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Correction of mouse models of sickle cell disease and Î²-thalassemia using stem cell and genetic-based therapy.

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CORRECTION OF MOUSE MODELS OF SICKLE CELL DISEASE AND β-THALASSEMIA USING STEM CELL AND GENETIC-BASED THERAPY

by

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A DISSERTATION

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in partial fulfillment of the requirement for the degree of
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ABSTRACT OF DISSERTATION
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Inherited disorders of hemoglobin occur with a greater frequency than any other monogenic disease, with over 1,000 mutations now known to cause thalassemia. The recent development of mouse models for sickle cell disease (SCD) and β thalassemia created an important reagent to test stem cell and gene-based therapies for correction of these diseases. Gene therapy for the correction of blood disorders has been considered since the first molecular characterization of a monogenic disorder, an A to T transversion in the sixth codon of β-globin resulting in SCD. Early gene therapy vectors were derived from retroviruses because of their ability to integrate into the host genome. However, retroviruses require breakdown of the nuclear envelope for integration into chromatin. As a consequence, retroviral vectors can only deliver transgenes to actively dividing cells. Lentiviruses have redundant nuclear-localizing proteins that enable stable integration into nondividing cells. We anticipated that lentiviral vectors could be used to transduce the highly quiescent hematopoietic stem cell population. Work shown here demonstrates for the first time correction of a mouse model of SCD using unmobilized, highly purified bone marrow hematopoietic stem cells transduced with a self-inactivating (SIN) lentiviral vector. Future treatment of hemoglobinopathies in the clinic may proceed by the correction of embryonic stem (ES) cells derived from skin fibroblasts of

the patient using nuclear transfer and reimplanted following their differentiation into hematopoietic stem cells. In anticipation of this strategy, we have corrected our mouse model of β-thalassemia by rescuing the β-globin defect in ES cells with a lentiviral vector and cloned the animals by tetraploid embryo complementation. This is the first step toward curing a hemoglobinopathy by "therapeutic cloning."

DEDICATION

This dissertation is dedicated to my family – Lynn, Mike and my mother Lena – for their unlimited encouragement while I "figured out what I wanted to be when I grew up." If my father were still alive, I think he would smile at the irony of his young son growing up to become an educator like he was; but, as a scientist, one who would continue to ask many questions about the world around him. Finally, to the wonderful woman who will soon be my wife; Kelly, with your grace and patience you have helped restore balance to my life, and for that I am grateful.

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how much fun I was having and that that did not need to change when I moved to the next step in my career.

I also acknowledge my fellow lab members and all the friends I have made while in Birmingham. Tom Ryan was the bright and friendly individual that was the face of the Townes lab for me when I was doing my first rotation. He was a very large reason I wanted to join the lab. It has been a fantastic place to work not only because of the wonderful science here but also because everybody is very friendly and Tim's perpetually positive outlook seems to rub off on everyone. I will miss the bike rides, TDF talk, and beers with Kevin and Tom. They have been wonderful guides in the lab and good friends. I am very pleased that Joe-Sun has returned to the lab from Taiwan, and he has brought Jane too! Kumar, Dewang, Doug, Andy, Jeff, WenYong, Jinxiang, Vladimir, Clark, Dominic, Susan, Aki, Howard, Evans, and Dakin are all people who have made this a very enjoyable place to work during my time here in Birmingham. The dedication of people working here is unwavering, but I will also fondly remember the generosity, warmth, and laughter. I realized very early that graduate school did not need to be just about spending every waking minute working in the laboratory. The Kaul Building Happy "Hour" (Cindy Vied, Gary Spradley, Walker Jackson, and Kelly Morrison) and roadtrips to New Orleans for Mardi Gras (Nate Kelly, Les Dupuy, Chris Berkhahn, and Rachel Reiman) and Texas (Dave Aiello, Bill Marion, and Ed Hinzman) for a Boston Red Sox game and evenings in the Southside will be additional reminders of a very rewarding period of my life.

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AS3	anti-sickling 3
Att	attachment
BMT	bone marrow transplantation
cPPT	central polypurine tract
CTS	central termination sequence
cDNA	complementary deoxyribonucleic acid
CFU-GEMM	colony-forming units-granulocyte/erythrocyte/macrophage/megakaryocyte
CFU-S	colony-forming unit-spleen
CMV	cytomegalovirus
DNase	deoxyribonuclease
ES	embryonic stem
EGFP	enhanced green fluorescent protein
EKLF	erythroid kruppel-like factor
5-azaC	5-azacytidine
5-FU	5-fluorouracil
FACS	fluorescence-activated cell-sorting
GVHD	graft versus host disease
G-CSF	granulocyte colony stimulating factor

Hb	hemoglobin
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HS	DNase-I hypersensitive site
HSC	hematopoietic stem cells
IEF	isoelectric focusing
LMO-2	LIM domain only-2
LCR	locus control region
LTR	long terminal repeat
MoMLV	moloney murine leukemia virus
MAPC	multipotential adult progenitor cell
MOI	multiplicity of infection
MEL	murine erythroleukemia
neo	neomycin phosphotransferase
NOD/SCID	non obese diabetic/severe combined immunodeficiency
PCR	polymerase chain reaction
RRE	rev reverse element
RNA	ribonucleic acid
SIN	self-inactivating
SCID	severe combined immunodeficiency
SCD	sickle cell disease
Sca-1	stem cell antigen-1

SCF	stem cell factor
TP	transplant
TSA	trichostatin A
VSV-G	vesicular stomatitis virus glycoprotein
WPRE	woodchuck hepatitus virus post-transcriptional regulatory element

INTRODUCTION

The study of hemoglobin during the last half century has yielded a wealth of information toward the understanding of gene regulation; consequently, it has recently become possible to use that knowledge toward treatments for hemoglobin disorders. Inherited disorders of hemoglobin occur with a greater frequency than any other monogenic condition in humans. Conservative estimates by the World Health Organization suggest that approximately 5% of the global population are genetic carriers for these hemoglobinopathies and that approximately 370,000 severely affected homozygotes are born every year [1]. These disorders were the first to be analyzed by recombinant DNA technology, and more is known about their molecular pathology than any other disease; however, there is still no adequate long-term treatment or cure. Current treatments focus only on prevention of infections, palliative measures to control pain, and surgical treatment of complications. Allogeneic bone marrow transplantation is the only available cure, but this procedure is limited to a minority of patients with an available, histocompatible donor. The development of a genetic-based therapy that targets a corrective globin gene to hematopoietic stem cells would facilitate a more widely available cure for patients with hemoglobinopathies.

Hemoglobin

The noted seventeenth century chemist Robert Boyle was the first to document the significance of oxygen. When he pumped air out of a chamber containing a burning

candle or a mouse, the candle was extinguished, and the animal expired. One hundred years later, Antoine Lavoisier determined that the element in air critical for the support of life was oxygen [2]. The proof that hemoglobin was responsible for oxygen delivery was assembled over the next two centuries, culminating with its elegant structural determination [3]. These and other studies have revealed that hemoglobin is a tetrameric protein comprised of two pairs of alpha-beta globin peptide dimers. The two salient properties that confer hemoglobin the ability to transport oxygen are the reversible and cooperative binding of oxygen. Coordination of one molecule of oxygen by each of the four heme rings allows the reversible release of oxygen in areas of low oxygen tension and retension in areas of high oxygen concentration. Because of the cooperativity of hemoglobin, when it is partially saturated with oxygen, the affinity of the remaining hemes in the tetramer increases substantially. Following the sequential oxygenation of two or three heme groups, there is a transition from a tense deoxygenated state to an oxygenated relaxed conformation [4].

The physiological importance of hemoglobin's allosteric cooperativity is significant. Cooperativity allows oxygenated hemoglobin to release its cargo over a very small drop in oxygen tension. Importantly, this same property also allows the heme groups to chelate and deliver the carbon dioxide byproduct of oxidation back to the lungs so that it can be expelled.

Developmental Hemoglobin Switching

All vertebrates change production of their hemoglobin variants at different developmental stages. This well characterized change is known as hemoglobin switching [5, 6].

During human development, hemoglobins are first produced in embryonic yolk sac erythrocytes during the third through eighth weeks of gestation. These variants are distinguished by their α- and β-like globin gene composition and are commonly known as Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$), and Portland ($\zeta_2\gamma_2$) (Table 1). At approximately 6

Table 1. Composition of human hemoglobins during development

Yolk sac (3 weeks):	Embryonic hemoglobins	
	Hb Gower 1	$\zeta_2\varepsilon_2$
	Hb Gower 2	$\alpha_2\varepsilon_2$
	Hb Portland	$\zeta_2\gamma_2$
Fetal liver (6 weeks):	Fetal hemoglobin	
	Hb F	$\alpha_2\gamma_2$
Bone marrow (30 weeks):	Adult hemoglobins	
	Hb A	$\alpha_2\beta_2$
	Hb A2	$\alpha_2\delta_2$

Subunit compositions of the human hemoglobin tetramers at each developmental stage are listed. The origin of each tetramer and its corresponding time of appearance during development is shown on the left. Three embryonic hemoglobins are produced in the yolk sac. A single hemoglobin tetramer is produced in the fetal liver and spleen. By the first year of age adult hemoglobin is predominantly composed of HbA with HbA2 representing 2-3% and HbF <1% of the tetramers.

weeks of development, red blood cell production shifts from the yolk sac to the fetal liver, and this organ becomes the major site of hemoglobin synthesis. The embryonic hemo-globins are rapidly replaced by the fetal hemoglobin HbF ($\alpha_2\gamma_2$), with a small contribution from Hb Portland; these variants constitute nearly all hemoglobin by 9 weeks of development. Concurrent with this switch, adult hemoglobin HbA ($\alpha_2\beta_2$) production gradually increases until the 30th week of development when there is a concerted switch

from HbF to HbA. Hemoglobin synthesis shifts from the fetal liver to the bone marrow, where erythropoiesis will take place throughout adulthood. By birth, the levels of HbF begin a steady decline with a concomitant increase in HbA (Fig. 1). Once the infant has reached 3 to 6 months of age, HbA reaches a steady state level of approximately 97%, and HbF levels decline to a baseline of less than 1%. Additionally, the other adult hemoglobin HbA_2 ($\alpha_2\delta_2$) is expressed at levels of approximately 2-3% throughout life. The expression levels of the minor variants HbF and HbA_2 will be important below when discussed in the context of therapies for the hemoglobinopathies.

Globin Genes

Current estimates suggest that the α- and β-globin genes diverged from a common ancestor nearly 500 million years ago. Gene duplication events and further divergence resulted in formation of the current α- and β-globin gene clusters [7]. In humans and mice, the genes are located on different chromosomes and in divergent chromosomal environments. However, coordinated regulation of the genes by an unknown mechanism results in balanced expression of α- and β-globin peptides. Within each locus, the genes are arranged in the order that they are expressed during development (Fig. 2). The human α-globin locus resides approximately 150 kb from the end of the short arm telomere of chromosome 16. Although the corresponding mouse α-globin locus is non telomeric and located interstitially on chromosome 11, the loci reside in similar environments (discussed below). Within the human α-globin locus there are three genes that encode functional polypeptides (ζ2, α1, and α2). These active genes flank three nonfunctional pseudogenes (ζ1, ψα2, and ψα1). The θ gene is a third evolutionarily

Figure 1. Human globin chain synthesis during development. Globin chain levels during prenatal development and adult life are shown as a percentage of total globin subunits. The predominant site of erythropoiesis at each stage (yolk sac, YS; fetal liver, FL; bone marrow, BM) is indicated above the graphs. Human α (top graph) and β (bottom graph) globin synthesis before and after birth is shown. Age is indicated in months. Subunit associations are listed in Table 1.

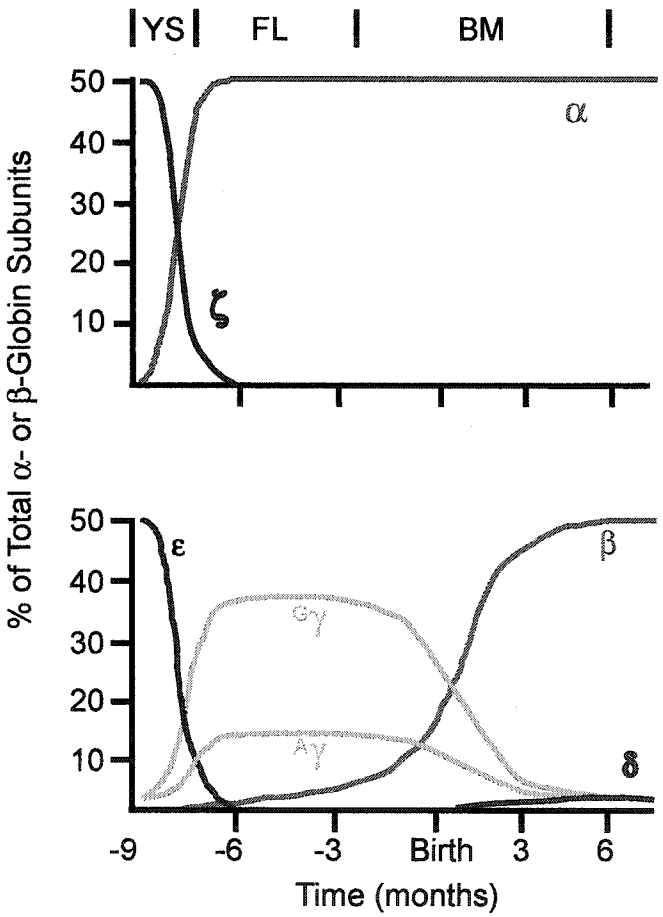
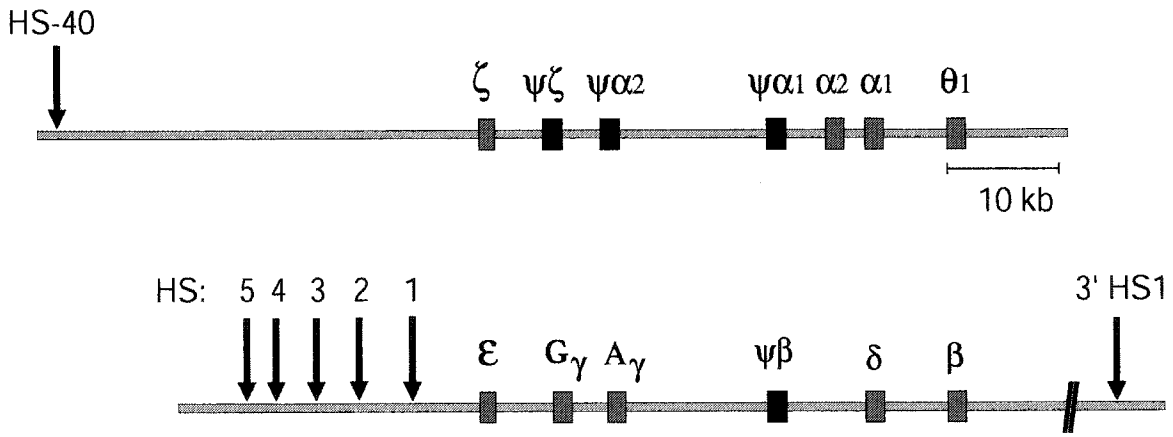


Figure 2. Human α- and β-globin loci. DNase I Hypersensitive site (HS) cores within the β-globin Locus Control Region and the corresponding α-globin HS-40 are indicated by arrows. Genes and pseudogenes are illustrated by blue and black boxes, respectively. Each gene locus is drawn according to scale. These regions act as enhancers and are required for high-level expression of the loci. The genes are arrayed in the order that they are expressed during development. Shown at top is the α-globin locus, and the β-globin locus is depicted at the bottom.



well-conserved α-globin that expresses low levels of mRNA but fails to encode globin polypeptide [8]. Deletion of the θ gene in humans does not result in any detectable phenotype [9], and the predicted amino acid sequence of this gene suggests that its product would not result in a functional polypeptide [10]. Any role of functional importance for the θ gene is unknown at this time. Consistent with a high level of conservation among mammals, the organization of the mouse α-globin locus closely patterns that of the human locus. It is comprised of three functional genes (ζ, α1, and α2), three pseudogenes (5'ψα, ψα2, and ψα1) and a non functional θ gene [11].

The human β-like globin gene family members are located on chromosome 11. They are comprised of five functional genes (ε, $^{G}\gamma$, $^{A}\gamma$, δ, β) and one nonfunctional pseudogene (ψβ) [12]. The genes are relatively small (~2 kb), and each contains three exons and two introns. The orthologous mouse β-globin cluster is located on chromosome 7 and contains four genes that encode protein (εy, βh1, β^{major}, and β^{minor}) and three non-expressed pseudogenes (βh0, βh2, and βh3) [13, 14]. In both species the globin genes are arranged along a 55 kb segment of DNA and are flanked on their 5' and 3' ends by an additional 35 kb of sequence. Although they are arranged on different chromosomes, the murine and human beta globin loci contain a high degree of homology over a 200 kb distance [15, 16].

Analysis of the α- and β- globin loci reveals that they reside in strikingly different chromosomal environments. Different compartments of the human genome can be categorized according to their gene density and content of G and C nucleotides (GC). These discrete regions of homogeneity are known as isochores and typically span approximately 300 kb [17]. Regions of the highest gene density correspondingly have a high GC con-

tent, and the highest gene density is found near telomeres [18]. Although there is currently active debate about generalized organization of the genome, widely expressed or "housekeeping" genes are often found in GC rich isochores, whereas tissue-specific genes are generally in isochores that are GC poor. The α-globin genes are in a gene rich isochore that contains a very high GC content (~54%) [19]. The erythroid and non erythroid housekeeping genes within this 300 kb region of open chromatin are constitutively expressed, and the surrounding chromatin is primarily in an "open" configuration [20]. DNase I sensitivity is an indicator of transcriptionally active or "open" chromatin; when genes are being transcribed, they are sensitive to digestion by DNase I. Although there are erythroid specific DNAse I hypersensitive (HS) sites in the promoters and upstream of the expressed genes that indicate important regulatory elements, there are also many non-erythroid sites that correspond with the expression of non erythroid genes [11, 19]. Consistent with constitutive expression, the α-globin genes lie in unmethylated CpG-rich islands [21] and are replicated early in S phase of the cell cycle in erythroid and non-erythroid cells [22]. In contrast, the β-globin genes lie in an AT-rich region of closed chromatin and are subject to tissue specific DNA methylation [23]. The β-globin locus replicates early in S phase only in erythroid cells [24]. Interestingly, the mutation rate of the α-globin genes is much higher than their β-globin. The composite structural differences of these two loci almost certainly affect transcription, DNA repair, and recombination in ways that may become clear with future study.

Since the α- and β-globin loci exist in dissimilar genomic environments, it would be expected that they would have different requirements for gene activation. The major regulatory erythroid specific hypersensitive site (HS-40) for the human α-globin genes

lies 40 kb upstream of the ζ2 gene in the intron of a gene of unknown function [11, 25]. Injection of a minimal HS-40 (0.5-2 kb) flanking human α-globin or 150 kb P1-derived artificial chromosome (PAC) or bacterial artificial chromosome (BAC) fragments containing the whole human locus into transgenic mice affords erythroid-specific expression of the α-genes; however, expression levels are low compared to endogenous murine alpha and were not copy number dependent [25-27]. In contrast, β-globin genes linked to very small [28] regulatory fragments (approximately 3 kb, discussed below) or fragments containing most of the locus [29] confer high level, near copy number dependent expression levels and overcome negative position effects at most sites of integration. Interestingly, when the human α1-globin gene was linked to a 13 kb β-globin regulatory element and injected into transgenic mice, the transgene overcame position effects at most ectopic loci, and human α-globin was expressed at levels similar to endogenous mouse α [30]. These results suggest that the α- and β-globin gene regulatory elements might function differently to afford high-level expression of their downstream genes. The mouse homolog of HS-40 (mouse alpha regulatory element or mαRE) has also been characterized. This element also affords expression to a linked human α-globin transgene when injected into transgenic mice [31]. Recently, homologous recombination was used to disrupt the mαRE in its endogenous locus. Deletion of the mαRE resulted in a 50% reduction in transcription, but homozygous mice were healthy with only mild hematological changes [32]. In contrast, the disruption of the β-globin regulatory element in our lab and others resulted in a 95% reduction in β-globin levels [33]. This led to a severe anemia that closely mimicked β-thalassemia resulting from a disruption of both adult β-globin genes in the mouse or in human patients [34, 35]. The data suggest that the HS-40 element acts

more like a classical enhancer, whereas the full β-globin regulatory element may additionally insulate the downstream genes from the effects of repressive chromatin at its endogenous locus.

Beta Globin Gene Regulation and the Locus Control Region

Successive levels of genetic regulation can be revealed by the DNase I sensitivity of a chromosomal locus and its component genetic elements. The 90 kb of chromatin containing the β-globin genes is 5 to 10 times more sensitive to DNase I digestion in erythroid cells [36, 37]. As expected, this sensitivity is erythroid specific; the globin genes are not transcribed in non erythroid tissues. The next layer of DNase I sensitivity suggests a more specific developmental stage-specific control of the globin genes and is marked by a greater sensitivity to DNase I digestion. DNase I "minor" HS appearance is coincident with the 5' promoter ends of individual globin genes. These sites are found only at the promoters of globin genes being expressed during the appropriate stage of development. Sites are present upstream of the epsilon globin gene in tissues and cultured cells that correspond to embryonic development but not in adult tissues [38, 39]. Consistent with this observation, DNase I hypersensitivity has been discovered in the promoters of the duplicated fetal γ-globin genes and adult δ- and β- globin genes during development in the fetal liver and adult bone marrow, respectively [37, 40]. The final level of control governing the β-globin genes was revealed when five DNase I super HS were discovered throughout a 20 kb region of chromatin 5-8 kb upstream of the embryonic globin genes and 20 kb downstream of the adult β-globin gene (3' HS1; see Fig. 2) [39-41]. The four upstream hypersensitive sites (HS1-4) are erythroid specific; however,

only HS 2, 3, and 4 appear essential for β-like globin gene expression [28, 42]. Recent studies have demonstrated that HS5 is also detected predominantly in hematopoietic tissue [43]. Two additional sites, HS6 and HS7, have recently been discovered, but it is not clear if they are essential components of this regulatory region [16]. Interestingly, human HS5 contains high sequence homology and known transcriptional factor binding sites that resemble those found in chicken HS4, a known insulator element, and this similarity invited speculation that HS5 might function as a boundary element [44-46]. The region encompassing HS1-5 collectively became known as the Locus Control Region (LCR) for its ability to regulate globin expression in a precise temporal fashion during development. Genetic dissection of the LCR in cell lines, transgenic mice, deletions in human patients, and targeted deletions of the endogenous mouse locus has advanced understanding of β-like globin gene regulation (reviewed in [47-49])

The precise role the LCR plays in regulating its downstream genes is currently under debate. Deletions encompassing the LCR in humans result in δβ- or γδβ-thalassemias that are characterized by near complete extinction of downstream β-like globin gene expression [50, 51]. The seminal discovery that the LCR might be necessary and sufficient for activation of the downstream globin genes came from studies of a Hispanic γδβ-thalassemic patient with a deletion containing HS 2-5 and removal of an addition 25 kb further upstream [52]. This deletion resulted in near complete extinction of β-like globin gene expression and loss of DNase sensitivity throughout the locus concomitant with replication late in S phase of the cell cycle [53]. In contrast, the full disruption of HS 1-6 in the endogenous mouse locus results in a 95% (92-99% depending on the particular stage of de-

velopment) loss of β-globin expression; however, DNase sensitivity at the promoters of the downstream genes is retained, albeit at lower levels [54].

Appropriate caution has always been exercised in the interpretation of the Hispanic deletion because it removed an additional 25 kb of upstream sequence that overlaps with neighboring odorant receptor genes and contains additional hypersensitive sites [16]. The suggestion remains that this deletion may have excised sequences necessary for the opening of the entire β-globin chromosomal domain. Alternatively, the loss of these sequences may have brought the β-like globin genes in proximity to repressive chromatin that could shut down the locus. These are questions that will likely be addressed in future targeting experiments in the endogenous mouse β-globin locus. Certainly, it will be interesting to determine if disruption of the 25 kb region 5' of HS1-6 results in any detectable phenotype alone or in combination with the Groudine HS1-6 knockout or the HS1-4 targeted animal that our laboratory has generated. The embryonic stem cells used to make these deletions can be retargeted to answer this question. Although the β-globin loci are highly conserved in humans and mice, there is another way this question could be answered. Using homologous recombination, two sequential "knock-in" experiments could be employed to replace the mouse sequence with the human LCR and the 5' HS containing upstream homology. The upstream region could then be disrupted by homologous recombination, followed by further targeting of the human sequences that correspond to murine HS1-6. This will determine whether the enhancement of β-like globin gene activation and opening of the β-globin locus are separable activities in the human locus.

The importance of the LCR for correct tissue and temporal expression of the β-like globin genes became apparent in studies of transgenic mice. Human γ- or β-globin genes with only 2 kb of their flanking 5' and 3' sequence initiated expression only in erythroid tissues and with correct developmental specificity. However, some animals failed to express the transgene, whereas the majority had very low levels (0.3%) compared to the endogenous mouse β-globin genes [55, 56] These results were consistent with the positional effects previously observed due to shutdown of transgenes by repressive flanking mouse chromatin [57-59]. Linking the LCR to a β-globin gene relieved these position effects and resulted in restoration of high-level human γ- or β-globin production in all transgenic mice that received an intact transgene [28, 60]. Unfortunately, correct developmental control was lost, resulting in β-globin expression in the fetal liver. Gamma or β-globin genes linked separately to the LCR and coinjected into transgenic mice also lost correct developmental control, resulting in high level β-globin expression in yolk sac and fetal liver and human γ-globin expression in the adult blood [61, 62]. Correct developmental specificity was restored by linking the γ- and β-globin genes in cis- to the full-length LCR [61, 63]. This final result led to a model for β-like globin gene regulation based on competition of the globin genes for interaction with the LCR [61]. The major supposition of the model is that developmental stage specific and ubiquitously expressed transcription factors bound to the promoter, and proximal LCR elements aid in the formation of stable transcriptional complexes during development. The preferential expression of specific β-like globin genes during different stages of development could be controlled in part by a changing milieu of positively and negatively acting transcription factors.

Characterization of the exact sequences within the LCR critical for function has being actively investigated by our group and others for the past 17 years. For the eventual correction of hemoglobinopathies, a minimal cassette of HS sequences was sought that could be moved into a gene therapy vector. Investigators began dissecting the LCR into smaller components that could confer near position independence to linked adult beta globin genes injected into transgenic mice or transfected into cell lines (reviewed in [64]). Constructs containing the full-length LCR and 3' HS1 afforded high-level expression (equivalent to 100% of the endogenous mouse β-globin) to a linked β-globin gene, but this 42 kb fragment was prohibitively large for insertion into a gene therapy construct [60]. Two groups independently analyzed 2.5 or 6.5 kb "microLCR" constructs containing elements of HS 2, 3 and 4 for β-globin expression in stably transfected murine erythroleukemia (MEL) cell lines. Amazingly, these cut down constructs yielded high level β-globin expression equivalent to the full length LCR [50, 65].

