therapies.
DEDICATION

For my lovely wife Christine,

inquisitive sons Aiden and Finn,

and sweet daughter Kiera-

I would be lost without your boundless love.

You helped me persevere and succeed,

and I pray that I can be the husband

and father that you deserve.
ACKNOWLEDGMENTS

First I must thank my mentor, Dr. Thomas Ryan, for offering me a chance to work in his laboratory. His knowledge, enthusiasm and patience have been remarkable.

I thank the members of my thesis committee- Dr. Anupam Agarwal, Dr. Peter Burrows, Dr. Peter Detloff and Dr. Tim Townes for their support and helpful discussions.

Former and current members of the Ryan laboratory that contributed to this work include Dr. Yongliang Huo, Dr. Susan Farmer, Tingting Zhang, Dr. Shanrun Liu, Rui Yang and Jackie McLeroy. Yongliang shared a bench with me for many years- now his skills, insight and wit are already missed.

Members of the Townes laboratory, both former and current, contributed a great deal materially and intellectually to this work. We could not ask for better colleagues and neighbors. Members of the Townes laboratory that initiated experiments that formed the foundation of this work include Dr. Kevin Pawlik, Dr. Jinxiang Ren, Dr. Chiao-Wang (Joe) Sun, Dr. Tim Townes and last but not least, my mentor, Dr. Thomas Ryan.

Other friendship and help came from Townes laboratory members Doug Morgan, Dr. Dewang Zhou, Dr. Li-Chen (Jane) Wu, Dr. Chia-Wei Chang, Yi-Shin Lai, Dr. Andy Svendsen, Dr. Dana Levasseur, Dr. Kaimao Liu, Chao Li and Clark Kelley.
Members of the Detloff, Agarwal, Schneider, Renfrow, Broker, Pritchard and Klug laboratories kindly made their equipment, reagents and support available. Technical expertise was provided by Marion Spell, Maya Spivey, Mary Hamilton, Trenton Schoeb, Steven Pittler, and Enid Keyser. Administrative support was indispensable from Diane Vickers, Sandy Pawlik, Gary Spradley, Gary Sunderland, Jimmy Alston, Audrey Moore, Lyn Harrison and Melvia Walton. I also would like to thank the T32 training grant and Carmichael scholarship committees for their support.

Additional assistance, useful discussions, direction and friendship came from too many individuals to mention but they include Rochelle Biffle, Dr. Edward Meehan, Dr. Pam Twigg, Dr. Thomas Hock, Wei-Jye Lin, Dr. Seung-ah Lee, Dr. Thomas Broker, Dr. Wayne Wang, Dr. Aaron Duffy, Dr. Robert Carter, Dr. Nilam Banerjee, Dr. Heui-Yun Joo, David Stella, Dr. Andy Crouse, Dr. Jesse Hunter, Dr. Sushmit Maitra, Monica Stinnett, Dr. Yelena Ginzburg, Dr. Shannon McKinney-Freeman, Lori and Dr. Tim Thompson, Jenny and Bert Myrick, Dr. Becky Belcher, Natalie, Terresa and Bill Lambert, Richard Pickrell, Jennifer and Dr. Chuck Sands, Janelle and Preston Hite, Jennifer and Dr. Todd Harrington, Katie and Dr. Rob Eaker, Kristen and Mark Comer, Erik Webb, Gay Johnson, Olivia and Jim Howard, Ginny and Doug Mills, Jane Cormack, Liesl and Nathan Varner, Matt Hobson, Tracy and Paul Podraza, Linda and Kendall Davis, Claudia and Patrick Sullivan, Melissa and Sean Sanderson, Sally and Kevin Young, Traci and Jerry McDuffie, Brian Turner, Jeff Cornelius, Angela and Scott Mains, Sarah and Jason Cook, Betsy and Marland Hayes, and Theresa and George Hayes.
Finally I must acknowledge the steadfastness, sacrifice and encouragement of my family—first my wife Christine, sons Aiden and Finn, and daughter Kiera. This also includes, both for the time leading up to my studies and for their duration, my parents—Kim and John Goldinger, Lowell McConnell, Beatrice and Larry Carney; brothers and sisters—Melissa and Byron McConnell, Scarlet and Ryan Spain; aunts and uncles—Mary Lou and Gary Carney, Vicky and Steve McConnell, Deborah VerBeek, Mike Grindberg, Cindy VerBeek, Nancy and Robert VerBeek, Ruth Wood, Dorothy and Vernon Oliver, Libby Hansel, Vicky and Joe Ed Moore; cousins—Amy Jo and Kirk Redman, Stacy and Brett Carney, Kyler and Kaci Moore, Seth VerBeek, Paul Hopkins; grandparents—Dorothy and John VerBeek, Ramie and David McConnell, Opal and Ray Carney, Erna and Manfred Baier. Though several are no longer with us, none have been forgotten. Each contributed in diverse ways, both small and large, but above all by believing in me with love. Such incredible support made everything possible.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Glorious Globins</td>
<td>1</td>
</tr>
<tr>
<td>Structural Sensations</td>
<td>2</td>
</tr>
<tr>
<td>With Friends like Globin</td>
<td>7</td>
</tr>
<tr>
<td>Sickle and Hyde (from Malaria)</td>
<td>9</td>
</tr>
<tr>
<td>Thalassemic Tale</td>
<td>11</td>
</tr>
<tr>
<td>A Globin for Every Task</td>
<td>13</td>
</tr>
<tr>
<td>Globin Gene Regulation</td>
<td>15</td>
</tr>
<tr>
<td>Globin Gene Competition</td>
<td>20</td>
</tr>
<tr>
<td>Erythroid Lineages</td>
<td>21</td>
</tr>
<tr>
<td>Globin of Mice and Men</td>
<td>24</td>
</tr>
<tr>
<td>Thalassemia in the Real World</td>
<td>24</td>
</tr>
<tr>
<td>Silent and Dominant Thalassemia</td>
<td>25</td>
</tr>
<tr>
<td>Basis for Beta Thalassemia</td>
<td>26</td>
</tr>
<tr>
<td>Analogies with Alpha Thalassemia</td>
<td>27</td>
</tr>
</tbody>
</table>
LIST OF TABLES

\begin{tabular}{ll}
\hline
\textit{Table} & \textit{Page} \\
\hline
HUMAN GLOBIN KNOCK-IN MICE COMPLETE FETAL-TO-ADULT & \\
HEMOGLOBIN SWITCHING IN POSTNATAL DEVELOPMENT & \\
1 Human $\gamma$ to total $\beta$-like globin mRNA during adult erythroblast differentiation & 57 \\
2 Postnatal F-cell and F-reticulocyte analysis in humanized mice & 61 \\
\hline
CORRECTION OF SEVERE $\beta$ THALASSEMIA AFTER TARGETING & \\
STEM CELLS WITH HUMAN $\gamma$ AND $\beta$ GLOBIN GENES & \\
1 Hematology of thalassemic alleles in mice and correction by human $\gamma$ $\beta^A$ knockin & 84 \\
2 Hematology of Humanized Mice and Humanized Trait Mice with $\beta^0$ Allele & 90 \\
\hline
$\alpha$ THALASSEMIA AMELIORATES SICKLE CELL DISEASE IN HUMANIZED & \\
KNOCKIN MICE INDEPENDENTLY OF FETAL HEMOGLOBIN & \\
1 Hematology of human $\alpha$ globin, trait, and $\gamma$ $\beta^S$ knockin mice & 112 \\
2 Hematology of humanized and sickle knockin mice with $\alpha$ thalassemia trait & 118 \\
\hline
\end{tabular}
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1 Structure of myoglobin</td>
<td>3</td>
</tr>
<tr>
<td>2 Structure of human sickle hemoglobin</td>
<td>5</td>
</tr>
<tr>
<td>3 Equilibria of hemoglobin ligand binding</td>
<td>8</td>
</tr>
<tr>
<td>4 Globin gene mutations are now a global phenomenon</td>
<td>10</td>
</tr>
<tr>
<td>5 Diagram of human globin gene regulation</td>
<td>14</td>
</tr>
<tr>
<td>6 Human $\beta$-like globin gene switch throughout development</td>
<td>16</td>
</tr>
<tr>
<td>7 Mouse $\beta$-like globin gene switch throughout development</td>
<td>17</td>
</tr>
<tr>
<td>8 Human and mouse $\beta$ globin gene loci</td>
<td>18</td>
</tr>
<tr>
<td>9 Human and mouse $\beta$-like globin gene switch through development</td>
<td>19</td>
</tr>
<tr>
<td>10 Human globin gene switching through erythroid lineages</td>
<td>22</td>
</tr>
<tr>
<td>11 Mouse globin gene switching through erythroid lineages</td>
<td>23</td>
</tr>
<tr>
<td>12 Overview of alpha thalassemia genetics</td>
<td>29</td>
</tr>
<tr>
<td>13 Human $\alpha^+$ globin alleles are caused by gene deletion</td>
<td>30</td>
</tr>
<tr>
<td>14 Humanized knockin mice</td>
<td>34</td>
</tr>
</tbody>
</table>

**HUMAN GLOBIN KNOCK-IN MICE COMPLETE FETAL-TO-ADULT HEMOGLOBIN SWITCHING IN POSTNATAL DEVELOPMENT**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Diagrams of the Human, Mouse, and Humanized $\gamma\beta^+$ Knockin Mouse $\beta$-Globin Loci</td>
<td>47</td>
</tr>
</tbody>
</table>
1. Correction of Thalassemia Intermedia with a Human γβA Globin Gene Cassette in Embryonic Stem Cells ................................................................. 81
2. Histology of Thalassemia Corrected with Human Globin Genes .................. 85
3. Osmotic Lysis Data for Humanized β Thalassemia Trait Mice .................. 89
4. Histology of Humanized β Thalassemic Mice ........................................... 91

α THALASSEMIA AMELIORATES SICKLE CELL DISEASE IN HUMANIZED KNOCKIN MICE INDEPENDENTLY OF FETAL HEMOGLOBIN
1. Targeting Human γβS, α2α1 and α1 Globin Gene Cassettes .................. 107
2. Human α Globin Knockin Expression in Primitive and Definitive RBCs ........ 113
3. Human α Globin Expression Levels from α2α1 and α1 Knockins ............... 116
4. Humanized α Thalassemia Trait RBCs Resist Osmotic Lysis .................... 120
5. Histology of Humanized α Thalassemic Trait mice .................................. 121
6. Human Fetal to Adult Hemoglobin Switching in Humanized Sickle Mice .... 123
7. Survival of Humanized Sickle Mice with α Thalassemia ............................. 124
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAC</td>
<td>Bacteria Artificial Chromosome</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>%BW</td>
<td>percentage of total Body Weight</td>
</tr>
<tr>
<td>dL</td>
<td>deciLiter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo-Nucleic Acid</td>
</tr>
<tr>
<td>ES cell</td>
<td>Embryonic Stem cell</td>
</tr>
<tr>
<td>EMSA</td>
<td>ElectroMobility Shift Assay</td>
</tr>
<tr>
<td>F-cell</td>
<td>Fetal hemoglobin positive cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Flurescein Isothiocyonate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft Versus Host Diseases</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>HbA</td>
<td>Adult Hemoglobin</td>
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<tr>
<td>HbF</td>
<td>Fetal Hemoglobin</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit (Packed Cell Volume)</td>
</tr>
<tr>
<td>HPFH</td>
<td>Hereditary Persistence of Fetal Hemoglobin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-Guanine Phosphoribosyltransferase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>hyg</td>
<td>hygromycin</td>
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<tr>
<td>iPS cell</td>
<td>induced Pluripotent Stem cell</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>KI</td>
<td>Knock-In</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-Out</td>
</tr>
<tr>
<td>LCR</td>
<td>Locus Control Region</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean Corpuscular Volume Hemoglobin</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean Corpuscular Hemoglobin Concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean Corpuscular Volume</td>
</tr>
<tr>
<td>n</td>
<td>number of mice</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate Kinase</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>retic</td>
<td>reticulocyte</td>
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<tr>
<td>RDW</td>
<td>Red blood cell Distribution Width</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
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<tr>
<td>TO</td>
<td>Thiazole Orange</td>
</tr>
<tr>
<td>μL</td>
<td>microLiter</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast Artificial Chromosome</td>
</tr>
</tbody>
</table>
INTRODUCTION

From antiquity, mankind has considered breath to be synonymous with life itself. This understanding of our place in the world has only been made more profound by groundbreaking scientific discoveries linking oxygen, hemoglobin, and several common genetic diseases. In this dissertation we explore the functional roles of normal and mutant human globin genes in humanized knockin mice, a novel model system that has great potential to provide additional insights into human hemoglobin disorders.

Glorious Globins

Globin proteins support life by facilitating small molecule acquisition, storage, and transport. Diverse globin family members have been discovered in bacteria, plants, and invertebrates on the deep sea floor, often displaying affinity for unique ligands (32). Yet the oxygen binding protein hemoglobin remains the most celebrated ambassador, comprising the red pigment and over 95% of the dry weight of mammalian red blood cells (RBCs). Blood from a normal person carries over a pound of hemoglobin, making possible the exchange of oxygen and carbon dioxide between the lungs and tissues. RBCs greatly outnumber all other cell types in circulation and can be relatively easily collected from peripheral blood. This fortuitous combination of availability and significance has helped hemoglobin research claim its disproportionate share of pivotal advances in biology and medicine (84).
**Structural Sensations**

Observations of hemoglobin crystals (colorfully described as a 'molecular lung') responding to oxygen tension differences (36) provided inspiration for Max Perutz's Nobel Prize-winning structural work (75). Whale myoglobin (50) and horse hemoglobin (76) became the first protein crystal structures examined in atomic detail, a feat that has profoundly shaped our understanding of life (74). Structural comparisons between oxygenated and deoxygenated conformations further shed light on globin function. Mechanistic studies further validated this structural work, augmenting its far-reaching impact.

The molecular basis for the divergent functions of myoglobin and hemoglobin was revealed with the determination of their three-dimensional structures. Myoglobin is a monomeric protein (Figure 2) that does not exhibit cooperative oxygen binding. Instead of oxygen transport in blood, myoglobin appears to be used primarily within muscle cells, improving oxygen availability and helping to safeguard against hypoxic conditions (117).

In contrast to monomeric myoglobin, hemoglobin is a multi-subunit protein (2, 93) composed of two α-like and two β-like globin chains (Figure 3). Following pairing of α-like and β-like globin chains to form a hetero-dimer, two dimers further assemble to form stable tetramers. All four globin chain subunits function together within hemoglobin (1), conferring the critical property of cooperative oxygen binding (8, 113). This cooperative binding facilitates efficient oxygen delivery from blood cells to tissues within a broad range of conditions.
Figure 1. Structure of myoglobin.

X-ray diffraction structure of protein crystals from sperm whales ( Physeter macrocephalus) solved to 2.0 angstroms (96). Protein database file 5MBN was rendered with backbone ribbons in Chimera (77), with a different color for each α helix. The heme prosthetic group (white) contains a central iron atom (brown) that binds oxygen.
A major function of the lungs is to saturate hemoglobin with oxygen (5, 35, 90). Encapsulated within RBCs, oxygen-saturated hemoglobin is then carried throughout the body via the circulatory system. As blood flows from arteries to capillaries, oxygen is released from hemoglobin as needed, meeting the constant demand of tissues such as the brain. Veins complete the circuit, returning oxygen-depleted blood back to the lungs for respiratory exchange. Life is sustained as hemoglobin acquires, transports, and then releases oxygen in a timely manner.

Hemoglobin, with its reversible binding of oxygen, uses a remarkable set of mechanisms to achieve a delicate balance of high affinity binding but full release in the proper context. The α and β globin chains in hemoglobin have extensive inter-subunit contacts that allow for conversion between two closely-related main structural forms, each stabilized in part by the presence or absence of oxygen. The crystal structures of both the oxygenated and deoxygenated forms helped to make this mechanism clear (74). After binding of oxygen to one of four sites, structural conversion at the remaining sites makes oxygen binding easier, and thus cooperative through this extended influence.

Allosteric effectors such as the small molecule 2,3-DPG (7, 13) also influence hemoglobin structure by stabilizing a particular conformation of hemoglobin upon binding. Binding of 2,3-DPG favors the deoxygenated form of hemoglobin, inducing the so called right-shift of the oxygen binding curve to a lower affinity. Other effectors collaborate to exert similar control over the oxygen affinity of hemoglobin. After release from cells, carbon dioxide waste is transported back to the lungs in part by binding to
Figure 2. Structure of human sickle hemoglobin.

The X-ray diffraction structure of protein crystals containing human sickle hemoglobin tetramers, protein database file 2HBS solved to 2.05 angstroms (33). The backbone ribbons were rendered in Chimera (77), with green and medium blue color for β globin subunits, while dark blue and light blue labels the α globin subunits. The four heme prosthetic groups (white) contain iron atoms (brown) that are used to bind oxygen cooperatively as the entire molecule is sensitive to structural changes induced upon binding at any one of the four subunits. Additional binding sites on the hemoglobin tetramer for small molecules such as carbon dioxide, hydrogen ions and 2,3 DPG help provide allosteric control over hemoglobin’s oxygen affinity. The sickle hemoglobin tetramer has a structure quite similar to normal adult hemoglobin, the main difference being a change at position six of the β globin chain from glutamate to valine. While this amino acid substitution slightly lowers the oxygen affinity of sickle hemoglobin, it more importantly allows sickle deoxyhemoglobin tetramers to interact and form long polymers that damage sickle red blood cells (30).
globin chains at various sites peripheral from the oxygen-binding core. Interestingly, carbon dioxide binding favors the deoxygenated conformation of hemoglobin and provides a signal for oxygen release, thus affecting hemoglobin function. The loss of bound carbon dioxide after return to the lungs primes hemoglobin molecules once again to accept oxygen by favoring the oxygenated conformation (Figure 4). Hemoglobin oxygen affinity is ultimately guided by structural cues between neighboring chain subunits that influence cooperative binding, along with allosteric responses to environmental cues. In this instructive manner, hemoglobin is highly optimized for oxygen transport.

**With Friends like Globin**

Globin gene defects are considered the most common monogenic disorders worldwide (109), warranting further explanation. More than a thousand globin gene mutations have been described (15, 26), often isolated within families, but a few mutant globin alleles are disproportionately common worldwide. Strikingly, several appear to have been under strong positive selection over thousands of years, indicated in part by their high frequencies within human populations inhabiting tropical regions. Malaria is thought to be the principle driving factor (29), as the mutant allele frequencies are highest in areas of the world where malaria is endemic.

Studies indicate that specific globin mutations affect the ability of *Plasmodium* parasites to maintain productive life cycles after infecting RBCs. Some mutations appear to confer resistance to the most severe forms of malaria (60, 63, 115, 116), notably *P. falciparum*.
Deoxygenated hemoglobin tetramers have a slightly different structure from oxyhemoglobin, and this structure helps favor cooperative oxygen (O$_2$) binding for O$_2$ molecules one through four. The entire hemoglobin tetramer is sensitive to structural changes induced upon O$_2$ binding at a single globin chain subunit. RBCs efficiently deliver O$_2$ during their transit through capillary beds. At this time waste carbon dioxide (CO$_2^-$) molecules and acid (H$^+$) are collected as they preferentially bind to deoxyhemoglobin molecules (the Haldane effect (14)) at amino groups away the O$_2$ binding site. The binding of H$^+$ and CO$_2^-$ favors a shift from oxyhemoglobin to deoxyhemoglobin that aids further oxygen release at tissues (the Bohr Effect (8, 113)). When H$^+$ and CO$_2^-$ saturated hemoglobin molecules return to the lung, they release their bound H$^+$ and CO$_2^-$ and this helps favor transition to the oxyhemoglobin structure for a return to maximal O$_2$ loading.
that causes cerebral malaria. Alterations to membranes of RBCs with hemoglobin disorders may limit RBC lifespan and ability of the parasite to maintain an infection. Thus, globin mutations can provide survival benefits in malarial environments that compensate for any relatively minor loss of RBC function, helping to explain their high prevalence in many human populations.

**Sickle and Hyde (from Malaria)**

The sickle cell anemia mutation is a common globin allele that appears to be under strong selective pressure, with heterozygote frequencies reaching over 10% in some regions of Africa (53, 114). Blood smears from patients displayed characteristic crescent-shaped RBCs (38), leading to the sickle moniker. Significantly, sickle cell anemia was the first disease linked to a change in primary sequence (69), ushering in a new era of molecular medicine (84). The sickle mutation is a single base pair change at codon six that causes an amino acid substitution, coding for an uncharged valine rather than a charged glutamate (42-44) on the globular surface of the β globin chain. This hydrophobic valine on one hemoglobin tetramer fits neatly into an existing hydrophobic pocket within a neighboring deoxygenated hemoglobin molecule, allowing tetramers to polymerize into long chains. These polymers, called sickle fibers, distort the shape of RBCs and damage their membranes, leading to frequent sickle RBC hemolysis and severe anemia. Additionally, sickle RBCs are responsible for painful and sometimes deadly vaso-occlusive crises as resistance to membrane deformation and adherence to blood vessels contribute to sickle RBC blockage of circulation.
Figure 4. Globin gene mutations are a global phenomenon.

Globin gene mutations appear to have arisen and been distributed predominantly in the tropical and subtropical regions of African and Eurasia (indicated with orange color). The colonial slave trade through West Africa and recent immigration (depicted across oceans with light blue color) have now made globin gene mutation population screening important in other regions of the world such as America and Northern Europe (areas with mixed orange and blue). Modified from (34), under Creative Commons License 2.0.
Homozygous sickle mutations (\(\beta^S/\beta^S\)) typically lead to devastating results, with very few children able to survive sickle cell disease in the developing world. Most of the 200,000 born each year with sickle cell disease in Africa do not reach five years of age (89, 108, 114), frequently succumbing to complications such as bacterial infection (114). Yet even with access to modern medicine in the developed world, sickle patients still suffer from significant morbidity (4, 27, 78) and greatly reduced lifespans (79, 118).

Heterozygous individuals carrying a sickle allele along with a normal globin gene have sickle cell trait (\(\beta^S/\beta^A\)). In contrast to the severity of sickle cell disease, sickle cell trait leads to mild symptoms as abundant normal \(\beta^A\) globin chains serve to block \(\beta^S\) polymerization. However, the sickle mutation in heterozygotes can still confer resistance to the most severe forms of malaria by subtly altering RBC properties, therefore contributing a significant survival advantage for individuals with sickle cell trait (3). Incredibly, the sickle mutation alternates between roles as either a killer or savior depending upon the co-inherited genes and environment.

**Thalassemic Tale**

The \(\alpha\) and \(\beta\) thalassemias appear to play a similar protective role against of malaria, being deeply rooted in tropical, low-lying areas that have long endured its ravages. Thalassemia is derived in part from the Greek word \(\theta\alpha\lambda\alpha\sigma\sigma\alpha\) meaning 'the sea', after its discovery in patients of Mediterranean origin. When malaria eradication measures became effective within the last century, it was clear that some immigrants to America carried with them a characteristic anemia that appeared to be genetic, a finding that was
soon confirmed in several populations throughout the world. Thalassemia was found to be particularly prevalent in Southeast Asia, where allele frequencies can exceed 10%. Taken together, selection pressure appears to neatly explain the considerable impact of diverse globin mutations on human populations throughout millennia (110).

In contrast to sickle cell disease, thalassemia is typically associated with normal \( \alpha \) and \( \beta \) globin chain sequences. Chain imbalance is the molecular basis of thalassemia (111), with reduced production of a particular globin chain relative to the other. Deficiency in \( \alpha \) globin is labeled \( \alpha \) thalassemia (88). Similarly, \( \beta \) globin deficiency causes \( \beta \) thalassemia, a disease resulting from surplus \( \alpha \) globin chains. Unpaired globin chains can precipitate in the cytoplasm or on membranes (23), leading to RBC dysfunction (87). Toxic globin inclusions that block normal red blood cell development in the bone marrow lead to ineffective erythropoiesis (85). In the mature erythroid cells that reach circulation, residual unpaired globin chains can damage the membrane and lead to removal from the defective RBCs from circulation or hemolysis. The severity of thalassemia depends largely on the degree of imbalance derived from excess \( \alpha \) or \( \beta \) globin chains, however in many cases, \( \alpha \) and \( \beta \) thalassemia outcomes remain distinct even with similar levels of imbalance (86, 87).

Thalassemia is a rather non-specific term since it spans a broad spectrum of pathologies in humans ranging from asymptomatic cases to lethality in utero. Classification of thalassemia takes into account clinical indications such as transfusion dependence, but fortunately this is in general agreement with the underlying genetic lesions. The most
severe form of thalassemia is hydrops fetalis, when absence of functional $\alpha$ globin chains can inhibit early fetal development and only rarely results in live births (16). Profound deficiency of $\beta$ globin chains causes Cooley’s anemia (17, 83), when disease becomes evident only by a few months following birth. This relative delay in onset for disorders caused by $\beta$ globin gene defects is due to the $\gamma$ to $\beta$ globin gene switch that is completed in the first year of postnatal life (48, 107). At first it was thought that the excess $\gamma$ globin chains were the cause of disease in infants with $\beta$ globin deficiency, but soon their protective role was realized. This discovery led to uncovering an array of globin chain forms possessing developmentally restricted expression patterns.

A Globin for Every Task

Hemoglobin in mammals and other higher organisms is comprised of several distinct forms that are tightly regulated during development. Globin chains are classified based on when they are expressed, such as during embryonic, fetal and/or adult stages of life. Early embryos express embryonic globins, which appear to function even prior to the onset of circulation by harnessing diffusion via their extremely high oxygen affinities. Following the onset of circulation, embryonic globins are eventually replaced by fetal/adult globins in maturing RBCs as hematopoiesis shifts from the yolk sac to the fetal liver and bone marrow. Hemoglobin switching describes the changing patterns of globin expression through development.
Figure 5. Diagram of human globin gene regulation.

The human β globin gene locus is shown, with upstream enhancer DNaseI hypersensitive sites (HS) shown to be important for high level expression of each developmentally appropriate downstream gene. Globins are transcribed from genes that contain three exons. The promoters are unique for each globin gene and help to control developmental specificity. Several mutations in the γ globin promoter (represented by blue asterisk) lead to hereditary persistence of fetal hemoglobin in adults. Mutations in the β globin gene can lead to defects including structural changes in the molecule (red asterisk) with different outcomes such as allowing sickle tetramer polymerization, or loss of proper exon splicing (purple asterisk) that leads to a deficit in mRNA produced from the mutant gene, causing thalassemia.
Globin Gene Regulation

Immature RBCs are factories for hemoglobin synthesis, relying on high-level transcription of stable globin messenger RNA from the requisite globin genes. Interestingly, the α and β globin gene loci are located on separate chromosomes in vastly different chromatin domains. Such independence of gene regulation at the two loci poses a challenge for developing RBCs that require high but relatively balanced levels of α and β globin for ample hemoglobin production.

DNaseI hypersensitive sites upstream of the α and β globin gene loci were mapped (102, 103) and the discovery of thalassemic patients lacking these sequences demonstrated their importance (24). While required for downstream globin gene expression, their more specific functions are still being worked out. Particular attention has been paid to the β globin locus as a model of enhancer function. For historical reasons the enhancers upstream of the human β globin locus, composed of five core hypersensitive regions (HS1-5) occupying roughly 20 kb, are collectively referred to as the locus control region (LCR), a label that has now also been used in reference to enhancers at other gene loci.

Transgenic mice produced by injection of human α or β globin genes into the male pronuclei of fertilized eggs expressed variable, low levels of the globin transgenes (100). Experiments soon showed that the co-injection of a 20 kb DNA fragment containing the human β globin LCR was sufficient to fill mature mouse RBCs with high levels of human globin chains (101). Important for high-level expression, the LCR sequences also
Figure 6. Human β-like globin gene switch throughout development.

The LCR within human β globin gene locus is shared through competitive interactions with the downstream genes. Some genes can also silence autonomously, such as ε in early embryonic life even without a competitor gene in the locus. By the time of birth β globin has begun to increase at the expense of γ globin. During adult life β globin is normally maintained at levels of 97%, while δ globin is 2.5% and γ globin is only 0.5%.
Figure 7. Mouse β-like globin gene switch throughout development.

The embryonic βh1 and εY globin genes are silenced by mid to late gestation in mice. By the time of birth the embryonic globins are essentially undetectable, with only adult β globin present. Mice have no minor δ or fetal γ globin gene equivalents to express in adult life.
Figure 8. Human and mouse α globin gene loci.

The hypersensitive site upstream and to some extent the functional analog for the β globin LCR is labeled HS40 within human α globin gene locus, and HS26 in mice. The α-like globin genes consist of embryonic ζ upstream of the fetal/adult α1 and α2 globins.
Figure 9. Human and mouse α-like globin gene switch through development.

The globin gene switch from embryonic $\zeta$ to the fetal/adult $\alpha_1$ and $\alpha_2$ globins is completed in early development in both mouse and man.
conferred resistance to the position effects seen with naked globin genes that otherwise did not express or randomly expressed at low levels in RBCs of transgenic mice.

**Globin Gene Competition**

Downstream of the enhancers, each globin locus contains genes for embryonic and fetal/adult globin that are tightly regulated with respect to lineage and developmental timing. Temporal control depends in part upon protein binding sequences located in the promoters of the genes, but also on their relative position to the other globin genes within the locus. Embryonic globin genes are located immediately downstream of the LCR, followed by fetal/adult globin genes. In the early embryo, close proximity to the LCR appears to be dominant until the embryonic globin genes become repressed, allowing high-level expression of the next globin gene in line. Competition appears to be particularly important in regulating the fetal to adult globin gene switch that occurs in humans around the time of birth (6).

The human α globin locus similarly contains upstream enhancers but these appear less powerful than the human β globin LCR at driving high-level human globin gene expression in transgenic mice or protecting from position effects. Functional properties of the α globin enhancer sequences remain poorly understood. Nevertheless, the array of downstream α-like globin genes is controlled by a set of shared enhancer sequences that appear necessary at each stage for high-level α-like globin gene expression. This enhancer sharing model neatly explains why expression of one globin at a locus comes at
the expense of the other globins linked under control of the same enhancer, leading to our current competition model of globin gene switching.

**Erythroid Lineages**

Two distinct lineages of erythroid cells arise *in utero* with unique anatomical and temporal origins (51, 68). Primitive erythroid cells are a transient population circulating from the yolk sac during early gestation. Nucleated primitive erythroid cells continue to divide in circulation, where they mature and provide support until the definitive erythroid lineage becomes established. Although recent evidence shows that primitive erythroid cells can enucleate, they soon become diluted by the deluge of definitive cells and then disappear altogether (25). Purification of each erythroid cell type has allowed demonstration that embryonic globin chains are restricted to primitive cell lineage.

A second wave of hematopoiesis establishes production of the definitive erythroid cells. Definitive erythroid cells are ultimately derived from hematopoietic stem cells (HSCs) that arise in the aorto-gonado-mesonephros (AGM) region of the embryo. Nascent HSCs migrate to developing hematopoietic organs such as the fetal liver during gestation, where they self-renew, differentiate and amplify to produce a large pool of erythroid progenitors. Around the time of birth HSCs colonize the bone marrow, where they sustain definitive erythropoiesis for the remainder of life. Definitive RBCs contain exclusively fetal/adult globins and are enucleated when they reach circulation.
**Human Hemoglobin Switching**

<table>
<thead>
<tr>
<th>Site</th>
<th>Primitive</th>
<th>Definitive Hematopoiesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk Sac</td>
<td>Fetal Liver</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>wks 3 - 10</td>
<td>month 2 - 7</td>
<td>month 7 on</td>
</tr>
<tr>
<td>ζ₂ε₂, α₂ε₂, α₂γ₂</td>
<td>α₂γ₂ (HbF)</td>
<td>α₂β₂ (HbA) α₂δ₂ (HbA₂)</td>
</tr>
</tbody>
</table>

**Figure 10. Human globin gene switching through erythroid lineages.**

Human primitive erythroid cells derived from the yolk sac express mainly embryonic and fetal globins- ζ, α, ε, and γ (while only low levels of β and δ globin are expressed). The primitive erythroid lineage disappears by the time bone marrow hematopoiesis is established. Human fetal γ to adult β (and the less expressed δ) globin gene switching occurs within the definitive erythroid lineage near the time that hematopoiesis transitions from fetal liver to bone marrow.
## Human Hemoglobin Switching

### Table: Human Hemoglobin Switching

<table>
<thead>
<tr>
<th>Site</th>
<th>Primitive</th>
<th>Definitive Hematopoiesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>Yolk Sac</td>
<td>Fetal Liver → Bone Marrow</td>
</tr>
<tr>
<td>Gestation</td>
<td>E7 - 15</td>
<td>E11 - birth → From birth</td>
</tr>
<tr>
<td>Globin</td>
<td>ζ₂β₁h₂, α₂ε₂Y₂</td>
<td>α₂β₂ (HbA) → α₂β₂ (HbA)</td>
</tr>
</tbody>
</table>

**Figure 11. Mouse globin gene switching through erythroid lineages.**

Mouse primitive erythroid cells, derived from the yolk sac, express all of the embryonic and fetal/adult genes- ζ, α, β₁h₁, εY and β (with only low levels of β globin). The primitive erythroid lineage disappears by the time bone marrow hematopoiesis is established. Only the fetal/adult α and β globin genes are expressed in mouse definitive erythroid cells derived from the fetal liver and bone marrow (unlike the definitive human γ to β and δ globin gene switch).
**Globin of Mice and Men**

Human definitive RBCs contain predominantly fetal hemoglobin (HbF, $\alpha_2\gamma_2$) until birth, and then switch to adult hemoglobin (HbA, $\alpha_2\beta_2$). In contrast, mouse RBCs do not have HbF, with their definitive erythroid cells instead relying on HbA during both fetal and adult life. This difference means that mutations in the $\beta$ globin gene can lead to prenatal anemia and lethality in mice, instead of the postnatal onset typical of human disease. Although mice transgenic for the human $\beta$ globin gene locus undergo $\gamma$ to $\beta$ globin gene switching, their switch is completed early in gestation unlike humans. Furthermore, expression of the human $\gamma$ globin gene appears to be restricted to primitive erythroid cells, meaning that it is regulated in transgenic mice similar to an embryonic globin gene.

**Thalassemia in the Real World**

Decades of patient study concerning the mechanisms of thalassemia have supported a causative role for globin chain imbalance (112). However, significant gaps in our understanding of thalassemia still exist. Certain mutations can have little if any effect on the hematological phenotype. The globin chain imbalance they cause appears to be of insufficient magnitude to overcome compensatory mechanisms within maturing erythroid cells. Additional genes can modify the phenotype, most notably with $\alpha$ and $\beta$ thalassemia co-inheritance. Thalassemia, while considered a monogenic disorder, exhibits additional characteristics of more complex genetic diseases that have less clearly defined relationships between genotype and phenotype.
Silent and Dominant Thalassemia

Thresholds form distinctions between accepted categories of thalassemia—silent, minor, intermedia, major, and dominant. Both silent thalassemia and thalassemia minor may go unnoticed by the patient and are distinguished by the degree to which they can be uncovered during routine hematological screening (silent thalassemia may only be evident by globin chain synthesis studies). Interestingly, thalassemia minor (also called trait) patients can be screened in populations based on the increased resistance of thalassemic RBCs to osmotic lysis in dilute saline solutions (91, 92). Thalassemia minor may be associated with a slight anemia that becomes somewhat more pronounced with age, but most patients live normal lives free of complications.

In contrast, thalassemia intermedia describes cases that fall in between the two extremes of unaffected trait and those most severely affected. Thalassemia intermedia patients have significant to severe anemia but they can survive without chronic blood transfusions. This degree of untreated anemia can begin to affect their quality of life (65).

Patients with β thalassemia major (also referred to as Cooley's anemia) acquire severe anemia when their fetal hemoglobin production diminishes in the months following birth. Their profound anemia becomes lethal without intervention, with these patients becoming dependent upon frequent blood transfusions to survive. β thalassemia major patients accumulate massive iron overload as a result of their chronic RBC transfusion therapy and this excess iron must be chelated in order to prevent organ failure. Lack of
compliance with chelation therapies has been a pressing issue for many patients. The costs of each of these long-term therapies remain high for society and the patients.

A rare set of alleles can cause thalassemia when heterozygous, without classic inheritance of an additional recessive allele (49, 65). These dominant thalassemia alleles typically mutate the carboxy-terminus of globin chains, causing an unstable hemoglobin molecule that precipitates and directly leads to RBC dysfunction in a manner less strictly related to the chain imbalance. Dominant thalassemia alleles have largely been restricted to small family studies.

Clinical observations regarding thalassemia have important implications for prenatal genetic counseling (45, 47). Mutations inactivating all adult globin chain production from an allele are generally considered recessive in nature. Heterozygous carriers have only minor hematological changes, and are typically unaware of their status until routine blood examination (which is not always informative). Fortunately, clinicians can often assign thalassemia patients to functional categories with reference to the underlying genetics. The main goal for screening and prenatal counseling is to help parents identify when both are carriers and inform them of potential reproductive risks.

**Basis for β Thalassemia**

Universal screening programs have uncovered a vast array of changes to the globin loci with wide-ranging effects. Most mutations of the β globin gene are non-deletional, single base pair changes (66, 67, 71). Over 200 such mutations have been described (15, 26),
but only a handful of frequently occurring forms occur within a given population. Often these mutations impair processing of β globin message by affecting splicing.

For thalassemia two general classes of β globin mutations have been defined. Some mutations abolish all β globin production and render β0 alleles nonfunctional. β+ alleles instead retain partial function and are thus an intermediate category between and β0 and normal βA alleles. β+ mutations encompass alleles with only minimal output (<5%) as well as others that have only slight reductions in β globin production. As expected, these differences in β globin output can strongly affect the thalassemic phenotype including their anemia (112).

Homozygous individuals provide the most stringent test cases for these definitions. Inheritance of two β0 alleles generally yields β thalassemia major. However, β+ mutations contribute a wide range of possible outcomes, from unaffected to a lethal anemia comparable to β0 alleles. Inheritance of distinct alleles in compound heterozygous individuals leads to further variation in thalassemic phenotypes. A detailed genetic analysis can help account for these complexities.

**Analogies with α Thalassemia**

In contrast to the non-deletional mutations typical at the β globin locus, mutations within the α globin locus are usually deletional and can affect either one or both of the adult α globin genes within the allele (46). α0 mutations inactivate both the adult α2 and α1 globin genes and are most prevalent in southeast Asia, with the major form being labeled
\( \alpha^{\text{SEA}} \). Other less common \( \alpha^0 \) deletions with unique regional distributions have also been described such as \( \alpha^{\text{FIL}} \) and \( \alpha^{\text{THAI}} \). Individuals homozygous for \( \alpha^0 \) alleles produce no functional adult \( \alpha \) globin, resulting in Barts hydrops fetalis with death prior to or shortly following birth. The unusual \( \gamma^d \) globin homotetramers observed in Bart’s hydrops fetalis are called Hb Barts and although they provide inadequate oxygen delivery, they can be used for prenatal screening. Fetal loss and stillbirths can be traumatic for families, but although they occur even more frequently, severe \( \alpha \) thalassemia forms have received less attention than their \( \beta \) thalassemia counterparts. One reason is that no consistently effective therapy has yet been demonstrated for hydrops fetalis (although transplantation and/or transfusion have been tried with mixed results) in part due to the strong possibility of accompanying developmental defects. With such limited treatment options, parents usually opt to terminate affected pregnancies whenever prenatal diagnosis is available (16).

Another class of mutations, \( \alpha^+ \), typically remove a single \( \alpha \) globin gene within the locus. Interestingly, the widespread \( \alpha^{3.7} \) and less common \( \alpha^{4.2} \) deletional mutations both remove a single \( \alpha \) globin gene but have distinct outcomes. The breakpoints of their deletions differ, with homologous recombination leaving the \( \alpha 1 \) globin gene intact for \( \alpha^{4.2} \). Depending upon the exact region involved in homologous recombination, the \( \alpha^{3.7} \) deletions can replace the \( \alpha 1 \) globin gene promoter with the more active \( \alpha 2 \) globin gene promoter and therefore lead to increased \( \alpha \) globin production from this allele compared to the \( \alpha^{4.2} \) deletion.
Human α globin gene copy number influences severity of α thalassemia. Because the human α globin genes are duplicated as α2 and α1, the normal diploid human α globin gene copy number is 4. A decrease to 3 α globin genes, particularly when the α1 globin gene has been deleted rather than the more dominant α2, typically results in no hematological phenotype but can be uncovered through sophisticated biochemical or genetic testing. Inheritance of 2 α globin genes results in α thalassemia trait, with only a mild hematological phenotype similar to β thalassemia minor. HbH disease is caused by mutation of 3 out of 4 α globin genes and has moderate to severe hematological changes similar to β thalassemia intermedia. Finally, the most severe form of α thalassemia is disruption of all 4 α globin genes, called Bart’s hydrops fetalis, which results in death in utero or sometimes shortly following birth. Modified from (34), under Creative Commons License 2.0.
Figure 13. Human $\alpha^+$ globin alleles caused by gene deletion.