Subsequent studies were directed at determining the sequences and transcription factor binding sites within the HS responsible for β-like globin gene expression. Our laboratory was the first to characterize activity of a single HS. *Ryan et al.* defined a 1.9 kb HS2 element that expressed β-globin levels equivalent to 40% of the full LCR [28]. Mutation of an 18 bp sequence within this 1.9 kb fragment demonstrated 95% of activity was attributed to a duplicated activator protein 1 (AP-1) site shared by the erythroid-enriched transcription factors Nuclear Factor Erythroid 2 (NFE-2) and Locus Control Region Factor 1 (LCRF1) [66, 67]. Further mutational analysis of the 373 bp "core" of the 1.9 kb HS2 fragment revealed transcription factor binding sites for ubiquitous and hematopoietic-enriched transcription factors. Individual disruption of any one of these sites

failed to completely abrogate position-independent expression of a linked β-globin gene and suggested that multiple protein binding sites were important for maximal HS2 enhancer activity [68, 69]. Interestingly, only HS2 enabled high-level β-globin gene expression in both transgenic mice [28] and transiently transfected MEL cells [70]. These findings led our group and others [71, 72] to postulate that HS2 alone might be necessary and sufficient to drive high-level β-globin expression from a gene therapy cassette. Unfortunately, HS2 could not maintain high level position-independent globin expression if delivered from adeno-associated virus (AAV) or retroviral vectors.

Analysis of the minimal 373 bp HS2 fragment and similar core sequences for HS3 and HS4 using DNase I footprinting revealed evolutionarily conserved transcription factor binding sites [64], suggesting that these minimal elements might be necessary and sufficient for high level β-globin activation. However, linkage of each core to the β-globin gene failed to yield maximal enhancer activity in transgenic assays [68, 73, 74]. Another role of core sequences has been postulated from studies of a 150 kb yeast artificial chromosome (YAC) containing the full β-globin locus. Disruption of the 225 bp HS3 core in this fragment resulted in loss of 90% of globin gene expression [75]. This result was observed in two independent founder lines, suggesting the data may not be due to site-specific integration effects; however, large transgenes with the entire human β-globin locus can still demonstrate a greater than twofold range of expression per copy [76, 77]. These results underlie the importance of examining many different lines of transgenic mice if possible. *Bungert et al.* postulated that sequences outside the HS3 core can lead to a dominant negative phenotype. The authors further surmised that if the HS3 element lacked its transcriptional activating property but could still interact with the other HSs or

the β-globin genes, the mutant HS3 could inhibit the activity of the entire LCR. Collectively, studies of the human HS in transgenic mice suggested that full-level LCR enhancement was derived only if 1-2 kb fragments from HS2, 3, and 4 were included in the construct.

Studies of the corresponding murine LCR suggested a similar requirement for β-like globin gene activation [78]. Targeted disruption of each HS in the endogenous mouse locus suggests the effect of each HS is additive, and loss of any one site does not result in a decrease of more than 30% expression in the endogenous murine locus [33, 79-81]. These results also demonstrate that HS2 and HS3 enhance β-globin expression equivalently; their disruption causes a 30% loss of β-globin in adult blood. Analysis of a HS1-4 knockout mouse created in our laboratory suggests that the homologous human HS2 and HS3 sequences used in previous transgenic studies may not be equivalent. Using homologous recombination in embryonic stem cells, these sequences were reinserted back into the mutant ΔHS1-4 mouse locus, and mice containing the HS2 or HS3 elements were created. Insertion of HS2 or HS3 resulted in unequal restoration of β-globin expression levels. Mice created with a HS2 insertion expressed β-globin at levels 36% of endogenous wild-type levels, but animals receiving HS3 expressed β-globin 90% of endogenous wild-type levels. As expected with such a high level of expression, the 1.9 kb human HS3 completely rescued the mice from severe β-thalassemia. These observations suggest the HS3 element may be a candidate for future cell and gene-based therapies.

Additional sequences within and flanking the β-globin gene are equally important for high level gene expression. Regulatory elements and enhancers are located within the β-globin promoter, the second intron, and 500 bp downstream of the polyadenylation site

[82-84]. The second β-globin intron is necessary for β-globin expression from transgenes injected into mice [85] or from a Simian virus 40 (SV40) viral expression cassette delivered into cultured cells [86]. It appeared that insertion of LCR HS 2, 3, and 4; β-globin regulatory sequences; and the adult globin gene into a gene therapy cassette would be sufficient to correct the β-globin polypeptide imbalance seen in β-thalassemia. However, previous work suggested that a hemoglobin variant other than adult HbA would be necessary to disrupt sickle hemoglobin (HbS; $\alpha_2\beta^S{}_2$) fiber formation and ameliorate SCD.

The Protective Effect of HbF and Recombinant Hemoglobins

Sickle cell disease is caused by a single mutation at the sixth residue of the adult β-globin polypeptide resulting in the substitution of a valine residue for glutamic acid. This valine creates a hydrophobic projection that inserts into a natural hydrophobic pocket created by Phe 85 and Leu 88 on the surface of a second hemoglobin tetramer. The interaction of tetramers results in the formation of long HbS polymers/fibers that cause red blood cells to become rigid and nondeformable and to occlude small capillaries, leading to all of the associated pathology of SCD. It has been known for 50 years that fetal hemoglobin (HbF; $\alpha_2\gamma_2$) confers a greater anti-sickling effect on the polymerization of deoxygenated HbS than that exerted by the normal adult hemoglobin [87]. The protective effect of HbF on HbS polymerization is achieved through the formation of hybrid tetramers of $\alpha_2\beta^S\gamma$. A corresponding effect is not seen with the formation of $\alpha_2\beta^S\beta$ hybrids.

The basis of HbF protection can be partially explained by several particular amino acid interactions between interacting polypeptides in the hemoglobin tetramer (Fig. 1,

paper 3). The adult hemoglobin variant HbA_2 ($\alpha_2\delta_2$) has also been shown to have potent anti-sickling abilities. Previous analyses of mutated hemoglobins and differences between HbA, HbF, and HbA_2 highlighted the importance of residues at positions β87 and β22. Hemoglobins F and A_2 both differ from HbA at these positions, and both significantly inhibit HbS polymerization [88].

The clinical course of sickle cell disease can be highly variable. The level of HbF in patients is thought to contribute significantly to this variation. Patients with levels of 20-30% typically have very mild disease [89-92]. However, large clinical trials suggest that levels as low as 8-10% correlate with increased life span [93] and decrease the likelihood of strokes from vaso-occlusive episodes [94]. Our group designed a recombinant β-globin molecule (β anti-sickling 2 or β^{AS2}) that incorporated several of these substitutions [95]. A β87 glutamine, normally resident in HbF and HbA_2, was used to replace the β87 threonine of HbA, and the glutamic acid at β22 was substituted with alanine. These two changes were designed to interrupt two important contacts along the length and sides of the HbS tetramer. These alterations to the β-globin molecule resulted in potent anti-sickling properties in vitro.

Another important consideration for the development of a successful anti-sickling β-globin molecule is the charge of α- and β-polypeptides. Under normal physiological conditions, α-globin is positively charged (isoelectric point of 8.4), and the β-globin is negatively charged [96]. Mutations that increase the negative charge of β-globin subunits enhance their ability to bind α-globin and complete tetramer formation [97]. Based on a charge lost with the substitution of valine for glutamic acid, the mutant β^S-polypeptide suffers a competitive disadvantage against a normal β^A polypeptide. This phenomenon is

observed in heterozygous sickle cell patients; the expected equal ratio of HbS and HbA is not seen, and only 42-44% HbS is manufactured [98]. To create a more negatively charged molecule, our laboratory created a second generation β-globin (β anti-sickling 3 or β^{AS3}) that contained an aspartic acid substitution for alanine at position 16. This naturally occurring mutation is known as HbJ-Baltimore and was employed to increase the ability of β^{AS3} to preferentially interact with α-globin molecules to form tetramers of HbAS3. In vitro mixing experiments using a 3:1 ratio of HbS and HbAS3 confirmed that HbAS3 impeded HbS polymer formation with greater efficiency than HbF. This finding strongly suggested that HbAS3 would inhibit HbS polymerization in vivo if HbAS3 levels constitute 25% of total hemoglobin. Unfortunately, gene therapy vectors able to efficiently deliver β^{AS3} to bone marrow or hematopoietic stem cells required further development to achieve these levels of HbAS3 (see below).

Pharmacological Induction of HbF

Although the mechanism of hemoglobin switching continues to be poorly understood, a concerted effort has been made in the last 20 years to upregulate HbF using pharmacological agents. Cytosine methylation of DNA was one of the first mechanisms of gene regulation to be postulated [99], and DNA hypomethylation was thought to be a way of upregulating HbF synthesis in adults. McGhee and Ginder demonstrated that expressed chicken β-globin genes were surrounded by hypomethylated DNA; but, when the same sites were methylated, these sites were silenced [100]. Flavell and coworkers then discovered that the human fetal globin gene was hypomethylated in fetal tissue [23]. Based on these observations, the DNA demethylating compound 5-azacytidine (5-AzaC)

was tested in baboons and found to upregulate HbF levels robustly [101]. Subsequently, 5-AzaC was administered to patients with β-thalassemia or SCD, and the drug increased HbF levels significantly [102]. However, the toxicity and carcinogenic potential of 5-AzaC necessitated a search for a drug without such untoward effects.

The ribonucleotide reductase inhibitor hydroxyurea (HU) became the preferred pharmacological treatment for the hemoglobinopathies during the next 20 years because of its ability to modestly upregulate HbF with limited short-term side effects [103]. HU therapy has been an effective treatment for many patients with SCD; it provides partial relief for some of their symptoms, decreases the number of hospitalizations, and improves patient survival [104]. Unfortunately, HU has many side effects that make prolonged administration difficult for children and adults, and its long-term safety is questionable (reviewed in [105, 106]). Additionally, levels of protective HbF that are induced by HU treatment do not approach the 20-30% level necessary for a substantial reversal of the disease in the majority of patients.

Concurrent with widespread HU administration in patients, there was a search for a new agent that could upregulate HbF. Sodium butyrate became a strong candidate based on its ability to reversibly induce gene expression in cultured cells [107]. The precise mechanism of gene upregulation by butyrate is unknown; however, this compound is a known inhibitor of histone deacetylase (HDAC). Changes in chromatin structure following treatment with butyrate or another known HDAC inhibitor Trichostatin A (TSA) include hyperacetylation and subsequent activation of previously silenced genes [72, 108]. The first suggestion that butyrate might upregulate HbF was noted with the observation that diabetic mothers had a delayed switch from γ- to β-globin gene expression

[109]. The cause of this delayed switch was due to high levels of circulating butyric acid. Delay of the γ- to β-globin switch was confirmed in erythroid cells and in fetal sheep. Shortly thereafter, sodium butyrate administration successfully increased HbF in baboons and upregulated γ-globin expression in erythroid progenitors of patients with SCD or β-thalassemia [110]. Although these results were encouraging, human clinical trials of continuous infusion of butyrate were disappointing, and long-term nonhuman primate studies demonstrated a toxic myelosuppression upon prolonged exposure to butyrate. A regimen of intermittent "pulse" therapy showed encouraging increases in HbF from 7 to 21%, with 9 of 11 patients experiencing relief from these protective HbF levels for 1 to 2 years [111]. Unfortunately, the documented toxicity and general anti-proliferative effects of butyrate have kept this compound from further clinical trials. Parallel research in the field of bone marrow transplantation suggested this approach might be a more attractive alternative for therapy of hemoglobin disorders and other blood diseases. More importantly, this therapy would only need to be administered once and had the potential to cure the patient for the duration of his or her lifetime.

Bone Marrow Transplantation: An Available Cure

The clinical application of bone marrow transplantation dates back to 1945, shortly after civilian populations were exposed to lethal doses of radiation. Shortly after, parallel studies in mice and human cancer patients demonstrated that bone marrow transplantation could rescue the recipient from doses of radiation that are normally lethal [112, 113]. Early progress was slow, and it would be several decades before bone marrow transplantation (BMT) would be used for the successful treatment of β-thalassemia [114].

BMT is currently the only available cure and continues to be the preferred treatment for severely affected patients with β-thalassemia or SCD (reviewed in [115, 116]. Unfortunately, allogeneic BMT is only available for patients who have an available histocompatible donor; an identical histocompatibility leukocyte antigen (HLA) match occurs only 20 to 30% of the time. Data from clinical trials indicate that approximately 80-90 percent of patients receiving allogeneic (genetically non-identical) BMT are cured; however, there is significant morbidity and mortality associated with this kind of BMT. Because most patients do not have an identical twin, rejection of an allogeneic bone marrow graft occurs almost 10% of the time in the form of graft-versus-host disease (GVHD). Immunosuppressive therapy with cyclosporin A often alleviates this complication; however, uncontrolled chronic or acute GVHD results in an unacceptably mortality rate following BMT. Although the amelioration of hemoglobinopathies in some patients by allogeneic BMT is encouraging, a more efficient and less toxic form of transplantation awaits development.

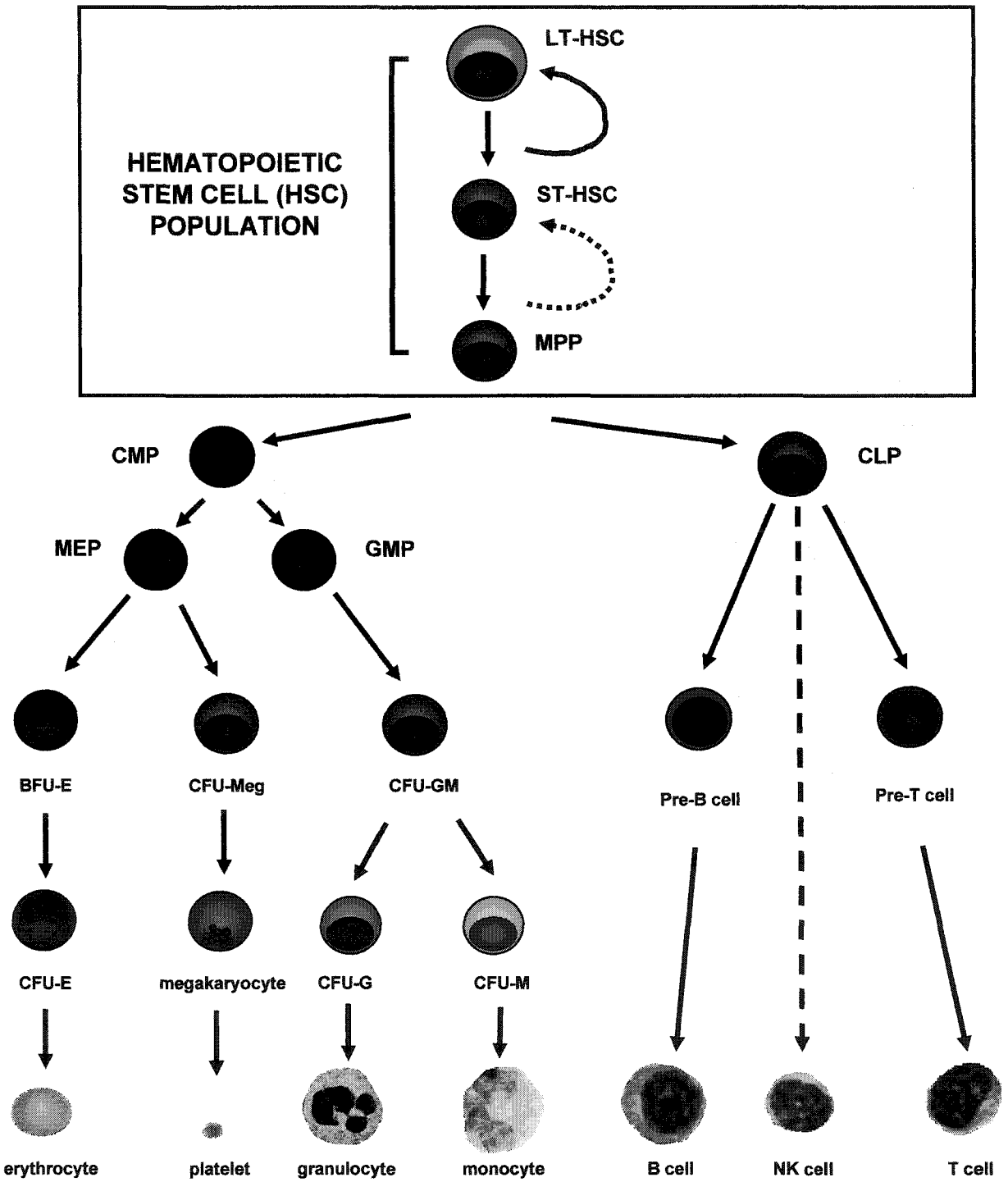
Hematopoietic Stem Cells

Early research in mice revealed that the radioprotection derived from a bone marrow transplant came from a rare pluripotent cell type [117, 118]. Whole bone marrow injected into irradiated mice formed distinguishable colonies on the surface of their spleens. A proportion of these spleen colony-forming units (CFU-S) could form new CFU-S in serially transplanted recipients. Later studies confirmed that these "stem cells" could be characterized by their ability to self-renew and differentiate into the more committed progenitors and functionally specialized cells that constitute the hematopoietic

system. These features remain the hallmarks that distinguish all tissue-specific stem cells (see discussion). The most important consequence of this asymmetric cell division is that these rare hematopoietic stem cells (HSC) must be able to reconstitute all blood cells for the life time of the host.

Work over the next several decades suggested that the majority of CFU-S were actually derived from a more committed progenitor and that the true pluripotent HSC had not yet been discovered [119, 120]. Using fluorescence-conjugated monoclonal antibodies coupled with fluorescence-activated cell sorting (FACS), the mouse HSC was identified by Weissman and colleagues [121]. This population of cells is very rare, occupying approximately 0.05% of mononuclear bone marrow cells. The cells were characterized by positive staining for the markers Thy-1, Sca-1, and c-Kit and negative or low staining for differentiation markers typically seen on B and T cells, macrophages, granulocytes and erythroid cells (Thy-1^{+}, Sca-1^{+}, c-Kit^{+} and $Lineage^{-/low}$). These studies showed that as few as 30 of these HSCs could rescue lethally irradiated recipients, and most importantly, this reconstitution was long term. Later experiments showed that this highly purified and rare population of cells was heterogeneous and could be further separated into cells capable of long-term (4 months or greater) reconstitution and self-renewal, short-term reconstitution (approximately 6-8 weeks), and limited self-renewal and multipotent progenitors that have no self renewal capability and can only give rise to differentiated progeny (Fig. 3)[122]. Further differentiated progenitor cells that give rise to the myeloid (Common Myeloid Progenitor) and lymphoid (Common Lymphoid Progenitor) lineages have also been characterized by their surface markers and ability to form defined colony types in semi-solid medium [123, 124].

Figure 3. Hematopoietic Stem Cell Lineage Specification. The current model for lineage specification of all cells of the hematopoietic system. The cascade is initiated by the long-term renewing hematopoietic stem cell (LT-HSC) which differentiates and self-renews for the lifetime of the host. The short-term renewing (ST) HSC has limited self-renewal properties which are exhausted after approximately 8 to 10 weeks. Multipotent progenitors (MPP) persist for an even shorter time in the circulation and are thought to lack the ability to self-renew. The common lymphoid (CLP) and common myeloid progenitors (CMP) further differentiate into all cells of the lymphoid and myeloid pathways, respectively. The recent discovery of megakaryocyte/erythroid (MEP) and granulocyte/macrophage (GMP) places these progenitors downstream of the CMP for differentation into mature cells of the myeloid lineages. The population of cells with a surface marker expression of c-kit^{+}, Sca-1^{+}, lin$^{-/low}$ (KLS) is 1000-fold enriched for HSC activity. These cells are predominantly ST-HSCs, MPPs, and LT-HSCs. The goal for autologous cell therapy of the hemoglobinopathies is to target the HSC pool with a corrective β-globin gene to permit expression in the erythroid lineage for the lifetime of the recipient.

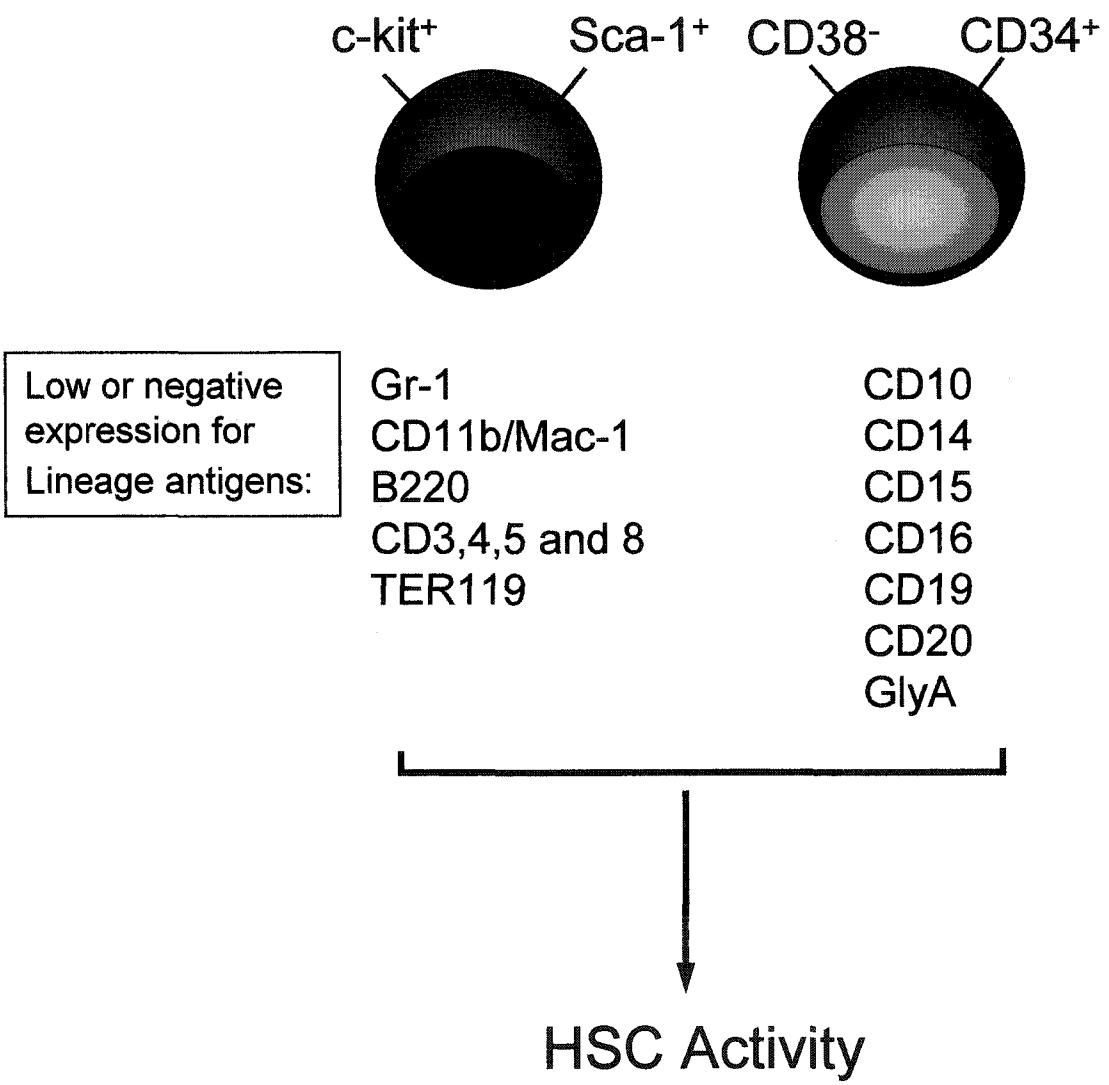


Purification and characterization of the mouse HSC was soon followed by isolation of a putative human HSC. This cell was identified by surface expression of CD34, Thy-1, and the absence of lineage marker expression, demonstrating that expression of surface markers on human and mouse HSCs overlap but are not completely redundant (Fig. 4). Early studies on this cell type confirmed that it could efficiently repopulate severe combined immunodeficient (SCID) mice [125]. Recently, clinical studies using autologous (derived from the patient) transplantation for treatment of leukemia and breast cancer have confirmed that purified HSCs provide a long-term rescue for patients who have had the equivalent of a lethal form of radiation [126, 127]. Most importantly, patients receiving purified HSCs had a vastly increased rate of survival than their counterparts receiving whole bone marrow or partially purified progenitors. This survival advantage is due to removal of contaminating cancerous cells during purification that are normally retained in the bone marrow compartment and do not express the same markers as purified HSCs [128, 129]. These results were very encouraging for other conditions that could benefit from autologous transplantation of genetically corrected stem cells. If stem cells from each patient could be harvested, corrected by gene therapy ex vivo and reinfused, issues of graft versus host disease (GVHD) and its associated toxicity would be obviated.

Gene Therapy: A Brief Historical Background

Gene therapy for the correction of blood disorders has been considered since the first molecular characterization of a monogenic disorder, which is the single base mutation that results in SCD [130]. This time period preceded the advent of recombinant

Figure 4. Overlapping surface phenotypes of human and mouse hematopoietic stem cells. The human and murine hematopoietic stem cell (HSC) compartment shares expression of certain surface markers and the absence of mature lineage markers for granulocytes (Gr-1, Mac1/CD11b), B (B220) and T cells (CD3, 4, 5, and 8), and erythroid cells (TER119 and GlyA). Sca-1 and c-kit are essential markers for murine HSC activity, but there is active debate regarding the status of CD34 expression on human hematopoietic stem cells. This marker may only be expressed on human HSC that are activated to "home" to the bone marrow.



DNA technology and methods for genetic manipulation; these tools would arrive in the early 1970s. Early study of the RNA and DNA tumor viruses Rous Sarcoma Virus and Simian Virus 40 focused on understanding mechanisms of RNA- or DNA-dependent nucleic acid replication and would eventually provide insight on how these simple agents could give rise to cancer [131]. Armed with the discovery of reverse transcriptase [132, 133] and a growing understanding of the retroviral life cycle, investigators proposed that a therapeutic gene could replace the resident viral oncogene and be delivered by the recombinant retrovirus to result in a heritable "gene therapy" [134].

Another decade would pass before recombinant spleen necrosis virus and Moloney murine leukemia (MoMLV) retroviral vectors were produced and transmission of a thymidine kinase gene to thymidine kinase negative cultured cells was demonstrated [135, 136]. Unfortunately, these early vectors were contaminated with replication-competent helper virus that could result in multiple integrations and increase the likelihood of transformation by insertional activation or mutagenesis. Early modifications of these vectors focused on modification of the wild-type packaging signal necessary for encapsidation of viral RNA and production of packaging cell lines containing the separated retroviral *gag/pol* and *env* genes stably integrated into their genome [137, 138]. This combination of modifications greatly diminished production of intact replication-competent retrovirus and increased the level of biosafety from these vectors. The host range of retroviral vectors was expanded by replacing the murine-specific ecotropic envelope of MoMLV vectors with the *env* gene from amphotropic virus 4070A [139]. This modification allowed transduction of murine or mammalian hematopoietic stem cells; however, the surface expression of the amphotropic receptor on mammalian HSCs was

low [140], necessitating a search for a new retroviral envelope protein. Retroviral particles can incorporate the glycoprotein envelope from vesicular stomatitis virus (VSV-G) by a poorly understood mechanism termed "pseudotype formation." VSV-G pseudotyped retroviral vectors can transduce mammalian (murine, human, and primate) and nonmammalian cell types (fish) normally refractory to infection by amphotropic vectors. The receptor is thought to be a ubiquitous phospholipid transporter. This rigid envelope allows the retroviral particles to be concentrated by ultracentrifugation to titers greater than 10^9 per milliliter [141]. Most importantly, retroviral vectors with the VSV-G envelope transduce murine hematopoietic stem cells and their progenitors more efficiently than their amphotropic counterparts [142]. Even with the characterization of a competent envelope, retroviral vectors would still have several obstacles to overcome to deliver sustained high-level expression of globin genes in hematopoietic stem cells.

Early Transduction of Hematopoietic Stem Cells

An effective gene-based therapy for blood disorders or other genetic diseases has several requirements. Ideally, the chosen vector would be used to transduce a population of hematopoietic stem and progenitor cells once, and this single treatment would deliver the corrective transgene to the appropriate hematopoietic lineage(s) and provide therapeutic protein expression for the lifetime of the patient. For certain disorders such as lysosomal storage diseases or severe combined immunodeficiency (SCID) due to adenosine deaminase deficiency (ADA-SCID), transgene expression does not need to be lineage specific and can be very low but still result in therapeutic relief. SCID caused by a mutated lymphocyte gamma chain (γ_c) or Janus kinase 3 gene (X-linked and Jak3-SCID, re-

spectively) can also be rescued by unregulated expression of a normal transgene. Unfortunately, this approach may be harmful because forced expression of a cytokine signaling component without normal *tight* regulation could result in abnormal lymphocyte development or leukemia (see below). In contrast, treatment of the hemoglobinopathies requires high-level and erythroid-specific expression of the corrective globin transgene that is not extinguished over time.

The earliest use of retroviral vector to transduce hematopoietic cells employed marking genes to demonstrate transfer of the bacterial gene for neomycin resistance (neo) [143]. Although these cells gave rise to hematopoietic colonies of the granulocyte-macrophage and erythroid lineages in G418-containing semisolid medium, they did not definitively demonstrate targeting of a pluripotent hematopoietic stem cell (HSC). As mentioned above, a more thorough demonstration of transduction of long-term renewing HSCs is the reconstitution of a lethally irradiated recipient by these donor cells. Using a marker gene of human HPRT or neo, several groups were able to achieve such a rescue by transducing whole bone marrow that could give rise to cells of the lymphoid and myeloid lineages in irradiated recipients [144-146]. With the detection of human hypoxanthine phosphoribosyl transferase (HPRT), the study by *Miller et al.* was the first to document protein expression from a retroviral vector in a reconstituted recipient. Conclusive proof that bone marrow-derived HSCs could be transduced by a retroviral vector came with the serial transplantation of bone marrow from a rescued primary recipient that could reconstitute all lymphoid and myeloid lineages of a lethally irradiated secondary recipient for a period of greater than 4 months [147]. This currently remains the most

rigorous demonstration of hematopoietic stem cell transduction. Following these early successes, investigators began testing retroviral transduction of β-globin genes.

Early experimentation with globin-containing retroviral vectors resulted in tissue-specific expression of human β-globin in mouse bone marrow, but expression was low and highly variable; levels were usually between 0 and 2% of endogenous mouse β^{major} RNA levels [148-151]. These experiments were initiated before the importance of up-stream β-globin regulatory elements was fully appreciated. The discovery of the LCR and fine mapping of its HS prompted groups to insert these sequences with β-globin into retroviral constructs in an attempt to increase expression of the linked transgene. Unfortunately, studies using LCR subfragments either failed to generate high titers [152-154], resulted in low expression [154] or were susceptible to transmission of rearranged proviral genomes [152]. Cryptic splice sites and polyadenylation signals within the second intron of the β-globin gene and LCR were eliminated, and small core LCR subfragments were incorporated into vectors in efforts to overcome vector rearrangement [155, 156]. Although these vectors resulted in higher titer and faithful transfer of an unrearranged transgene, the small core LCR elements failed to provide position-independent β-globin expression from the integrated vector. Furthermore, expression was extinguished over time.

A further barrier restricting efficient retroviral transduction of stem cells with globin transgenes is the silencing of virally transduced genes. Mammalian genomes have co-evolved with transposable elements such as transposons, retrotransposons, and endogenous retroviruses, with conservative estimates suggesting these elements comprise 45% of our genome [157]. Although there are abundant data ([158] and references

therein) suggesting metazoan genomes evolve with the assistance of retrotransposable elements, the genome is thought to have an elegant defense against retroviral insertion that involves methylation of invading sequences [159]. Retroviruses and retroviral vectors are rapidly inactivated following infection or insertion into fertilized mouse zygotes [160], blastocysts [161], or HSCs [162], and this inactivation is associated with methylation of the retroviral sequences [160, 162]. Integrating AAV vectors are also rapidly inactivated upon insertion into the genome [72]. The discovery that the methyl CpG binding protein MeCP2 interacts with a HDAC-containing core complex revealed that MeCP2 might function in part by recruiting repressive deacetylase activity to methylated DNA [163]. Interestingly, silencing of an AAV vector could only be relieved by treatment with the HDAC inhibitor TSA, but MoMLV retroviral vectors required treatment with both the DNA methylation inhibitor 5-azaC and TSA [164]. Host genomic silencing mechanisms may also differ for the two viruses. AAV repression is associated with histone H4 deacetylation; however, retroviral shutdown is concurrent with deacetylation of histone H3, but not histone H4 [108, 165]. *Lorincz et al.* did not see reactivation of heavily methylated retroviral vectors using TSA. This result may not be surprising. The histones of silenced chromatin are regulated by other posttranslational modifications, including methylation of histone H3 lysine 9 [166, 167] and ubiquitination of histone H2B [168]. The causal sequence of DNA methylation and histone tail modification responsible for heritable chromatin states remains to be elucidated; however, the silencing of virally delivered genes remains a serious problem for retroviral gene therapy.

Another difficulty with retroviral gene transduction is the failure of these vectors to transduce nondividing cells. Retroviruses require cell transit through mitosis and nu-

clear envelope breakdown for integration into host chromatin [169, 170]. Unfortunately, hematopoietic stem cells are highly quiescent and divide infrequently. Early attempts to overcome this problem relied on treatment with cytokines that could mobilize HSCs into cell cycle [171, 172]. Cytokine mobilization enables more efficient transduction but compromises stem cell function, causing loss of pluripotency and impairing proper hematopoietic lineage specification (see paper 2 and references therein).

Lentiviruses contain nucleophilic proteins that obviate the need for nuclear envelope breakdown and cell division to occur for efficient transduction [173-177]. The development of lentiviral vectors [178-180] has enabled efficient transduction of non-dividing human hematopoietic stem and progenitor cells [181, 182]. Human progenitor $CD34^+$ cells can be transduced by lentiviral vectors in the absence of cytokine stimulation, and these cells can repopulate nonobese diabetic severe combined immunodeficient (NOD-SCID) mice; however, hematopoiesis is abnormal in these animals, and more than 90% of the human cells develop into B lymphocytes [183]. Our group was the first to demonstrate that unmobilized murine HSCs were efficiently transduced with a lentiviral vector in the absence of cytokine stimulation and that the transduced gene was expressed in all hematopoietic cell lineages examined for at least 5 months after transplantation into lethally irradiated recipients (see paper 1). However, several studies suggested that efficient lentiviral transduction of quiescent cells was cell-cycle dependent [183, 184]. This observation has led most groups to incorporate mobilization regimens that stimulate HSC cell division, even though cytokine stimulation can compromise stem cell function.

Another advantage of lentiviral-based vectors is the transport of full-length RNA transcripts by the Rev accessory protein [185]. Since LCR-globin containing transgenes

were incorrectly processed by retroviral vectors, we predicted that rev would mediate efficient nucleocytoplasmic export of intact transgenes. Addition of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to the lentiviral vector increases the efficiency of RNA processing and contributes to the production of full-length viral transcripts (Fig. 1, paper 2).

Stable Introduction of Globin Genes into Hematopoietic Stem Cells

As mentioned earlier, an effective treatment for SCD would require upregulated or virally forced expression of HbF or a recombinant molecule with equivalent anti-sickling properties. Unfortunately, achieving high-level expression of γ-globin is hampered by several difficulties. Experiments in transgenic mice demonstrate that LCR γ-globin constructs are expressed four times less efficiently than LCR β-globin constructs [61, 62]. These data suggest that the adult transcription factor expression profile is not capable of driving high levels of a gene normally active only in the fetal environment; alternatively, there may be repressive transcription factors or cofactors that downregulate γ-globin expression during adult life. In recent years, other groups have created γ-β or δ-β globin hybrids containing combinations of the γ- or δ-gene with β-globin promoters and introns [71, 186-188]. These constructs have all failed to yield the high levels of HbF or γ-like globin necessary for protection from β-thalassemia or SCD. This failure is often due to globin transgene rearrangement directed by cryptic splicing, but the cause is not always known.

Because forced expression of γ-globin in transgenic animals was low, our group designed a recombinant human β-globin gene that would combine the anti-sickling prop-

erties of HbF but also maintain correct control in the adult recipient. In vitro mixing experiments (3:1 ratio of HbS and HbAS3) confirmed that HbAS3 impeded HbS impeded polymer formation with greater efficiency than HbF. This finding strongly suggested that HbAS3 would inhibit HbS polymerization in vivo if HbAS3 levels constituted 25% of total hemoglobin. This prediction was borne out by the recent correction of our mouse model of SCD using a lentiviral vector to transduce purified, unmobilized hematopoietic stem cells; HbAS3 levels of 20-25% resulted in correction of all hematological parameters and reversed organ pathology associated with the disease (paper 2).

Alternative Strategies for Correction of SCD

As mentioned above, the adult HbA_2 ($\alpha_2\delta_2$) variant has potent anti-sickling properties. Unfortunately, expression of δ-globin expression is low and results in HbA_2 levels of only 2 to 3% in normal adults and sickle cell patients [189]. Previous work by our group revealed the molecular basis of this downregulated expression. The consensus sequence and spacing of the δ-globin promoter CACCC box is disrupted, and this prevents binding and transactivation by the β-like globin transcription factor erythroid kruppel-like factor (EKLF). Correction of the CACCC sequence and spacing allowed restoration of δ-globin expression from transfected DNA templates [190]. Although the δ-globin gene can not be readily altered in vivo, an alternative approach was suggested by this work. If a modified EKLF could be designed that would bind the δ-globin gene at the mutant CACCC box, then HbA_2 could rise to levels that would inhibit HbS polymerization in sickle cell patients. Phage display technology has been used to select modified zinc-finger proteins that bind to novel sites in vivo [191]. *Choo et al.* validated the physio-

logical relevance of this approach by demonstrating binding of a designed transcription factor could bind to the BCR-ABL translocation break point and inhibit of BCR-ABL oncogene expression [192]. More recently, this approach was used for specific upregulation of erythropoietin [193] and vascular endothelial growth factor (VEGF) [194] in vitro, raising the possibility that these critical regulators of erythroid cell production and blood vessel growth could be attenuated in vivo. This was achieved when controlled upregulation of VEGF by a designed transcription factor was used to demonstrate in vivo induction of angiogenesis (blood vessel production) in a mouse model [195].

In collaboration with Carl Pabo, who pioneered the use of designed transcription factors, we have begun testing modified δ-globin binding EKLF variants for the ability to upregulate the delta globin gene in MEL cells. Initial experiments demonstrate a fivefold upregulation of δ-globin gene expression using several different modified EKLF-like transcription factors. These results validate this alternative genetic approach for correction of SCD.

Other Gene Delivery Systems

Retroviral vectors were the first gene delivery systems to gain wide appeal; however, other vector systems based on adenovirus, AAV, and DNA transposons are being developed for applications of gene therapy. Adenoviral vectors do not integrate into the host genome but persist as episomal DNA. They have been used to transduce quiescent muscle or dividing liver cells [196, 197], which made this system attractive for gene delivery to hematopoietic stem cells. However, effective use of these vectors is hampered by a potent immune response directed in response to low levels of viral gene expression

that results in destruction of transduced cells [198]. Second [199] and third generation vectors [200] focused on deletion of several or all viral genes, but these vectors still failed to eliminate destructive cellular or humoral immune responses. Transduction efficiency is limited further by low levels of the adenoviral-coxsackie receptor envelope [201] and cellular surface integrins on target cells [202]. Additionally, adenoviral transduction of hematopoietic stem cells appears to be very toxic, whether at high MOIs of 500-1,000 [203] or at low MOIs of 10 or less [204]. These barriers will most likely preclude the use of adenoviral vectors for globin gene transduction into hematopoietic stem cells.

AAV vectors can deliver episomally expressed or stably integrated transgenes into the host genome and have been used to transduce quiescent myocytes or actively dividing cells. AAV has been used to successfully deliver intact human globin genes with regulatory LCR elements into cultured human erythroleukemia cells [205, 206], but expression levels were low. High level globin expression could be seen in cultured cells, but, like retroviral vectors, AAV is readily inactivated by epigenetic silencing mechanisms [72]. Low-level globin expression is also observed with hybrid Adenoviral-AAV vectors containing large HS2 and HS3 globin regulatory elements [188]. However, in the latter two studies it is not clear if low-level expression is due exclusively to silencing of the transgene or to transcriptional interference [207] caused by inclusion of a second transcriptional unit for expression of lacZ or EGFP marker genes. Expression of globin genes, even in the context of the entire LCR, are extremely susceptible to transcriptional interference from LTR [208] or phosphoglycerate kinase [79] promoters.

The propensity of AAV to integrate within a defined region of chromosome 19 suggested AAV-derived vectors might offer a way to target insertion of transgenes into

the host chromosome. The development of a hybrid lentiviral-AAV vector could combine the genomic targeting specificity of AAV with the transgene stabilizing properties of the lentivirus. However, high MOIs of approximately 10,000 are needed to transduce 25% of HSCs by the hybrid Adenoviral-AAV vector used by *Shayakhmetov et al.* Although immunogenicity is not a hallmark of AAV, toxic humoral resonses have been associated with this vector system [209]. Additionally, host genome targeting by AAV may not be completely innocuous. Although the majority of integrations by AAV occur on chromosome 19, mapping of junctions reveals targeting into genes throughout the genome. More importantly, integration results in chromosomal rearrangements and deletions up to 2 kb in size [210, 211]. Gene targeting by AAV is thought to occur by the double-strand break (DSB) model of homologous recombination, because this can explain the higher targeting frequencies observed when DSBs are present in the targeting construct or target locus [210]. Integration by AAV may require DNA DSBs and is enhanced 60- to 100-fold by induced DNA DSBs [210].

DNA transposons have been explored recently as an alternative vehicle for gene delivery. Transposons of the Tc1/mariner family are transpositionally inactive due to acquired mutations. Recombinant correction of these mutations rescued activity, resulting in the transpositionally competent Sleeping Beauty (SB) transposon capable of insertion into fish, mouse, and human cells [212]. Targeting efficiency of naked SB DNA is low; therefore, an AAV-SB transposon hybrid vector was designed to take advantage of the AAV delivery system and transposon integration. This AAV-SB hybrid and a less immunogenic gutless AAV-SB hybrid have both been used to correct Factor IX deficiency in mouse models of hemophilia [213, 214]. However, factor IX levels of only 1 to 5 per-

cent from gene-targeted liver are sufficient for correction of this mouse model. Higher levels of protein may likely be necessary to rescue the disease in humans, and levels significantly higher would be required for correction of hemoglobinopathies. Interestingly, the AAV-SB system described above had a propensity for integration into repetitive DNA elements.

Targeted Gene Delivery

The ideal gene delivery system would target the desired gene to a precise location within the genome. Such a system has been developed. In nature, the bacteriophage ϕC31 inserts its DNA by union of its *att*P (attachment) sites with the host *att*B site. Pseudo-*att*P sites exist within the genomes of mice and humans, and these sites have enough homology with wild-type *att*P sites to support persistent integrase-mediated recombination [215]. Tail vein coinjection of plasmids containing the bacteriophage ϕC31 integrase and a Factor IX cassette flanked by an *att*B site resulted in integration at two pseudo *att*P sites, therapeutic expression levels of Factor IX from targeted hepatocytes, and correction of a mouse model of hemophilia [216]. There are thought to be 100-1,000 pseudo *att*P sites in the human genome and a hotspot region of ϕC31 integrase targeting on human chromosome 8 [215], suggesting this might be a useful gene delivery system in human patients. Using DNA "shuffling" to direct evolution of the integrase in vitro, Calos and colleagues were able to create a ϕC31 integrase that could bind the chromosome 8 ψA hotspot 30% of the time, a sixfold increase over the wild-type frequency of integration. The majority of pseudo *att*P sites within the genome have not been se-

quenced; therefore, some of these sites may exist within genes or in proximity close enough to coding sequences to exert harmful position effects.

More recent studies have focused on developing even greater transgene insertion site specificity using the yeast Ty retrotransposons. Yeast Ty5 insertions target heterochromatin, specifically within a 3 kb window flanking the silent mating loci (*HML* and *HMR*) Saccharomyces cerevisiae [217]. A small targeting domain of 6 amino acids within the C-terminus of Ty5 integrase has been mapped, which is essential for targeting [218], and this targeting domain mediates a specific interaction with the C-terminus of Sir4p (SIR4C) [219]. Using a LexA-SIR4C fusion protein that would bind LexA operators on a transfected plasmid flanked by 3 kb of Arabadopsis DNA, Voytas and colleagues were able to demonstrate specific binding of the native Ty5 retrotransposon TD with SIR4C in vivo [220]. In addition, they were able to engineer multiple different targeting specificities by replacing the targeting domain with other heterologous DNA-binding protein domains. In all instances, the engineered Ty5 targeting domain was able to bind its protein partner and integrate within 120 bp of the the LexA binding sites. It is not clear how Ty5 accesses the DNA after docking to Sir4p, but these studies show that integration site specificity can be controlled. It would seem that reengineering of the Ty1, 2, 3, and 4 retrotransposon integrases might also be an attractive target for directing transgene site specificity. Ty1, 2, and 4 typically reside within 750 bp of the 5' end of tRNA genes transcribed by RNA polymerase III (Pol III) [221, 222]. Specificity is even more discriminating with Ty3 elements; integration occurs within 1-2 bases of Pol III transcriptional initiation. The target site of these elements is harmless because they are in gene-poor regions, and their integration does not disrupt Pol III transcription [223, 224].

Development of gene delivery systems using the ϕC31 or Ty integrases will be very promising approaches toward achieving safe and efficient gene transfer in vivo.

Improved transposon systems may be promising vectors if they are found not to integrate preferentially into genes or their flanking promoter elements. Studies of endogenous mobile DNA and de novo transpositions suggest that retroelements that were once thought to be innocuous can also cause genetic lesions within the human genome. Approximately 1% of endogenous L1 retrotransposons can actively retrotranspose [225], and insertional inactivation by these transposable elements has been identified as the disease-causing mutation in at least 12 patients, including one with β-thalassemia [226]. The movement of mobile DNA within mammalian genomes occurs infrequently at best; however, it is important to remember that retroelements and retroviral genomes have likely evolved to select genomic insertion sites that favor their propagation. Although these locations are often innocuous, it is clear from the retroviral life cycle that the virus can leave a damaged genome with impunity to move on to the next host.

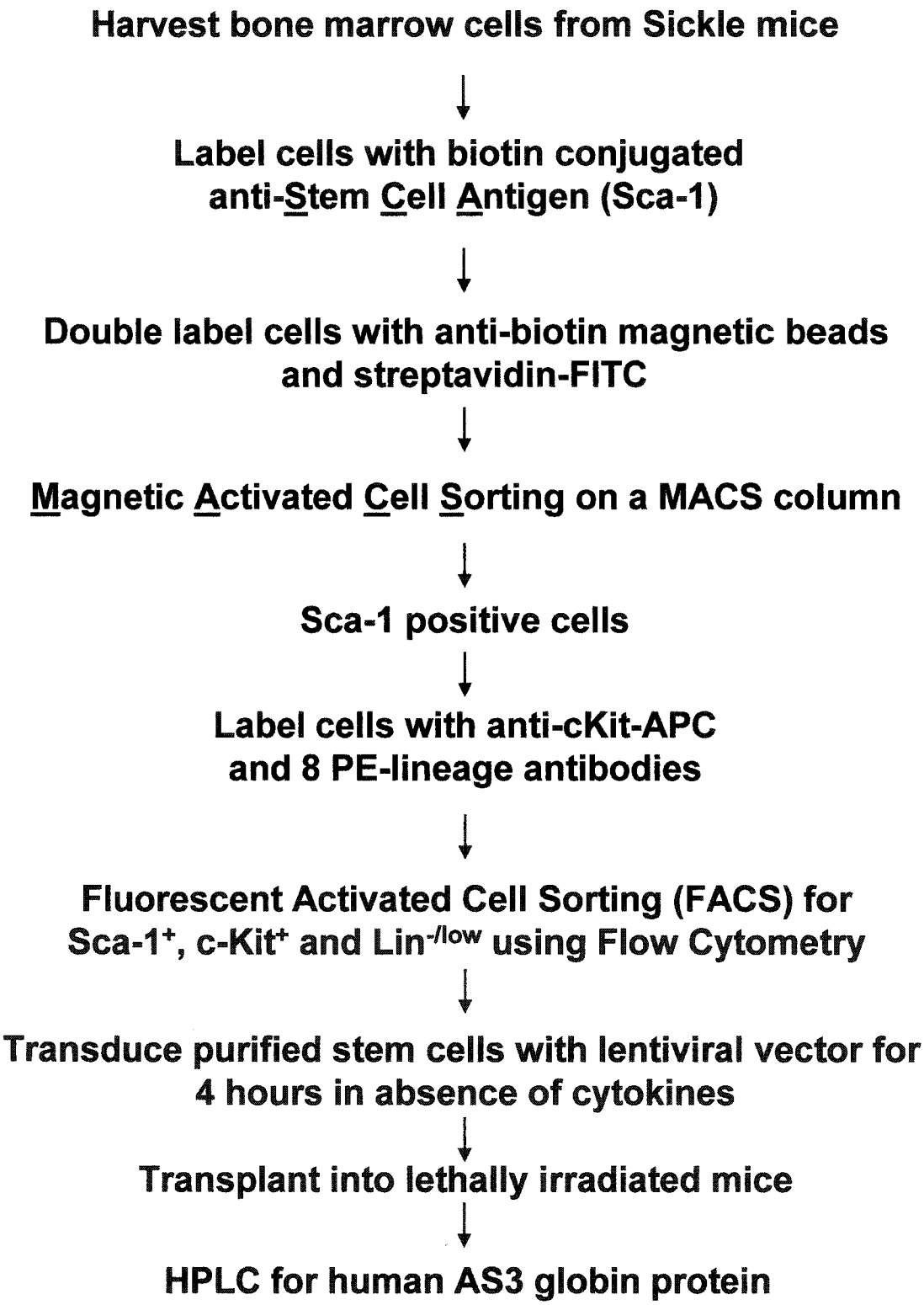
Concerns about viral transduction of hematopoietic cells have recently been raised by the tragic development of leukemia in two SCID patients who were treated by retroviral gene therapy [227, 228]. Insertion of viral vectors near the LMO-2 gene apparently stimulated expression of the LIM domain only-2 (LMO-2) protooncogene that normally regulates crucial steps in hematopoiesis [229]. It is interesting to note that translocation caused by illegitimate recombination between the T-cell receptor and LMO2 occurs with a high frequency in the thymus of healthy patients, as well as in patients suffering from T-cell receptor/LMO2 translocating leukemias [230]. Patients with SCID would likely be more susceptible to leukemogenesis initiated by cells with this translocation because of

their deficient or absent immune response. The potential hazards associated with retroviral insertional mutagenesis were not without precedent [231, 232]. Activation of oncogenes by enhancer sequences present in the LTRs of retroviruses has been reported in the literature [232], and these LTR enhancers can activate genes at distances approaching 100 kb [233]. Many viruses that integrate into the host genome as part of their lifecycle insert nonrandomly into genes or regions of DNase I hypersensitivity, suggesting a requirement for open chromatin [234, 235]. Numerous studies have demonstrated that retroviruses [234-236], lentiviruses [236, 237], and AAV [211] integrate into active genes. Recent data analyzing a large number of integrants suggests that HIV-1 and MoLV integrate into transcriptionally active regions [236, 237]. However, these studies analyzed rapidly dividing human cell lines. It is possible that target site specificity of nonmobilized bone marrow or quiescent stem cells may be different from cell lines undergoing exponential growth. Other studies suggest that transcriptionally active regions are not preferred targets for oncoretroviral integration in vivo [235, 238]. Analysis of Human T-cell Leukemia Virus-1 (HTLV-1) integration sites in patients with adult T-cell leukemia/lymphoma suggests that most regions of the genome appear to be accessible to HTLV-1 and that integration is random [235]. Similar studies need to be performed in relatively quiescent HSCs to determine the pattern of integration. We utilized a self-inactivating (SIN) lentiviral vector in our studies in order to prevent insertional activation events. Other groups have used clever gene delivery systems to target genes to specific loci within the genome [216, 220]. However, none of these protocols can assure that an insertional event leading to leukemogenesis can be prevented. The introduction of cor-

rective genes into adult or embryonic stem cells by homologous recombination will likely be the safest option for future cell-based therapies.