$\alpha^+$ deletions typically remove a single $\alpha$ globin gene from the human $\alpha$ globin locus. $\alpha^{4.2}$ deletional mutations (top red line) remove the $\alpha_2$ globin gene. $\alpha^+$ deletions can also create fusion gene products depending upon their homologous recombination breakpoints. The more common (especially in African populations) $\alpha^{3.7}$ deletions frequently combine the $\alpha_2$ globin gene promoter with sequence from the $\alpha_1$ globin gene. Preservation of the more active $\alpha_2$ globin gene promoter can result in higher output from the $\alpha^{3.7}$ deletional allele compared with the $\alpha^{4.2}$ deletion. The genes located between the embryonic $\zeta$ and adult $\alpha_2$ and $\alpha_1$ globin genes are considered pseudogenes, along with $\theta$ globin. Modified from (34), under Creative Commons License 2.0.
Analogous to inheritance of β globin gene mutations, individuals heterozygous for α⁰ or α⁺ alleles are recessive carriers with either silent α thalassemia or α thalassemia minor. Similarly, individuals with homozygous α⁺ mutations have α thalassemia minor when retaining two partially functional α globin genes. Co-inheritance of α⁰ and α⁺ alleles results in a condition called HbH disease, with significant anemia analogous to β thalassemia intermedia. HbH disease leads to cellular inclusions derived from β⁴ globin homotetramers also called HbH. α⁺ globin alleles (predominantly α³.7) are quite common within African populations, but α⁰ is rare, meaning that births within African populations are considered to be at relatively low risk for HbH disease or hydrops fetalis.

The deletional form of HbH disease resulting from a combination of alleles such as α⁰ and α³.7 is generally not transfusion dependent. On the other hand, non-deletional α⁰ forms of α globin mutations have been described which interact even more strongly with α⁰ alleles. The most common form is α⁰CS, with a mutant α globin gene that forms extended, unstable globin chains called hemoglobin Constant Spring (HbCS). Non-deletional HbH patients inheriting α⁰ alleles are often transfusion dependent, with more severe anemia than deletional HbH patients. These differences point to an important role for globin chain stability in modulating thalassemia phenotypic severity.

**Mouse Thalassemia**

Unlike human β thalassemia, non-functional β globin alleles are inherited dominantly in mice. Mice inheriting a single allele with either an inactivating mutation (deletional or non-deletional) have comparatively severe β thalassemia intermedia. In general, similar
anemia is observed in humans only when both β globin alleles are mutated. Globin chain balance difference between humans and mice appears to be insufficient to account for this observation. However, some mutations have been described in humans that produce unstable globin chains and a similar dominant inheritance pattern, providing an alternate explanation with a proposed differential stability of human and mouse chains.

Homozygous β globin knockout mice expire in utero with their definitive erythropoiesis almost completely ineffective. Nevertheless, transplant of fetal liver cells from these homozygous β knockout mice into lethally-irradiated adult mice produced the first transfusion-dependent mouse model of Cooley's anemia. However, RBCs did not develop from the transplanted β knockout cells, as reticulocyte production was undetectable, being defective in part due to the lack of any fetal γ or minor adult δ globin that would serve to defend against the toxic excess mouse α globin chains.

Previous mouse models of human hemoglobin disorders display additional characteristics that limit their utility but remain poorly understood. Somewhat paradoxically, even heterozygous β knockout mice have a relatively severe thalassemia intermedia, markedly different from the thalassemia minor caused by analogous mutations in man. Finding a convincing explanation for these discrepancies may help improve our understanding of thalassemia pathology.
Thesis Overview

We hypothesized that knockin human globin genes in mice could lead to improved models of human hemoglobin disorders. Beginning with human $\alpha_2$ and $\alpha_1$ globin knockins at the mouse $\alpha$ globin locus and $^\gamma\gamma$ and $\beta^\Lambda$ at the mouse $\beta$ globin locus, we bred animals to produce $\alpha_2\alpha_1 / \alpha_2\alpha_1 \gamma\beta^\Lambda / \gamma\beta^\Lambda$ compound homozygotes (humanized knockin mice). In this thesis we also describe humanized knockin mice with an assortment of mutant alleles that have been described in man. One goal of these studies is to produce faithful animal models of human disease. Availability of these mouse models is anticipated to advance our understanding of the corresponding human disorders and stimulate development of novel therapies.

In chapter one, we hypothesized that humanized knockin mice would display perinatal switching from fetal to adult globins, similar to humans. Focusing on the developmental timing of globin gene regulation, we demonstrate that the switch from the human $\gamma$ globin gene to the human $\beta$ globin gene is completed after birth in humanized knockin mice. Furthermore, we show that this process occurs within mouse definitive erythroid cells. Our work provides a platform for further advances in understanding $\gamma$ globin gene regulation.
Fully Humanized Knockin Mice

HS26  ζ α2 α1
       α2α1 Knockin

HS26  ζ α2 α1
       α2α1 Knockin

LCR   εY βh1 Λγ βA
       γβA Knockin

LCR   εY βh1 Λγ βA
       γβA Knockin
Figure 14. Humanized knockin mice.

Human globin gene cassettes are targeted into the mouse genome to replace the mouse fetal/adult globin genes. The LCR in the mouse β globin gene locus regulates the downstream human γ and β globin genes. Human α globin genes are similarly controlled by upstream mouse HS26. By breeding together different knockins, fully humanized knockin mice can be produced that express 100% human fetal and adult hemoglobin in their mouse red blood cells, a model system amenable to several avenues of study.
In chapter two, we hypothesized that human \( \gamma \) and \( \beta \) globin genes could repair a \( \beta \) thalassemia allele in mouse embryonic stem cells. After replacement of the mutant allele, we demonstrated that human \( \beta \) globin gene expression corrected their thalassemia intermedia. When complemented with human \( \alpha \) globin genes, thalassemia in humanized knockin mice more closely follows clinical findings rather than the disproportionately severe disease found in previously described thalassemic mice.

In Chapter three we hypothesized that \( \alpha \) thalassemia is sufficient to alleviate sickle cell disease in humanized knockin mice. We compare hematology and survival with alleles containing a single human \( \alpha_1 \) globin gene or both the human \( \alpha_2 \) and \( \alpha_1 \) globin genes. Co-inheritance of \( \alpha \) thalassemia in humanized sickle mice ameliorates several aspects of their anemia, providing protection that is independent of the effects of fetal hemoglobin.

The conclusion analyzes our work in the context of ongoing research. We provide an outlook based on developments within two critical areas: gene regulation and stem cell therapy. Further advances in these fields will give much needed hope for patients with hemoglobin disorders.
HUMAN GLOBIN KNOCK-IN MICE COMPLETE FETAL-TO-ADULT HEMOGLOBIN SWITCHING IN POSTNATAL DEVELOPMENT

by

SEAN C. MCCONNELL, YONGLIANG HUO, SHANRUN LIU, AND THOMAS M. RYAN


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Format adapted for dissertation
Abstract

Elevated levels of fetal $\gamma$-globin can cure disorders caused by mutations in the adult $\beta$-globin gene. This clinical finding has motivated studies to improve our understanding of hemoglobin switching. Unlike humans, mice do not express a distinct fetal globin. Transgenic mice that contain the human $\beta$-globin locus complete their fetal-to-adult hemoglobin switch prior to birth with human $\gamma$-globin predominantly restricted to primitive erythroid cells. We established humanized knock-in mice that demonstrate a distinct fetal hemoglobin (HbF) stage, as $\gamma$-globin is the dominant globin chain produced during mid- to late gestation. Human $\gamma$- and $\beta$-globin gene competition is evident around birth, and $\gamma$-globin chain production diminishes in postnatal life with transient production of HbF reticulocytes. Following completion of the $\gamma$-to-$\beta$-globin switch, adult erythroid cells synthesize low levels of HbF. We conclude that the knock-in globin genes are expressed in a pattern strikingly similar to human development, most notably with postnatal resolution of the fetal-to-adult hemoglobin switch. Our findings are consistent with the importance of BCL11A in hemoglobin switching, as removal of intergenic binding sites for BCL11A results in human $\gamma$-globin expression in mouse definitive erythroid cells.
Introduction

Mammalian globin gene family members are temporally regulated to produce different hemoglobins during embryonic, fetal, and adult life. Humans are born with high levels of fetal hemoglobin (HbF) in their red blood cells (RBCs) and complete the switch to adult hemoglobin (HbA) during the first year of life (32, 43). Patients with mutations in their adult β-globin genes that cause thalassemia or hemoglobinopathies like sickle cell anemia are clinically normal at birth, but become progressively anemic upon completion of their fetal-to-adult hemoglobin switch. Understanding this hemoglobin switch is of great clinical importance, because disease in these patients could be averted by reactivation of the fetal globin gene or by preventing the switch to the adult gene altogether. Generation of an in vivo animal model that recapitulates the postnatal human fetal-to-adult hemoglobin switch would be a valuable research tool for examining the mechanism of hemoglobin switching.

Hemoglobin is a tetrameric protein composed of two α-like and two β-like globin chains. The genes encoding these α- and β-like chains are grouped into separate loci that are regulated by distant enhancers such as the locus control region (LCR) located upstream of the β-like globin structural genes (16, 18, 19, 26). The human β-globin locus (Fig. 1A) is comprised of five functional genes (ε, Gγ, Aγ, δ, and β) organized 5’ to 3’ in the order of expression during development. The murine β-globin locus (Fig. 1B) has four highly expressed genes (εY, βh1, β1, and β2) arranged roughly in their order of expression. Similarly, the α-globin loci of humans and mice are organized with a single embryonic ζ-
globin and two adult α-globin genes. The high-level expression of each of the above genes is temporally regulated through development to produce balanced amounts of α- and β-like globin chains for the production of hemoglobin specifically in erythroid cells.

Erythroid cells can be classified into two distinct lineages which arise in utero with unique anatomical and temporal origins. First to appear are primitive erythroid cells that arise in the yolk sac blood islands during early gestation. Nucleated primitive erythroblasts enter the circulation where they continue to divide and mature until the definitive erythroid lineage becomes established. In the mouse primitive erythroblasts undergo a single maturational globin gene switch from embryonic βh1-globin to εY-globin (10, 25, 48). Humans switch from embryonic ε-globin to fetal γ-globin gene expression when the site of erythropoiesis shifts from the yolk sac to the fetal liver (15, 20). A maturational switch from embryonic ζ-globin to α-globin occurs in both the human and murine α-globin loci (25, 49). Importantly, expression of embryonic globin genes (βh1, εY, and ζ in mice; ε and ζ in humans) is restricted to the primitive erythroid lineage (43, 45).

The onset of the definitive erythroid lineage is heralded by the emergence of hematopoietic stem cells (HSCs) at the aorta-gonad-mesonephros region of the late embryo (28). The first wave of definitive erythropoiesis in humans and mice occurs after HSCs seed the developing fetal liver. Around the time of birth HSCs migrate to the bone marrow where definitive erythropoiesis is maintained for the remainder of adult life. Definitive erythroblasts proliferate, differentiate, mature, and finally enucleate to become...
reticulocytes before entering the peripheral blood. In humans the γ-globin genes are the major β-like globin genes expressed during definitive fetal liver erythropoiesis. These γ-globin chains pair with α-globins to form HbF (α2γ2) the dominant hemoglobin present in fetal life. Around birth there is a gradual switch from fetal γ-globin expression to the adult β-globin genes. Accordingly, adult hemoglobin, HbA (α2β2), gradually replaces HbF to become the dominant hemoglobin during adult life. Mice on the other hand, do not have distinct fetal and adult type hemoglobins. Mice simply express their adult β1- and β2-globin genes during both fetal liver and adult bone marrow definitive erythropoiesis (2, 45).

Transgenic mice have been used extensively for the study of human hemoglobin switching (2, 6, 9, 14, 17, 33, 38, 39, 41). However, generating mouse models with fetal-stage hemoglobin has been challenging. Mice transgenic for the human β-globin locus produce a definitive erythroid lineage that appears deficient for HbF (41). In this report, we examine the regulation of human γ- and β-globin genes that are inserted into the mouse β-globin locus. We demonstrate that humanized (100% human hemoglobin) knock-in mice complete fetal-to-adult hemoglobin switching in postnatal development, thus modeling this hallmark of human hematopoiesis in mouse definitive erythroid cells.
Materials and Methods

Development of humanized γβΑ mice

Mouse β-globin knockout mice (5) were backcrossed more than ten generations to C57BL/6J to make congenic β-thalassemic mice (B6.Cg-Hbbθ). Congenic β-thalassemic males were bred to female 129S1/SvImJ mice (Jackson Laboratories, Bar Harbor, ME) in which ovulation was hormonally induced, and 4.5-day blastocysts were plated onto mouse embryonic fibroblasts to generate F1 hybrid β-thalassemic embryonic stem cells (129SB6.Cg-HbbθF1). The human γβΑ-globin gene cassette (39) that contains 383 bp of the human γ-globin gene promoter was targeted to the mouse β-globin knockout locus in these F1 embryonic stem cells using the previously described targeting construct (50). Correctly targeted human γβΑ knock-in embryonic stem cells were microinjected into 8 cell blastocysts to clone heterozygous γβΑ knock-in mice (36). Heterozygous γβΑ knock-in mice were bred to cytomegalovirus (CMV)-Cre transgenic mice (30) to delete their loxP-flanked hygromycin marker gene. Human α2α1-globin knock-in mice (22, 23; T.M. Ryan, unpublished data) were bred to γβΑ knock-in mice to produce doubly heterozygous knock-in mice, which were interbred to obtain homozygous α2α1/α2α1 γβΑ/γβΑ knock-in mice (humanized mice) for analysis.

Collection of peripheral blood cells

Timed pregnancies were set up, with day of vaginal plug designated as embryonic day 0.5 (E0.5). Peripheral blood was isolated by fetal decapitation in 0.5 mL of phosphate-buffered saline (PBS) with 5 mM EDTA, or by tail vein or cardiac puncture into EDTA
blood collection tubes for postnatal time points. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Erythroid cell purification and flow cytometry analysis**

Flow cytometry sorting was performed on FACS Aria or FACS Vantage SE Diva (BD Biosciences, San Jose, California) instruments. Primitive and definitive erythroid cells from E14.5 fetal blood were sorted by gating on their forward and side scatter properties (25). Bone marrow was isolated by flushing femurs and tibias with PBS followed by repeated passage through a 26-gauge needle to make a single-cell suspension. Bone marrow erythroid cells at various stages of differentiation were stained with a combination of phycoerythrin (PE)-conjugated Ter119 and fluorescein isothiocyanate (FITC)-conjugated CD71 antibodies (BD Biosciences), and sorted into various erythroblast populations as previously described (23, 42).

We used HbF-PE antibody (BD Biosciences) and thiazole orange (Sigma-Aldrich, St. Louis, MO) for enumerating the HbF cellular distribution and reticulocytes (1, 12). Briefly, cells were fixed in fresh 0.05% glutaraldehyde for 10 min, washed in PBS, and permeabilized in 0.1% Triton X-100 for 5 min. After two washes with PBS, cells were stained with HbF antibody for 30 min. Antibody-stained cells were incubated 15 min with thiazole orange and then washed and resuspended in PBS. All incubations were at room temperature. Stained cells were analyzed on a FACSCalibur instrument (BD Biosciences). Thiazole orange was poorly compensated in the PE channel; thus, we determined the portion of HbF-positive reticulocytes from the difference between HbF-
positive cells stained with and without thiazole orange. Similarly, HbF-negative reticulocytes were determined from the difference between HbF-negative cells with and without thiazole orange.

Cytospin preparation and morphological analysis

Sorted primitive and definitive erythroid cells were resuspended in PBS with 5% mouse serum, loaded into cytospin funnels, and centrifuged at 500 × g for four minutes on a Shandon Cytospin II instrument (Thermo Fisher Scientific, Waltham, MA). Slides were air dried and stained using JorVet Dip Quick (Jorgensen Laboratories, Loveland, CO). Images were captured at ×1,000 magnification using an AX70 microscope (Olympus America, Center Valley, PA) fitted with an Axiocam camera (Carl Zeiss MicroImaging, Thornwood, NY).

Quantitative real-time RT-PCR (qPCR) expression analysis

RNA was purified from peripheral blood or sorted cells using Trizol LS (Invitrogen, Carlsbad, California). cDNA was prepared using the High Capacity cDNA Kit (Applied Biosystems, Carlsbad, California). Real-time PCR reactions contained ABI Taqman Mastermix (Applied Biosystems), 900 nM probe, 200 nM primers, and typically 1 to 10 ng of cDNA as described. PCR reactions for cDNA prepared from sorted cells, were similar except that FastStart Universal Probe Mastermix (Roche Applied Science, Indianapolis, IN) was used. Reactions were run on a 7900HT Real-Time PCR System (Applied Biosystems). Measurements were performed in triplicate using a 384 well optical plate and the standard error of the mean from each set of biological replicates was
calculated. At least four biological replicates were used for each qPCR experiment. Expression was calculated by the $\Delta\Delta C_T$ method using experimentally determined primer efficiencies as described (22, 34).
Results

Description of humanized $\gamma\beta^A$ knock-in mice

The aim of our study was to develop a humanized mouse model with perinatal HbF-to-HbA switching similar to humans. We hypothesized that a human $\gamma$- and $\beta$-globin gene construct that demonstrated delayed hemoglobin switching in transgenic mice (38, 39) would effectively generate the desired expression pattern when knocked into the mouse $\beta$-globin locus. This delayed switching human $\gamma\beta^A$-globin gene cassette was knocked into the mouse $\beta$-globin locus, replacing both adult mouse $\beta$-globin genes (Fig. 1C). In order to produce mice that synthesize complete human HbF and HbA, we bred the human $\gamma\beta^A$ knock-in mice to animals with a similar targeted knock-in of the human $\alpha_2$- and $\alpha_1$-globin genes into the murine $\alpha$-globin locus (22, 23). Mice homozygous for both the human $\alpha_2\alpha_1$ and $\gamma\beta^A$ knock-ins expressed exclusively human hemoglobins within their definitive erythroid cells. A detailed description of globin gene switching in these humanized mice is presented below.

Hemoglobin switching in humanized $\gamma\beta^A$ knock-in mice

We analyzed globin gene switching at the RNA and protein levels in humanized mice through development. Total RNA and protein hemolysates were prepared from peripheral blood cells at various gestational and postnatal time points. This permitted us to measure globin mRNA levels by qPCR and globin chain levels by HPLC for each of the human and mouse $\beta$-like globin genes during development. These analyses revealed that
Figure 1. Diagrams of the human, mouse, and humanized γβA knock-in mouse β-globin loci. (A) Human β-globin locus has five functional genes (black rectangles) located downstream of the LCR enhancer delineated by a series of DNaseI hypersensitive sites (black circles). (B) Mouse β-globin locus has four highly expressed genes (grey rectangles) situated downstream of the murine LCR similarly composed of DNaseI hypersensitive sites (grey circles). (C) Humanized γβA knock-in mouse β-globin locus has the adult mouse β1- and β2-globin genes replaced by the human fetal Aγ- and adult β-globin genes. The human genes must interact with the mouse LCR enhancer sequences at a distance. Eighty kilobases of each locus is shown.
humanized $\gamma^{A}$ knock-in mice had three distinct switches of their $\beta$-like globin genes (Fig. 2A).

Primitive erythroid cells displayed two $\beta$-like globin gene switches in early gestation. The first switch that occurred between E9 and E10.5 (Fig. 2A) was the expected switch from mouse embryonic $\beta h1$-globin to mouse $\varepsilon Y$-globin. Embryonic $\beta h1$-globin message levels drop from 51% of total $\beta$-like globin at E9 down to 28% at E10.5. During this same interval, there was a reciprocal increase in embryonic $\varepsilon Y$-globin from 33% to 58%. Low human $\gamma$-globin levels (16% and 13%) and very low human $\beta$-globin levels (below 1%) were present in these early primitive erythroid cells.

The second switch observed in primitive erythroid cells, from mouse $\varepsilon Y$-globin to human $\gamma$-globin, occurred between E10.5 and E14.5 (Fig. 2A). The high mouse $\varepsilon Y$-globin levels at E10.5 (58%) declined by E14.5 (28%) while human $\gamma$-globin levels rose during the same time interval (13% to 63%). Mouse $\beta h1$ levels continued their decline (0.3%) in E14.5 fetuses and human $\beta$-globin began to increase (9%). Finally, primitive erythroid cell expression levels of both murine embryonic genes were silenced by E16.5. Beginning with the E14.5 time point the levels of human $\gamma$- and $\beta$-globin mRNA represent the combined levels from both primitive and definitive erythroid cells present in fetal blood. The determination of the relative distribution of each human mRNA that occurs in either the primitive or the definitive erythroid cells requires the prior separation of these cell types before measurement (see “Erythroid cell lineage and maturation analysis”).
Figure 2. Globin gene switching in humanized $\gamma\beta^A$ mice through development. (A) Three globin gene switches were observed in humanized mice. The first switch occurred in the early embryo from mouse embryonic $\beta_h1$ (□) to mouse embryonic $\epsilon Y$ (△). Mouse $\epsilon Y$ then switched to human fetal $\gamma$ (■) that became the dominant gene expressed during most of fetal life. The final switch from human fetal-to-adult $\beta$ (▲) was completed after birth. Mouse and human globin mRNA levels in embryonic, fetal, and adult blood were measured by qPCR and plotted as a percentage of total $\beta$-like globin mRNA versus gestational age. (B) Mouse and human globin chain levels were measured in fetal blood hemolysates by HPLC. Human $\gamma$-globin chains became the dominant $\beta$-like protein in fetal erythroid cells by E15.5. (C) Human fetal-to-adult hemoglobin switching was completed around three weeks after birth. Human $\gamma$- and $\beta$-globin chains in fetal and postnatal blood hemolysates were quantified by HPLC. Time point values in panels A to C represent the means ± SEM (n ≥ 4). E, embryonic day; NB, newborn; P, postnatal day.
The final globin gene switch occurred in definitive erythroid cells between E18.5 and birth (Fig. 2A). Human \( \gamma \)-globin expression levels were the dominant globin RNA throughout the majority of fetal life (63% to 77% from E14.5 to E18.5). The high fetal \( \gamma \)-globin levels were replaced by human adult \( \beta \)-globin gene expression. Competition with the adult \( \beta \)-globin gene accelerated in the last days of gestation. Human \( \beta \)-globin expression levels increased from 8% at E14.5 to 20% at E18.5 before rapidly rising to become the principal \( \beta \)-like globin mRNA (83%) at the time of birth, with the remainder consisting of human \( \gamma \)-globin (17%). At the completion of the switch in adult humanized mice, \( \beta \)-globin represented 99.7% of peripheral blood globin mRNA with only 0.3% \( \gamma \)-globin message. Compared to wild-type mice, humanized \( \gamma \beta^A \) knock-in mice had acquired an additional distinctly fetal pattern of globin gene expression.

Hemoglobin switching in humanized \( \gamma \beta^A \) knock-in mice was also analyzed at the protein level by quantifying \( \beta \)-like globin chains in hemolysates prepared from fetal and adult blood. Between E14.5 and E16.5, there was a clear globin chain switch from mouse embryonic chains to human globin chains (Fig. 2B). The major \( \beta \)-like globin chains synthesized in E14.5 fetuses were the protein product of the mouse \( \varepsilon \)-Y-globin gene. E14.5 mouse \( \varepsilon \)-Y chain levels decreased from 52% to 4% of total chains over the next two days of gestation. Likewise, the low levels of mouse chains synthesized from the mouse \( \beta h1 \) gene decreased from 6% to undetectable levels over the same time period. Human \( \gamma \)- and \( \beta \)-globin chains did the converse. Fetal \( \gamma \) chains increased from 31% to 55% to become the predominant globin chain present in E16.5 fetuses. Human \( \beta \) chains rose
from 11% at E14.5 to 41% in E16.5 fetal blood. Thus, similar to the qPCR expression data described above for primitive erythroid cells, humanized γβA knock-in fetuses switch from mouse embryonic to human fetal globin chains. The only major difference was a temporal delay of about two days between the measured RNA and protein levels.

Completion of the postnatal human γ- to β-globin switch was assessed by HPLC analysis of the human globin chains in postnatal peripheral blood hemolysates (Fig. 2C). Again the hemoglobin switch at the protein level reflected the overall pattern that was observed by qPCR, albeit with a slight delay. Newborn humanized mice had γ-globin chain levels that were 42% of total human chains. Postnatal γ-chain levels declined over the first 3 weeks after birth to 13% at one week, 4% at two weeks, and 0.4% at three weeks of age with little variability. No significant γ-globin chain levels were detectible by HPLC analysis of older adult humanized γβA hemolysates.

Thus, humanized γβA knock-in mice have a distinct period of fetal hemoglobin expression in which human γ-globin expression is the dominant β-like chain produced through most of fetal gestation. Furthermore, these humanized mice display a gradual fetal-to-adult hemoglobin switch that is initiated before birth but completed in postnatal life. Both of these patterns of expression are similar to hemoglobin switching in humans.

**Erythroid cell lineage and maturation analysis**

A fetal hemoglobin switching pattern similar to humans implies robust expression in both primitive and definitive erythroid cells. To directly test the γ-globin expression profile within these two distinct erythroid populations, primitive and definitive erythroid cells
were purified from E14.5 fetal blood by flow cytometry. Total RNA from these sorted populations was analyzed by qPCR for expression of the \( \beta \)-like globin genes (Fig. 3). Figure 3A demonstrates the gating of the primitive and definitive erythroid cells based upon their forward scatter and side scatter properties. Morphological examination of these two sorted populations confirmed the separation of nucleated primitive cells from the mostly enucleated definitive cells (Fig. 3B).

Expression analysis of the sorted primitive erythrocytes demonstrated high level expression of murine embryonic \( \varepsilon \)Y-globin (19% of total \( \beta \)-like globin message) but little embryonic \( \beta h1 \)-globin (Fig. 3C). This was expected, since the murine maturational switch of embryonic \( \beta h1 \) to \( \varepsilon Y \) is essentially complete by E14.5 (10, 25, 48). The human \( \gamma \)-globin gene had the highest expression levels in the primitive cells at 67% of total \( \beta \)-like globin message. This confirms the second hemoglobin switch from murine embryonic to human fetal globin in primitive erythroid cells observed between E10.5 and E14.5 (Fig. 2A). There were also significant amounts of adult human \( \beta \)-globin gene expression (14%) similar to that reported for the adult mouse \( \beta \)-globin genes in sorted primitive cells from wild-type mice (25).

Expression analysis of the sorted E14.5 enucleated erythrocytes confirmed they were definitive cells (Fig. 3D). They did not express embryonic globins (<1% of total \( \beta \)-like globin mRNA) but instead expressed high levels of human fetal \( \gamma \)-globin (82% of total), with lesser amounts of human adult \( \beta \)-globin (18%). While clearly distinct, humanized
Figure 3. Humanized γβA mice express high levels of human γ-globin in both primitive and definitive erythroid cells. (A) Flow cytometry was used to sort primitive and definitive erythroid cells from E14.5 fetal blood of humanized mice. (B) Cytospin preparations of sorted cells from panel A demonstrate that primitive erythroid cells were mostly nucleated while definitive erythroid cells were mostly enucleated. (C and D) Expression of human and mouse β-like globin genes in sorted primitive (C) and definitive (D) erythroid cells. Globin mRNA levels were determined by qPCR of E14.5 fetal blood RNA. Both primitive and definitive erythroid cells express high levels of human γ-globin. Definitive cells did not express murine embryonic globin genes.
primitive and definitive erythroid populations both express high levels of human fetal hemoglobin.

Low postnatal HbF levels led us to further examine human γ-globin gene expression during adult erythroblast differentiation/maturation. The human fetal-to-total β-like globin expression ratios were calculated in early and late erythroblasts to see if there was a maturational globin gene switch occurring during definitive bone marrow erythropoiesis. Early and late stages of erythroblast differentiation were sorted by flow cytometry of adult bone marrow cells after staining with fluorescently labeled antibodies to the transferrin receptor, CD71, and the erythroid-specific antigen, Ter119. The ratio of human γ- to β-like globin message in the RNA from the sorted populations was determined by qPCR (Table 1). There was no significant change measured in the relative γ-globin levels as differentiation progressed from pro-erythroblasts (CD71\textsuperscript{HIGH} Ter119\textsuperscript{LOW}) to basophilic/polychromatic (CD71\textsuperscript{HIGH} Ter119\textsuperscript{HIGH}) and polychromatic/orthochromatic (CD71\textsuperscript{LOW} Ter119\textsuperscript{HIGH}) erythroblasts and circulating reticulocytes (CD71\textsuperscript{NEG} Ter119\textsuperscript{HIGH}). Therefore, unlike the primitive erythroid cells, the adult bone marrow definitive erythroblasts in human γβ\textsuperscript{A} knock-in mice did not exhibit a maturational globin gene switch.

**Postnatal F-cell and F-reticulocyte analysis**

The postnatal completion of the human γ- to β-globin switch in humanized γβ\textsuperscript{A} knock-in mice was analyzed at the individual RBC level. The percentages of peripheral blood cells that contained fetal γ-globin chains (F-cells), RNA (reticulocytes), and F-cells with RNA
Table 1

Human γ-globin to total β-like globin mRNA during adult erythroblast differentiation.

<table>
<thead>
<tr>
<th>Erythroblast Population</th>
<th>γ / (γ + β)(^a)</th>
<th>P value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD71(^{\text{HIGH}}) - Ter119(^{\text{LOW}})</td>
<td>0.344 ± 0.067</td>
<td></td>
</tr>
<tr>
<td>CD71(^{\text{HIGH}}) - Ter119(^{\text{HIGH}})</td>
<td>0.232 ± 0.011</td>
<td>0.15</td>
</tr>
<tr>
<td>CD71(^{\text{LOW}}) - Ter119(^{\text{HIGH}})</td>
<td>0.339 ± 0.063</td>
<td>0.96</td>
</tr>
<tr>
<td>CD71(^{\text{NEG}}) - Ter119(^{\text{HIGH}})</td>
<td>0.329 ± 0.070</td>
<td>0.89</td>
</tr>
</tbody>
</table>

\(^a\) γ-globin message as a % of β-like message SEM

\(^b\) Statistical analysis was performed by student’s \(t\) test compared with those for CD71\(^{\text{HIGH}}\) - Ter119\(^{\text{LOW}}\) cells.
(F- reticulocytes) were measured over time in postnatal humanized mice by flow cytometry. HbF was present in the majority of erythroid cells in newborn humanized mice (Fig. 4). The fraction of F-cells dropped markedly in the weeks following birth. By postnatal day five (P5), F-cell levels had decreased from 81% at birth to 42% (Table 2). By weaning age, P21, F-cells had further declined to 11%. In mature adult humanized mice, F-cell levels were detected in about 1% of peripheral blood cells. While F-cell levels declined in a manner similar to that seen for the total \( \gamma \)-chain levels measured in hemolysates (Fig. 2C), the human \( \gamma \)-globin chains were not pancellularly distributed in all RBCs, but were confined to a subset of cells (F-cells) in a heterocellular distribution.

The circulating F-cells detected after birth were either produced \textit{in utero} or were generated after birth indicating that the human \( \gamma \)- to \( \beta \)-globin gene switch was not yet completed. To test for any postnatal production of nascent F-cells, we measured the production of F- reticulocytes (Table 2). At birth F- reticulocytes represented 29% of all peripheral blood cells. Furthermore, F- reticulocytes were 69% of total reticulocytes at birth, more than double the number of reticulocytes that contained only adult human \( \beta \)-globin chains (Non-F reticulocytes) (Table 2). The F- reticulocyte levels decreased to 7% at P5, 2% at P21, and were below the limit of detection in older adults (P140). The Non-F reticulocytes demonstrated a transient rise from 13% to 37% of total cells over the first 5 days of life before declining to 18% at P21 and reaching steady state level of 4.9% in adult humanized mice. The presence of F- reticulocytes for several weeks after birth demonstrated that the human \( \gamma \)-globin gene was still expressed and that the fetal-to-adult hemoglobin switch was completed after birth.
Figure 4. HbF cellular distribution during postnatal development of humanized γβ^A mice. Flow cytometry analyses of peripheral RBCs stained with PE-conjugated anti-HbF antibody. Contour plots of HbF staining versus forward scatter are shown from postnatal day 1 (P1) to day 140 (P140) mice. The F-cell percentages are indicated in the top part of each panel. High newborn F-cell levels are gradually replaced by RBCs with only human HbA (Non-F cells) in the weeks following birth.
Table 2

Postnatal F-cell and F- reticulocyte analysis in humanized mice$^a$

<table>
<thead>
<tr>
<th></th>
<th>% Non-F cells HbF- TO-</th>
<th>% Non-F retics HbF- TO+</th>
<th>% F cells HbF+ TO-</th>
<th>% F retics HbF+ TO+</th>
<th>Total retics HbF+ TO-</th>
<th>Total F cells HbF+ TO+</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>6.4±0.7</td>
<td>12.9±1.5</td>
<td>51.9±3.0</td>
<td>28.9±1.7</td>
<td>41.7±2.2</td>
<td>80.8±0.5</td>
</tr>
<tr>
<td>P5</td>
<td>21.1±1.1</td>
<td>36.7±2.7</td>
<td>34.8±2.0</td>
<td>7.4±2.1</td>
<td>44.1±1.8</td>
<td>42.2±1.9</td>
</tr>
<tr>
<td>P21</td>
<td>70.6±0.3</td>
<td>18.4±1.1</td>
<td>9.2±0.7</td>
<td>1.9±0.2</td>
<td>20.2±0.9</td>
<td>11.0±0.9</td>
</tr>
<tr>
<td>P140</td>
<td>94.4</td>
<td>4.9</td>
<td>0.7</td>
<td>0.0</td>
<td>4.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^a$ % gated cells ± SEM (n = 3 mice except in the case of P140, where n = 1). Retics, reticulocytes; HbF, fetal hemoglobin antibody staining; TO, thiazole orange staining.
Discussion

Humanized $\gamma^A\beta$-globin knock-in mice recapitulate the developmental timing of HbF-to-HbA switching that occurs in humans. This is remarkable because mice do not have a distinct fetal globin gene equivalent. Early knockout experiments clearly demonstrate that deletion of the adult mouse $\beta$-globin genes results in fetal death because there is no fetal hemoglobin to sustain life to birth (5, 52). In order to provide a fetal-stage hemoglobin and model human fetal-to-adult globin switching, we generated knock-in mice by targeted gene replacement of the adult mouse $\alpha_1\alpha_2$- and $\beta_1\beta_2$-globin genes with human $\alpha_2\alpha_1$- and $\gamma^A\beta$-globin genes, respectively. These knock-in mice express the human $\gamma$-globin gene in both primitive and definitive erythroid cells. Primitive erythropoiesis is marked by two maturational hemoglobin switches from murine embryonic $\beta_h1$ to $\epsilon Y$ around E10 followed by $\epsilon Y$ to human $\gamma$ around E12. Definitive erythroid cells synthesize exclusively human hemoglobins in fetal and adult RBCs.

High-level $\gamma$ expression occurs throughout fetal life to make it the major $\beta$-like globin expressed during mid to late gestation. There is a single human $\gamma$- to $\beta$-globin switch in definitive erythroid cells that is completed after birth. Newborn hemolysates showed HbF levels of 42%, similar to the low end of the range of HbF reported for human infants (32). There is a gradual decline in HbF levels until the steady-state level of 1% is reached by about three weeks of age. Adult bone marrow expression of $\gamma$-globin was not preferentially restricted to immature erythroblasts, but was consistent with low-level expression of $\gamma$-globin during all stages of erythroid differentiation.
HbF is present in most erythroid cells in newborn humanized mice (Fig. 4) and is present in a similar percentage of RBCs during fetal life (data not shown). While HbF did not appear to be distributed pancellularly, a study using human cord blood showed similar numbers of HbF-negative cells (1), implying that this type of staining procedure may underestimate the number of F-cells. The percentage of F-cells decreases markedly after birth; however, detection of F-reticulocytes in the weeks following birth demonstrates the sustained postnatal production of F-cells. The F-reticulocyte fraction drops to background levels by maturity, when the switch to adult human β-globin is complete. In contrast to previous models, humanized γβ^A mice exhibit a fetal-to-adult hemoglobin switch in definitive erythroid cells that begins in utero and is completed after birth similar to humans. Thus, even though wild-type mice lack a true fetal hemoglobin stage during development the requisite factors needed for perinatal human fetal-to-adult hemoglobin switching are apparently conserved between the species.

Transgenic mice are an important model system for understanding human hemoglobin switching. Early transgenic experiments demonstrate that individual γ- or β-globin transgenes are temporally regulated (27, 44), but their expression is low level and integration site dependent. Inclusion of LCR sequences with individual globin transgenes overcomes the low-level expression and position effects (16, 37), but results in the loss of correct temporal expression (2, 8, 9). Correct temporal expression returns when multiple globin genes are forced to compete for interaction with a single LCR (2, 9, 14, 33). Yet, transgenic animals that contain the entire human β-globin locus inappropriately complete
the fetal-to-adult hemoglobin switch in fetal development (14, 33, 35). Significantly, human \( \gamma \)-globin gene expression in these transgenic mice is predominantly restricted to primitive erythroid cells (41). Without robust \( \gamma \)-globin gene expression during fetal life, human \( \beta \)-globin locus transgenic mice that contain adult \( \beta \)-globin gene mutations die in utero after breeding to mouse \( \beta \)-globin knockout mice (4, 24). These experiments imply that the human \( \gamma \)-globin gene in the context of the entire \( \beta \)-globin locus is regulated similar to an embryonic globin gene, and the fetal-to-adult switch in the definitive erythroid lineage is not completed after birth as in humans.

This study is the first detailed analysis of the hemoglobin switching knock-in cassette, \( \gamma \beta^A \), responsible for the high \( \gamma \)-globin levels throughout fetal life. As depicted in Fig. 1, to generate this cassette we removed the intergenic region between the human \( ^A \gamma \)- and \( \beta \)-globin genes, placing the genes in close proximity while potentially removing key regulatory elements required for human hemoglobin switching. Transgenic mouse studies that link this cassette directly to human LCR sequences result in continued expression of \( \gamma \)-globin in fetal life (39; Ryan, unpublished). Although the spatial organization of the \( \gamma \beta \) cassette may contribute to loss of normal repression of the human \( \gamma \)-globin gene during fetal development, this is an advantage for modeling human hemoglobin disorders in mice. This delayed switching cassette is responsible for the in utero survival and high-level expression of HbF in newborn knockout-transgenic sickle cell mice (38). Similarly, knock-in of this delayed switching \( \gamma \beta^S \) cassette into the mouse \( \beta \)-globin locus results in the postnatal survival of the first humanized knock-in model of sickle cell anemia (50). Even in the absence of a functional \( \beta \)-globin gene, humanized \( \gamma \beta^0 \) knock-in mice can
survive to birth solely by synthesizing HbF (22). Interestingly, incorporation of hereditary persistence of fetal hemoglobin (HPFH) mutations into the \( \gamma \)-globin promoter furthers extends the postnatal life of these humanized Cooley’s anemia mouse models (21, 23). These experiments affirms the importance of the production of HbF during perinatal development in sickle cell disease and Cooley’s anemia. Moreover, the delayed switching \( \gamma \beta \) cassette mimics fetal-to-adult hemoglobin switching either as a transgene linked directly to human LCR sequences or after incorporation into the murine \( \beta \)-globin locus where it must interact with murine LCR sequences at a distance. This implies that these mouse enhancer sequences can interact with and drive the high-level, temporally regulated expression of the human \( \gamma \) - and \( \beta \)-globin genes in definitive erythroid cells.

Trans-acting factors involved in hemoglobin switching are known, such as KLF1 and BCL11A, that bind to cis regulatory elements located in the \( \beta \)-globin locus. Familial association studies demonstrate that mutation of either gene can result in persistent fetal globin gene expression in adults (3, 29, 47). Recent studies link BCL11A to the temporal regulation of \( \gamma \)-globin by binding to sequences in the intergenic region between \( \gamma \) - and \( \beta \)-globin (40, 41, 51). Different isoforms of BCL11A are produced during development. Human fetal liver erythroblasts that express \( \gamma \)-globin synthesize a short BCL11A isoform, whereas adult bone marrow erythroblasts synthesize a larger isoform and repress fetal gene expression. Mice synthesize a single Bcl11a isoform throughout definitive erythropoiesis and effectively restrict \( \gamma \)-globin gene expression to primitive erythroid cells in human \( \beta \)-globin locus transgenic mice (41). Our \( \gamma \beta^A \) knock-in cassette lacks the intergenic sequences that bind Bcl11a, providing one possible explanation for persistent
γ-globin expression in definitive erythroid cells during fetal life. Inclusion of the BCL11A binding sites in the γβ^A knock-in cassette with or without the erythroid-specific removal of the Bcl11a repressor isoform would be interesting future experiments.

In the absence of the intergenic BCL11A binding sites in the γβ^A knock-in, presumably Klf1, in addition to other factors (31, 53), is sufficient to regulate the perinatal human γ- to β-globin switch that still occurs. KLF1 not only regulates the level of BCL11A expression (3, 54) but influences the human fetal-to-adult hemoglobin switch by binding directly to the β-globin promoter (7, 11).