The work discussed in this dissertation begins with the description of a lentiviral vector and protocol (Fig. 5 and paper 1) that enables efficient and stable transduction of highly quiescent, unmobilized hematopoietic stem cells without impairing self-renewal and normal lineage specification in vivo. This protocol was developed as an alternative to current protocols that utilize ex vivo stimulation of hematopoietic stem cells with cytokines. Cytokine mobilization increases the number of stem cells that are actively progressing through the cell cycle and, therefore, increases the efficiency of retroviral or lentiviral transduction. Unfortunately, cytokine stimulation of HSCs and premature exit from quiescence can decrease self-renewing capacity, homing efficiency, and, therefore, long-term engraftment in mice and in humans (see references within paper 2). More importantly, mobilization of bone marrow in human patients is toxic and can result in death [239]. We have demonstrated for the first time that cytokine stimulation is not required for high-level stable HSC transduction. Following these encouraging results using a lentiviral-GFP marking vector, we decided to employ a similar methodology to transduce HSCs from our mouse model of SCD. A potent anti-sickling globin gene developed in our laboratory was inserted into an improved SIN lentiviral vector that we term lenti/ β^{AS3}. Using lenti/ β^{AS3}, we were able to achieve high level β^{AS3}-globin levels that resulted in the sustained correction of our mouse model of SCD. This study was the first correction of a hemoglobinopathy using a protocol that may be translatable to human patients. The third paper documents the development of the anti-sickling β-globin, β^{AS3}

Figure 5. Protocol for isolation of unmobilized hematopoietic stem cells. Shown is a schematic of bone marrow extraction and purification of long-term renewing hematopoietic stem cells (HSC). Bone marrow is isolated from the long bones of unmobilized sickle mice, and purified HSCs are not exposed to cytokine stimulation regimens that are known to alter the homing and self-renewal properties of HSCs. For the work here, only animals that were greater than 99% reconstituted with sickle HSCs were analyzed to preclude the artifactual anti-sickling effects that are provided by residual murine red blood cells.



and the use of HbAS3 to genetically correct sickle animals by breeding a knockout-transgenic mouse expressing exclusively human HbAS3 with a sickle mouse to confirm the anti-sickling efficacy of this molecule in vivo. Finally, we show correction of β-thalassemia mice created by tetraploid embryo complementation using lentiviral transduction of embryonic stem cells. This work is particularly promising toward the development of therapies for hemoglobinopathies using "therapeutic cloning."

LENTIVIRAL VECTOR TRANSDUCTION OF HEMATOPOIETIC STEM CELLS THAT MEDIATE LONG TERM RECONSTITUTION OF LETHALLY IRRADIATED MICE

by

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ABSTRACT

Lentiviral vectors efficiently transduce human CD34+ cells that mediate long-term engraftment of NOD/SCID mice. However, hematopoiesis in these animals is abnormal. Typically, 95% of the human cells in peripheral blood are B lymphocytes. To determine whether lentiviral vectors efficiently transduce stem cells that maintain normal hematopoiesis in vivo, we isolated Sca-1$^+$c-Kit$^+$Lin$^-$ bone marrow cells from mice without 5-fluorouracil treatment and transduced these cells in the absence of cytokine stimulation with a novel lentiviral vector containing a GFP (green flourescent protein) reporter gene. These cells were transplanted into lethally irradiated C57Bl/6 mice. In fully reconstituted animals, GFP expression was observed in 8.0% of peripheral blood mononuclear cells for 20 weeks posttransplantation. Lineage analysis demonstrated that a similar percentage (approx. 8.0%) of GFP-positive cells was detected in peripheral blood B cells, T cells, granulocytes and monocytes, bone marrow erythroid precursor cells, splenic B cells, and thymic T cells. In secondary transplant recipients, up to 20% of some lineages expressed GFP. Our results suggest that quiescent, hematopoietic stem cells are efficiently transduced by lentiviral vectors without impairing self-renewal and normal lineage specification in vivo. Efficient gene delivery into murine stem cells with lentiviral vectors will allow direct tests of genetic therapies in mouse models of hematopoietic diseases such as sickle cell anemia and thalassemia, in which corrected cells may have a selective survival advantage.

INTRODUCTION

Efficient transduction of hematopoietic stem cells is essential for a genetic treatment of many blood diseases. Non-cycling, quiescent [1] stem cells are poorly transduced with retroviral vectors because breakdown of the nuclear membrane during mitosis is required for efficient retroviral integration into host chromatin [2]. Poor transduction of stem cells is also correlated with low-level expression of receptors for amphotropic retroviral vectors [3]. Although adeno-associated viral (AAV) vectors have been shown to efficiently transduce postmitotic skeletal muscle or liver cells [4-6], transduction of hematopoietic stem cells is low [7]. In addition, we have demonstrated that virally transduced genes are silenced after integration into host chromosomes [8], and that the mechanism of silencing involves histone deacetylation and chromatin condensation [9].

In contrast to retroviruses, lentiviral vectors efficiently transduce quiescent postmitotic cells [10, 11]. In a recent study, *Miyoshi et al.* [12] demonstrated that human $CD34^+$ cells are efficiently transduced by a lentiviral vector, and sustained expression of a green fluorescent reporter (GFP) reporter gene is detected in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice for 22 weeks. However, hematopoiesis in these animals is abnormal. The human cells in peripheral blood of these mice were predominantly B lymphocytes [12], and thus it is difficult to evaluate the maintenance of normal hematopoiesis after lentiviral transduction. We now report the efficient transduction of highly purified murine bone marrow stem cells with a novel lentiviral vector. We demonstrate that normal hematopoiesis is preserved after lentiviral transduction of stem cells and reconstitution of lethally irradiated recipient mice. In addition, we demonstrate

that expression of transduced genes persists after long-term engraftment and in secondary transplants.

MATERIALS AND METHODS

Purification of Stem Cells

C57Bl/6 Hbb^d donor mice were obtained from the Jackson Lab (Bar Harbor, ME; http://www.jax.org) and bred in our mouse facility. Bone marrow was flushed from femurs and tibia of 8- to 16-week-old donor mice with Iscove's modified Dulbecco's medium (IMDM) medium containing 5 mM EDTA, 2% fetal bovine serum (FBS) and antibiotics. After washing once with separation buffer (phosphate-buffered saline [PBS] containing 5 mM EDTA and 0.5% charcoal treated bovine serum albumin), cells were labeled with biotin-conjugated Sca-1 antibody (Pharmingen; San Diego, CA; http://www.pharmingen.com) in label buffer (phosphate-buffered saline [PBS] containing 5 mM EDTA) for 15 min on ice. Cells were then washed once with separation buffer to remove free Sca-1 antibody, and labeled with magnetic bead-conjugated streptavidin (Miltenyi Biotech GmbH; Bergisch Gladbach, Germany; http://www.miltenyibiotec.com) for 15 min on ice. This step was immediately followed (without washing) by addition of fluorescein isothiocyanate-conjugated streptavidin (Caltag; South San Francisco, CA; http://www.caltag.com) for another 15 min on ice. After labeling, cells were washed once with separation buffer, and magnetic bead-labeled cells were enriched, using the maximum allowable concentration, on a column (Miltenyi) as the manufacturer suggested. Cells were eluted from the column, pelleted, and resuspended in labeling buffer. Cells were then simultaneously labeled with allophycocyanin-conjugated c-Kit antibody

(Pharmingen) and a cocktail of phycoerythrin(PE)-conjugated lineage antibodies containing B-220, CD3, CD4, CD5, CD8, Mac-1, Gr-1, and Ter-119 (Pharmingen) for 15 min on ice. Cells were washed once with separation buffer and resuspended in IMDM medium for sorting on a Becton-Dickinson (Franklin Lakes, New Jersey; http://www.bd.com) FACSVantage SE. Sca-1^{+}c-$Kit^{+}Lin^{-}$ cells were collected into a 5-ml tube with IMDM containing 1% FBS for transduction.

Production of Lentiviral Vector

To construct the pPCW-eGFP gene transfer vector, a polymerase chain reaction (PCR)-amplified DNA fragment containing the EGFP gene (derived from pEGFP-C1, Clontech Laboratories; Palo Alto, CA) was ligated into the BamHI/XhoI sites of the pHR-cytomegalovirus (CMV)-*LacZ* plasmid [10], generating pHR-CMV-eGFP. Then, a 150-bp sequence of DNA (coordinates 4327 to 4483) containing the central polypurine tract (cPPT) and central terminal site (CTS) was PCR-amplified from the HIV-1 pSG3 molecular clone [13] and ligated into the unique ClaI site of pHR-CMV-eGFP. To increase eGFP expression in the transduced cells, a post-transcriptional regulatory element derived from the woodchuck hepatitis virus (WPRE) was inserted downstream of eGFP, generating the pPCW-eGFP gene transfer vector.

Transduction of Stem Cells

Sorted Sca-1^{+}c-$Kit^{+}Lin^{-}$ stem cells were centrifuged at 300 x *g* for 10 min, and resuspended in IMDM medium containing 10 μg/ml dextran sulfate and 1% FBS. One

thousand stem cells were infected in a total volume of 100 µl for 4 h at 37°C and transplanted into a single, lethally irradiated mouse as described below.

Transplantion

The congenic recipient mice (C57Bl/6 Hbb^s) were purchased from the Jackson Lab and maintained in our transgenic facility. Mice were lethally irradiated with 1,250 RADS in two doses of 625 RADS, each with a Picker Cyclops Cobalt-60 unit. Anesthetized mice were transplanted with 1,000 stem cells per mouse in 100 µl IMDM by retro-orbital injection. Transplants were maintained on antibiotic water containing 1.1 g/l neomycin sulfate (Sigma; St. Louis, MO; http://www.sigma-aldrich.com) and 1 x 10^6 units/l polymyxin B sulfate (Sigma) for two months posttransplantation. For secondary transplantation, five million unfractionated bone marrow cells from primary transplants were retro-orbitally injected into each recipient mouse. Hematopoietic recovery of transplants was monitored by analysis of diffuse hemoglobin using high performance liquid chromatography (HPLC) as described previously [14].

Mononuclear Cell GFP Analysis

Fifty microliters of peripheral blood from each mouse was collected from the tail vein and mixed with 1 ml PBS containing 2.5 mM EDTA. The cells were further diluted to 3 ml with PBS immediately before gradient separation. Three milliliters of Histopaque-1077 (Sigma) was loaded into a 15-ml conical tube, and 3 ml of diluted blood cells were carefully layered on the top. Cells were centrifuged at 300 x *g* for 10 min. The opaque layer of mononuclear cells that formed at the interface was carefully transferred

to a new tube and washed once with PBS. Cells were then aliquoted and labeled with PE-conjugated antibodies as described above, and GFP expression was analyzed using FACS. The same gradient procedure was also used to prepare bone marrow mononuclear cells for analysis. Single cell suspensions of spleen or thymus were used directly for labeling and analysis.

Progenitor Assay

Bone marrow cells were mixed with methylcellulose medium M3434 (Stem Cell Technology; Vancouver, BC; http://www.stemcell.com) to 3 x 10^4/ml, plated onto 35 mm plates, and cultured at 37°C for 12 days as the manufacturer suggested. Colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) colonies were examined using an inverted microscope, and fluorescent images from the colonies were captured using an Olympus IX70 inverted microscope with epifluorescence optics and a Hamamatsu charged-coupled device camera.

RESULTS

Reconstitution of Lethally Irradiated Mice with Sca-1$^+$C-Kit$^+$Lin$^-$ Cells

Bone marrow was isolated from femurs and tibias of C57Bl/6 donor mice containing the diffuse hemoglobin (Hbb^d) haplotype. No 5-fluorouracil was administered to the mice to mobilize stem cells prior to marrow isolation. Sca-1$^+$c-Kit$^+$Lin$^-$ cells were isolated as described in the **Materials and Methods** section and transplanted into lethally irradiated, wild-type C57Bl/6 recipient mice containing the single hemoglobin (Hbb^s) haplotype. As few as 50 of these highly purified cells were capable of fully reconstitut-

ing hematopoiesis (data not shown). Reconstitution with donor stem cells was followed by HPLC of hemolysates. For the transduction experiments described below, 1,000 Sca-1^{+}c-$Kit^{+}Lin^{-}$ cells per recipient mouse were routinely infected with a lentiviral vector prior to transplantation. Figure 1 illustrates reconstitution in a representative animal transplanted with 1,000 transduced stem cells. Within eight weeks post-transplantation, all erythroid cells were derived from the donor as indicated by the replacement of Hbb^{S} (β^{s}, β^{t}) with Hbb^{D} (β^{maj}, β^{min}).

Design of the Lentiviral Vector

The lentiviral vector used in this study was an HIV-1 vector pseudotyped with vesicular stomatitis virus G (VSV-G) glycoprotein. The vector contained a CMV promoter driving a GFP reporter gene, cPPT and CTS derived from a molecular clone of HIV-1 to increase packaging efficiency [13], and a posttranscriptional regulatory element derived from Woodchuck hepatitis virus (WPRE) (Fig. 2A). Zufferey et al. [15] recently demonstrated that the WPRE enhances retroviral and lentiviral transduction efficiency in cultured cells by increasing the efficiency of RNA processing. In our experiments, lentiviral vectors with or without WPRE were able to transduce cultured murine erythroleukemia (MEL) cells efficiently (data not shown); however, only the vector with a WPRE efficiently transduced purified murine bone marrow stem cells as described below. We were unable to detect any GFP expression in hematopoietic cells in vivo when using the vector without WPRE (data not shown).

Figure 1. Reconstitutiton of lethally irradiated mice with Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. Lethally irradiated recipient mice were transplanted with 1000 virally transduced Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. Peripheral blood was collected from donor (diffuse hemoglobin haplotype), recipient (single hemoglobin haplotype), and transplants at indicated time points. Hemolysates were analyzed by HPLC using a 35% to 41% acetylnitrile gradient. Full reconstitution was achieved 8 weeks after transplantation.

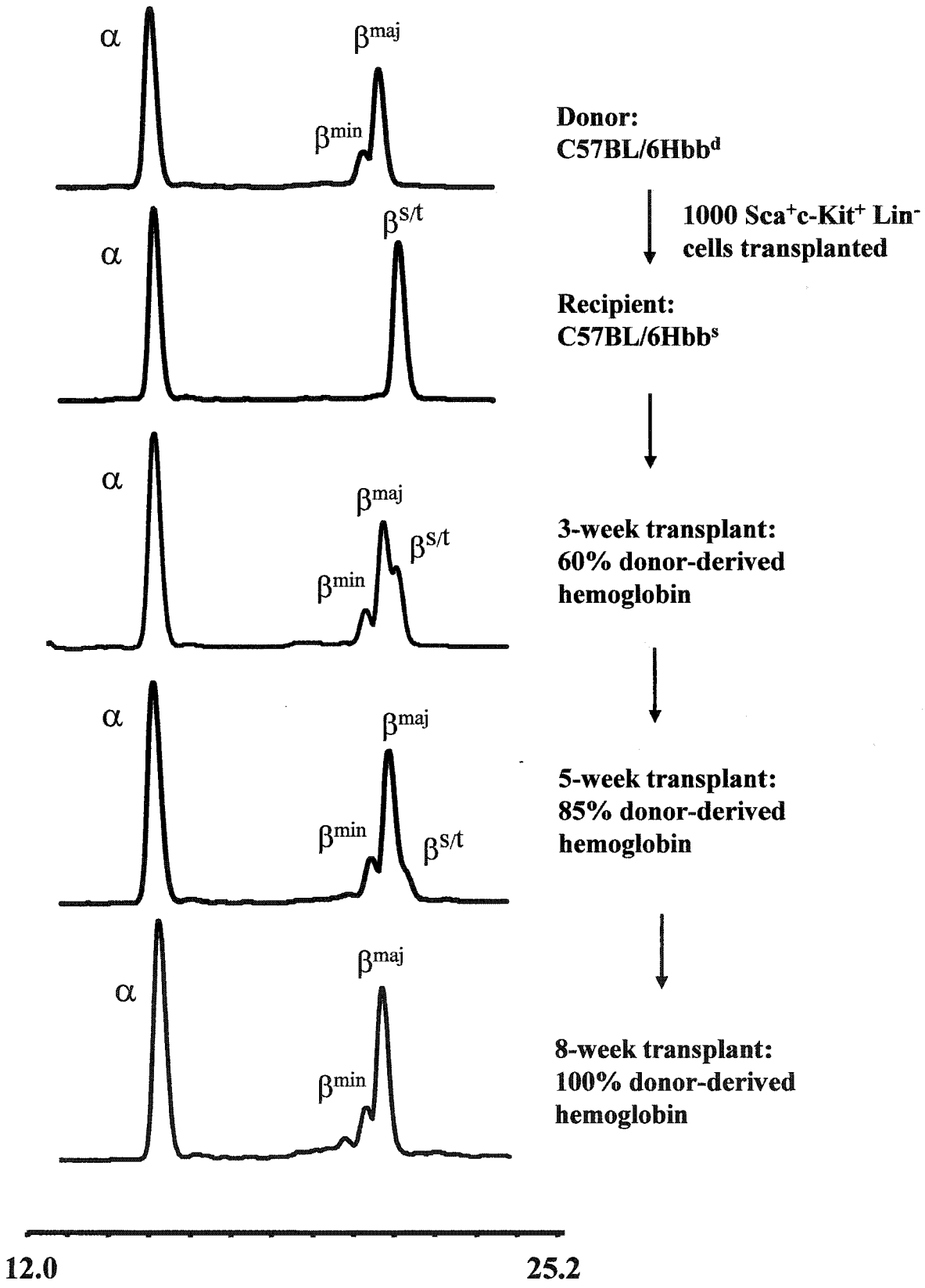
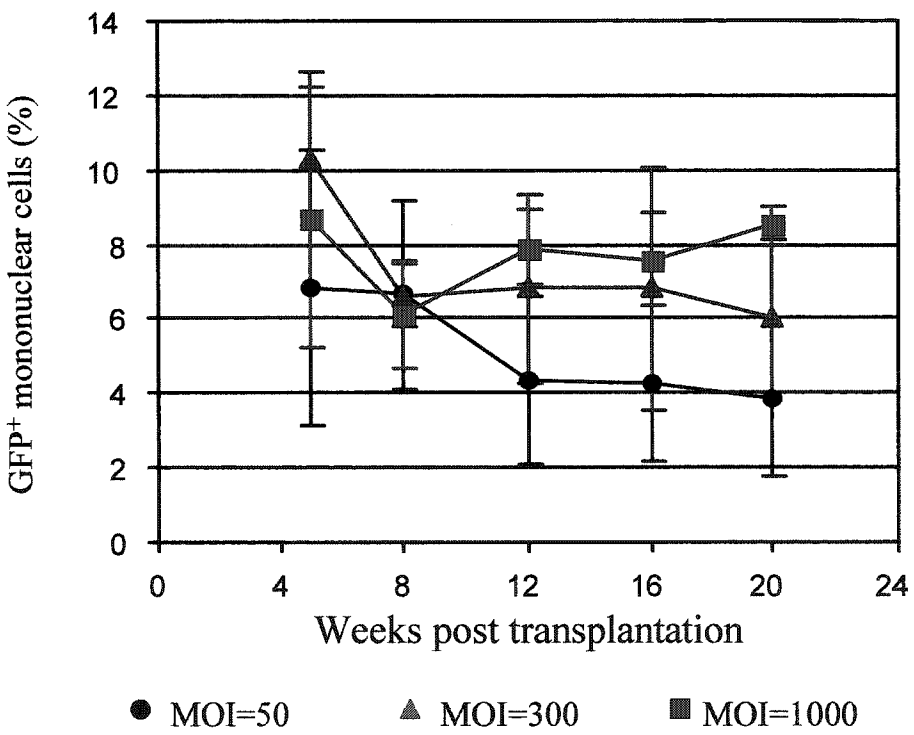


Figure 2. Stable transduction of Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells by a lentiviral vector. (A) Map of lentiviral vector PCW-eGFP used in this study. A cPPT and a CTS derived from a molecular clone of HIV-1 were inserted in the vector to increase packaging efficiency. A post-transcriptional regulatory element of WPRE was placed downstream in the sense orientation with CMV/eGFP to increase GFP expression. (B) Persistence of GFP expression in mononuclear cells from peripheral blood. One thousand Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells were transduced with the vector at an MOI of 50, 300, or 1,000, and these cells were transplanted into a single, lethally irradiated mouse. Four mice were reconstituted with stem cells infected at an MOI of 300 and four at an MOI of 1,000. Three mice were reconstituted with cells infected at an MOI of 50. At the time points indicated, 50 μl of blood from each transplanted mouse was collected from the tail vein. Mononuclear cells were then isolated and analyzed for GFP expression by FACS. Starting at 16 weeks posttransplantation, some mice were sacrificed for secondary transplantation and bone marrow cell analysis. At least two mice from each group (MOI= 50, 300, or 1,000) were analyzed after 20 weeks. GFP expression persisted for 20 weeks in all reconstituted mice. These data demonstrate that lentiviral vectors mediate stable transduction of stem cells which are capable of long-term reconstitution in vivo. The results also demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells.

A

pPCW-eGFP

B



Transduction of Sca-1$^+$C-Kit$^+$Lin$^-$ Cells and Persistent Expression of Vector-Derived GFP in Hematopoietic Lineages in Vivo

Although lentiviral vectors are able to transduce quiescent cells [10, 11], transduction efficiency is enhanced when cells are induced to enter the cell cycle [16, 17]. However, induction of hematopoietic stem cell replication in vitro may result in loss of pluripotency. Therefore, we chose to infect purified stem cells for only 4 h in the absence of cytokines. Cells were then transplanted into lethally-irradiated recipients in which stem cells could home to the marrow and replicate *in vivo* under conditions that favor maintenance of pluripotency. Transduction efficiency was measured by FACS analysis for GFP expression.

Figure 2B illustrates the results of GFP expression in peripheral blood mononuclear cells of mice at 5 to 20 weeks posttransplantation. These animals received stem cells transduced at multiplicities of infection (MOIs) of 50, 300 or, 1,000. Full reconstitution with donor stem cells was achieved at eight weeks (data not shown). At an MOI of 50, an average of 4% of peripheral blood mononuclear cells were GFP positive, and the percentage was increased to 6% and 8% with MOIs of 300 and 1000, respectively. Few GFP-positive mononuclear cells were detected when stem cells were transduced with an MOI of 5 (data not shown). These results demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells. The data also demonstrate that this lentiviral vector mediates stable transduction of stem cells that are capable of long-term reconstitution in vivo.

We next examined whether transduced hematopoietic stem cells maintained pluripotency after long-term reconstitution. Mice were sacrificed at 16 and 20 weeks posttransplantation and mononuclear cells were labeled with PE-conjugated, lineage-

specific antibodies for B cells (α-B220), T cells (α-CD3, CD4, and CD8), neutrophils, monocytes and granulocytes (α-Mac-1 and GR-1), and erythroid cells (*a*-Ter-119). Results from a representative mouse are shown in Figure 3. In peripheral blood, 7.9% of B cells, 9.6% of T cells, and 12.6% of neutrophils, granulocytes, and monocytes were positive for GFP expression (Fig. 3A). Similar values were observed at all time points for peripheral blood mononuclear cells (data not shown). These results demonstrate that transduced hematopoietic stem cells maintain the capacity for normal lineage specification in fully reconstituted mice

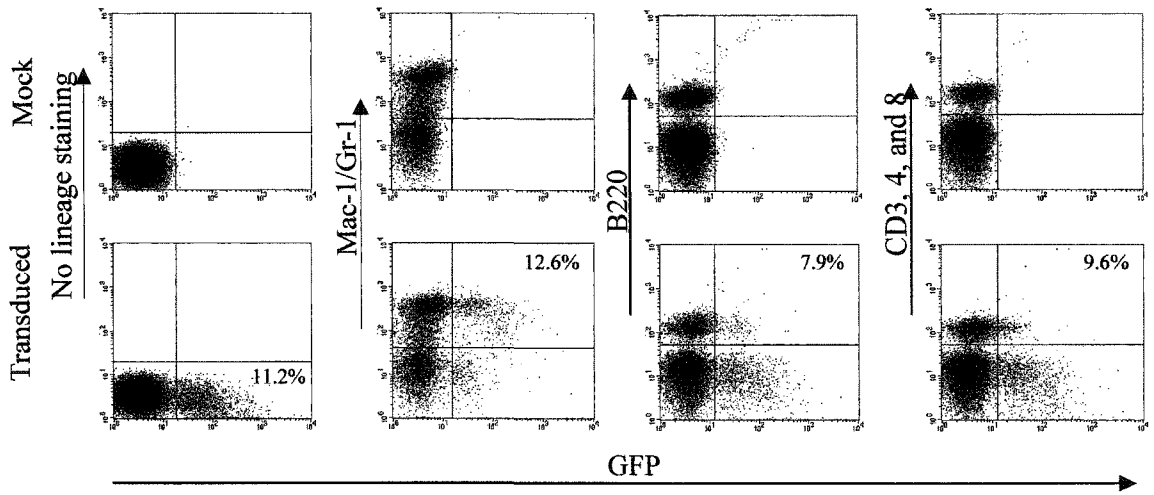
Interestingly, less than 1% of erythroid cells in peripheral blood were positive for GFP (data not shown). Although this result was surprising, we speculated that erythroid progenitors were stably transduced, but that little GFP persists in enucleated red blood cells. Therefore, we examined Ter-119^{+} bone marrow mononuclear cells for GFP expression and observed that 9.7% of these erythroid precursors were GFP positive. This result demonstrated that transduced stem cells are also capable of normal erythroid lineage differentiation. Bone marrow B cells, neutrophils, granulocytes and monocytes (Fig. 3B), as well as splenic B cells and thymic T cells (Fig. 3C) were all GFP positive at similar percentages. Again, these results demonstrate that lentiviral vectors efficiently transduce hematopoietic stem cells and do not alter normal properties of self-renewal and lineage specification in fully reconstituted mice.