Integration of the roles of different trans-acting factors together with their binding sequences (such as those removed in deletional HPFH or mutated in non-deletional HPFH) is needed for the complete picture of globin gene regulation to emerge. Understanding the mechanisms regulating human fetal-to-adult hemoglobin switching will be important for the therapeutic reactivation of the human γ-globin gene, an important clinical goal for β-thalassemia and sickle cell anemia.
Acknowledgements

This work was supported by National Institutes of Health grants R01 HL072351 (to T.M.R.), R01 HL073440 (to T.M.R.), UAB CMB Training Grant T32 GM008111 (to S.C.M.), and a Carmichael Scholarship (to S.C.M.).

We thank Marion Spell in the UAB Center for Aids Research and Enid Keyser from the UAB Analytic Preparative Cytometry Facility for cell sorting expertise. We thank Tim Townes, Louis Chow, and Peter Detloff for access to equipment. We thank the Cooley’s Anemia Foundation, UNICO Foundation Inc., and Joseph Ruisi for their support.
References


HAPLOINSUFFICIENCY FOR β GLOBIN IN HUMANIZED KNOCKIN MICE
RESULTS IN β THALASSEMIA MINOR SIMILAR TO HUMAN CLINICAL FINDINGS

by
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Manuscript in progress
Format adapted for dissertation
Abstract

In mice, heterozygous knockout of the adult mouse $\beta$ globin genes results in a dominant $\beta$ thalassemia intermedia phenotype that is more severe than the human equivalent, recessive $\beta$ thalassemia minor. To understand why, we examined $\beta$ thalassemia phenotypes in the context of knockin mice following replacement of the adult mouse globin genes with human globin genes. First, we demonstrate that, similar to control $\beta$ globin knockout mice, mice heterozygous for a nonfunctional human $\beta^0$ globin allele have severe $\beta$ thalassemia intermedia. In contrast, replacement of the thalassemic allele with a normal human $\beta^A$ globin knockin produced mice that were completely rescued from severe anemia. After interbreeding human $\beta$ globin knockins to human $\alpha$ globin knockin animals, we generated fully humanized knockin mice that express exclusively human globin chains. Interestingly, with heterozygous inheritance of a nonfunctional human $\beta^0$ globin knockin allele, fully humanized trait mice presented relatively mild hematological changes, but had increased resistance of their humanized red blood cells to osmotic lysis similar to clinical findings in human $\beta$ thalassemia minor individuals. Taken together, these results are consistent with more deleterious effects of excess mouse $\alpha$ globin chains compared with human $\alpha$ globin chains. Humanized knockin mice represent a novel platform to advance our understanding and therapeutic approaches toward human $\beta$ thalassemia.
Red blood cells (RBCs) deliver oxygen with hemoglobin, a tetramer composed of equal parts of α- and β-like globin chains. Balanced amounts of these chains are critical for RBC function, as unpaired globin chains form inclusions (7), oxidatively damage the cell and lead to its premature destruction (27). Excess α globin chains cause β thalassemia (12, 36), a disorder with broad clinical spectrum and rising significance due to demographic changes in the population (35).

β thalassemia minor, also called trait, is found in individuals with single β0 globin alleles inactivated by mutation (37). These heterozygous individuals are asymptomatic or have relatively mild anemia, often unaware of their status, but can be identified by decreased RBC osmotic fragility (32). Thalassemia trait patients have long been considered recessive carriers for the more severe forms of thalassemia caused by mutation of both β globin alleles (2). Among patients homozygous for mutations of both β globin alleles, chronic transfusion dependence distinguishes β thalassemia major, also labeled Cooley's anemia (5), from the moderate to severe but less life-threatening β thalassemia intermedia (20). Although regular blood transfusion and iron chelation therapies greatly improve the prognosis for severe cases, they are associated with significant long-term costs and risks. β thalassemia major can be cured by allogeneic bone marrow transplantation, but most patients lack a suitable transplantation donor and the procedure is associated with significant morbidity and mortality. Considerable interest is now focused on genetic repair of thalassemic stem cells as an ideal way to overcome these obstacles.
Mouse models of human hemoglobin disorders have proven indispensable for advancing novel therapies (8, 14-16, 23). However, previously described experimental mouse models of β thalassemia have an anemia that is markedly more severe than the analogous human thalassemia being modeled (4, 23, 38). Unlike humans, β thalassemia major mice die in utero from profound anemia due to the lack of any fetal hemoglobin equivalent in the mouse. Likewise, heterozygous β globin knockout mice have a dominant β thalassemia intermedia phenotype that is more severe than the human equivalent, recessive β thalassemia minor (4, 10, 38). This difference in disease severity between human thalassemia patients and comparative mouse models can be attributed to unique species-specific properties of hemoglobin chain sequences. These include interactions between globin chains and the RBC membrane (3), or the solubility and proteolysis of unpaired globin chains (25). Another factor is the low-level presence of fetal γ and/or minor adult δ globin chains present in human erythrocytes. Previous transgenic and knockout models of anemia might be considered poorly suited to address these differences, having significantly divergent genetics with little ability to directly control globin chain levels or balance (6).

Recently, we began to address these issues by producing knockin mice by targeting human α, γ, and β globin genes directly into the mouse α and β globin loci. By replacing the adult mouse α and β globin genes with human α globin genes and a γ-to-β globin switching cassette, respectively, humanized knockin mice were generated that synthesize 100% human hemoglobin in their definitive RBCs (17). These mice mimic human
hemoglobin switching by synthesizing high-levels of fetal hemoglobin during fetal development and completing their fetal-to-adult switch after birth (17). This also enables humanized knockin mice with homozygous, nonfunctional human $\beta^0$ globin gene mutations to survive postnatally. Similar to Cooley’s anemia ($\beta$ thalassemia major) patients, these mice become dependent on blood transfusions for survival into adulthood (9-11). Humanized knockin mice provide a stable experimental platform that allows precise control of globin gene location, copy number, and expression level, thus offering advantages over transgenic animals. They also maintain standard globin allele inheritance patterns that are compatible with studying the effects of variant globin alleles in the context of complete replacement of their mouse hemoglobin with human hemoglobin. In this report we examine the ability of humanized knockin mice to accurately model essential aspects of human $\beta$ thalassemia.
Materials and Methods

Targeting of Embryonic Stem Cells to Generate $\gamma^\beta^A$ Knockin (KI) mice

A targeting construct, $\gamma^\beta^A$, was built in a pBluescript vector (Stratagene) containing from 5’ to 3’ a phosphoglycerate kinase (pgk) promoter driving thymidine kinase, 1.7 kb of mouse homology upstream of the mouse $\beta^{\text{maj}}$ globin gene (HindIII fragment), 4.7 kb human $\Lambda\gamma$ globin gene fragment, 4.1 kb human $\beta$ globin gene fragment, a pgk promoter driving hygromycin (pgk-hyg) resistance gene flanked by two loxP sites, and 7 kb of mouse homology downstream of the mouse $\beta^{\text{min}}$ globin gene (BamHI fragment). The plasmid was linearized by NotI digestion before electroporation into an F1 129S1vImJ-C57BL/6J mouse embryonic stem (ES) cell line that was derived by breeding a 129S1vImJ female to a heterozygous $\beta$ knockout male congenic on the C57BL/6J background. The targeted ES cells were selected in Hygromycin B (125$\mu$g/mL) and ganciclovir (1mM) in ES cell 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 $\mu$g/mL streptomycin, 0.1 mM $\beta$-mercaptopethanol, and 1000 U/mL leukemia inhibitory factor]. Following selection, DNA from drug resistant ES cell colonies was screened by PCR to identify the homologous recombinants. Mice were cloned by injecting the ES cells into eight-cell D2-C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) blastocysts that were transferred into the uteri of outbred CD1 (Charles River Laboratories, Inc., Wilmington, MA) pseudopregnant recipient mice. The cloned mice were bred to human $\alpha_2\alpha_1$ globin knockin mice containing the hCMV-Cre transgene (19; T.M. Ryan, unpublished data) to generate
doubly heterozygous $\gamma^A\beta$ and $\alpha_2\alpha_1$ knockin mice and delete the hygromycin marker genes. Fully humanized mice were produced by breeding two heterozygous knockin mice. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Hematological Indices and Histopathology**

Peripheral blood was collected from anesthetized mice into Microtainer® EDTA collection tubes (Becton Dickinson, Franklin Lakes, NJ). RBC counts and RBC distribution widths (RDW) were measured on a HemaVet® 1700 (Drew Scientific, Waterbury, CT) hematology analyzer. Packed cell volume (PCV) was measured in a JorVet J503 (Jorgenson Laboratories Systems, Loveland, CO) micro-hematocrit 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. Reticulocyte counts were determined by flow cytometry after staining with thiazole orange. Tissues were fixed in 70% alcoholic formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin or Prussian blue by standard methods at the UAB Comparative Pathology Laboratory.

**Resistance to Osmotic Lysis**

Fresh RBCs from humanized knockin mice or humanized $\beta$ thalassemia trait mice were incubated in either deionized water or a 0.36% phosphate buffered saline solution for 30
minutes. After centrifugation, the supernatant was collected and a spectrophotometer was used to measure amount of released hemoglobin at OD 540 nm. Percent lysis was calculated as the ratio of lysis in 0.36% salt to lysis in water as described (21).
Figure 1. Correction of Thalassemia Intermedia with a Human γβ^A Globin Gene Cassette in Embryonic Stem Cells. Schematic of targeted gene replacement of the adult mouse β globin gene knockout allele by a human γβ^A globin gene cassette in β thalassemic mouse embryonic stem cells. The hygromycin marker gene (hyg) was deleted by breeding to CRE transgenic mice.
Results

Correction of \( \beta \) Thalassemic Mouse Stem Cells with Human Globin Genes

Through targeted correction of the mouse \( \beta \) thalassemic allele (4) by homologous recombination in embryonic stem cells (Figure 1), human \( \gamma \) and \( \beta^A \) globin genes were introduced into the mouse \( \beta \) globin locus. After microinjection of correctly targeted embryonic stem cells into tetraploid or eight cell blastocysts, heterozygous \( \gamma \beta^A \) knockin mice (\(+/+\ \gamma \beta^A/+\)) were cloned. As controls, heterozygous \( \beta \) knockout mice (\(+/+\ 0/+\)) were also cloned from the starting embryonic stem cell line. Cloned mice were bred to C57BL/6J animals and heterozygous offspring were analyzed from each line of mice. Mice heterozygous for the \( \gamma \beta^0 \) knockin (\(+/+\ \gamma \beta^0/+\)) have been previously described (10).

Phenotype of \( \beta \) Thalassemia with Mouse Globin Chains

In the context of mouse \( \alpha \) and \( \beta \) globin chains, haploinsufficiency for \( \beta \) globin (a single \( \beta \) globin inactivated in \(+/+\ \gamma \beta^0/+\) or \(+/+\ 0/+\) mice) resulted in thalassemia intermedia, with significant anemia indicated in part by a 22% reduction in RBC number (Table 1). This reduction is consistent with reduced RBCs in human thalassemia intermedia patients (37), rather than the slightly increased or normal RBC number for thalassemia minor caused by haploinsufficiency for \( \beta \) globin in humans. Along with reduced RBC number, hematology values indicate that RBCs from \(+/+\ \gamma \beta^0/+\) and \(+/+\ 0/+\) mice were hypochromic and microcytic to a degree that may interfere with RBC function. \(+/+\ \gamma \beta^0/+\) and \(+/+\ 0/+\) mice have a 50% reduction in hemoglobin, 32% reduction in hematocrit, 35% reduction in MCH and 12% reduction in MCV compared with control mice (\(+/++/+\),
MCH and MCV not shown but can be derived by standard calculations using the data presented in Table 1). These values in +/- $\gamma^\beta_0^+/+$ and +/- 0/+ mice are more consistent with a moderate to severe thalassemia, even more so for the reduction in MCV when considered in the context of a 13 fold increase in reticulocytes (to greater than 20% of circulating blood cells) that have high MCV. Consistent with reticulocytosis and also associated with fragmentation and destruction of microcytic circulating RBCs, extreme RBC size variation is apparent in the two-fold increase in RDW for +/- $\gamma^\beta_0^+/+$ and +/- 0/+ mice compared with +/- +/+ mice.

**Correction of Mouse $\beta$ Thalassemia with a Human $\beta$ Globin Knockin**

Hematological indices for heterozygous $\gamma^\beta_A$ knockin mice (+/+ $\gamma^\beta_A^+/+$) were almost identical to wild-type mouse (+/+ +/+ ) hematology, demonstrating that correction of the knockout allele with human $\gamma$ and $\beta^A$ globin genes rescued the mice from $\beta$ thalassemia (Table 1). Significant differences in RBC number, hemoglobin, hematocrit, and reticulocyte count were observed between +/- +/+ controls and +/- 0/+ mice RBC number. Similarly, these values for +/- $\gamma^\beta_A^+/+$ mice were all significantly different from +/- 0/+ mice. Similar to +/- $\gamma^\beta_0^+/+$ mice (10), +/- 0/+ mice had marked anemia. Displaying reticulocytosis, polychromia and anisocytosis, thalassemia mice had microcytic and hypochromic RBCs (Figure 2). +/- $\gamma^\beta_0^+/+$ and +/- 0/+ mouse spleen size was 12 fold higher than +/- +/+ controls, due to expansion of thalassemic mouse spleens as erythropoietic organs (Table 1). On the other hand, +/- $\gamma^\beta_A^+/+$ mouse spleen size was not significantly different from wild-type controls (+/+ +/+ ), demonstrating, along with
Table 1. Hematology of Thalassemic Alleles in Mice and Correction by Human $\gamma\beta^A$ Knockin. Hematology parameters of wild-type, mouse $\beta$ knockout, $\gamma\beta^0$ knockin and $\gamma\beta^A$ knockin mice were obtained at 2 months of age.

<table>
<thead>
<tr>
<th>Locus Genotype</th>
<th>$\alpha$ globin</th>
<th>$\beta$ globin</th>
<th>n</th>
<th>RBC ($10^6/\mu l$)</th>
<th>Hct (%)</th>
<th>Hb (g/dL)</th>
<th>Retic (%)</th>
<th>RDW (%)</th>
<th>Spleen (% BW)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
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<td>$+/+$</td>
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<td>$</td>
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<td>$7.9^{*}$</td>
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<td>$2.2^{*}$</td>
<td>$16.0^{**}$</td>
<td>$0.3^{*}$</td>
<td>$51.1^{*}$</td>
<td>$14.7^{*}$</td>
</tr>
</tbody>
</table>

* $p<0.05$ by student t-test compared with heterozygous $\beta$-knockout mice ($+/+\ 0/+$).
** $p<0.0001$. ns not significant

Mice were analyzed at eight weeks of age.

Values represent Mean ± SEM.

n, number of mice; RBC, red blood cell; Hct, hematocrit; Hb, hemoglobin; Retic, reticulocyte; RDW, red cell distribution width; $\mu$L, microliter; dL, deciliter; % BW, percentage total body weight.
Figure 2. Histology of Thalassemia Corrected with Human Globin Genes.

‘+/+’ denotes wild-type mice (+/+ +/+), ‘0/+’ denotes heterozygous thalassemic mice (+/+ 0/+), and ‘γβA/+’ denotes mice heterozygous for the corrected allele (+/+ γβA/+).

Peripheral blood smears (×1,000 magnification) show marked hypochromia, polychromia, microcytosis, anisocytosis and poikilocytosis for RBCs from +/+ 0/+ mice. In contrast, RBC morphology from +/+ γβA/+ mice was rescued and appears similar to +/+ +/+.

Spleen sections stained with hematoxylin and eosin show that splenic white pulp (×100) was disrupted in +/+ 0/+ mice, while it was restored after correction of the mutant allele. +/+ γβA/+ animals were also rescued from extramedullary hematopoiesis (erythroid cells displayed in liver shown at ×1,000), and lacked accumulation of liver iron (Prussian blue staining, ×1,000) found in thalassemic mice.
their hematology and red blood smear that appeared normal, correction of their thalassemia. Histology of +/+ γβA/+ mice also closely matched wild-type controls (+/+ +/+), including lack of extramedullary hematopoiesis and liver iron staining (Figure 2). In contrast, +/+ 0/+ mice had nucleated erythroid progenitors apparent in the liver and increased iron staining. +/+ γβ0/+ mice also had extramedullary hematopoiesis and increased liver iron similar to +/+ 0/+ mice. The phenotype of these mice is extremely similar, as expected due to a non-functional human β globin gene combined with functionally insignificant expression levels produced from the wild-type human γ globin allele in adult γβ0 knockin mice (10).

Similarly, the γβA allele expressed almost exclusively human β globin (only about 0.3% human γ globin) (17). Compared with complete absence of expression from the knockout allele in +/+ 0/+ mice, high levels of human β globin in +/+ γβA/+ mice could apparently contribute to a significant reduction in excess mouse α globin chains. The dramatic rescue of the thalassemic phenotype in +/+ γβA/+ mice indicated that they benefited from restored globin chain balance. Although human β chains from the corrected allele paired with mouse α globin chains to form hybrid human β-mouse α hemoglobin with high oxygen affinity (26) in this context, the function of these heterotetramers proved sufficient to correct the mice from thalassemia intermedia.
**Phenotype of Humanized Knockin Mice**

Addition of human α globin knockin alleles to human β globin knockin alleles allowed us to investigate the role of human hemoglobin in mice. When the human α2α1 knockin was bred to the γβ^A knockin to produce doubly homozygous, humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A), a relatively normal hematological phenotype was apparent. Histology for humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A) was comparable to wild-type controls (+/+ +/+), including lack of extramedullary hematopoiesis and liver iron staining (Figure 4). The hematology was perhaps surprisingly similar to wild-type mouse hematology, considering the replacement of mouse hemoglobin with human hemoglobin (Table 2). Spleen size for humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A) was not significantly different from wild-type controls (+/+ +/+), p>0.5.

Hemoglobin and hematocrit for humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A) were also within normal ranges (Table 2). Only minimal reticulocytosis or RDW increase were apparent, particularly in comparison to the large changes observed with the mouse globin knockout models of thalassemia (0/+ or +/+). RBC number increased slightly in humanized knockin (α2α1/α2α1 γβ^A/γβ^A) relative to wild-type controls (+/+), possibly as a compensatory response to the increased oxygen affinity of human hemoglobin. In addition, the resistance to osmotic fragility test was negative for humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A, Figure 5), indicating minimal or no thalassemia, while their blood smears revealed no overt RBC pathology apart from slight hypochromia (Figure 3).
β Thalassemia in Humanized Knockin Mice

We then examined the phenotype of β thalassemia in the context of human globin chains in mice. β thalassemic humanized knockin mice heterozygous for a non-functional β⁰ allele (α2α1/α2α1 γβ⁰/γβ⁰) have blood smears consistent with β thalassemia minor, displaying microcytic cells, target cells and poikilocytosis (Figure 4). Hemoglobin and hematocrit values for β thalassemic humanized knockin mice (α2α1/α2α1 γβ⁰/γβ⁰) are reduced compared with humanized knockin mice (α2α1/α2α1 γβ⁰/γβ⁰) by 17% and 11%, respectively (Table 2). MCV and MCH are reduced by 17% and 22%, respectively, while RBC number increased 6%, all consistent with data from human thalassemia minor patients.
Figure 3. Osmotic Lysis Data for Humanized β Thalassemia Trait Mice.

Humanized β thalassemia trait mouse RBCs had increased resistance to osmotic lysis, with a reduced percentage of lysis compared with RBCs from humanized knockin mice.
Table 2. Hematology of Humanized Mice and Humanized Trait Mice with $\beta^0$ Allele

<table>
<thead>
<tr>
<th>Locus Genotype</th>
<th>$\alpha$ globin</th>
<th>$\beta$ globin</th>
<th>n</th>
<th>RBC ($10^6/\mu L$)</th>
<th>Hct (%)</th>
<th>Hb (g/dL)</th>
<th>Retic (%)</th>
<th>RDW (%)</th>
<th>Spleen (% BW)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
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<tbody>
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<td>$\alpha_2\alpha_1/\alpha_2\alpha_1$</td>
<td>$\gamma\beta^A/\gamma\beta^A$</td>
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<tr>
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</table>

Hematology of human $\alpha_2\alpha_1$ knockins paired with $\gamma\beta^A/\gamma\beta^A$ or $\gamma\beta^0/\gamma\beta^A$ knockins.

* $p<0.05$ by student t-test compared with humanized trait ($\alpha_2\alpha_1/\alpha_2\alpha_1$ $\gamma\beta^0/\gamma\beta^A$).

** $p<0.0001$

ns not significant

Mice were analyzed at eight weeks of age. Values represent Mean ± SEM.

n, number of mice; RBC, red blood cell; Hct, hematocrit; Hb, hemoglobin; Retic, reticulocyte; RDW, red cell distribution width; $\mu$L, microliter; dL, deciliter; % BW, percentage total body weight.
Figure 4. Histology of Humanized β Thalassemic Trait Mice.