Persistent GFP Expression in Secondary Transplants

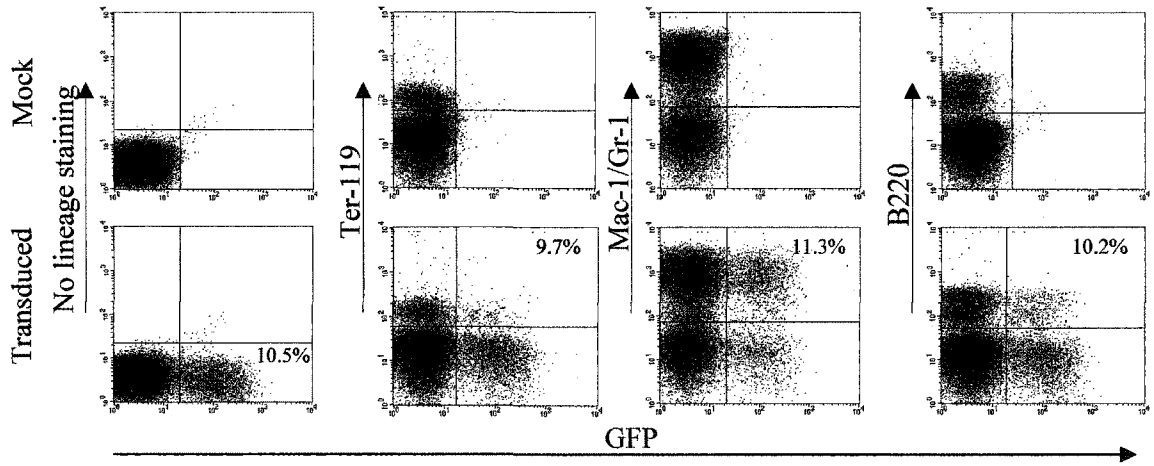
To determine whether GFP expression persists in secondary transplants, five million bone marrow cells from primary transplants were injected into lethally irradiated

Figure 3. GFP expression in hematopoietic cell lineages. At 16 weeks posttransplantation, mononuclear cells were collected from peripheral blood (A), bone marrow (B), and spleen and thymus (C). This primary recipient initially received 1000 stem cells transduced at an MOI of 300. Mononuclear cells were analyzed by FACS without staining or after staining with PE-conjugated lineage antibodies (B220 for B cells, a mixture of CD 3, 4, and 8 for T cells, a mixture of Mac-1 and Gr-1 for neutrophils, monocytes and granulocytes, and Ter-119 for erythroid cells). The percentage of GFP positive mononuclear cells is shown on each FACS profile. These results demonstrate that transduced hematopoietic stem cells maintain the capacity for normal lineage specification in fully reconstituted mice.

A. Peripheral blood



B. Bone marrow



C. Spleen and thymus

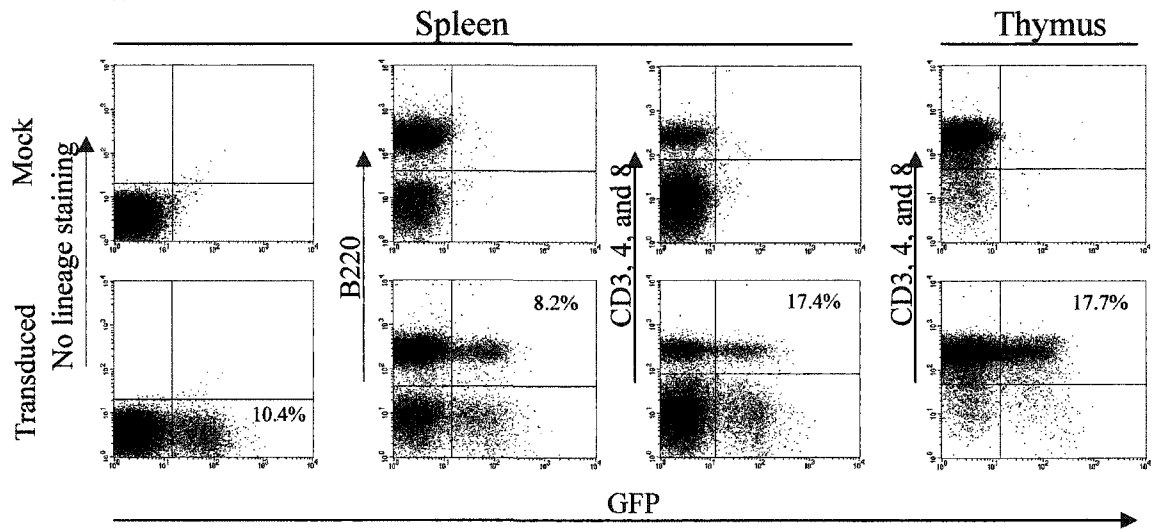


Table 1 GFP expression in peripheral blood mononuclear cells of secondary transplants

	Secondary transplants							Primary transplant
	TP85	TP86	TP87	TP88	TP89	TP90	Average	TP64
No staining	10.8	12.4	14.3	8.4	4.1	9.8	10.0	11.2
B220	7.2	5.8	9.7	3.9	3.0	7.8	6.2	7.9
CD3,4&8	16.3	19.3	20.8	12.4	7.1	15.4	15.2	9.6
Mac-1/Gr-1	6.1	12.6	7.6	8.0	3.5	5.9	7.3	12.6

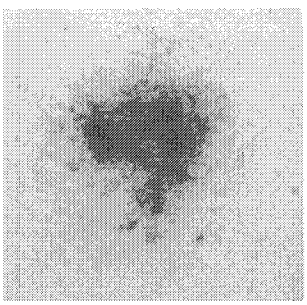
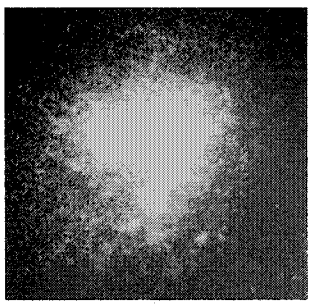
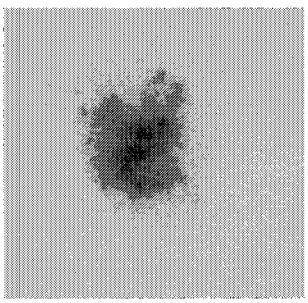
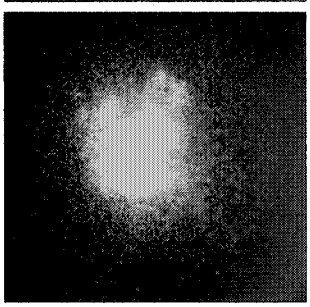
Five million bone marrow cells from a 16-week primary transplant were used to reconstitute lethally irradiated secondary recipients. The percentages of GFP-positive cells in peripheral blood mononuclear cells, unstained or stained with lineage-specific antibodies, are shown for six secondary transplants at 12 weeks posttransplantation. The percentage of GFP-positive, peripheral blood mononuclear cells in the primary recipient at 16 weeks posttransplantation is also shown for comparison.

C57BL/6Hbb^d recipient mice. Table 1 illustrates one set of secondary transplants derived from a 16-week primary transplant. G FP expression was detected in all reconstituted secondary recipient mice at 12 weeks posttransplantation, and the average percentages of GFP positive mononuclear cells, B cells, T cells, and neutrophils and monocytes were consistent with those from the primary transplant. These results further support the conclusion that long-term, self-renewing stem cells were transduced by the lentiviral vector and that the pluripotency of these cells was preserved in the fully reconstituted recipients.

Silencing of Lentivirally Transduced Genes

We also examined GFP expression in CFU-GEMM derived from long-term reconstituted mice (Fig. 4). Bone marrow cells were obtained from animals at 16 weeks posttransplantation and plated (3 x 10^4/plate) in methylcellulose. Colonies were counted after 12 days of culture. As expected, the number of CFU-GEMM derived from reconstituted and wild-type mice was similar (data not shown). Interestingly, the GFP-positive colonies were not sectored into expressing and nonexpressing cells (Fig. 4); the colonies were either completely positive or negative. In transgenic mice that express the *lacZ* gene specifically in erythroid cells, early erythroid colonies are sectored into LacZ expressing and nonexpressing cells [18]. This result in transgenic mice suggested that silencing of the transgene occurred during erythroid differentiation. The colonies derived from mice that were transduced with the lentiviral vector were not sectored; therefore, silencing of the transduced gene apparently does not occur during lineage specification. However, silencing may occur in stem cells or early progenitors. PCR analysis demonstrated that 40% of CFU-GEMM contained GFP DNA (data not shown); however, only

Figure 4. GFP expression in CFU-GEMM colonies. Bone marrow cells (3 x 10^5 per 35-mm plate) from fully reconstituted recipient mice at 16 weeks (mouse #TP 54) or 20 weeks (mouse #TP 62) posttransplantation were plated in methylcellulose and cultured for 12 days at 37°C. CFU-GEMM colonies were scored and examined by fluorescence microscopy. TP 54 and TP 62 each received 1,000 stem cells that were transduced at an MOI of 1,000 and 300, respectively. The percentages of GFP-positive CFU-GEMM colonies from the two recipients are shown. GFP-positive mononuclear cells from peripheral blood (PBL) and bone marrow (BM) of the same mice are also shown for comparison. Colonies were either fully positive or negative; no sectoring was observed. This result suggests that silencing of the transduced gene does not occur during lineage specification. However, some silencing may occur in early progenitors. The percentage of GFP-positive CFU-GEMM was approximately 2 times higher than the percentage of GFP-positive mononuclear cells in peripheral blood and bone marrow. Nevertheless, a high percentage of GFP positive cells persists for 16 and 20 weeks in fully reconstituted mice.

	Bright field	Fluorescence	% GFP+ CFU-GEMM	% GFP+ PBL MNC	% GFP+ BM MNC
TP54-1			17.6	8.0	6.5
TP62-2			22	11.2	10.5

20% of these colonies and 10% of peripheral blood cells expressed GFP. These data suggest that silencing occurs at two separate stages after transduction. One-half of all vector integration sites are silenced in stem cells soon after transduction. Subsequently, one-half of the remaining sites are silenced in early progenitors. Nevertheless, a high percentage (10%) of GFP-positive cells persist in bone marrow and peripheral blood for 20 weeks posttransplantation in fully reconstituted mice and for at least 12 weeks in secondary recipients.

DISCUSSION

We have demonstrated that highly purified murine bone marrow stem cells are efficiently transduced with a novel lentiviral vector and that the normal pluripotency of these cells is preserved in fully reconstituted, lethally irradiated mice. Although efficient transduction of human CD34+ cells has been reported previously [12], this is the first report to our knowledge of stable transduction of murine hematopoietic stem cells with a lentiviral vector. In the system reported here, normal hematopoiesis is maintained after transplantation; therefore, we can conclude that stable lentiviral transduction does not alter normal cell lineage specification. The CD34+ cells transduced previously were transplanted into NOD/SCID mice in which normal hematopoiesis is not maintained; therefore, it is difficult to determine whether normal lineage specification is altered by lentiviral transduction.

Interestingly, high MOIs were required for efficient transduction in our experiments. At an MOI of 5, few cells were transduced. An MOI of 50 was required for significant transduction and an MOI of 1,000 increased the efficiency of transduction two-

fold. High MOIs were not required to transduce human CD34+ in the experiments cited above [12]. The high MOIs required in the present studies may result from the use of highly purified stem cells that were transduced without cytokine stimulation. We used unstimulated stem cells in an attempt to preserve pluripotentiality, and these undifferentiated cells may express fewer receptors for the VSV-G glycoprotein.

Persistent expression of virally transduced genes has been problematic for many viral vectors. Our previous studies have demonstrated that histone deacetylation and chromatin condensation are involved in the silencing of virally transduced genes [8,9]. In several cases expression of transduced genes was completely extingished. In the present studies stable expression was established in 10% of hematopoietic stem cells. Apparently, lentiviral vectors are not as sensitive to silencing as AAV and Moloney-based retroviral vectors. However, some silencing does occur. Analysis of bone marrow obtained from long-term reconstituted mice demonstrates that 40% of GEMM colonies contain the transduced gene, but the gene is expressed in only 10% of peripheral blood mononuclear cells. Similar results were observed when human CD34+ cells were transduced with a lentiviral vector and transplanted into NOD/SCID mice [12]. We are presently determining whether genes that are transduced by lentiviral vectors can be reactivated by histone deacetylase inhibitors in vivo.

Efficient gene delivery into murine hematopoietic stem cells will provide a powerful tool for genetic correction of thalassemia and sickle cell disease in mouse models [14, 19-21] and will provide a foundation for similar protocols in man. The life span of sickle and thalassemic red blood cells is significantly shorter than normal; therefore, we speculate that correction of approximately 10% of erythroid precursors in the marrow

may translate into a major fraction in the peripheral blood. If silenced, lentivirally transduced genes can be reactivated by deacetylase inhibitors, a combination of lentiviral transduction and drug treatment may result in high-level, therapeutic gene expression and thus provide a powerful treatment for hereditary blood diseases.

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Dr. T.T. holds equity in Erythrogen, Inc. and Drs. *J.K.* and *X.W.* hold equity in Transzyme, Inc.

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CORRECTION OF A MOUSE MODEL OF SICKLE CELL DISEASE: LENTIVIRAL/ANTI-SICKLING β-GLOBIN GENE TRANSDUCTION OF UNMOBILIZED, PURIFIED HEMATOPOIETIC STEM CELLS

by

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ABSTRACT

Although sickle cell anemia was the first hereditary disease to be understood at the molecular level, there is still no adequate long-term treatment. Allogeneic bone marrow transplantation is the only available cure, but this procedure is limited to a minority of patients with an available, histocompatible donor. Autologous transplantation of bone marrow stem cells that are transduced with a stably expressed, anti-sickling globin gene would benefit a majority of patients with sickle cell disease (SCD). Therefore, the development of a gene therapy protocol that corrects the disease in an animal model and is directly translatable to human patients is critical. A method is described in which unmobilized, highly purified bone marrow stem cells are transduced with a minimum amount of self-inactivating (SIN) lentiviral vector containing a potent anti-sickling β-globin gene. These cells, which were transduced in the absence of cytokine stimulation, fully reconstitute irradiated recipients and correct the hemolytic anemia and organ pathology that characterize the disease in humans. The mean increase of hemoglobin was 4.6 g/dL, and the average lentiviral copy number was 2.2; therefore, a 2.1 g/dL/vector copy increase was achieved. This transduction protocol may be directly translatable to human sickle cell patients who cannot tolerate current bone marrow mobilization procedures and may not be safely exposed to large viral loads.

INTRODUCTION

SCD is an autosomal recessive disorder that affects over 300,000 individuals worldwide and over 70,000 in the United States. The molecular basis of the disease is an A to T transversion in the sixth codon of the β-globin gene [1, 2]. This mutation results

in the substitution of a valine residue for glutamic acid on the surface of sickle hemoglobin (HbS, $\alpha_2\beta^S_2$) tetramers. The valine creates a hydrophobic projection that fits into a natural hydrophobic pocket formed on Hb tetramers after deoxygenation [3, 4]. The interaction of tetramers results in the formation of HbS polymers/fibers that cause red blood cells to become rigid and nondeformable and to occlude small capillaries [5-10]. These vasoocclusive events cause severe tissue damage that can result in strokes, splenic infarction, kidney failure, liver and lung disorders, painful crises, and other complications [11, 12]. Cycles of erythrocyte sickling also cause the cells to become fragile, and lysis produces chronic anemia. Although sickle cell anemia was the first hereditary disease to be understood at the molecular level [1, 2], there is still no adequate long-term treatment or cure. Current measures focus on induction of fetal hemoglobin to inhibit polymer formation, treatment and prevention of infections, palliative measures to control pain, and surgical treatment of complications [12]. Allogeneic bone marrow transplantation is the only available cure, but this procedure is limited to a minority of patients with an available, histocompatible donor [13].

Autologous transplantation of bone marrow stem cells that are transduced with a stably expressed, anti-sickling globin gene would benefit a majority of patients with SCD. Recently, several groups have utilized lentiviral vectors for globin gene transfer to bone marrow mononuclear cells, and this work has resulted in the correction of sickle cell anemia and β-thalassemia in relevant mouse models of these human diseases [14-17]. Although these studies represent major advances in gene therapy for hemoglobinopathies, the transduction protocols that were used cannot be directly translated to human patients. In all of the cases referenced above, donor bone marrow cells were mobilized by treating

the mice with 5-Fluorouracil. The equivalent treatment in humans is mobilization of hematopoietic stem cells with G-CSF (granulocyte-colony stimulating factor) and/or SCF (Stem Cell Factor). This treatment is effective for mobilizing bone marrow hematopoietic stem cells (HSCs) into peripheral blood and these HSCs successfully engraft in recipients [13]. Unfortunately, G-CSF cannot be used safely in patients with sickle cell disease. *Abboud et al.* [18] have described severe sickle cell crisis in a patient receiving G-CSF, and *Adler et al.* [19] have reported the death of a sickle cell patient whose bone marrow was mobilized with G-CSF. This latter patient was 47 years old and had no previous history of sickle cell crisis. Stem cell factor, which has also been used to mobilize bone marrow, is associated with anaphylaxis and other serious adverse effects which preclude its use in a transplantation protocol for sickle cell patients [20].

Another difficulty with the transduction protocol utilized in recent gene therapy studies for hemoglobinopathies is the ex vivo stimulation of hematopoietic stem cells with cytokines. This treatment is included in the protocols to increase the number of stem cells that are actively progressing through the cell cycle and, therefore, to increase the efficiency of retroviral or lentiviral transduction. Unfortunately, cytokine stimulation of HSCs and premature exit from quiescence can decrease self-renewing capacity, homing efficiency, and, therefore, long-term engraftment in mice and in humans [21-25]. Although retroviral transduction requires cell division [26, 27], efficient lentiviral transduction can occur in quiescent cells [28-31]. We recently demonstrated that unmobilized HSCs derived from murine bone marrow are efficiently transduced with a lentiviral vector in the absence of cytokine stimulation and that the transduced gene is expressed in all hematopoietic cell lineages examined for at least 5 months after transplantation into

lethally irradiated recipients [32]. These results suggest that quiescent, unmobilized hematopoietic stem cells are efficiently transduced by lentiviral vectors without impairing self-renewal and normal lineage specification in vivo. Therefore, the protocol that we have adopted for our globin gene transfer studies described below does not include cytokine stimulation.

A final problem with the lentiviral transduction protocols used previously is the large amount of virus utilized. Several million bone marrow mononuclear cells are transduced at a multiplicity of infection (MOI) of 60 or more and transplanted into recipients; therefore, each animal is exposed to more than 1 x 10^8 lentiviral particles [15, 16]. Replication defective lentiviral vectors appear to be safe in animal models; however, clinical trials in humans will most likely be approved only for protocols utilizing minimal amounts of virus. Therefore, we have developed a protocol that exposes the animals to at least 3,000 times less lentivirus than previous protocols by transducing highly purified HSCs (Sca-1^+c-$Kit^+Lin^{-/low}$) at an MOI of approximately 30.

MATERIALS AND METHODS

Anti-sickling Lentiviral Vector Design and Production.

A 2.3 kb recombinant human β^{AS3} gene (Ryan, Levasseur, McCune, Reilly, Asakura, and Townes, unpublished) and 3.4 kb of human β-globin Locus Control Region (LCR) sequences were subcloned into pWPT-GFP (a kind gift of Didier Trono) replacing the elongation factor-1 alpha promoter and GFP. This self-inactivating (SIN) vector contains a deletion in the U3 region of the 3' long terminal repeat (LTR) from nucleotide 418 to nucleotide 18 that inhibits all transcription from the LTR [33]. The β^{AS3}-globin gene

contains 266 bp of promoter, the 260 bp PstI 3' globin enhancer [34], and a 375 bp RsaI fragment deletion of IVS2 [35]. DNase I Hypersensitive Site (HS) fragments, 5' HS4, 3, and 2 were amplified by PCR from a 22 k fragment of the LCR [36,37]. Nucleotide co-ordinates from Genbank accession number U01317 are HS4 592-1545, HS3 3939-5151, and HS2 8013-9215. The entire HS4, 3, 2 β^{AS3}-globin gene construct was verified by sequencing. Vectors were produced by transient transfection into 293T cells as previously described [38] with the following modifications. A total of 2.5 x 10^6 293T cells were seeded in 10 cm-diameter dishes containing Dulbecco's minimal essential medium with 10% fetal bovine serum (FBS) 24 h prior to transfection. Forty micrograms of plasmid DNA was used for transfection of one 10-cm dish. The DNA cocktail contained 5 μg of envelope-coding plasmid pMD.G, 15 μg of the packaging plasmid pCMVΔR8.91 (which expresses Gag, Pol, Tat, and Rev), and 20 μg of SIN transfer vector plasmid. Transfection medium was removed after 14 to 16 h and replaced with Dulbecco's minimal essential medium/F12 without phenol red (Invitrogen, Carlsbad, CA) containing 2% FBS. Viral containing supernatant was collected after an additional 24 h, cleared by low-speed centrifugation and filtered through a low protein binding 0.22-μm polyethersulfone filter (Millipore, Bedford, MA). The virus was concentrated 1,000-fold by one round of centrifugation at 26,000 RPM for 90 min at 8°C using a SW-28 rotor (Beckman, Palo Alto, CA), resuspended into serum-free stem cell growth factor medium (Cellgenix, Freiburg, Germany), and allowed to incubate on ice for 2 h before storage at -80°C. Virus titer was determined by infecting murine erythroleukemia (MEL) cells, plating individual cells into wells of a 96-well plate, and assaying DNA from the cultures by PCR for the human β^{AS3}-globin gene.

Murine Bone Marrow Cell Preparation and Stem Cell Sorting

Hematopoietic stem cells from our SCD mice [39] were obtained as previously described with minor modifications [32]. Briefly, bone marrow cells were flushed from the femurs and tibias of SCD mice and stained with a biotinylated Sca-1 antibody (clone E13-161.7; BD-Biosciences, San Diego, CA) followed by an anti-biotin magnetic bead (Miltenyi Biotec, Auburn, CA) and fluorescein isothiocyanate-conjugated streptavidin (Caltag, Burlingame, CA). Sca-1 cells were then isolated on a MACS cell sorter (Miltenyi Biotec,). The Sca-1 enriched cells were then stained with phycoerythrin-conjugated antibodies directed against lineage antibodies CD3, 145-2C11; CD4, RM4-5; CD5, 53-7.3; CD8, 53-6.7; CD11b (Mac-1), M1/70; Gr-1, RB6-8C5; CD45RA, RA3-6B2; and TER119/Ly-76, TER119 (BD-Biosciences) followed by staining with allophycocyanin-conjugated c-Kit (clone 2B8; BD-Biosciences). Cells were washed once and resuspended in Iscove's modified Dulbecco's medium (IMDM) medium containing 2% FBS for sorting on a FACSVantage SE (BD-Biosciences) flow cytometer. Sca-1^{+}c-$Kit^{+}Lin^{-/low}$ cells were collected into IMDM containing 1% FBS for transduction. Purification of bone marrow $Ter119^{+}$ and $CD11b/Mac\text{-}1^{+}$ and $Gr1^{+}$ cells from secondary transplants was accomplished by staining bone marrow mononuclear cells with phycoerythrin-conjugated antibodies directed against Ter119 or against CD11b/Mac-1 and Gr1 and sorting on a FACSVantage SE (BD-Biosciences) flow cytometer. DNA was obtained from approximately 1×10^{5} purified cells using the Qiagen (Valencia, CA) DNeasy kit.

Congenic Knockout-Transgenic Sickle Mice and Hematopoietic Stem Cell Preparation

Congenic knockout-transgenic (KO-TG) SCD mice were generated from Line HbS5 [39] by backcrossing mouse α-globin knockout [40], mouse β-globin knockout [41], and LCRα/LCR$^{A}\gamma\beta^{s}$ transgenic [39] mice to the C57Bl/6 (B6) strain in parallel. After eight successive backcross generations, these three B6 congenic lines were inter-bred to generate KO-TG-B6 SCD mice that synthesize solely human hemoglobin in their adult red blood cells. These backcrosses were performed to inhibit graft versus host reactions in long-term reconstituted recipients. The animals are extremely anemic on the C57Bl/6 background; hemoglobin levels are as low as 2.3 gms/dL. We backcrossed both the HbS5 and HbS3 lines that we originally reported [39]. The HbS3 line, which has balanced α- and β-globin chains, was so severely anemic on the C57Bl/6 background that they could not be used for these experiments. The HbS5 line is slightly β-thalassemic, similar to the BERK mice [42], and survives longer on the C57Bl6/ background. Therefore, we utilized this line for these studies.

Stem Cell Transduction and Transplantation

Sorted Sca-1^{+}c-Kit^{+}Lin$^{-/low}$ stem cells were resuspended in IMDM medium containing 10 μg/ml dextran sulfate and 1% FBS. One thousand stem cells were infected at an MOI of 30 in a total volume of 100 μl at 37°C in 5% CO_2 for 4 h. Mock or lenti/β^{AS3} transduced stem cells were injected into the retro-orbital sinus of anesthetized eight week old C57Bl/6 mice that were lethally irradiated (1325 cGy administered in a split dose). Hematopoietic reconstitution with donor HSCs from the sickle mice was monitored by analyzing the replacement of murine hemoglobin polypeptides with human hemoglobin

polypeptides using high performance liquid chromatography (HPLC) as described previously [39]. Secondary transplantations were performed with one million unfractionated mononuclear cells isolated from primary transplants. The cells were injected into lethally irradiated C57Bl/6 mice, and hemoglobins were analyzed at 4 months posttransplantation.

Lenti/β^{AS3} Vector Copy Number Determination

The lenti/β^{AS3} vector copy number in transplanted mice was determined by an allele specific assay. Transplant bone marrow DNA was obtained using the Qiagen DNeasy kit. Bone marrow DNA was amplified by PCR with primers that anneal to identical sequences in the human β^S and β^{AS3}-globin genes. These primers do not amplify mouse β-globin. The sequence of the forward and reverse primers are 5'-ACATTTGCTTCTGACACAACT-3' and 5'-GGGAAAATAGACCAATAGGC-3', respectively. Twenty-five cycles of PCR were performed with unlabeled primers followed by one cycle of primer extension with 5' [^{32}P]-labeled reverse primer. Twenty five cycles of PCR were within the linear range of amplification. The resulting 264 bp amplicon was digested with Bsu36I that only cuts the β^{AS3}-globin gene, producing a 194 bp restriction fragment. After complete digestion, the resulting radiolabeled fragments were separated on a 2% TBE agarose gel. The copy number of the human β^S allele was determined by the same method as for lenti/β^{AS3} except that β^S-globin was compared to mouse β-globin in sickle animals that were heterozygous for the mouse β-globin knockout. Forward and reverse primers complementary to identical sequences in the mouse and human β-globin genes were 5'- ATGAAGTTGGTGGTGAGC-3' and 5'-

AGACTCACCCTGAAGTTCTC-3', respectively. Twenty-seven cycles of PCR were performed with unlabeled primers followed by one cycle of primer extension with 5' [^{32}P]-labeled forward primer. Twenty-seven cycles of PCR were within the linear range of amplification. The resulting 389 bp amplicon was digested with SacI that only cuts the mouse β-globin gene, producing a 337 bp restriction fragment. Using this allele-specific PCR methodology to analyze transgene ($LCR^{A}\gamma\beta^{s}$) copy number, we determined that the mice contain three copies of the human β^{S} gene. DNA bands were quantified with a Molecular Dynamics Storm Phosphorimager (Amersham Biosciences, Piscataway, NJ) and the ratio of lenti/β^{AS3} to $LCR^{A}\gamma\beta^{s}$ was determined by IMAGEQUANT software (Amersham Biosciences, Piscataway, NJ).