γβA denotes humanized knockin mice (α2α1/α2α1 γβA/γβA), and γβ0 denotes humanized β thalassemic trait mice (α2α1/α2α1 γβ0/γβA). Humanized β thalassemic trait mice (α2α1/α2α1 γβ0/γβA) had target cells and poikilocytosis evident in their peripheral blood smears, and a slight degree of liver iron staining, unlike control humanized knockin mice (α2α1/α2α1 γβA/γβA). Magnification power is identical to the Figure 2 descriptions.
Discussion

After generating humanized knockin β thalassemia trait mice, we found that their thalassemic pathology is mild and actually quite similar to the minimal or no pathology found in human β thalassemia trait patients. In contrast, β globin inactivation alleles confer dominant β thalassemia inheritance in mice (4, 38), rather than recessive β thalassemia inheritance typical of β globin inactivation alleles in humans. The relative severity of the blood smear of +/+ 0/+ and +/+ γβO/+ mice is perhaps even more consistent with human β thalassemia major (due to functional loss of two alleles) instead of human thalassemia intermedia. Lack of protective minor adult δ globin chains or fetal γ globin chains in mouse thalassemia offers one explanation for the marked anemia of +/+ 0/+ and +/+ γβO/+ mice compared with mild clinical findings for an analogous β globin inactivation mutation in humans, or in humanized β thalassemia trait mice. However, β thalassemia alleles that delete the human γ, δ and β globin genes are also inherited recessively in patients (33). On the other hand, knockin alleles with an inactivated human β globin gene but that produce moderate levels of γ and δ globin (with up to 10% of β like chains in adults) are inherited dominantly in the background of mouse globin chains but recessively in the background of human globin chains (11; Y. Huo, unpublished data). Collectively, these findings indicate that the protective effects of minor adult δ globin chains or fetal γ globin chains are unlikely to offer a convincing explanation for the phenotypic disparity between the globin chain backgrounds. Instead, three additional lines of evidence indicate that the distinct thalassemias of mouse and man arise due to intrinsic human and mouse globin chain differences.
First, even with similar haploinsufficiency for globin alleles in humans and mice (and reciprocal degrees of thalassemia), phenotypes for α and β thalassemia can significantly diverge (1, 4, 22, 37, 40). Thalassemic severity is largely determined by the degree of chain imbalance, ranging from asymptomatic carriers to death in utero without intervention (37). Both α and β thalassemic RBCs are microcytic and hypochromic due to significant deficiency of either chain, while excess globin chain amount is critical in determining the phenotypic outcome. However, the identity of the chains in excess plays an important role in determining membrane pathology and alterations in RBC development (28-31). Pathologies of heterozygous β and α knockout mice are quite different (1), demonstrating that unique properties of the globin chains found in excess can greatly influence disease outcomes. Ineffective erythropoiesis is a hallmark of moderate to severe β thalassemia, with the effects of chain imbalance becoming evident in the marrow as nucleated erythroblasts undergo apoptosis (39). In contrast, erythroid development is relatively normal in moderate to severe α thalassemia, while RBCs in circulation are sensitized to premature destruction as their membranes accumulate damage at an accelerated rate (18).

Second, several rare mutant alleles in human patients cause dominant thalassemia (33). Dominant thalassemias are frequently linked to amino acid substitutions in the third exon of β globin that destabilize the protein fold or α-β dimer interface (13), providing a link between hemoglobin instability and increased thalassemic severity. Thus, if mouse hemoglobin is more unstable than human hemoglobin, this might explain some of the phenotypic difference for thalassemia. Interestingly, the proper storage of mouse
hemolysates is more critical than for human hemolysates (6), consistent with an observed lower stability measured for mouse hemoglobin compared with human hemoglobin (34). Mouse RBCs may be sensitized by increased dissociation of mouse hemoglobin compared with the more stable human hemoglobin, helping to explain the dominant inheritance of β thalassemia in mice with a mouse globin chain background. This may be contrasted with recessive thalassemia observed after inactivation of one β globin allele in human patients, indicating a protective role for stability of excess α globin chains.

Third, mouse α globin chains are less soluble than human α globin chains (24). Reduced proteolytic removal of these insoluble, excess mouse α chains may further impede their clearance, exacerbating the thalassemic phenotype. These features conspire to form inclusions in +/+ γβ⁰/+ thalassemic mouse RBCs (10) similar to those found in developing human RBCs that carry unstable human hemoglobin variants. However, excess α globin may be present at relatively high levels in bone marrow cells, while human RBCs in circulation have achieved approximate globin chain balance. One mechanism may be the pitting of circulating thalassemic RBCs by the spleen, removing insoluble globin inclusions from their membranes. Since heterozygous β thalassemic (+/+ 0/+ ) mouse RBCs often do not reach circulation due to ineffective erythropoiesis (1), this indicates that their excess mouse α globin chains cannot be effectively removed through such a process. Thus, compensatory mechanisms both intrinsic and extrinsic to the thalassemic RBCs appear to play a role in β thalassemia, influencing severity.
Taken together, reduced stability of mouse α globin chains may contribute to dominant mouse β thalassemia, as more highly toxic mouse α globin inclusions ravage β thalassemic mouse RBCs. Thus we hypothesized that these negative effects on RBCs would be reduced for thalassemia in the context of excess human α globin chains (having greater stability). Excess human α globin chains in humanized β thalassemia trait mouse RBCs allowed us to explore this hypothesis, since no minor hemoglobin chains (δ) and only negligible levels of fetal hemoglobin (γ) were available to ameliorate the β thalassemia. Our data for β thalassemia in humanized trait mice are more consistent with the mild thalassemia observed in human β thalassemia trait patients rather than thalassemia of β knockout mice. This indicates reduced severity for excess human α globin compared with mouse α globin chains, supporting a protective role for human hemoglobin in thalassemic mouse RBCs. In conclusion, replacement of mouse hemoglobin with human hemoglobin provides β thalassemic mice with an improved hematologic outcome, quite similar to thalassemic patients but unlike previous mouse models, promoting humanized knock-in mice as a model of human β thalassemia.
Acknowledgments

This work was supported by National Institutes of Health grants R01 HL072351 (T.M.R.) and R01 HL073440 (T.M.R.), a Carmichael Fellowship (S.C.M.) and UAB CMB Training Grant T32 GM008111 (S.C.M.).

Authorship

Author contributions: S.C. designed experiments, performed experiments, analyzed research, made figures and wrote the paper; Y.H. designed experiments, performed experiments and analyzed research; S.R., R.Y. and T.Z performed experiments and analyzed research; T.M.R. designed experiments, analyzed research and wrote the paper.
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α THALASSEMIA AMELIORATES SICKLE CELL DISEASE IN HUMANIZED KNOCKIN MICE INDEPENDENTLY OF FETAL HEMOGLOBIN

by

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Manuscript in progress

Format adapted for dissertation
Abstract

Sickle cell anemia is monogenic yet the clinical picture is heterogeneous as genetic modifiers that affect fetal hemoglobin levels and α thalassemia strongly influence clinical diversity. Indeed, experiments in knockout transgenic sickle cell mice indicate that persistent levels of fetal hemoglobin and/or some degree of thalassemia are necessary for their viability. In order to overcome the problems inherent in transgenic mouse experiments, we generated α and β globin locus knockin mice that survive on 100% human hemoglobins. Knockin of both adult human α globin genes produced expression levels comparable to endogenous mouse α globin, while knockin of a single human α globin gene resulted in reduced expression and thalassemia trait when homozygous. Replacement of the mouse adult β globin genes with a human fetal-to-adult globin gene switching cassette rescued humanized knockin sickle mice from in utero death by synthesizing high levels of human fetal hemoglobin throughout fetal development. After completing their fetal-to-adult hemoglobin switch postnatally, humanized sickle mice became severely anemic and most did not survive beyond four months of age. In contrast, humanized sickle mice with α thalassemia had reduced anemia and most survived beyond one year of age. Similar to findings in humans, α thalassemia increased the resistance of humanized trait red blood cells to osmotic lysis, identifying a property of α thalassemia that could protect adult humanized sickle mice from hemolytic anemia even in the absence of significant levels of fetal hemoglobin. Our work highlights the beneficial effects of α thalassemia in sickle cell disease and provides a novel model system for mechanistic and therapeutic studies.
Introduction

Sickle cell disease is an inherited disorder (60) that causes severe hemolytic anemia as well as the hallmark painful crises and complications associated with vaso-occlusion (36, 90). The majority of the 200,000 Africans born each year with sickle cell disease are not expected to reach five years of age (87, 108, 109). Even sickle patients with the benefit of modern medicine (75, 106) face high morbidity (2, 30, 73) and mortality (74, 113) with limited treatment options available. Although the molecular basis of sickle cell disease was elucidated over five decades ago (44, 68), the complex chain of events unleashed by this simple amino acid change remains a topic of extensive research (27, 94). A better understanding of how patients homozygous for the sickle mutation can have either a mild or severe clinical course is required (19, 92). While several genetic modifiers have been identified in sickle patients (25, 85, 93), most notably affecting \( \alpha \) globin gene expression (22, 38) or fetal hemoglobin levels (50, 52, 98), their direct effects are challenging to study within diverse patient populations. Thus, a critical need remains for the development of improved \textit{in vivo} models to test disease mechanisms and advance therapies (23, 82).

Many challenges to modeling human hemoglobin disorders in mice have been overcome over the last 25 years. A robust mouse model of sickle cell disease not only requires the high-level, erythroid tissue-specific, and temporally correct expression of the human \( \alpha, \gamma, \) and \( \beta^S \) globin genes, but the endogenous mouse \( \alpha \) and \( \beta \) globin chains, which have “anti-sickling” properties (76), must be removed in order for the \textit{in vivo} sickling of RBCs to
occur (32, 81). Current knockout-transgenic sickle cell anemia mouse models largely
overcame these issues to become an invaluable research tool, but most of these models
appear to require either the presence of polymerization inhibitors such as mouse globin
chains, persistent high levels of fetal hemoglobin, or the added presence of thalassemia to
remain viable (23). All transgenic sickle mouse models vary due to the particular
transgene used, position effects from random chromosomal integration, and random
transgene copy numbers that produce variable globin expression levels. Imbalanced
levels of human $\alpha$ and $\beta^s$ globin transgene expression are typical, a critical feature that is
difficult to control in these models but that can strongly influence the sickle cell disease
phenotype. In addition, transgenic sickle mice lack classical globin gene inheritance
patterns. The variability inherent in transgenic methodology insures that no two sickle
mouse models will be the same and any research findings will need to be evaluated in the
context of the model that is used.

An alternative approach to building a mouse model of sickle cell disease is to make site-
specific modifications to the genome such that the precise gene number and location can
be controlled. Starting with embryonic stem cells, we replaced the adult mouse $\alpha$ and $\beta$
globin genes with human fetal/adult $\alpha$ and $\beta$ globin genes, respectively, which were
designed to rely on endogenous mouse enhancer sequences. We demonstrate that human
$\alpha$ globin knockin alleles are expressed at high levels in both primitive and definitive
erythroid cells. Likewise, a human $\gamma$ and $\beta$ globin knockin allele switches expression
from high levels of human fetal-to-adult globin through development. We demonstrate
that fully humanized knockin mice with the normal $\beta^A$ globin allele are healthy, but
sickle $\beta^s$ globin knockin mice suffer from severe anemia. Finally, we show that $\alpha$ thalassemia ameliorates the anemia and increases the lifespan of humanized sickle mice.
Materials and Methods

Targeting of Embryonic Stem Cells to Generate α2α1, −α1, γβS, Knockin (KI) mice

A targeting construct, γβS, was built in a pBluescript vector (Stratagene) containing from 5’-to-3’ a phosphoglycerate kinase (pgk) promoter driving thymidine kinase (TK), 1.7 kb of mouse homology upstream of the mouse βmaj globin gene (HindIII fragment), 5.7 kb human γ globin gene fragment, 4.1 kb human βS globin gene fragment, pgk promoter driving hypoxanthine phosphoribosyltransferase (HPRT) resistance gene flanked by two loxP sites, and 7 kb of mouse homology downstream of the mouse βmin globin gene (BamHI fragment). The plasmid was linearized by NotI digestion before electroporation into an HM1 129 strain mouse embryonic stem (ES) cell line that lack HPRT. The targeted ES cells were grown in HAT selection media with ganciclovir (1mM) in ES cell 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]. Following selection, DNA from drug resistant ES cell colonies was screened by PCR to identify the homologous recombinants.

α2α1 and −α1 targeting plasmids were similarly constructed, containing from 5’-to-3’, a pgk promoter driving TK, 4.0 kb of mouse homology upstream of the mouse α1 globin gene (containing part of the mouse ζ globin gene), a 7.7 kb human α2α1 globin gene fragment (HUMHBA4: 5,047-12,847 bp) amplified by PCR primers containing NotI adaptors, or alternatively a 3.8 kb fragment containing only the human α1 globin gene,
A

Mouse $\beta$ locus

Human $\gamma^{\beta S}$ targeting

$\gamma^{\beta S}$ knockin allele

B

mouse $\alpha$ locus

Human $\alpha 2\alpha 1$ gene targeting

CRE

$\alpha 2\alpha 1$ Knockin

$-\alpha 1$ Knockin
Figure 1. Targeting Human $\gamma^{\beta S}$, $\alpha_2\alpha_1$ and $-\alpha_1$ Globin Gene Cassettes.

(A) Schematic of targeted gene replacement of the adult mouse $\beta$ globin genes by a human $\gamma^{\beta S}$ globin gene cassette in HM1 mouse embryonic stem cells. The HPRT marker gene was deleted by breeding to CRE transgenic mice. (B) Targeting diagrams for the $\alpha_2\alpha_1$ knockin that replaces the adult mouse $\alpha$ globin genes with two human $\alpha$ globin genes. The human $-\alpha_1$ knockin cassette (bottom row) was similarly targeted to the mouse $\alpha$ globin locus with identical 5’ and 3’ homology regions but instead replaced the adult mouse $\alpha$ globin genes with only a single human $\alpha$ globin gene. The loxP-flanked hygromycin marker gene ($hyg$) was deleted by breeding to CRE transgenic mice.
a pgk promoter driving hygromycin (hyg) resistance gene flanked by two loxP sites, and 6.4 kb of mouse homology downstream of the mouse α2 globin gene. The two plasmids were linearized before separately being electroporated into the HM1 129 strain mouse embryonic stem (ES) cell line. After targeting, each human α2α1 and −α1 globin knockin line of ES cells was grown in ES cell media with hygromycin selection, and colonies were PCR screened for homologous recombinants.

Chimeras were produced by injecting correctly targeted ES cells into D2-C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) blastocysts that were transferred into the uteri of outbred CD1 (Charles River Laboratories, Inc., Wilmington, MA) pseudopregnant recipient mice. Chimeras were bred to C57BL/6J mice to test for germline transmission and offspring βS knockin mice were bred to mice carrying the hCMV-Cre transgene to delete the HPRT marker gene. The different heterozygous knockin mice were interbred, for instance γβS and α2α1 or γβA and −α1 knockins, to produce humanized (doubly homozygous) sickle knockin mice and humanized trait knockin mice, respectively. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Hematological indices, histopathology and expression analysis**

Peripheral blood was collected from anesthetized mice into Microtainer® EDTA collection tubes (Becton Dickinson, Franklin Lakes, NJ). RBC counts and RBC distribution widths (RDW) were measured on a HemaVet® 1700 (Drew Scientific, Waterbury, CT) hematology analyzer. Packed cell volume (PCV) was measured in a
JorVet J503 (Jorgenson Laboratories Systems, Loveland, CO) micro-hematocrit centrifuge. Hb 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 Hb standards. Reticulocyte counts were determined by flow cytometry after staining with thiazole orange. Tissues were fixed in 70% alcoholic formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin or Prussian blue by standard methods at the UAB Comparative Pathology Laboratory. Quantitative real-time expression analysis and flow cytometry experiments were performed as described (56).

**Resistance to Osmotic Lysis**

Fresh RBCs from humanized α thalassemia trait mice or humanized knockin mice were incubated in either a 0.36% phosphate buffered saline solution or in deionized water for 30 minutes. Centrifugation was used to pellet residual cell debris, and the supernatant was collected. A spectrophotometer at OD 540 nm provided measurements of the amount of hemoglobin released in the supernatant for both conditions. Percent lysis was calculated as a ratio of lysis in 0.36% salt to lysis in water, as previously described (64).
Results

Generation of Heterozygous $\alpha2\alpha1, -\alpha1, \gamma\beta^A$, and $\gamma\beta^S$ Knockin Mice

Human $\alpha$ and $\beta$ globin gene knockin mice were generated from ES cells after targeted gene replacement of the endogenous mouse $\alpha$ and $\beta$ globin genes, respectively (Figure 1). Both adult mouse $\alpha$ globin genes were replaced with either both human $\alpha$ globin genes ($\alpha2\alpha1$) or a single human $\alpha$ globin gene ($-\alpha1$) (Figure 1A). Similarly, both adult mouse $\beta$ globin genes were replaced with a human fetal ($\gamma$) to adult ($\beta^S$) globin gene switching cassette (Figure 1B). Heterozygous human globin gene knockin mice were produced from each targeted ES cell line and backcrossed onto the C57BL/6 strain.

Phenotype of Heterozygous Human Knockin Mice

Heterozygous human globin gene knockin mice were phenotypically normal. Analysis of adult peripheral blood demonstrated that the majority of the RBC indices were not significantly different from control animals (Table 1). Small but significant increases were observed in the RDW and MCH values of the human $\gamma\beta^A$ knockin mice ($+/+ \gamma\beta^A/+$) and in the RDW of human $\alpha1$ knockin mice ($-\alpha1/+ +/+$). Normally heterozygous deletion of the adult mouse $\alpha$ ($0/+ 0/+$) or mouse $\beta$ globin genes ($+/+ 0/+$) would result in a moderate $\alpha$ thalassemia or severe $\beta$ thalassemia, respectively (6). The absence of any significant anemia in these human globin gene knockin mice suggests that the knockin alleles are expressed at relatively high levels. Indeed, we demonstrate by expression analysis in $\alpha2\alpha1$ knockin mice ($\alpha2\alpha1/+ +/+$) that the two human $\alpha$ globin genes were expressed at levels equivalent to the mouse $\alpha$ globin genes (Figure 2).
Table 1- Hematology Indices Indicate α Thalassemia in Homozygous but not Heterozygous –α1 knockin mice.

<table>
<thead>
<tr>
<th>Locus Genotype</th>
<th>n</th>
<th>RBC (10^6/μl)</th>
<th>Hct (%)</th>
<th>Hb (g/dL)</th>
<th>Retic (%)</th>
<th>RDW (%)</th>
<th>Spleen (% BW)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2α1 / +</td>
<td>7</td>
<td>8.7 ± 0.1</td>
<td>42.7 ± 0.5</td>
<td>14.3</td>
<td>2.2 ± 0.3</td>
<td>17.6 ± 0.2</td>
<td>0.4 ± 0.0</td>
<td>49.1 ± 0.5</td>
<td>14.5 ± 0.7</td>
<td>29.4 ± 1.4</td>
</tr>
<tr>
<td>−α1 / −α1</td>
<td>5</td>
<td>10.4 ± 0.3</td>
<td>42.3 ± 1.3</td>
<td>11.8</td>
<td>3.3 ± 0.8</td>
<td>24.6 ± 0.6</td>
<td>0.5 ± 0.1</td>
<td>40.6 ± 0.7</td>
<td>11.3 ± 0.7</td>
<td>27.9 ± 1.7</td>
</tr>
</tbody>
</table>

Hematology Wt, heterozygous α2α1, heterozygous –α1 and heterozygous γβS, homozygous α2α1 and homozygous –α1.

* p<0.05 by student t-test compared with wild-type mice (+/+/ +/+),

** p<0.0001, ns not significant, Values represent Mean ± SEM.

Heterozygous knockin and wildtype mice were analyzed at three to eight months of age after at least ten generations of backcrossing to the C57BL/6J genetic background.

Homozygous knockin mice were analyzed at two to four months of age with mixed 129SvImJ/S1 and C57BL/6J genetic backgrounds.

n, number of mice; RBC, red blood cell; Hct, hematocrit; Hb, hemoglobin; Retic, reticulocyte; RDW, red cell distribution width; μL, microliter; dL, deciliter; % BW, percentage total body weight.
Figure 2. Human α Globin Expression Levels from α2α1 and −α1 Knockins. Human α globin expression (red) compared with mouse α globin (blue), normalized to mouse β globin expression, in heterozygous α2α1 knockin and heterozygous −α1 knockin adults.
However, in $-\alpha 1$ knockin mice ($-\alpha 1/+ +/+$) the single human $\alpha 1$ globin gene was expressed at reduced (about 60%) levels compared with the mouse $\alpha$ globin genes, consistent with an allele causing $\alpha$ thalassemia trait.