Globin Protein Analysis

Although the β^{AS3} polypeptide differs from β^{S} by four amino acids, we were unable to discriminate the two species using HPLC. Therefore, hemoglobins were separated by isoelectric focusing (IEF) of hemolysates as described previously [43, 44]. Briefly, IEF was performed using the Isothermal Controlled Electrophoresis system (Fisher Scientific, Pittsburgh, PA) with precast agarose IEF gels (Isolab, Akron, OH). Hemoglobin bands were quantitated by densitometry with a BioRad (Hercules, CA) GS-800 scanner using Quantity One software. HbS and HbAS3 bands focused at the same isoelectric point as HbS and HbAS3 purified from KO-TG mice that synthesize these hemoglobins exclusively. HbAS3 bands were also confirmed by tryptic digestion and analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Single Cell Expression Level Analysis

Analysis of mRNA levels was performed on peripheral blood reticulocytes isolated from animals transplanted with the lenti-β^{AS3} vector. Reticulocytes were stained with Thiazole Orange [45], purified on a FACScan flow cytometer (BD-Biosciences), and plated after limit dilution at one cell per three wells in a 96-well plate. Plates were stored at -80°C for 1 h, and cells were thawed into 2.5 µl lysis buffer (1.6% IGEPAL/NP-40; Sigma, St. Louis, MO). Reverse transcription (RT) was performed with Superscript II RT (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Two microliters of the RT reaction was used to perform PCR with ExTaq (Panvera-TaKaRa, Madison, WI). As described above for genomic DNA PCR, the primers used for RT-PCR anneal to sequences that are identical in the human β^S and human β^{AS3} globin genes. The forward primer sequence was 5'-ACATTTGCTTCTGACACAACT-3' (5' untranslated region), and the reverse primer was 5'-CCATAACAGCATCAGGAGTG-3' (2nd exon). Thirty-five cycles of PCR were performed with unlabeled primers, and then one cycle of primer extension was performed with 5' γ-[^{32}P]-labeled reverse primer. This cycle number was within the linear range of amplification. The resulting 222 bp PCR product was digested with Bsu36I that cuts β^{AS3} and yields a 151 bp fragment. After complete digestion, the resulting radiolabeled fragments were separated on a 8% acrylamide gel

Hematological Indices and Histopathology

Blood was collected from anesthetized animals into Microtainer EDTA collection tubes. RBC count was measured on a HemaVet 1500 (CDC Technology, Oxford, CT)

hematology analyzer. Hemoglobin concentration was determined spectrophotometrically after conversion to cyanmethemoglobin with Drabkin's reagent (Sigma). Before determining the hemoglobin concentration, red cell membranes were pelleted at 14000 RPM for 5' in an Eppendorf centrifuge. Pelleting the membranes inhibits artifactually high values caused by membrane-bound, denatured hemoglobin. Packed Cell Volume was measured with a JorVet J503 (Jorgenson Laboratories Systems, Loveland, CO) micro-hematocrit centrifuge. Reticulocyte count was determined by flow cytometry after staining with Thiazole Orange. Urine osmolality was measured with the Wescor Vapro Vapor Pressure Osmometer 5520 (Logan, UT) after food and water were withheld from the mice for 4 h. Tissues were fixed in 70% alcoholic formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin or Gomori iron stain by standard methods.

RESULTS

Insertion of Potent Anti-Sickling Gene into a Lentiviral Vector

We previously demonstrated that substitution of an alanine at position 22 and glutamine at position 87 of the human β-globin polypeptide (β^{AS2}) significantly inhibits HbS polymerization [46]. We have now introduced an additional modification that increases the affinity of β-globin subunits for α-globin polypeptides and results in a molecule that impedes polymer formation with greater efficiency than fetal hemoglobin (HbF) (Ryan, Levasseur, McCune, Reilly, Asakura, and Townes, unpublished data). This anti-sickling gene, β^{AS3}-globin, was inserted into a SIN lentiviral vector [33] to form the construct designated lenti/β^{AS3} (Fig. 1B). The selection of β-globin promoter, enhancer and LCR elements was based on our previous studies analyzing the expression of human β-

globin genes in transgenic mice [34, 36, 47, 48] and unpublished data. Lenti/β^{AS3} contains the central polypurine tract/DNA flap for increased packaging efficiency, viral titer, and transduction efficiency [49,50] and the woodchuck hepatitis virus posttranscriptional egulatory lement (WPRE) that increases the efficiency of RNA processing and contributes to the production of full-length viral transcripts [51]. Lenti/β^{AS3} was pseudotyped with the vesicular stomatitis virus G (VSV-G) glycoprotein and concentrated to a final titer of 1 x 10^8 infectious units/ml as described in the **Materials and Methods** section.

Transduction and Transplantation of Sca-1$^+$c-Kit$^+$Lin$^-$ SCD Hematopoietic Stem Cells

As described in the introduction, mobilization of bone marrow stem cells with cytokines such as G-CSF and SCF can not be safely performed in sickle cell patients. Therefore, we utilized a transduction protocol that does not include mobilization [32]. Bone marrow was isolated from untreated KO-TG-B6 SCD (sickle) mice and HSCs that were positive for Stem Cell Antigen (Sca-1$^+$) and c-Kit$^+$, and negative (or low) for hematopoietic lineage antigens (lin$^{-/low}$) were isolated by preparative flow cytometry (Fig. 2) [52, 53]. Without cytokine pre-stimulation, 1000 Sca-1$^+$, c-Kit$^+$, lin$^{-/low}$ (KLS) stem cells were transduced for 4 h with lenti/β^{AS3} at a MOI (multiplicity of infection) of 30. This relatively low amount of virus was used to minimize exposure of the animals to lentivirus and to reduce the possibility of insertional mutagenesis. Transduced HSCs were transplanted into lethally irradiated (1,325 cGy), wild-type C57Bl/6 recipients, and reconstitution was quantified by HPLC analysis of hemoglobins (see supplementary material). Animals that expressed greater than 99% human hemoglobin 12 weeks post- transplantation were identified and retained for long-term analysis. This high level of

Figure 1. Insertion of a potent anti-sickling human β-globin gene into a lentiviral vector. (A) Schematic of the human β-globin LCR and downstream genes. HS cores are indicated by arrows. Genes are illustrated by filled boxes and are represented according to scale. (B) The lentiviral/anti-sickling beta globin expression construct is shown. HS2 (1203 bp), HS3 (1213 bp), and HS4 (954 bp) sequences, the 3' globin enhancer, the 266 bp beta globin promoter (βp), and the β^{AS3} globin gene are drawn to scale. The HIV-1 LTR is shown with a 3' SIN deletion; ψ, packaging signal; SD and SA, splice donor and acceptor sites; RRE, Rev-responsive element; cPPT/CTS, central polypurine tract or DNA flap/central termination sequence; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (C) Lentiviral vector copy number determination. Lane 1 is the PCR product obtained from bone marrow of a KO-TG sickle mouse after amplification, labeled primer extension, and Bsu36I digestion; lanes 2, 3, and 4 are products of representative lenti/β^{AS3} transduced mice; lane 5 is a knockout-transgenic animal expressing exclusively human HbAS3.

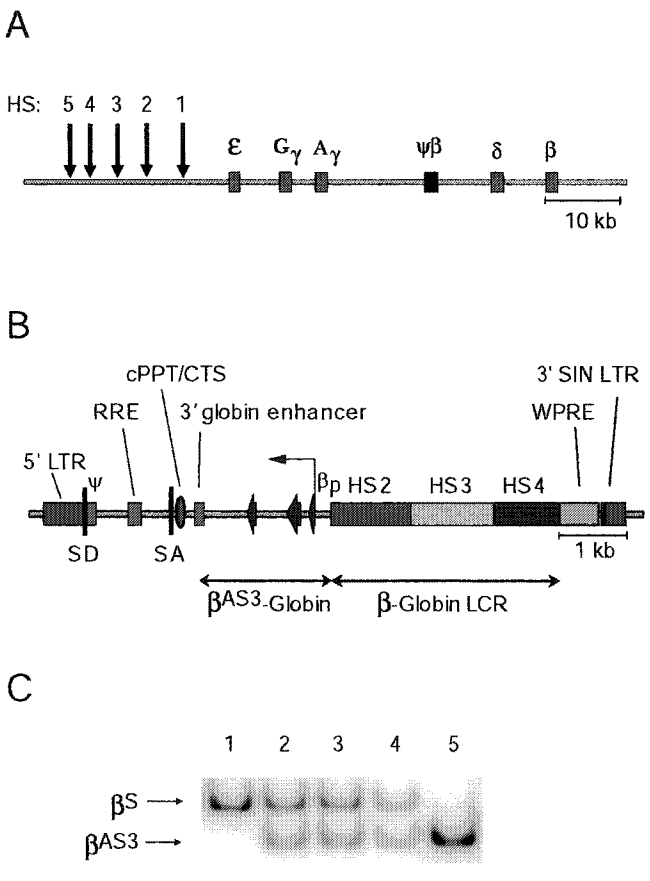
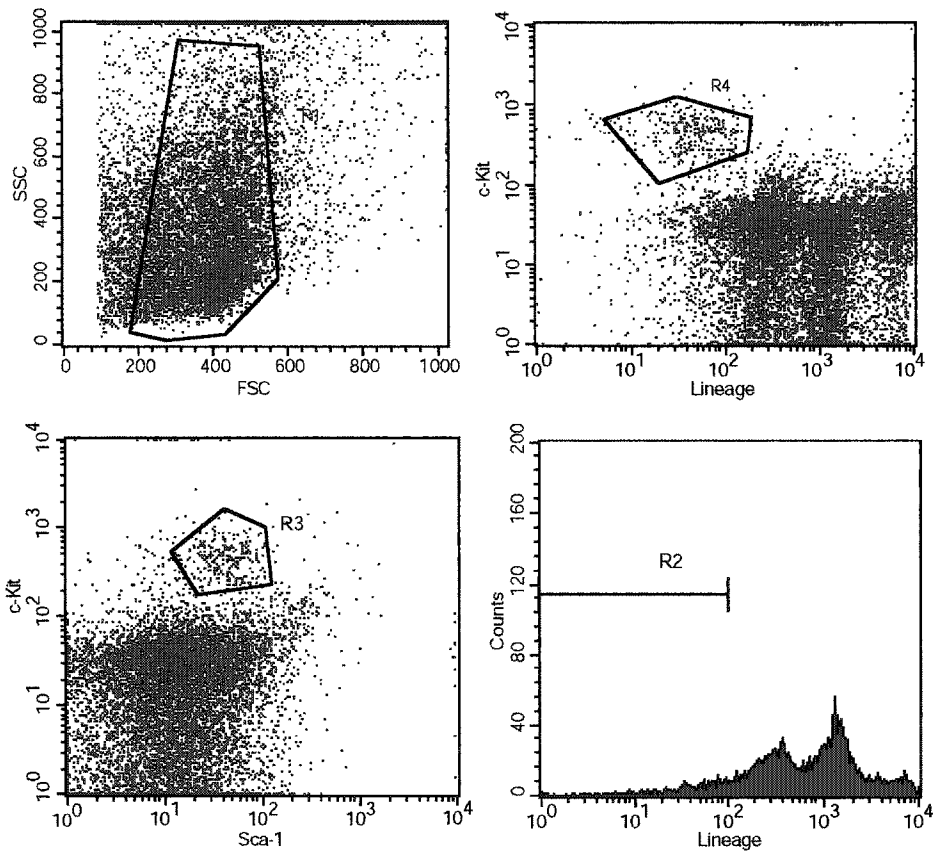


Figure 2. Isolation of highly purified Sca-1^{+}c-Kit^{+}Lin^{-} hematopoietic stem cells from sickle mice. Whole bone marrow was sorted on a magnetic-activated cell sorting column to obtain Sca-1 enriched cells. Three-color preparative FACS was then performed to isolate Sca-1^{+}c-Kit^{+}Lin$^{lo/-}$ hematopoietic stem cells. Purified Sca-1^{+}, c-Kit^{+}, Lin$^{lo/-}$ cells satisfy gating requirements for R1 (upper left panel), R2 (lower right), and R3 (lower left).



donor cell engraftment precludes artifactual amelioration of anemia resulting from the presence of residual murine red blood cells.

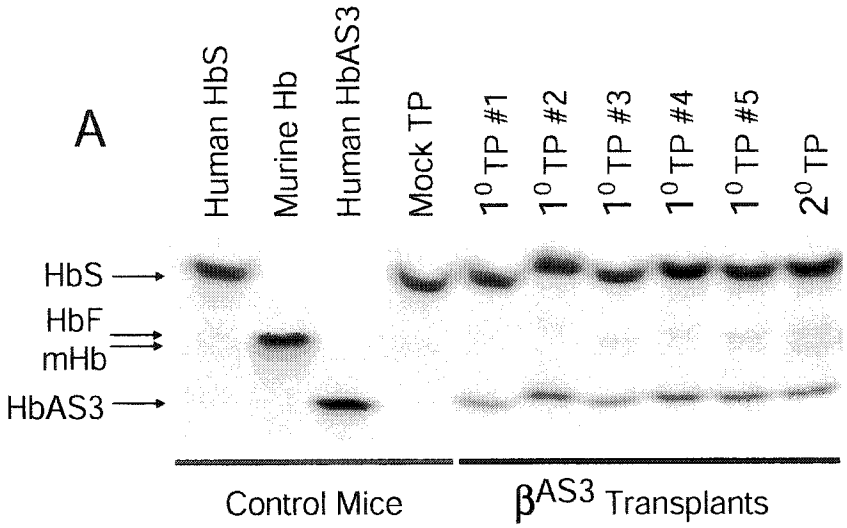
Vector copy number was determined by allele-specific PCR of bone marrow DNA. Lenti/β^{AS3} was present at 2.2 ± 0.3 (SE) proviral units per transduced cell (Fig. 1C).

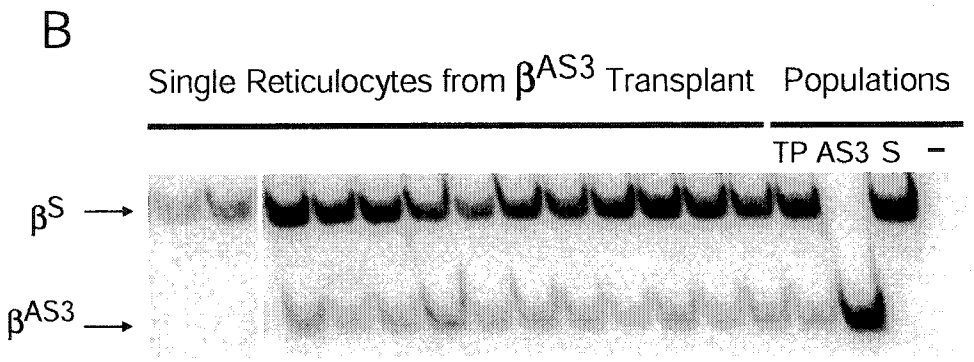
Therapeutic Expression Levels of Human β^{AS3} in Murine Erythrocytes Following Lentiviral Gene Transfer into Purified Hematopoietic Stem Cells

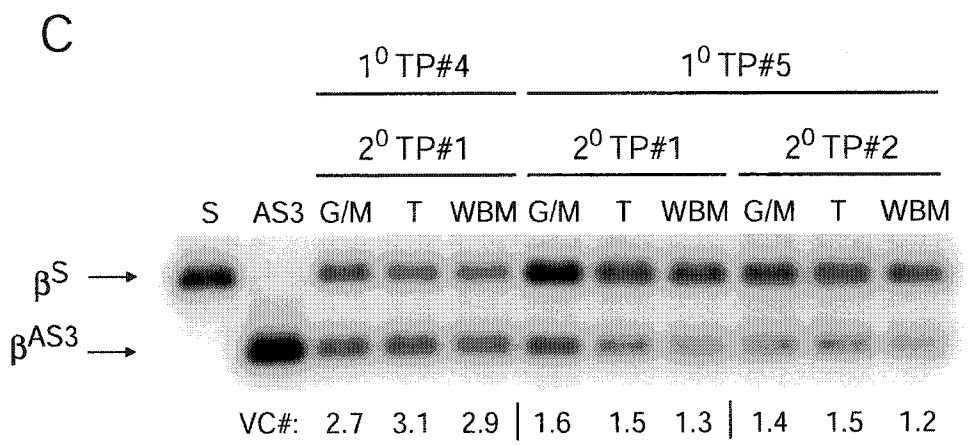
At 5 to 7 months posttransplant, peripheral blood was collected from lenti/β^{AS3} and mock-transduced animals. Hemolysates were prepared and hemoglobins (HbS and HbAS3) were separated by IEF. All of the fully reconstituted animals expressed 20-25% HbAS3 and 75-80% HbS (Fig. 3A). Bone marrow was isolated from several of these animals and transplanted into lethally irradiated secondary recipients. The last lane of Figure 3A (TP2°) is a hemolysate from a representative secondary recipient at 7 months posttransplantation; HbAS3 is 20% of total hemoglobin. These results demonstrate that unmobilized, unstimulated HSCs are stably transduced for up to 14 months with lenti/β^{AS3}.

The fraction of erythroid cells that express β^{AS3} was determined by single-cell, allele-specific RT-PCR. Reticulocytes were isolated by FACS, and individual cells were analyzed for β^{AS3} and β^{S} mRNA. The primers for β^{AS3} and β^{S} amplification were identical, and PCR products were distinguished by digestion with Bsu36I which cuts β^{AS3} but not β^{S} amplicons. Figure 3B illustrates the results for several individual reticulocytes and for a population of cells from a representative lenti/β^{AS3} mouse. Eighty-five percent (17/20) of the reticulocytes that were examined contained β^{AS3} mRNA. Individual reticu-

Figure 3. Therapeutic β^{AS3}-globin expression levels in murine erythrocytes following lentiviral gene delivery. (A) IEF of hemolysates from recipient mice at 5 to 7 months posttransplantation. The first four lanes are human HbS, murine Hb, human HbAS3, and mock-transduced controls. The next 5 lanes are five primary lenti/β^{AS3} mice reconstituted with >99% donor cells. The final lane is a representative secondary transplant at 4 months post transplantation. (B) Single cell expression analysis of lenti/β^{AS3} reticulocytes. Following Thiazole Orange staining, reticulocytes were sorted by flow cytometry. mRNA was isolated from individual lysed cells, reverse-transcribed, PCR amplified, radioactively labeled by primer extension, and digested with Bsu36I which digests β^{AS3} but not β^{S}. Data are from 13 representative reticulocytes. Population controls of 100 cells from the same transplant (TP), a HbAS3 knockout-transgenic (AS3), or a donor KO-TG sickle mouse (S) are also shown. (C) Lentiviral vector copy number determination in bone marrow granulocytes/macrophages, erythroid progenitors, and whole bone marrow from secondary recipients at 7 months post-transplantation. G/M is granulocyte/macrophage ($Gr1^+$ and $CD11b/Mac1^+$), T is erythroid progenitor ($Ter119^+$) and WBM is whole bone marrow.







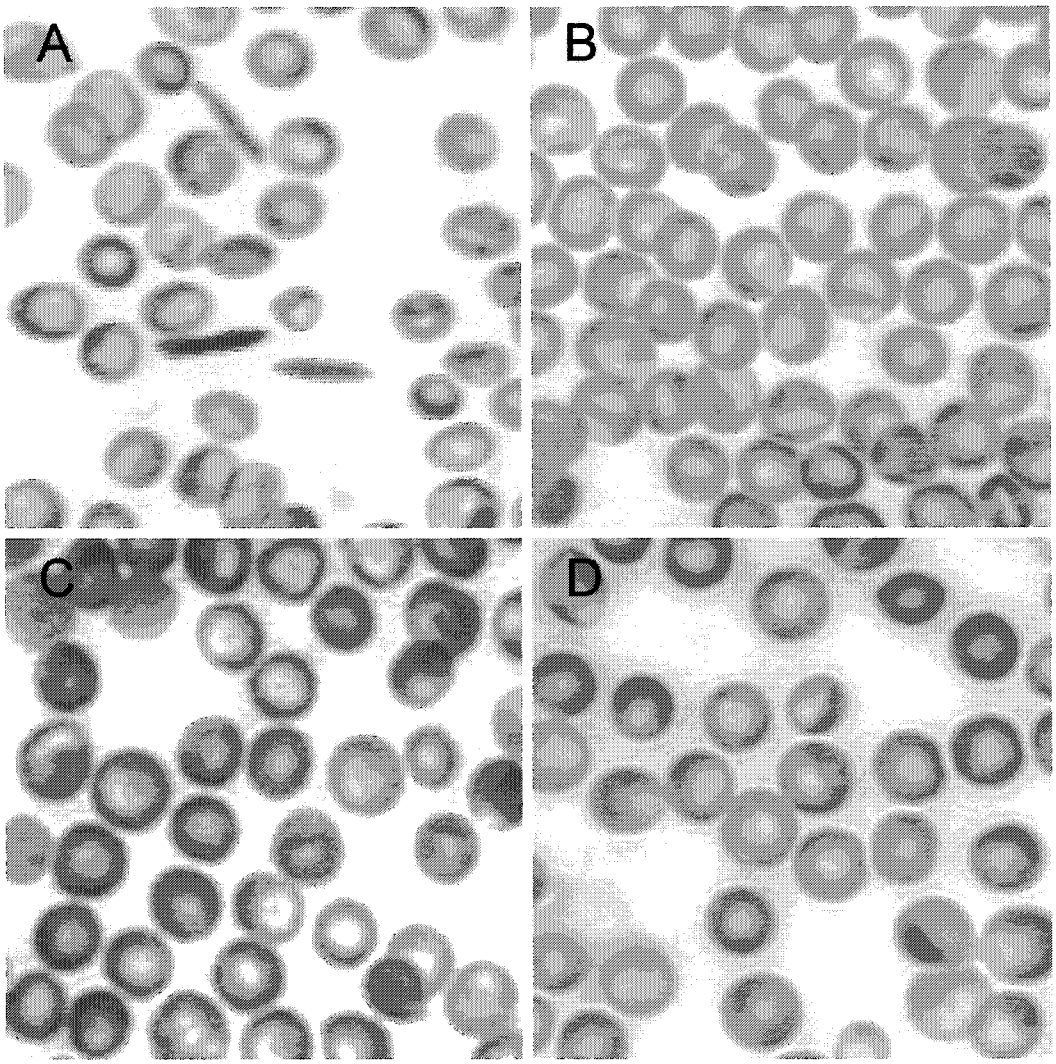
locyte levels of human β^{AS3}-globin mRNA ranged from a low of 10% to a high of 43% of human β^{S}-globin mRNA. The level of β^{AS3}-globin mRNA expression in the entire population was 24%, and this level is consistent with the level of HbAS3 detected in the IEF gel described above.

All secondary recipients expressed between 20-25% HbAS3 7 months post-transplantation (see representative sample in Fig. 3A). Figure 3C demonstrates that FACS purified bone marrow granulocytes/macrophages ($Gr1^{+}/Mac1^{+}$), erythroid progenitors ($Ter119^{+}$) and whole bone marrow from three of these secondary recipients (7 months posttransplantation) contain an average lenti/β^{AS3} copy number of 1.9. Similar vector copy numbers for granulocyte/macrophages, erythroid progenitors, and whole bone marrow in individual recipients suggest that hematopoietic stem cells are transduced and that corrected erythroid progenitors are not selectively amplified in the marrow.

Correction of Abnormal RBC Morphology and Hematological Parameters in Lenti/β^{AS3} Corrected SCD Mice

Figure 4 illustrates blood smears from animals transplanted with mock-transduced and lenti/β^{AS3}-transduced sickle HSCs 5 months posttransplant. Many rigid, elongated cells are observed in the mock-transduced control (Panel A); however, no sickled cells are observed in lenti/β^{AS3} mice (Panels C and D; two representative animals). Panel B is a wild-type control. The blood smears of lenti/β^{AS3} animals also lack the anisocytosis, poikilocytosis, and polychromasia characteristic of the erythrocyte morphology observed in SCD mice and HbSS patients [11, 39]. Table 1 compares hematological indices of control, sickle, mock-transduced, and lenti/β^{AS3}-transduced mice. Lenti/β^{AS3} animals have a marked increase in RBC counts, Hemoglobin levels, and hematocrits and a sig-

Figure 4. Correction of abnormal RBC morphology in lenti/β^{AS3}-rescued transplants. (A) Blood smear of a mock transplanted animal with characteristic sickled erythrocytes and a pronounced reticulocytosis. (B and C) Two representative primary transplant recipients of β^{AS3} transduced stem cells. No sickled cells were observed in any fields examined. (D) Wild-type C57Bl6 control.



nificantly reduced reticulocytosis compared to mock transduced controls. The 4.6 gm/dL rise in hemoglobin level was achieved with a lenti/β^{AS3} copy number of 2.2 proviral units per cell. This increase of 2.1 gm/dL/proviral copy compares favorably with the values reported for correction of another sickle mouse model by *Pawliuk et al.* (1.5 gm/dL/copy) [15] and for correction of a β-thalassemia mouse model by *May et al.* (3.8 gm/dL/copy) [14], *Rivella et al.* (2.3 gm/dL/copy) [54], *Imren et al.* (1.5 gm/dL/copy) [16], and *Persons et al.* (1.1 gm/dL/copy) [17]. The last line of Table 1 demonstrates that secondary recipients at 7 months posttransplantation retain the marked increases in red blood cell counts, hemoglobin levels, and hematocrits and reduced reticulocytosis compared to mock-transduced controls.

Sickled erythrocytes are not observed in blood smears or in tissues of lenti/β^{AS3} mice; however, the animals remain significantly anemic with hemoglobin concentrations of 7.3 gm/dL. This persistent anemia in spite of a 4.6 gm/dL rise in hemoglobin is a consequence of the extremely low baseline hemoglobin concentrations in sickle animals that are bred onto the C57B/6 background (2.7 gm/dL). We do not know the reasons for this strain-dependent, severe anemia, but we are currently examining genes that modify disease severity. A 4.6 gm/dL increase in hemoglobin in human sickle patients would dramatically reduce anemia.