**Phenotype of Homozygous Human $\alpha$ Globin Knockin Mice**

Heterozygous $\alpha 1$ globin knockin mice were then bred together to produce offspring with a further reduction in human $\alpha$ globin gene copy number to two functional genes. Hematological analysis might be expected to be more informative in these animals since $\alpha^+$ globin alleles typically must be homozygous in order to produce $\alpha$ thalassemia trait in humans. This is highlighted by the fact that a reduction in human $\alpha$ globin gene copy number from four to two genes is usually necessary to produce clinical findings. Interestingly, homozygous $-\alpha 1$ knockin animals ($-\alpha 1/-\alpha 1 +/+$) had a hematological phenotype indicating mild $\alpha$ thalassemia trait, with significantly increased RBC number and significantly reduced MCH (Table 1). In contrast, homozygous $\alpha 2\alpha 1$ knockin mice ($\alpha 2\alpha 1/\alpha 2\alpha 1 +/+$) maintained a hematological phenotype similar to wild-type mice, despite their reliance on a human $\alpha$ globin / mouse $\beta$ globin inter-species tetramer with extremely low oxygen affinity (8). These hematology results for the homozygous $\alpha$ globin knockin mice are thus consistent with the marked difference observed in human $\alpha$ globin expression for the two distinct knockin alleles.
Generation of Fully Humanized Knockin Mice

Human $\gamma\beta^A$ knockin mice were generated as described (56; T.M. Ryan, unpublished data). To generate humanized knockin mice, we bred human $\alpha_2\alpha_1$ knockin mice to human $\gamma\beta^A$ knockin mice. This allowed us to analyze compound homozygous mice living on 100% human hemoglobin in postnatal life.

Developmental Expression of Human $\alpha$ Globin Knockin Gene(s)

To determine the developmental expression pattern and hemoglobin switching driven by the human $\alpha_2\alpha_1$ globin knockin allele, we examined mouse embryonic $\zeta$ globin and human $\alpha$ globin switch by expression analysis in peripheral blood from humanized knockin mice. After sorting RBCs at embryonic day 14.5 into two distinct populations (Figure 3), we found that humanized knockin mice produce high level mouse embryonic $\zeta$ globin message in their primitive but not definitive RBCs. In contrast, humanized knockin mouse definitive RBCs contained only human $\alpha$ globin chains to complement their human $\beta$ globin chains. Similar results could be obtained by comparing embryonic day 10.5 with adult peripheral blood expression (data not shown). These results indicate that the human $\alpha$ knockin did not disrupt proper regulation of the mouse $\alpha$ globin locus, preserving a developmental switch from the mouse $\zeta$ globin gene to the downstream human $\alpha$ globin genes.

Characterization of Humanized Knockin Mice

Humanized knockin mice ($\alpha_2\alpha_1/\alpha_2\alpha_1 \gamma\beta^A/\gamma\beta^A$) mice were also phenotypically normal. Their hematology (Table 2) is similar to wild-type mouse controls despite the change to
Figure 3. Human α Globin Knockin Expression in Primitive and Definitive RBCs.

E14.5 humanized knockin mouse erythroid cells were sorted based on size measurements by flow cytometry and expression of the upstream mouse embryonic ζ globin gene was analyzed and compared with the knockin human α globin genes.
human hemoglobin with higher oxygen affinity. Furthermore, other than slight microcytosis, humanized knockin mouse (α2α1/α2α1 γβ^A/γβ^A) peripheral blood smears presented normal RBC morphology (Figure 5). These findings indicate that their globin chains are relatively balanced.

**Generation of Humanized α Thalassemia Trait Mice**

Starting with heterozygous –α1 and γβ^A knockin mice, we paired mutant human α^+ alleles with wild-type human β globin alleles. Thus we generated humanized α thalassemia trait mice (–α1/–α1 γβ^A/γβ^A genotype). We sought to determine whether their phenotype was consistent with α thalassemia (similar to the –α1/–α1 +/+ genotype but instead in combination with human β globin knockin alleles).

**Characterization of Humanized α Thalassemia Trait Mice**

Humanized α thalassemia trait mice (–α1/–α1 γβ^A/γβ^A) have mild deficiencies in hematological indices (Table 2) compared with control humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A). For instance, the RBC number and RDW increased significantly in humanized α thalassemia trait mice compared with humanized knockin mice, while slight but significant reductions in the MCV and MCH were evident, reflecting their use as markers for hematological screening of human patients for α thalassemia trait (37). Characteristic RBC morphological changes including target cells, microcytic cells, polychromasia, and anisopoikilocytosis were evident in humanized α thalassemic trait mouse peripheral blood smears (Figure 5). White and red pulp disruption in humanized α thalassemic trait mice was consistent with mouse spleen
Table 2- Hematology of humanized knockin mice with α thalassemia and/or sickle cell disease.

<table>
<thead>
<tr>
<th>Locus Genotype</th>
<th>n</th>
<th>RBC (10^6/μl)</th>
<th>Hct (%)</th>
<th>Hb (g/dL)</th>
<th>Retic (%)</th>
<th>RDW (%)</th>
<th>Spleen (% BW)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2α1 / α2α1γβA / γβA</td>
<td>11</td>
<td>10.8±0.5</td>
<td>44.9±1.6</td>
<td>13.7±0.6</td>
<td>2.3±0.7</td>
<td>19.4±0.7</td>
<td>0.3±0.0</td>
<td>41.6±1.4</td>
<td>12.7±0.4</td>
<td>30.5±0.6</td>
</tr>
<tr>
<td>α2α1 / −α1γβA / γβA</td>
<td>4</td>
<td>4.4±0.2</td>
<td>22.4±1.3</td>
<td>5.0±0.3</td>
<td>61.1±0.8</td>
<td>26.2±0.8</td>
<td>51.3±0.5</td>
<td>62.9±1.8</td>
<td>14.4±0.8</td>
<td>22.5±1.7</td>
</tr>
<tr>
<td>−α1 / −α1γβA / γβA</td>
<td>10</td>
<td>12.1±0.3</td>
<td>39.6±1.5</td>
<td>11.5±0.7</td>
<td>5.6±0.5</td>
<td>20.5±0.7</td>
<td>0.5±0.0</td>
<td>32.8±0.5</td>
<td>9.6±0.1</td>
<td>29.1±0.4</td>
</tr>
<tr>
<td>α2α1 / −α1γβS / γβS</td>
<td>8</td>
<td>3.3±0.2</td>
<td>20.6±1.3</td>
<td>4.7±0.3</td>
<td>75.7±3.6</td>
<td>24.7±1.3</td>
<td>6.7±0.5</td>
<td>62.9±1.8</td>
<td>14.4±0.8</td>
<td>22.8±1.1</td>
</tr>
<tr>
<td>α2α1 / α2α1γβS / γβS</td>
<td>5</td>
<td>3.3±0.3</td>
<td>20.6±1.3</td>
<td>4.7±0.3</td>
<td>75.7±3.6</td>
<td>24.7±1.3</td>
<td>6.7±0.5</td>
<td>62.9±1.8</td>
<td>14.4±0.8</td>
<td>22.8±1.1</td>
</tr>
<tr>
<td>−α1 / −α1γβS / γβS</td>
<td>9</td>
<td>5.3±0.3</td>
<td>26.0±1.2</td>
<td>5.7±0.4</td>
<td>57.6±3.5</td>
<td>31.5±1.0</td>
<td>6.3±0.5</td>
<td>51.0±1.2</td>
<td>11.2±1.3</td>
<td>21.9±0.8</td>
</tr>
</tbody>
</table>

Student t-test compared α2α1 / α2α1γβA / γβA with α2α1 / −α1γβA / γβA and −α1 / −α1γβA / γβA. Student t-test compared α2α1 / α2α1γβS / γβS with α2α1 / −α1γβS / γβS and −α1 / −α1γβS / γβS.

* p<0.05, ** p<0.0001, ns not significant, Values represent Mean ± SEM.

Mice of mixed 129SvImJ/S1 and C57BL/6J genetic backgrounds were analyzed at two to three months of age, with the exception of −α1/−α1γβS/γβS mice averaging one year of age (data similar with −α1/−α1γβS/γβS mice at two months old, in (111)).

n, number of mice; RBC, red blood cell; Hct, hematocrit; Hb, hemoglobin; Retic, reticulocyte; RDW, red cell distribution width; μL, microliter; dL, deciliter; % BW, percentage total body weight.
expansion as an erythropoietic organ. Humanized α thalassemic trait mice also had signs of extramedullary hematopoiesis and liver iron staining. None of these changes were found in control humanized knockin mice.

**Resistance of Humanized α Thalassemia Trait RBCs to Osmotic Lysis**

Humanized α thalassemia trait mice had increased resistance to osmotic lysis compared with control humanized knockin mice (Figure 4). Increased membrane redundancy associated with α thalassemia allows thalassemic RBCs to resist changes in osmotic pressure (20). This functional data demonstrates that humanized trait mice have a mild α thalassemic phenotype, analogous to clinical findings in individuals with human α thalassemia trait using this screening procedure (13).

**Severe Anemia in Humanized Knockin Sickle Mice**

We also bred together the α2α1, –α1 and γβS knockins to generate humanized sickle mice with and without α thalassemia trait. These compound homozygous sickle knockin mice both displayed marked anemia, but several differences were noted. Humanized knockin sickle mice (α2α1/α2α1 γβS/γβS) have the most severe anemia, with the RBC number and hemoglobin value both extremely low. In contrast, α thalassemic humanized sickle mice (–α1/–α1 γβS/γβS) have significantly reduced anemia, consistent with an improved sickle cell disease phenotype.
Figure 4. Humanized α Thalassemia Trait RBCs Resist Osmotic Lysis. Humanized trait mouse (−α1/−α1 γβ^A/γβ^A) RBCs resist osmotic lysis significantly more than RBCs from humanized knockin mice (α2α1/α2α1 γβ^A/γβ^A).
Figure 5. Histology of Humanized α Thalassemic Trait mice. Humanized α thalassemic trait mice (−α1/−α1 γβA/γβA genotype, denoted as ‘−α1’) have RBC morphological changes including target cells, microcytosis, polychromasia, poikilocytosis and anisocytosis (×1,000 magnification), compared with control humanized mice (α2α1/α2α1 γβA/γβA genotype, denoted as ‘α2α1’). The humanized α thalassemic trait mouse spleen (×100) has disrupted white and red pulp. Extramedullary hematopoiesis and liver iron staining are evident in humanized α thalassemic trait mice but not in control humanized knockin mice.
**Fetal Hemoglobin in Humanized Sickle Mice**

Previous work showed that a construct producing a fetal hemoglobin switch near the time of birth (7, 80) overcame the *in utero* lethality observed with other mouse models of sickle cell disease. A similar human $\gamma\beta^S$ globin switching allele was used to generate humanized sickle mice (Figure 1A). Humanized sickle mice completed their fetal-to-adult hemoglobin switch after birth (Figure 6). Adult humanized knockin mice expressed low levels of fetal hemoglobin (<1%), with survival relying entirely upon human sickle hemoglobin.

**Increased Survival in Humanized Sickle Mice with $\alpha$ Thalassemia**

Survival curve analysis shows that humanized knockin sickle mice ($\alpha2\alpha1/\alpha2\alpha1 \gamma\beta^S/\gamma\beta^S$) have a severely decreased lifespan with most animals succumbing within several months (Figure 7). In contrast, most $\alpha$ thalassemic humanized sickle mice ($-\alpha1/-\alpha1 \gamma\beta^S/\gamma\beta^S$) mice survived beyond one year, demonstrating that $\alpha$ thalassemia improved their sickle cell disease phenotype. As controls, most humanized knockin mice survived about two years, similar to wild-type mice.
Figure 6. Human Fetal-to-Adult Hemoglobin Switching in Humanized Sickle Mice.

RNA from humanized knockin sickle mice with α thalassemia (−α1/−α1 γβ^S/γβ^S) was analyzed at developmental timepoints (E10.5, E14.5, NB-newborn, adult). Expression of human γ globin (red) and human β globin (blue) were normalized to total β-like chains, as a ratio of human γ / (γ + β).
Figure 7. Survival of Humanized Sickle Mice with $\alpha$ Thalassemia.

Survival curve comparison between humanized knockin mice ($\alpha_2\alpha_1/\alpha_2\alpha_1 \gamma^A/\gamma^A$ genotype, denoted as ‘a2a1 gba’), humanized sickle mice ($\alpha_2\alpha_1/\alpha_2\alpha_1 \gamma^S/\gamma^S$ genotype, denoted as ‘a2a1 gbs’), and humanized sickle mice with $\alpha$ thalassemia ($-\alpha_1/-\alpha_1 \gamma^S/\gamma^S$ genotype, denoted as ‘a1 gbs’), demonstrating increased severity of humanized sickle mice without $\alpha$ thalassemia ($\alpha_2\alpha_1/\alpha_2\alpha_1 \gamma^S/\gamma^S$).
Discussion

Modeling human hemoglobin disorders in mice, while challenging, has advanced our fundamental understanding of gene regulation. Early experiments showed that human globin transgenes were expressed specifically in the erythroid lineage (12, 100), but at variegated, low levels, at about 1% of endogenous mouse globins. After discovery of the locus control region (LCR) upstream of the β-like globin genes (101), addition of these enhancer sequences to transgenes permitted high-level, position-independent expression of human β globin (33, 78, 95), averaging 100% endogenous levels. The first high-level expression of human α globin genes was also achieved by addition of LCR sequences (35, 79). These advances led to the development of transgenic mice that produced high levels of human sickle hemoglobin (32, 81), although residual mouse globin chains interfered with sickle hemoglobin polymerization (76). The next step was knockout of the adult mouse α (66) and β globin genes (14, 112). Sickle transgenes were then bred to these knockout mice to produce compound, homozygous deletions at both mouse globin loci, leading to production of the first knockout-transgenic sickle mice that expressed 100% human hemoglobin in their RBCs (65, 80). With the ability to form sickle fibers at physiological oxygen conditions, the pathology of knockout-transgenic sickle mice is well-studied (23, 54), providing important preclinical models for pharmaceutical intervention (40, 48, 89, 102) and gene therapy (53, 70, 71).

However, several problems have plagued knockout-transgenic sickle mouse models. Sufficient expression for globin transgenes can be difficult to achieve and may require
several integrated copies (103), without advance determination. No two integrated globin transgenes are the same due to random copy numbers, orientations and genomic positions. While transgenic loci have rarely been mapped in detail, the effects of tandem arrays, fragmentation, and unique sequence junctions (10, 28, 69, 72) have been implicated in unpredictable silencing. Unorthodox positions for enhancer sequences within multi-copy transgenic loci can potentially alter transgene regulation (24). Variegated expression frequently occurs (55), restricting human globin to a subset of the erythroid cells (31), even for transgenic globin loci under control of the LCR (1). Troubling loss of function mutations also can arise upon insertional disruption of important genomic sequences (59). These considerations necessitate the more costly production and examination of multiple transgenic lines to verify findings. Furthermore, transgenic loci lack classical globin gene inheritance patterns, with more complicated genotyping (83) and inefficient breeding schemes (23). Difficulty in controlling \(\alpha\) and \(\beta\) globin chain balance means that transgenic sickle mice typically also have thalassemia (23), which can strongly influence their phenotype. In summary, transgenic models are the sum of many parts that remain out of the control of investigators. To overcome these problems, a new approach is needed.

Our approach was to generate knockin alleles containing human globin genes in place of the related mouse globin genes. Knockin globin chain levels, type and balance could be rationally controlled, simply by including the relevant deletional and non-deletional gene mutations found in man (at the \(\alpha\) and \(\beta\) globin loci, respectively, for these experiments). By targeting specific chromosomal locations at the permissive mouse globin gene loci,
problems associated with randomly integrated transgenes could be overcome. The
knockin alleles follow simple Mendelian inheritance patterns that correspond with human
globin genes, and since hemoglobinopathies have polygenic aspects, distinct knockin
alleles can easily be bred together to produce humanized mice with the different globin
types and chain balance of human development and disease. Globin knockin mice are
advancing our ability to accurately model human hemoglobin disorders, in part by
bypassing problems associated with earlier transgenic models.

Enhancers within the $\alpha$ globin locus have been characterized in humans and mice (39),
but their precise roles in regulating $\alpha$ globin expression remain unresolved. Interestingly,
an experiment that replaced the entire mouse $\alpha$ globin locus with 100 kb of homologous
human $\alpha$ globin sequence, including enhancers, through recombinase-mediated genomic
replacement, demonstrated 2-3 fold reduced levels of human $\alpha$ globin expression
compared with mouse $\alpha$ globin (105). Likely contributing to this result are the
observations that human and mouse $\alpha$ globin loci are located in different chromosomal
contexts, and have distinct $\alpha$ globin enhancers and promoters (17, 26). Indeed, while
deletion of the human HS40 enhancer reduced $\alpha$ globin expression roughly 100-fold (88),
deletion of the homologous mouse HS26 enhancer reduced $\alpha$ globin expression by only
two-fold (3). For our knockin experiments the endogenous mouse $\alpha$ globin enhancers
were left in place, but human $\alpha$ globin genes replaced the adult mouse $\alpha$ globin genes.
Surprisingly, we found that expression of the human $\alpha$ globin genes from the $\alpha_2\alpha_1$
knockin was equivalent to the mouse $\alpha$ globin genes. Thus, mouse globin enhancer
sequences appear to be sufficient to interact with and regulate knockin human globin
genes to express at appropriate levels. Preservation of the original genomic context (e.g. endogenous enhancers) may thus be desirable as a general strategy for overcoming interspecies gene regulatory differences to ensure high expression levels for introduced genes.

α globin gene copy number can vary due to gene deletion in humans (15, 18), with α⁺ mutant allele frequencies exceeding 30% in some populations (91). In otherwise normal individuals, heterozygous deletion of a single α globin gene leads to silent carrier α thalassemia (9), with absence of findings during routine hematologic examination. Deletion of two out of four α globin genes causes α thalassemia trait, with a mild hypochromic, microcytic anemia, but little to no impact on quality of life (91). Our –α1 globin knockin allele was designed to mimic these human α thalassemia phenotypes. While previous reports either have examined humanized knockin mice homozygous for the human –α1 knockin (34, 45, 111), or control humanized knockin mice with the human α2α1 knockin (41-43, 56), here we present data highlighting the generation and properties of each human α globin knockin, as well as differences between them. We found that human α globin expression from the α2α1 knockin allele was equivalent to mouse α globin, but expression from the –α1 knockin allele was significantly reduced (at ~60%). Inheritance of single –α1 globin knockin alleles led to the silent carrier phenotype in the context of either mouse or human β globin chains, but inheritance of two –α1 alleles led to α thalassemia trait with microcytic anemia relative to wild-type or humanized mouse controls. Thus, our results for the human –α1 and α2α1 globin knockins are consistent with deletional α thalassemia in man.
Paradoxically, while α thalassemia trait leads to mild anemia for normal β globin alleles, it instead improves the severe anemia caused by sickle β globin alleles (22, 38). This phenotypic improvement can apparently extend to lifespan (57). Several explanations have been offered to account for the ameliorating effects of α thalassemia in sickle cell disease, including a reduced MCHC that would retard intracellular sickle hemoglobin polymerization (20), and thalassemic changes to the red cell membrane (21) that can prevent the rapid hemolysis associated with sickle cell disease (16). Our results are consistent with the latter hypothesis, since α thalassemia did not significantly reduce the MCHC in the context of humanized knockin sickle mice, but α thalassemia provided increased resistance of humanized α thalassemia trait mouse RBCs to osmotic lysis, possibly helping to explain the ameliorating effects of α thalassemia on the phenotype of humanized sickle mice.

In sickle cell anemia patients, soon after birth, a decline in fetal hemoglobin levels is accompanied by the onset of pathology (107). These findings have also been relevant for sickle mouse models (49, 99). Mice transgenic for the human β globin locus are deficient for human γ globin expression during fetal life, an important difference with humans that has limited transgenic sickle mouse viability in utero (23, 65, 114). However, direct linkage of human γ and β globin genes in transgenic mice could provide a delayed fetal-to-adult hemoglobin switch (7, 80), with improved gestational and perinatal survival. With a similar design, our γβS knockin construct also completed a fetal-to-adult hemoglobin switch after birth. Interestingly, during adulthood γ globin was expressed at
low levels (<1%) in humanized knockin sickle mice, apparently not responsive to the erythropoietic stress of sickle cell disease, but consistent with our earlier findings for Cooley's anemia knockin mice relative to control humanized knockin mice (41, 56). Adult γ globin levels in humanized knockin sickle mice were significantly lower than the 5-7% γ globin levels observed for our transgenic sickle mice (80). The presence of multiple enhancers or other factors unique to the LCR γβ transgenic loci may help to explain these regulatory differences in postnatal γ globin expression (unpublished data).