Amelioration of Spleen, Liver and Kidney Pathology and Restoration of Kidney Function in Sickle Mice

Recipients of lenti/β^{AS3}-transduced sickle HSCs developed little spleen, liver, and kidney pathology compared to recipients of mock-transduced cells. Histological sections

Table 1. Correction of hematological pathology and urine concentration defect in mice transplanted with lenti/β^{AS3}-transduced hematopoietic stem cells

Mice	RBCs, $10^6/\mu l$	Hb, g/dL	PCV, %	Reticulocytes, %	MCV, fL	MCH, pg	Urine concentration (mOsm)
C57Bl/6 controls (n = 5)	9.0 ± 0.2	11.9 ± 0.5	39.6 ± 1.0	4.1 ± 0.7	46.1 ± 3.2	15.8 ± 1.3	1879 ± 231
Sickle controls (n = 5)	3.3 ± 0.3	3.0 ± 0.2	18.2 ± 2.2	69.3 ± 8.6	53.7 ± 2.8	12.8 ± 1.1	849 ± 49
Mock transduced (n = 5)	2.4 ± 0.3	2.7 ± 0.4	14.0 ± 1.9	68.3 ± 3.4	54.4 ± 4.6	11.3 ± 1.0	777 ± 104
Lenti/β^{AS3} transduced (n = 5)	6.3 ± 0.2*	7.3 ± 0.7†	30.0 ± 2.3*	19.8 ± 2.8*	46.9 ± 2.3‡	11.5 ± 1.1	1633 ± 183‖
Secondary transplants (n = 5)	6.1 ± 0.2*	7.0 ± 0.3*	26.2 ± 0.9*	16.1 ± 2.0*	44.6 ± 1.4§	11.5 ± 0.1	1922 ± 129*

Sickle controls are KO-TG sickle mice that were crossed for eight generations onto the C57Bl/6 background. Lenti/β^{AS3} primary transplants were analyzed 5 (n = 2) or 7 (n = 3) months posttransplantation. Secondary transplants are derived from primary transplants, number 4 (n = 2) and 5 (n = 3). All secondary transplants were analyzed 7 months posttransplantation. Values represent the mean plus/minus the Standard Error of the Mean (SEM). Statistical significance is measured for primary and secondary transplants compared to mock transduced controls. Significance was determined by a two-tailed Student *t*-test assuming unequal variances. RBCs, red blood cell count; Hb, hemoglobin; PCV, packed cell volume or hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin

*Statistically significant difference with $P < 0.001$.

†Statistically significant difference with $P < 0.003$.

‡Not statistically different with $P = 0.32$.

§Not statistically different with $P = 0.13$.

‖Statistically significant difference with $P < 0.01$

of wild-type, mock-transduced, and lenti/β^{AS3} animals are shown in Figure 5 (panel A.) The spleens of mock-transduced mice are characterized by a massive expansion of red pulp, dramatic pooling of sinusoidal erythrocytes, vaso-occlusion, and a complete loss of lymphoid follicular structure. In lenti/β^{AS3}-transduced mice, normal splenic red and white pulp is observed, and virtually no pools of sickle erythrocytes or infarcts are evident. In addition, splenomegaly is substantially diminished in lenti/β^{AS3} mice (Fig. 5B); lenti/β^{AS3} spleens are approximately 0.7% of total body weight compared to mock-transduced spleens that are almost 2.0% of body weight.

Livers of mock-transduced animals are characterized by focal areas of necrosis and pronounced congestion of the intrahepatic vasculature with aggregates of sickled RBCs. Erythroid progenitors are evident in the sinusoids, and this extramedullary hematopoiesis is indicative of severe anemia. There is also abundant hemosiderin deposition subsequent to Kupffer cell erythrophagocytosis. In lenti/β^{AS3} animals, focal areas of necrosis and aggregation of sickled erythrocytes are not observed; also, extramedullary hematopoiesis and hemosiderin deposition are absent.

In the kidneys of mock-transduced mice, engorgement and occlusion of blood vessels results in vascular, tubular, and glomerular changes. Sequestration and occlusion are most obvious at the corticomedullary junction where dilated capillaries are easily observed in this region of reduced oxygen tension. Reduced medullary blood flow in HbS patients causes extensive tubular damage that results in hyposthenuria, and this same loss of urine concentrating ability is observed in the sickle mice. Kidneys of mock-transduced animals also accumulate abundant hemosiderin in the cortical region, and these aggregates are easily visualized with Gomori iron staining (see bottom panels of Fig. 5A). In

Figure 5. Amelioration of spleen, liver, and kidney pathology in lenti/β^{AS3} mice. (A) Spleen, liver, and kidney sections were analyzed at low (10X for spleen and kidney, 40X for liver; top 3 and bottom rows) and high magnifications (100X). In lenti/β^{AS3}-transduced mice, normal splenic red and white pulp is observed, and virtually no pools of sickle erythrocytes or infarcts are evident. In livers of lenti/β^{AS3} animals, focal areas of necrosis and aggregation of sickled erythrocytes are not observed; also, extramedullary hematopoiesis and hemosiderin deposition are absent. Kidneys of lenti/β^{AS3} mice appear normal and free of the disruptive vascular RBC pooling and hemosiderin deposits observed in mock-treated animals. (B) Correction of splenomegaly in lenti/β^{AS3}-transduced mice.

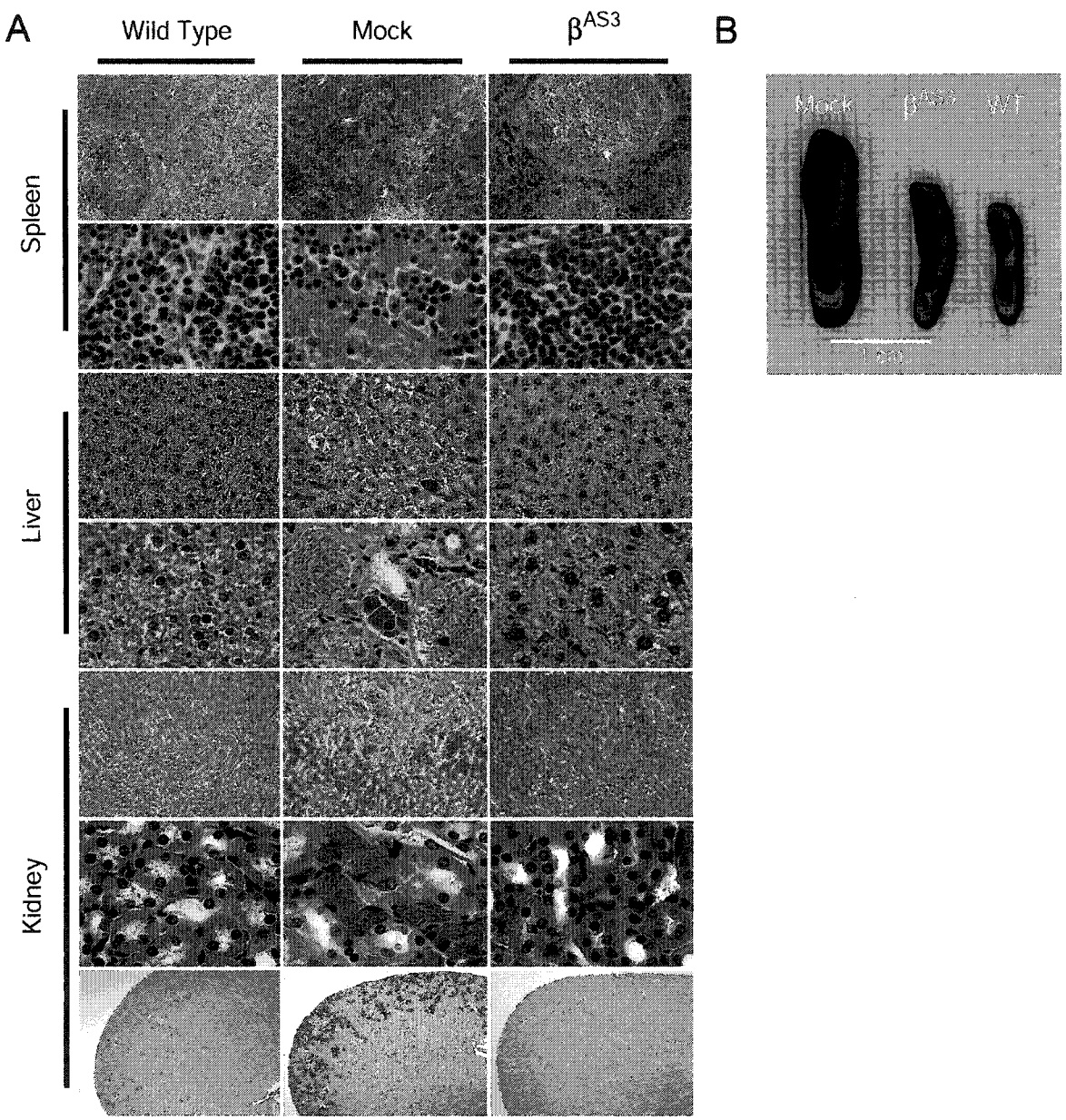
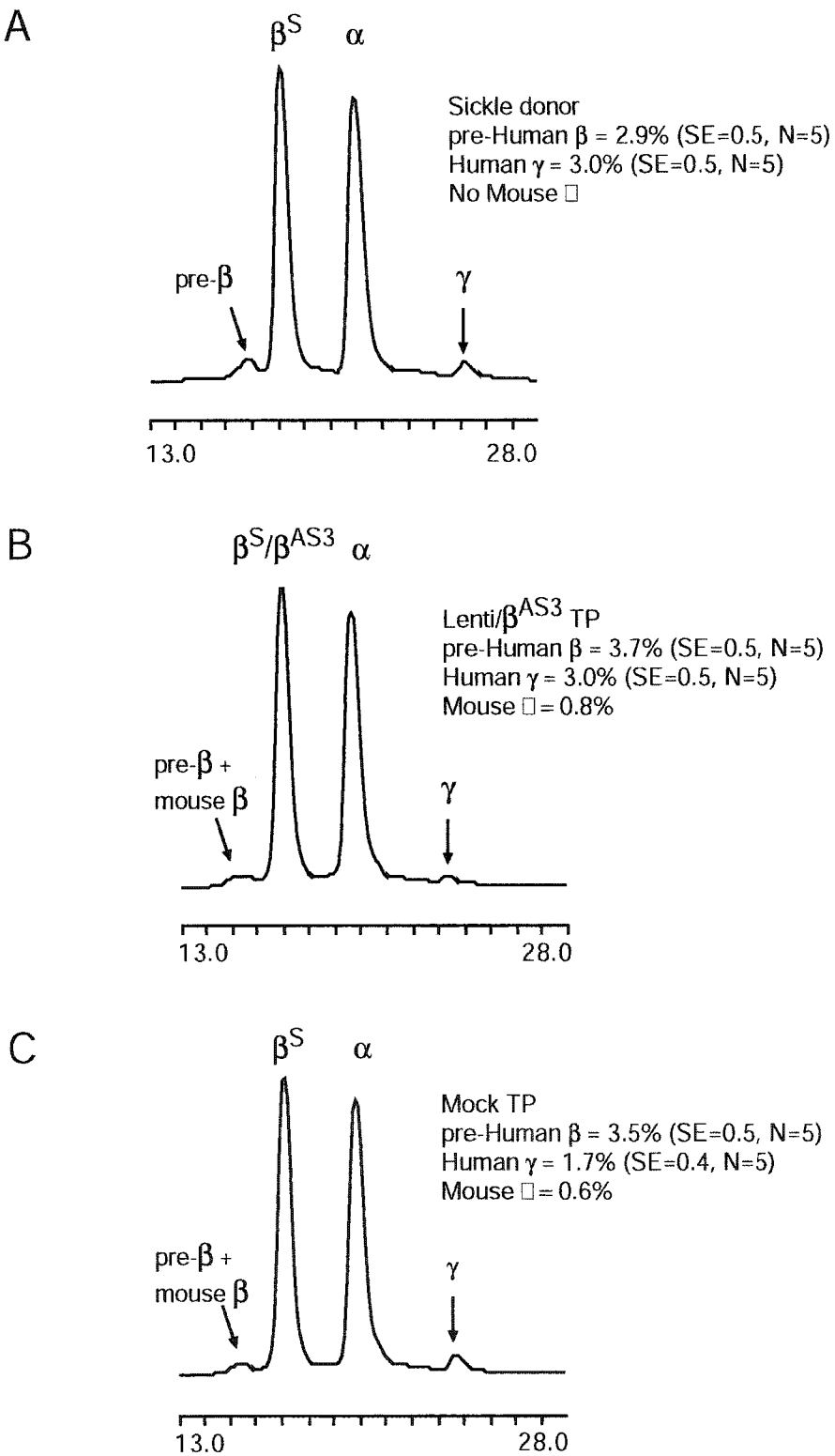


Figure 6. Recipients transplanted with purified hematopoietic stem cells are completely reconstituted with donor hemoglobin. (A) HPLC chromatogram of a sickle donor mouse hemolysate demonstrating exclusively human beta and gamma globin protein. A characteristic human pre-beta peak is also indicated (pre-β). (B) Representative lenti/β^{AS3} transplant with less than 1% mouse beta globin remaining after lethal irradiation and reconstitution with lenti/β^{AS3}-transduced stem cells. (C) Mock-transduced control.



contrast, kidneys of lenti/β^{AS3} mice appear normal and free of the disruptive vascular RBC pooling and hemosiderin deposits observed in mock-treated animals. Most importantly, urine concentrating ability is almost completely restored in lenti/β^{AS3} mice, and this restoration is maintained in secondary recipients at 7 months posttransplantation (Table 1).

DISCUSSION

We have corrected our mouse model of SCD utilizing minimal amounts of a SIN lentiviral-based vector designed to deliver a novel, anti-sickling human beta globin gene into purified hematopoietic stem cells. The correction was accomplished with a transduction protocol that should be translatable to human patients. The protocol does not include mobilization of bone marrow or cytokine stimulation of HSCs. Bone marrow mobilization protocols are not safe for sickle cell patients because rapid increases in leukopoiesis are associated with vascular crises that can result in death [18, 19]. Therefore, we collected bone marrow from animals in the absence of pretreatment with 5-FU or G-CSF. We also utilized quiescent HSCs that were not stimulated with cytokines prior to or during transduction. Most, if not all, cytokine pretreatment protocols induce some measure of HSC differentiation and loss of long-term repopulating activity [21-25]; therefore, autologous and allogenic bone marrow transplantation protocols in humans typically do not utilize cytokine pretreatments [13]. Our previous results with a lenti/CMV-GFP vector demonstrated that efficient lentiviral transduction of purified HSCs does not require cytokine induction of cell cycle progression [32]; therefore, we used the same protocol in the lenti/β^{AS3} studies described in this paper.

At 5 to 7 months posttransplantation, we analyzed lenti/β^{AS3} mice that were reconstituted with greater than 99% donor cells, and all of these animals were corrected; all mock-transduced controls developed severe disease. Secondary lenti/β^{AS3} transplants were also corrected for at least 7 months posttransplantation. These results demonstrate that long-term repopulating cells are stably transduced. Interestingly, 75-85% of reticulocytes in these animals synthesized β^{AS3} mRNA. This nearly pancellular expression was unexpected using a nonmobilizing transduction protocol in the absence of cytokine prestimulation. These results suggest that transduction of relatively quiescent HSCs was efficient with our protocol.

Hematological values of mice transplanted with lenti/β^{AS3}-transduced HSCs were dramatically improved. RBC counts, hematocrits and hemoglobin levels were markedly increased, and reticulocyte numbers were reduced from 69% to 14%. Although some anemia was still evident in these animals, the mice mimicked sickle patients with HPFH (Hereditary Persistence of Fetal Hemoglobin). HPFH patients who synthesize 20% HbF typically have mild disease and lead relatively normal lives.

Tissue pathology was almost completely corrected in the lenti/β^{AS3}-transduced animals. No splenic infarction, liver necrosis, or kidney corticomedullary damage was observed, and urine concentrating ability was restored to normal levels. These results demonstrate that a single dose of lenti/β^{AS3} can correct SCD and that this correction is stable for the lifetime of the animal and in secondary transplant recipients.

Concerns about viral transduction of hematopoietic cells have recently been raised by the development of leukemia in two SCID patients who were treated by gene therapy [55,56]. Insertion of viral vectors near the LMO-2 gene apparently stimulated expression

of this protooncogene that normally regulates crucial steps in hematopoiesis [57]. Activation of oncogenes by enhancer sequences present in the LTRs of retroviruses has been reported in the literature [58], and these LTR enhancers can activate genes at distances approaching 100 kb [59]. Although insertional activation of protooncogenes by lentiviral LTR enhancers has not been reported, we utilized a SIN lentiviral vector in our studies to minimize these events.

We also attempted to minimize insertional activation/mutagenesis by limiting the total amount of virus in our transduction protocol. Low levels of viral exposure were possible because highly purified hematopoietic stem cells were transduced instead of whole bone marrow or partially purified cells. At least 3,000 times less lentivirus was used compared to previous protocols by transducing and transplanting only 1,000 Sca-1^{+}c-$Kit^{+}Lin^{-/low}$ cells. This procedure limits vector integration into committed progenitors and mature cells that are abundant in total mononuclear cell preparations. Mature donor T and B cells can persist for long periods of time in transplant recipients. These cells replicate actively and are responsible for the acquisition of donor immunity in allogeneic recipients [60-62]. Vector insertions in these cells and in other committed lineages, which can be transformed [63, 64], may increase the risk of insertional activation of protooncogenes such as LMO-2. We did not observe leukemias in any of our primary or secondary transplant recipients.

Finally, the use of low levels of virus in our transduction protocol may also minimize the probability of generating replication competent lentivirus. Although the probability of recombination between the lentiviral vector and endogenous viral-like se-

quences is low, a 3,000-fold decrease in viral exposure should reduce the frequency of recombination even further.

In summary, we have corrected SCD in a mouse model that mimics most if not all of the pathology of this severe disease. Our transduction protocol, which utilizes highly purified, unmobilized hematopoietic stem cells and minimal exposure to the self-inactivating lenti/β^{AS3} vector, may be effectively and safely translated to human patients.

ACKNOWLEDGEMENTS

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A RECOMBINANT HUMAN HEMOGLOBIN WITH ANTI-SICKLING PROPERTIES GREATER THAN FETAL HEMOGLOBIN

by

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ABSTRACT

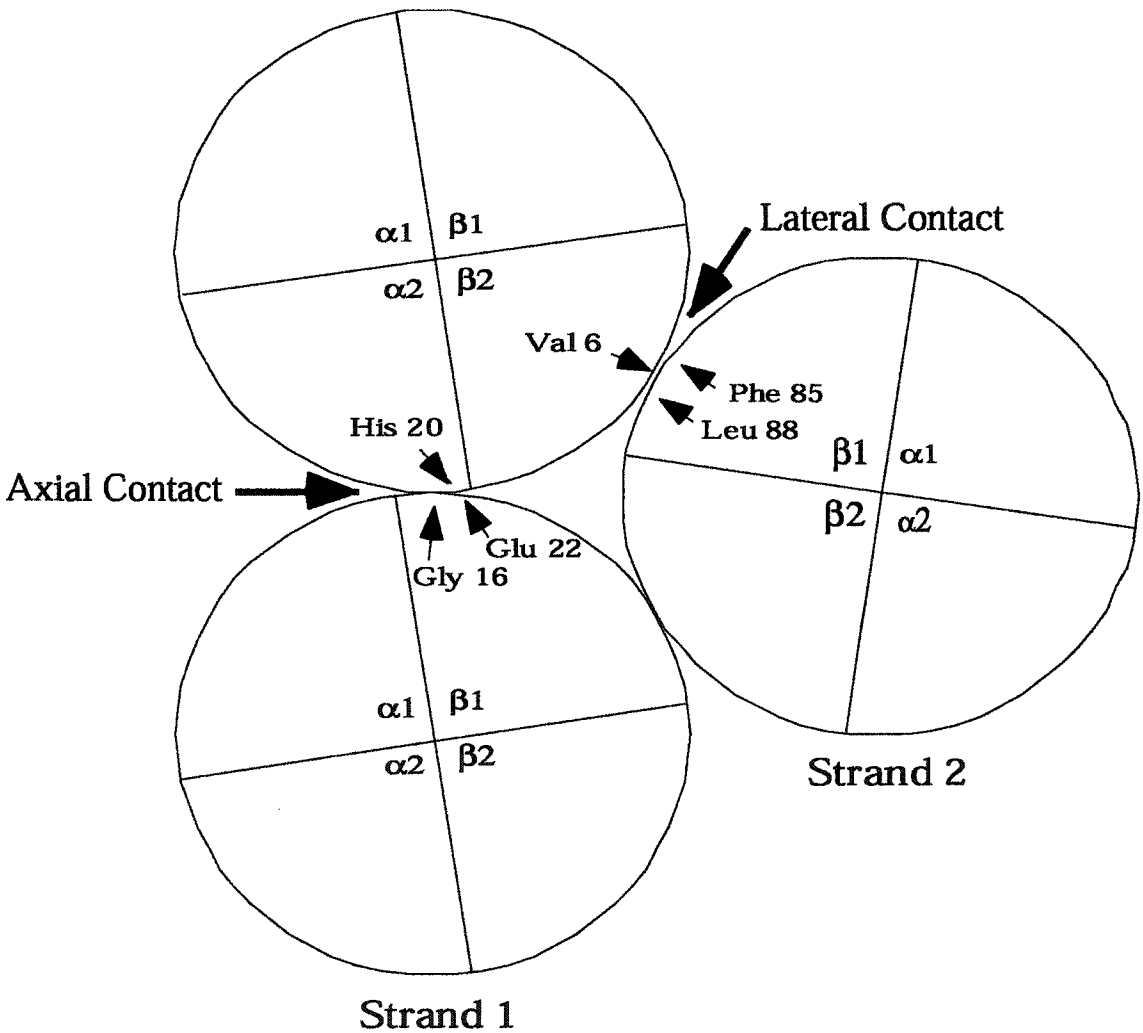
A new recombinant anti-sickling human hemoglobin, HbAS3 (β16 Gly->Asp, β22 Glu->Ala, β87 Thr->Gln), designed to increase affinity for α-globin, has been produced. The mutations at β22 and β87 are located at axial and lateral contacts of the sickle hemoglobin (HbS) polymers and strongly inhibit deoxy-HbS polymerization. The β16 substitution confers the recombinant β-globin subunit (β^{AS3}) with a competitive advantage over β^S for interaction with the α-globin polypeptide. Transgenic mouse lines that synthesize high levels of HbAS3 ($\alpha_2\beta^{AS3}{}_2$) were established, and recombinant Hb AS3 was purified from hemolysates and characterized. HbAS3 binds oxygen cooperatively and has an oxygen affinity that is comparable to fetal hemoglobin. Delay time experiments demonstrate that HbAS3 is a potent inhibitor of HbS polymerization. Subunit competition studies confirm that β^{AS3} has a distinct advantage over β^S for dimerization with α-globin. When equal amounts of β^S and β^{AS3}-globin monomers compete for limiting α-globin chains, up to 82% of the tetramers formed are Hb AS3. Transgenic-knockout animals expressing exclusively human HbAS3 were produced. Breeding HbAS3 animals to a mouse model of sickle cell disease corrected all hematological parameters and organ pathology associated with the disease. Expression of β^{AS3}-globin should effectively lower the concentration of HbS in erythrocytes of patients with sickle cell disease, especially in the 30% percent of these individuals who coinherit α-thalassemia. Therefore, constructs expressing the β^{AS3}-globin gene may be suitable for future clinical trials for sickle cell disease.

INTRODUCTION

Sickle cell disease (SCD) results from an A to T transversion at the sixth codon of the human β-globin gene on chromosome 11 [1, 2]. The mutation of a single DNA base leads to the substitution of a valine for a glutamic acid in the β-globin polypeptide of sickle hemoglobin (HbS). The positioning of a hydrophobic residue at this position permits an interaction with a hydrophobic pocket on another hemoglobin tetramer (Fig. 1). This interaction allows deoxy-HbS to polymerize in an entropy-driven process [3-5]. The polymerization of deoxy-HbS leads to erythrocyte deformation from a biconcave morphology into the sickle shapes for which SCD is named.

Polymerization of deoxy-HbS is effectively inhibited by fetal hemoglobin (HbF) and individuals who are homozygous for the sickle mutation but also express high levels of HbF are typically asymptomatic [6-8]. The level of HbF necessary to significantly reduce the symptoms of SCD ranges from about 20-25%; however, data showing an enhanced red cell survival with as little as 9% HbF have been reported [9]. The efficacy of HbF in inhibiting HbS polymerization suggests that transduction of fetal globin genes into hematopoietic stem cells might be an effective strategy for sickle cell disease gene therapy. However, high levels of γ-globin gene expression are difficult to achieve in adult erythroid cells even in the absence of competition with the β-globin gene for Locus Control Region (LCR) interactions [10, 11]. Low level expression of the γ-globin gene apparently results from the absence of fetal-specific positive regulatory factors in adult cells. Our approach to overcome this deficiency has been to utilize the β-globin

Figure 1. Structure of human HbS fibers. Two types of contacts occur between deoxy-HbS tetramers in the double-stranded fibers. Contacts along the long axis of the fiber are termed axial contacts while contacts along the sides of tetramers are termed lateral contacts. The β6 valine plays a crucial role in the lateral contact by interacting with the hydrophobic β85 phenylalanine and β88 leucine on a neighboring tetramer. An important axial contact is the interaction of the β22 glutamic acid with an α20 histidine on an adjacent tetramer. The recombinant hemoglobin Hb AS 3 has mutations at positions 16, 22, and 87 of the β-globin chain. The mutation at β16, glycine to aspartic acid, is known as J-Baltimore and provides Hb AS 3 with an enhanced ability to interact with the α-globin subunit to form hemoglobin dimers. The mutation at β22, glutamic acid to alanine, disrupts the axial contact interaction with the α20 histidine, and the mutation at β87, threonine to glutamine, disrupts the lateral contact of the β6 with the hydrophobic pocket.



gene as a backbone and to introduce γ-globin amino acid substitutions into this construct. We previously demonstrated that a β-globin gene containing alanine at position 22 and glutamine at position 87 (β^{AS2}) significantly inhibited HbS polymerization [12]. We have now introduced an additional modification into β^{AS2} to form β^{AS3}; this modification increases the affinity of β-globin subunits for α-globin polypeptides. In this paper we demonstrate that β^{AS3} significantly outcompetes β^{S} polypeptides for interaction with limiting α-globin subunits and that HbAS3 dramatically inhibits deoxy-HbS polymerization.

MATERIALS AND METHODS

Construction and Microinjection of Anti-Sickling β-Globin Genes

Plasmids were constructed by standard procedures [13]. Mutagenesis was performed using the Altered Sites system (Promega, Madison, WI) [14] and by megaprimer mutagenesis [15]. The mutagenic oligonucleotides were as follows:

β16, CTGCCCTGTGGGACAAGGTGAACGTG;

β22, GTGAACGTGGATGCCGTTGGTGGTGAG;

β87, GGCACCTTTGCCCAGCTGAGTGAGCTG.

Fragment preparation and microinjection were as described previously [16]. Transgenic animals expressing high levels of human hemoglobin were identified by isoelectric focusing (IEF) of hemolysates. IEF was performed using the Isothermal Controlled Electrophoresis system (Fisher Scientific, Pittsburgh, PA) with precast agarose IEF gels (Isolab, Akron, OH).