Nevertheless, low levels of fetal hemoglobin in adult humanized knockin sickle mice can help provide instructive isolation of α thalassemia's effects in modulating the sickle cell disease phenotype. In practical terms, this indicates that in addition to the proven approach of increasing fetal hemoglobin levels, other mechanisms towards improving sickle cell anemia are likely to still warrant further study.

Our findings, that sickle cell disease in humanized knockin mice is ameliorated by α thalassemia, support a beneficial role for α thalassemia that is independent of the effects of fetal hemoglobin. Reduced hemolysis of α thalassemic RBCs would lead to less erythropoietic stress, consistent with our observation of lower levels of reticulocytes in the humanized knockin sickle mice with α thalassemia (Table 2). Improved nitric oxide metabolism in plasma (4, 5, 67, 77, 110) may also result from reduced RBC hemolysis (16, 47), potentially providing some protection from sickle sequelae that include leg ulcers, priapism and pulmonary hypertension (62, 96, 97), although this mechanism has been the subject of recent debate (11, 29). In contrast, the altered membrane properties (21, 51, 61, 86) and greater number of α thalassemic RBCs (22, 38) may exacerbate the
vaso-occlusive sub-phenotype of sickle cell disease including greater incidence of pain crises and osteonecrosis (58, 97).

Conflicting data have also been reported in different studies for the effects of α thalassemia on many aspects of sickle cell disease such as the risk of pain crises, stroke, and death (20, 91). Besides environment (46, 63), one potentially confounding factor in these studies of sickle patients is the presence of additional epistatic influences within their genetic backgrounds. Of note, fetal hemoglobin distribution changes such as those caused by differences in patient β globin haplotypes may alter red cell survival and selection in circulation. Fortunately, by design these types of variables can more easily be removed from analysis of humanized sickle mice. In the quest for prospective indicators of sickle patient outcomes (84, 104), the improved hematology and increased survival that we observe in humanized sickle mice with α thalassemia might be of particular interest, validating their role as a useful model while inviting further exploration of hemolysis and its role in sickle pathophysiology. In conclusion, our findings in humanized knockin sickle mice with and without α thalassemia encourage further investigation of mechanisms involved in sickle cell disease, and provide a preclinical model for the development of targeted therapies.
Acknowledgments

This work was supported by National Institutes of Health grants R01 HL072351 (T.M.R.), R01 HL073440 (T.M.R.), UAB CMB Training Grant T32 GM008111 (S.C.M.), and Carmichael Scholarship (S.C.M.).

We thank Marion Spell in the UAB Center for AIDS Research and Enid Keyser from the UAB Analytic Preparative Cytometry Facility for cell sorting expertise. We thank Tim Townes, Louis Chow, and Peter Detloff for access to equipment. We thank the Cooley’s Anemia Foundation, UNICO Foundation Inc., and Joseph Ruisi for their support.

Authorship

Author contributions: S.C.M. designed experiments, performed experiments, analyzed research, made figures and wrote the paper; Y.H., T.T.Z., K.M.P., J.R., and T.M.T. designed experiments, performed experiments and analyzed research, T.M.R. designed experiments, performed experiments, analyzed research and wrote the paper.
References


DISCUSSION

Two Roads to a Happy Ending

Globin research has contributed many key advances in modern medicine and changed the way that we see the world. We now know the molecular basis of disorders such as sickle cell anemia, the first disease described with an observable connection with a primary sequence change. When the first multi-subunit protein structure was solved using hemoglobin crystals, atomic level detail illuminated the most fundamental mechanisms of biology, and soon helped reveal how sickle cell anemia causes its unique pathology. More recently sickle cell anemia was the first disease cured in a mouse model by therapeutic cloning with pluripotent stem cells, hematopoietic differentiation, and transplantation of corrected cells (31). Lamentably, such achievements have not yet led to sufficient improvements in the lives of patients with hemoglobin disorders, as many patients today find current therapies inadequate even if they are fortunate enough to have sufficient access (108). Fortunately, trails have already been blazed that offer hope.

Path #1- Fetal Globin Avenue

The main goal of this branch of globin research is essentially to reverse or indefinitely postpone the fetal to adult hemoglobin switch. A set of patients with hereditary persistence of fetal hemoglobin (HPFH) enjoy the benefits of fetal hemoglobin effectively replacing their defect in adult hemoglobin, a clinical finding that has provided quite sufficient rationale for fetal hemoglobin upregulation studies. In fact, the necessary levels of fetal hemoglobin for phenotypic improvement have already proven from these genetic changes. Thus, if a drug could be found that upregulated fetal hemoglobin levels
in a manner analogous to mutations that cause high level HPFH, it would cure patients with β thalassemia and sickle cell disease.

The promise for therapy in adult patients without such HPFH mutations was first realized in 1982, initially with reports by Joseph DeSimone et. al. in anemic baboons (19), and also in β thalassemic patients (55) by Tim Ley and colleagues. The astonishing finding was that an epigenetic drug being used for chemotherapy, 5-azacytidine, could upregulate the γ globin gene in adult patients. 5-azacytidine's mechanism of action was hypothesized to be demethylation of the γ globin promoter, leading to its activation in adults. However, toxicity concerns prevented 5-azacytidine's widespread application, prompting a search for similar molecules. Hydroxyurea soon became a leading candidate (80). George Stamatoyannopoulos proposed a more general cytotoxic mechanism for these drugs that were shown to upregulate γ globin (105), but hydroxyurea had the more favorable safety profile. Hydroxyurea is now the only FDA-approved drug for sickle cell disease. Interestingly, hydroxyurea may also improve sickle cell disease through mechanisms other than γ globin gene reactivation. However, while hydroxyurea helps many patients reduce disease burden, it leaves much to be desired in terms of efficacy and certainly is no cure.

In addition to the above drugs, following analysis of offspring with high γ globin levels from diabetic mothers (72), butyrate was shown by Susan Perrine and colleagues to help delay the fetal to adult hemoglobin switch (73). Propionate (57) and related compounds have also been shown to have similar effects. However, the necessary high
concentrations have generally been difficult for patients to tolerate, and often are not
effective. Many patients with hemoglobin disorders, particularly those with β
thalassemia major (also called Cooley’s anemia) do not adequately respond to these drugs
and thus the search for better candidates continues.

A PICh in the Dark

It is difficult to control something that you do not grasp. To upregulate the γ globin gene,
a better understanding of globin gene regulation remains a worthy goal. A major step
towards this would be obtaining a complete catalog of proteins that bind to the β globin
locus when the γ globin gene is either on or off. In spite of decades of reports heralding
dozens of candidate binding proteins, the role of a large proportion of these factors has
not been confirmed \textit{in vivo} or even by additional laboratories. Thus one obstacle to
gaining a fuller understanding of globin gene regulation remains our lack of knowledge
concerning which proteins bind to the locus.

The traditional approach has been to make an informed guess about which protein to
query, then obtain antibodies and test proteins one by one for DNA binding by EMSA or
with Chromatin Immuno-Precipitation (ChIP) experiments. An obvious drawback to this
method is that what isn’t being looked for isn’t being seen. Thus while we can ask which
DNA sequences can bind to a particular protein on a genomic scale (e.g. ChIP-chip), the
reverse experiment remains inaccessible, where we ask what proteins can bind to a given
sequence. There simply has simply been no effective, unbiased way to examine what
proteins (or RNA, etc.) are binding to a particular stretch of DNA \textit{in vivo}. 
Towards developing such a technique, Jérôme Déjardin and Robert Kingston published an elegant paper (18) that demonstrated proof of principle for telomeres. They called the method ‘Proteomics of Isolated Chromatin segments’ (PICh). A major caveat to their demonstration is the fact that the telomere sequences captured are actually comprised of hundreds of repetitive copies, and the analysis required large numbers (tens of liters) of cultured cells. Thus we may still need to improve the sensitivity by at least a couple of orders of magnitude before this technique can be readily applied to a single copy sequence such as the globin locus, and just as importantly, with small numbers of primary cells. Nevertheless, their success may turn out to be a significant milestone. Further work may improve the method to the point where it can begin shedding significant light on chromatin at the globin locus. By gaining a fuller understanding of globin gene regulation, we should have a better opportunity to upregulate the \( \gamma \) globin gene for therapy.

**Concerning Gene Therapy**

Rather than working around a broken part, sometimes it makes sense simply to replace it. Dysfunctional \( \beta \) globin genes can be compensated for by adding back a functional \( \beta \) globin gene copy through various gene delivery methods. An efficient and therefore currently important method is infection of cells with genetically engineered viruses. Through infection of bone marrow cells (59, 70) or purified HSCs (54) with globin mutations, globin gene addition can correct disease in the erythroid progeny of their infected HSCs. However, high viral copy numbers are sometimes needed in order to
insure adequate production of globin from the transgene in infected cells. Due to technical limitations, current human gene therapy protocols typically infect and transplant millions of cells. Each of these infected cells can have its own unique and potentially mutagenic insertions, meaning that using this large number of cells greatly multiplies the chance that something may go wrong.

The risk of insertional mutagenesis probably cannot to be overstated for gene therapy. Trials were halted in the gene therapy field after several patients receiving therapeutic retrovirus (11) to correct their severe combined immunodeficiency (SCID) later contracted leukemia (28). The cause was shown to be due to retroviral integrations that activated the LMO2 oncogene. Although the genomic location of viral integration appears to be somewhat random, it actually can have a significant preference for insertion near genes, which can increase the risk of gene disruption. Successful correction can require millions of infected cells for transplantation, implying a large number of different integration sites within the cell population that further increases the risk of oncogenesis.

Gene therapy has nevertheless soldiered ahead despite these setbacks. Innovations continue to be introduced that have the potential to incrementally improve safety. Thus, a new round of gene therapy trials is moving forward which hopes to beat the considerable odds. Recently a patient with β thalassemia received a transplant of infected cells that led to significant therapeutic benefit. The patient even became transfusion independent, but remained somewhat anemic (12). Unfortunately, safety concerns continue to cast a dark
shadow on these efforts as a clone containing an integration event near the HMG2A gene was reported to greatly expand within the patient’s bone marrow cells.

One method to improve safety for gene therapy would be to use only the progeny from a single infected cell for transplantation, making the therapy clonal. By infecting β thalassemic pluripotent stem cells with lentivirus carrying the human β globin gene, the infected stem cells can be subcloned from single cells, each with its own unique insertions. Individual stem cell lines can be amplified to large numbers of cells either for screening purposes or therapy. Expression of therapeutic levels of human β globin for each line can then be assays after differentiation of the stem cells into hemoglobin-containing cells by in vitro culture. Furthermore, integrations within each stem cell line can be mapped to screen for potentially harmful locations, and detect whether some integrations prove more functional than others. The effects of individual insertion sites at their unique genomic locations can be analyzed in vivo either for expression of therapeutic levels of human β globin or for disruption of important genomic sequences. This is a basic outline for a model system amenable to the clonal study of individual lentiviral inserts.

Random lentiviral integration events are sometimes located in repetitive portions of the genome. Interestingly, these lentiviral inserts may be silenced if not integrated nearby a gene, and thus will not provide therapeutic levels of human β globin gene expression. On the other hand, a considerable number of inserts are located in or nearby genes, which has the potential to disrupt gene regulation. These lentiviral inserts can also potentially
dysregulate the expression of additional neighboring genes, especially those located closest to the integration site. A preference for integrations with high level expression may be tantamount to getting out of the frying pan to play with fire, since in order to get high level expression one apparently must risk disruption of important genomic sequences. While these concerns linger for gene therapy, alternative therapeutic approaches have recently been established.

Path #2- Gene Correction Boulevard

Homologous recombination has been an enormously useful tool for altering genomic DNA sequences. The principle is that a provided DNA template contains sequences that match and flank the sequence of interest. This allows the provided DNA to find and pair with a matching target genomic sequence. Repair machinery can detect mismatches in base pairing between the target site and DNA template in the region of interest, and then use the provided DNA as a template to alter the genomic sequence to match.

Homologous recombination has been especially useful in the pluripotent stem cell field, as recognized by recent Nobel Prizes awarded for the pioneering work (20, 22, 98) of Oliver Smithies, Martin Evans and Mario Capecchi. Certain pluripotent stem cells, including embryonic stem (ES) cells derived from either fertilization or nuclear transfer, and the more recent induced pluripotent stem (iPS) cells derived from somatic cells (95), have a practically unlimited capacity to expand in culture. This allows stem cells to amplify to large numbers after subcloning to single cells, greatly expanding their utility and allowing selection schemes and screening to insure correct targeting.
Mouse ES cells have been a workhorse of mouse genetics, with gene targeting via homologous recombination responsible for the plethora of knockout and knockin mice available. ES cells can be micro-injected into fertilized blastocysts in order to make chimeric or cloned mice, depending upon the age and ploidy of the host blastocyst. Once generated, the knockout or knockin mice can be interbred to produce mice homozygous for the genes or mutations of interest. Alternatively, the starting ES cell line can be targeted multiple times to produce changes at different alleles.

Beginning with a corrected ES cell line, generation of cloned mice can be a highly efficient method to analyze the introduced genetic changes. Cloned mice can be derived from microinjection of ES cells into either 8-cell (82) or tetraploid blastocysts (106). The advantages of using cloned mice include facilitating direct analysis due to all of the cells of the cloned mouse being derived from the starting ES cell line. Chimeric mice instead contain mixed contributions from the ES cells and host blastocyst cells, and thus need to be bred to hopefully pass the targeted genome through the germline whereby its effects can be analyzed in the progeny. In another common approach, the introduced cells can contain a tag such as fluorescence that allows them to be isolated from the chimeric mouse for analysis. Altogether, gene targeting in mouse embryonic stem cells remains an important experimental approach.

Human ES cells (99), in contrast, have been shown to grow less efficiently than mouse ES cells and lack the native ability of mouse ES cells to form single cell colonies for subcloning (37). Human ES cells may be distinct developmentally from mouse ES cells,
as they are cultured in media containing factors that activate different signaling pathways, and appear to correspond to different embryonic developmental stages (56, 64). In support of this hypothesis, mouse epiblast stem cells have been derived that appear to more closely match human ES cells (9, 97, 119). Importantly, homologous recombination in human ES cells is much less efficient than mouse ES cells (120).

The lack of efficient gene targeting in human ES or iPS cells remains problematic at many levels. One major application is therapeutic cloning, where pluripotent stem cells are produced from differentiated somatic cells of a patient. Therapy then typically requires differentiation of the pluripotent stem cells into the lineage of choice to be used for therapy. Fortunately, for many therapeutic applications a plausible workaround exists where homologous recombination can be performed first in the starting cell population, such as in the initial fibroblasts used to generate iPS cells. The disadvantage to targeting these somatic cells with more limited capacity to expand is that it can be a challenge to carry out subcloning and selection schemes needed to screen for correct targeting.

However, the biggest challenge for therapeutic cloning with human pluripotent stem cells today is probably the lack of efficient differentiation protocols. Although in vitro culture has often been used to differentiate mouse ES cells into progeny that correspond with many different types of cells, differentiation into the most fundamental of hematopoietic cells, the HSC, is still an achievement that remains somewhat elusive (61). Experiments that differentiate ES cells into the hematopoietic lineage have indicated that although a subset of HSC-like cells appear to engraft after transplantation (62), they lack the surface
markers of both adult and embryonic HSCs, require unusual conditioning protocols to engraft, and apparently fail to provide any significant long term engraftment greater than six months. Protocols for differentiation of human pluripotent cells into HSCs are even less advanced. Further development of long-term culture methods for HSCs from both humans and mice may represent an important piece of this cell therapy puzzle. Concerns about the presence of residual pluripotent stem cells or less/alternatively differentiated cells among those to be transplanted, as well as the troubling genomic modification methods used to generate the pluripotent stem cells are also likely to persist for the foreseeable future. Thus for now, although therapies involving transplantation of cells differentiated from pluripotent stem cells remains a sought after goal, especially in conjunction with correction of globin gene mutations, it may be wise to focus also on alternative approaches that lack many of the aforementioned technical and expected regulatory hurdles.

**Focus on the Future**

As a bittersweet example of how far we have come but how far we have to go, Cooley’s anemia patients currently enjoy an increased lifespan (often from death in early childhood to beyond 30 years of age) due to significant advances made in transfusion medicine over the last century (52). However, lifelong dependence on blood transfusions places a heavy burden on Cooley’s anemia patients. These chronically transfused patients are at risk of contracting blood-transmitted diseases such as HIV. Transfusion also causes iron overload, meaning that intravenous chelation drugs must be administered to slow damage to many vital organs, and necessitating careful monitoring. Unfortunately, this level of
care may not be sustainable in many developing countries. Even the best standards of
care may not yet be able to offer a desirable quality of life. Some might argue, sadly, that
the single most effective measure implemented to date has been the widespread diagnosis
and termination of pregnancies. Yet, we desperately need alternatives with patients
living longer and affected births still occurring each year.

Bone marrow transplantation is currently the only available cure for Cooley's anemia and
sickle cell disease. However, current transplantation protocols with allogeneic bone
marrow cells carry considerable risks, as recipients must be carefully monitored for graft
versus host disease, and require immunosuppressive drugs. This would still be preferable
for many patients over lifelong dependence on transfusions and chelation therapies, yet
their options are limited without a histocompatible donor source available. Unfortunately,
only a small fraction of patients ever become eligible for bone marrow transplant due to
an inability to find suitably matched donors.

We must keep hope alive to find a better solution. While we can identify the causal
mutations for hemoglobin disorders such as Cooley's anemia, healthcare providers are
currently unable to address the root problem (i.e. an altered DNA sequence). Often the
problem is as isolated as a single base pair change. A straightforward therapy would
therefore be to simply target the altered DNA sequence and repair it, for instance by
homologous recombination using a provided DNA correction template. Fortunately, only
one allele would need to be corrected to cure sickle cell disease or β thalassemia.
Furthermore, although globin sequence changes are present in all cells of an individual,
the deleterious effects of globin mutations only appear within the erythroid lineage. Since erythroid cells are continually being derived from a population of resident HSCs, correction of the mutation would only need to take place in the HSCs. Correction of the mutation in autologous (patient-derived) HSCs thus represents an ideal cure. Transplantation and high level engraftment of the patient’s own corrected HSCs should then become a safe and lifelong curative therapy.

Targeting the Cooley's anemia mutation with homology-directed repair in HSCs is expected to provide a long-term cure without many of the drawbacks of alternative therapeutic approaches. One key benefit is that patients already have a ready supply of their own HSCs. Genetic repair of patient-derived HSCs bypasses immunological problems associated with traditional allogeneic transplantation. By simply restoring the mutated DNA sequence on either one or both alleles to normal, it should also be possible to circumvent the safety issues inherent with insertional mutagenesis in current viral gene therapy trials.

The major problem has been that, until recently, technologies for efficient and specific repair of a mutation in HSCs have not been available. Now that much research progress has been made towards this goal, including development of more efficient gene correction methods (10, 21, 58, 81, 94, 104), it is possible to test such methods using \textit{in vivo} models. Sufficiently high correction frequencies would offer the possibility of transplanting HSCs in the absence of long term culture or drug selection strategies. A good model system choice for these preclinical studies might be the Cooley's anemia
knockin mice recently developed by our lab that carry the relevant human globin gene sequences (39-41). Cooley's anemia knockin mice have classic signs of the disease, becoming transfusion dependent after birth and developing iron overload. Importantly, transplantation with HSCs is likely to require only a minimal level of corrected cell engraftment to be sufficient to rescue Cooley's anemia animals from transfusion dependence, due to the large survival advantage afforded to erythroid cells carrying a normal adult β globin gene.

Thus, now may be the right time to propose targeting the Cooley's anemia mutation in HSCs, followed by transplantation of the corrected cells. Beginning with mouse models, the successful completion of these studies will provide proof of principle for gene correction in HSCs from Cooley's patients. Progress is expected to highlight a promising avenue towards a long term cure not only for Cooley's anemia, but for other devastating monogenic diseases as well.

**Outlook**

With good fortune, adequate support, and no small amount of hard work, research advances will soon usher in a brighter future for these patients.
References


structure and replication across the entire beta-globin locus. Genes Dev 4:1637-49.


35. Harvey, W. 1628. Exercitatio anatomica de motu cordis et sanguinis in animalibus.


THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 4, 2010

TO: Ryan, Thomas M.
KAUL 568A 0024
906-2175

FROM: [Signature]
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Cell Therapies for Cooley’s Anemia
Sponsor: NIH
Animal Project Number: 100508769

On June 4, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>C</td>
<td>137</td>
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<tr>
<td>Mice</td>
<td>B</td>
<td>602</td>
</tr>
<tr>
<td>Mice</td>
<td>A</td>
<td>3702</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from May 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100508769 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7682.

Institutional Animal Care and Use Committee
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