Analysis and Purification of Recombinant Human Hemoglobins

Initial analysis of hemoglobin tetramers was performed by anion exchange high performance liquid chromatography (HPLC) utilizing a Synchropak AN 300 (4.6 mm x 25 mm) column (MICRA Scientific, Northbrook, IL) [17]. Preparative IEF was performed on 4% acrylamide gels with 2% Pharmalyte (Amersham Pharmacia Biotech, Uppsala, Sweden) (pH 6.7 to 7.7). Bands of hemoglobin were sliced from the gel and eluted in 0.1 M potassium phosphate buffer, pH 7.0 [18]. Mouse and human globins were separated by reverse-phase HPLC using a Series 4500i HPLC system (Dionex, Sunnyvale, CA). Approximately 25-30 μg of hemoglobin was injected into a C4 reversed phase (4.6 mm x 250 mm) column (Vydac, Hesperia, CA) and eluted with a linear gradient of acetonitrile and 0.3% trifluroacetic acid [19, 20].

Functional Analysis of Recombinant Human Hemoglobins

Oxygen equilibrium curves (OEC) were measured with a Hemox Analyzer (TCS Scientific, New Hope, PA) as described [21]. The OECs were determined in 0.1 M potassium phosphate buffer (pH 7.0) at 20°C with a hemoglobin concentration of 25 μM. Polymerization kinetics were determined in 1.8 M potassium phosphate buffer as described [22]. Polymerization was initiated using the temperature jump method in which the temperature of deoxygenated hemoglobin solutions is rapidly changed from 0°C to 30°C, and aggregation is monitored turbidimetrically at 700 nm [22, 23].

Analysis of Hemoglobin Subunit Recombination

Monomeric α- and β-globin subunits were prepared as described [24]. The hemolysates were treated with carbon monoxide prior to separation to reduce formation of methemoglobin. Isolated α- and β-globin monomers were allowed to combine at 0°C for 1 h. The amounts of HbS and HbAS3 formed were determined by HPLC using a Poly-CAT A cation exchange column (PolyLC, Columbia, MD). Hemoglobins were eluted from the column with a linear gradient of Buffer A (35 mM BisTris, 1.5 mM KCN, 3 mM ammonium acetate, pH 6.47) and Buffer B (35 mM BisTris, 1.5 mM KCN, 16.85 mM ammonium acetate, 150 mM sodium acetate, pH 7.0) [25]. The flow rate was 1 ml/min with detection at 415 nm. The relative amounts of each hemoglobin were calculated by integration of the area under each peak.

Analysis of Heterotetramer Formation

Equimolar amounts of purified oxygenated HbS and HbAS3 or HbA were mixed and allowed to equilibrate overnight at 0°C as previously described [17]. The mixtures were analyzed by cation exchange chromatography as described above, with the addition of 3 mM sodium dithionite to the elution buffers. Sodium dithionite was added to allow separation of hemoglobins under anaerobic conditions, which was necessary for detection of heterotetramers.

RESULTS AND DISCUSSION

Production of Recombinant Human Hemoglobin in Transgenic Mice

A recombinant human hemoglobin, HbAS3, was produced that inhibits HbS polymerization. Mutations were introduced into the human β-globin gene by site-directed mutagenesis, and the mutant sequences were inserted downstream of a 22 kb DNA fragment containing the DNase hypersensitive sites 1-5 (5' HS 1-5) of the β-globin LCR [16]. These constructs were injected into fertilized mouse eggs, and transgenic lines were established. Hemolysates obtained from several animals were analyzed by HPLC to quantitate the amounts of human, mouse, and hybrid hemoglobins [17]. The purity of the human hemoglobins was assessed by denaturing reverse-phase HPLC which separates the α- and β-globin subunits [19, 20].

Functional Analysis of Recombinant Human Hemoglobins

The OEC for purified Hb AS3 is sigmoidal (data not shown), demonstrating the cooperativity of oxygen binding. The P_{50} value is the partial pressure of oxygen at which hemoglobin is half-saturated and was determined for HbAS3 and compared with HbA and HbF (Table 1). The P_{50} for stripped HbAS3 is 7.2 mm Hg and is increased to 10.3 mm Hg in the presence of 2 mM bisphosphoglycerate. Although stripped HbAS3 has a higher oxygen affinity than HbA or HbF, its affinity in the presence of bisphosphoglycerate is similar to that of HbF. In a physiologic environment, HbAS3 should be indistinguishable from HbF, which is a protein that is known to function in vivo to provide protection against sickling crises.

Table 1: P_{50} values of recombinant and naturally occurring human hemoglobins.

	P_{50} Values (mm Hg)	
Sample	Without BPG	With BPG
HbA	8.8	13.3
HbF	8.8	10.0
HbAS3	7.2	10.3

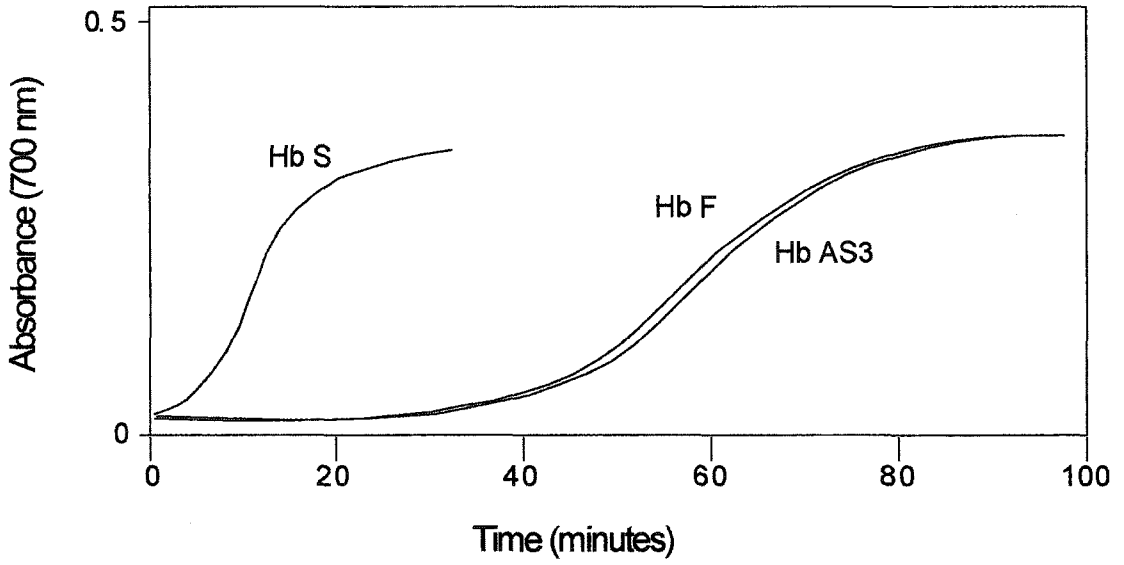
Inhibition of HbS Polymerization by Recombinant Human Hemoglobins

The ability of HbAS3 to inhibit deoxy-HbS polymerization was analyzed by delay time measurements [22]. Briefly, HbS (100%) or mixtures of HbS (75%) and HbF or HbAS3 (25%) were deoxygenated, and polymerization as a function of time was measured spectrophotometrically [22, 23]. Figure 2A demonstrates that HbS polymerizes rapidly but that Hbs F and AS3 markedly delay Hb S polymerization. Hb AS3 inhibits Hb S polymerization at approximately the same level as Hb F., which is known to inhibit sickling in vivo at a 3:1 ratio [6, 7, 26]. This finding strongly suggested that HbAS3 would inhibit HbS polymerization in vivo if HbAS3 levels constituted 25% of total hemoglobin. This prediction was recently borne out by the recent correction of our mouse model of using a lentiviral vector able to transduce purified, unmobilized hematopoietic stem cells; HbAS3 levels of 20-25% resulted in correction of hematological parameters and reversed organ pathology associated with the disease (*Levasseur et al.* Blood-in press).

The delay times determined in Figure 2A were all measured at a concentration of 60 mg/dL. Figure 2B illustrates the results of similar experiments performed using different concentrations of total hemoglobin. The ratio of HbS to HbF or HbAS3 in these

Figure 2. Polymerization delay times for deoxygenated mixtures of human hemoglobins. (A) Delay times for hemoglobin mixtures containing 100% HbS or 75% HbS with 25% HbAS3. Curves were determined at a concentration of 60 mg/dL using the temperature jump method. The delay time is an indication of the ability of a hemoglobin to inhibit polymerization of HbS. (B) Delay time vs. hemoglobin concentration. Hemoglobin concentration ranged from 30 mg/dL to 125 mg/dL. The progression of the plots from left to right demonstrates the increased hemoglobin concentrations that are required for polymerization to occur in the presence of either HbF or the recombinant anti-sickling HbAS3.

A



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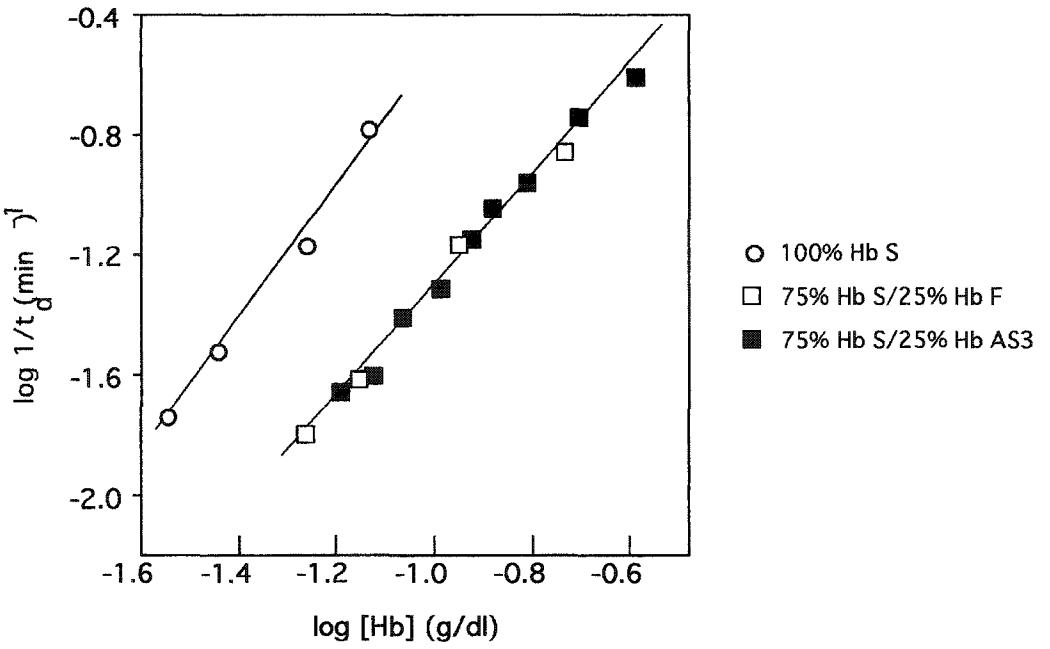


experiments was 3:1. In this figure the log of the reciprocal of the delay time and the log of hemoglobin concentration are plotted. As reported by *Hofrichter et al.* [27], an empirical relationship between delay time and hemoglobin concentration is described by the equation: $1/td = \gamma S^n$, where S = [Hb]total/[Hb]soluble, and γ is an experimental constant. The *n* value is related to the size of nuclei formed during polymerization. The *n* values of the data shown in Figure 2B are between 2 and 3, which agree well with those shown previously in high phosphate buffer [22]. The delay time plots for mixtures of HbS with HbF and HbS with HbAS3 overlap, indicating that HbAS3 has an ability to inhibit HbS polymerization that equals that of HbF.

Competition for Dimerization with the α-Globin Subunit

Previous work demonstrated that two mutations were sufficient to provide the β-globin subunit with a significant ability to inhibit the polymerization of sickle hemoglobin [12]. The anti-sickling HbAS2 described in that work was the starting point for the improved anti-sickling hemoglobin, which we have termed AS3. HbAS3 combines the mutations at position 22 and 87 of AS2 with a third mutation at position 16, which is known as HbJ-Baltimore [28]. This mutation increases the ability of a β-subunit to compete for dimerization with an α-subunit [29].

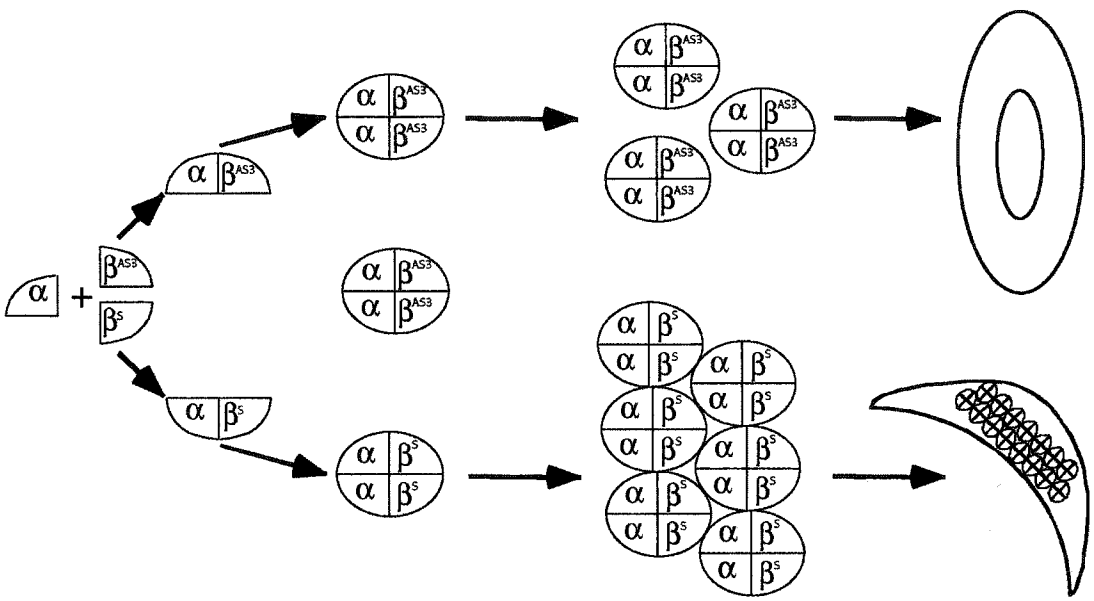
The parameters that determine how well a β-subunit will interact with an α-subunit have been defined using naturally occurring mutant hemoglobins [30]. The dimerization relationship is straightforward and is based primarily upon the charge of the α- and β-chains [31]. Under physiologic conditions, the α-subunit is positively charged (pI 8.4), and the β-subunit is negatively charged (pI 6.7), [32]. Mutations that increase

the net negative charge on the β-subunit increase its ability to dimerize with the α-subunit. Similarly, mutations that decrease the negative charge on the β-subunit decrease its ability to dimerize with the α-subunit. The β^S-subunit is particularly vulnerable to competition with a more negatively charged β-subunit because the sickle mutation, glutamic acid to valine, reduces the negative charge on the β^S-chain. For this reason, erythrocytes in individuals with sickle trait do not contain 50% HbS and 50% HbA but contain approximately 42-45% HbS [33]. Competition for the α-subunit, therefore, appears to be a potential method to increase the amount of anti-sickling hemoglobin in an erythrocyte at the expense of sickle hemoglobin.

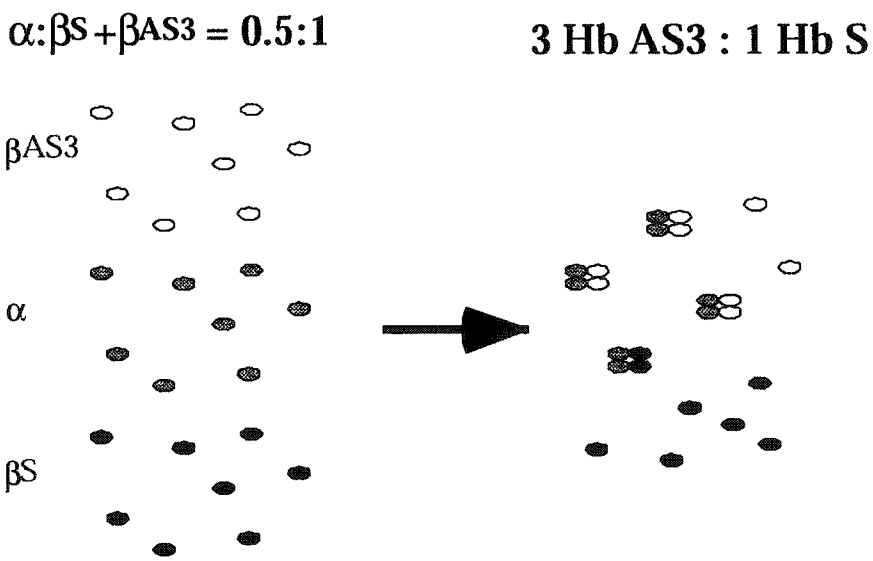
The addition of the J-Baltimore mutation causes the net charge on β^{AS3} to equal that of β^A; the addition of an aspartic acid at β16 compensates for the loss of a glutamic acid at β22. Based strictly upon charge, β^{AS3} should outcompete β^S for dimerization with the α-subunit. When α-, β^S-, and β^{AS3}-globin subunits are combined at an α-:β-globin ratio of 1:1, HbAS3 comprises 59.5% of total hemoglobin (Fig. 3A). As the α-globin subunit becomes limiting, the proportion of Hb AS3 increases dramatically, reaching a level of 75.1% of total hemoglobin at an α-:β-globin ratio of 0.5:1 and 82% of total hemoglobin at a ratio of 0.1:1. This interaction is depicted schematically in Figure 3B. These results demonstrate that β-subunits with the J-Baltimore mutation have a significant competitive advantage over β^S-polypeptides for interaction with α-subunits. The β^{AS3}-subunit interacts with the α-subunit even more efficiently than would be expected

Figure 3. β-globin competition for dimerization with the α-globin subunit. (A) The recombinant human hemoglobin HbAS3 outcompetes β^S-globin for interaction with the α-globin subunit. The higher affinity of β^{AS3}, relative to β^S, for dimerization with the α-globin subunits becomes even more pronounced as the amount of α-globin becomes limiting. (B) Schematic representation of subunit competition. When equal amounts of β^S and β^{AS3} are mixed with a limiting amount of α-globin, β^{AS3} will combine more readily with α-globin than does β^S. The recombined subunits are depicted as tetramers of HbAS3 and HbS since the chromatographic separation was done under oxygenated conditions which favor homotetramer formation. Heterotetramer formation is documented in Fig. 4.

A



B



on the basis of charge alone. Apparently, the specific combination of negative residues in β^{AS3}-subunits improves the ability of this polypeptide to interact with α-globin chains.

Additionally, a recombinant therapeutic hemoglobin must be able to form hetero-tetramers with deoxy-HbS in order to achieve effective inhibition of polymerization. Earlier studies have shown that the inhibitory effect of HbF on the polymerization of deoxy-HbS is dependent on the formation of heterotetramers ($\alpha_2\beta^S\gamma$) [34]. A similar mechanism was proposed to explain the polymerization inhibitory effects of the naturally occurring minor hemoglobin, HbA_2 ($\alpha_2\delta_2$) [35]. Unlike the $\alpha_2\beta^A\beta^S$ heterotetramer, the $\alpha_2\beta^S\gamma$ and $\alpha\beta^S\delta$ heterotetramers are excluded from the sickle polymer, which accounts for the increased inhibitory effects of Hb F and Hb A_2 relative to Hb A. To determine whether recombinant Hb AS3 could form heterotetramers ($a_2\beta^S\beta^{AS3}$), we mixed oxygenated Hb AS3 with oxygenated Hb S. At equilibrium, a binomial (1:2:1) distribution of parent hemoglobins and heterotetramers is observed (Fig. 4). Similar results were obtained for oxygenated mixtures of Hb A and Hb S.

Correction of Abnormal Red Blood Cell Morphology and Hematological Parameters in Sickle/AS3 Mice

SCD in humans and in relevant mouse models is associated with the presence of sickled erythrocytes (drepanocytes) and the characteristic features of anisocytosis, poikilocytosis, and polychromasia. Blood smears from sickle control and sickle-AS3 rescued animals are represented in Figure 5. Many drepanocytes are observed in the sickle control; however, no sickled cells are observed in sickle-AS3 mice (two representative animals). The upper left panel is a wild-type control. The blood smears of sickle-

Figure 4. Heterotetramer formation. Oxygenated forms of purified HbS and HbAS3 or HbA were mixed and allowed to equilibrate overnight at 0°C. The hemoglobin mixtures were separated by cationic exchange chromatography under anaerobic conditions, which allows detection of hybrid heterotetramers. The ratio of peak areas for HbAS3 (or HbA):heterotetramer:HbS was 1:2:1.

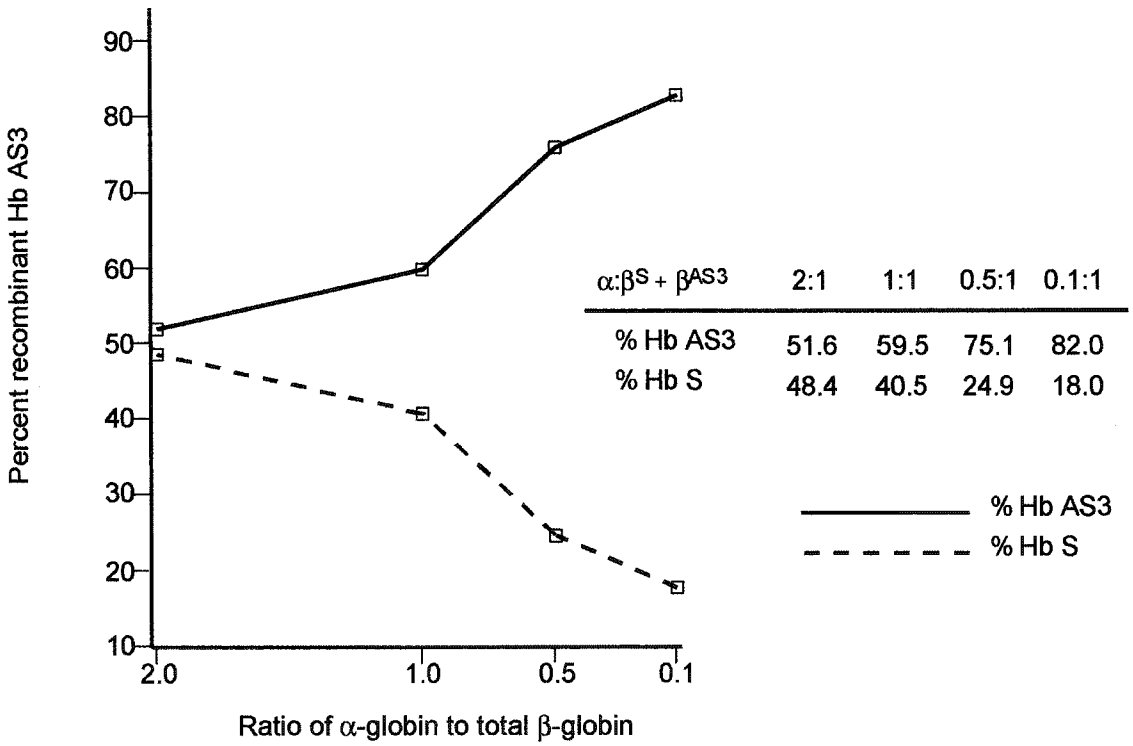


Figure 5. Correction of abnormal red blood cell morphology in sickle-AS3 mice. Shown is a blood smear of a sickle animal with characteristic sickled erythrocytes, anisopoikilocytosis, and a pronounced reticulocytosis (S). Two representative sickle-AS3 animals are shown (S/A #1 and S/A #2). No sickled cells were observed in any fields examined. Wild-type C57Bl6 control (WT).

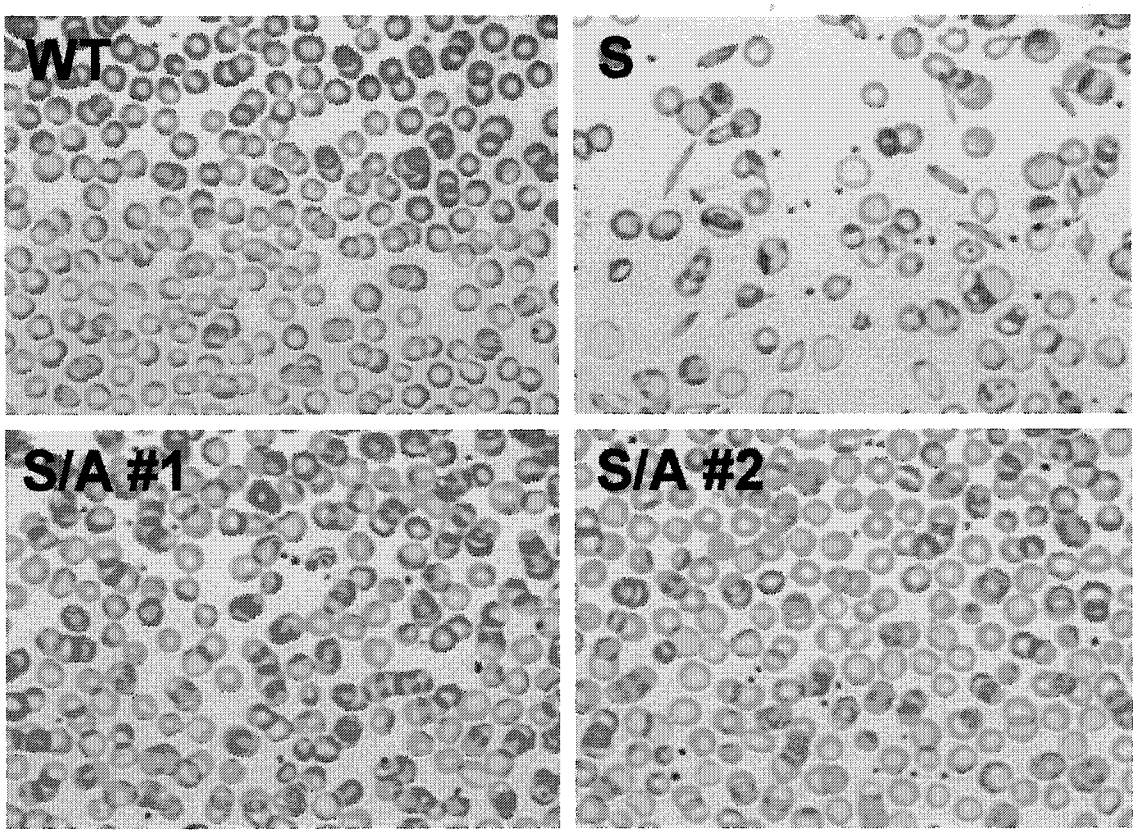
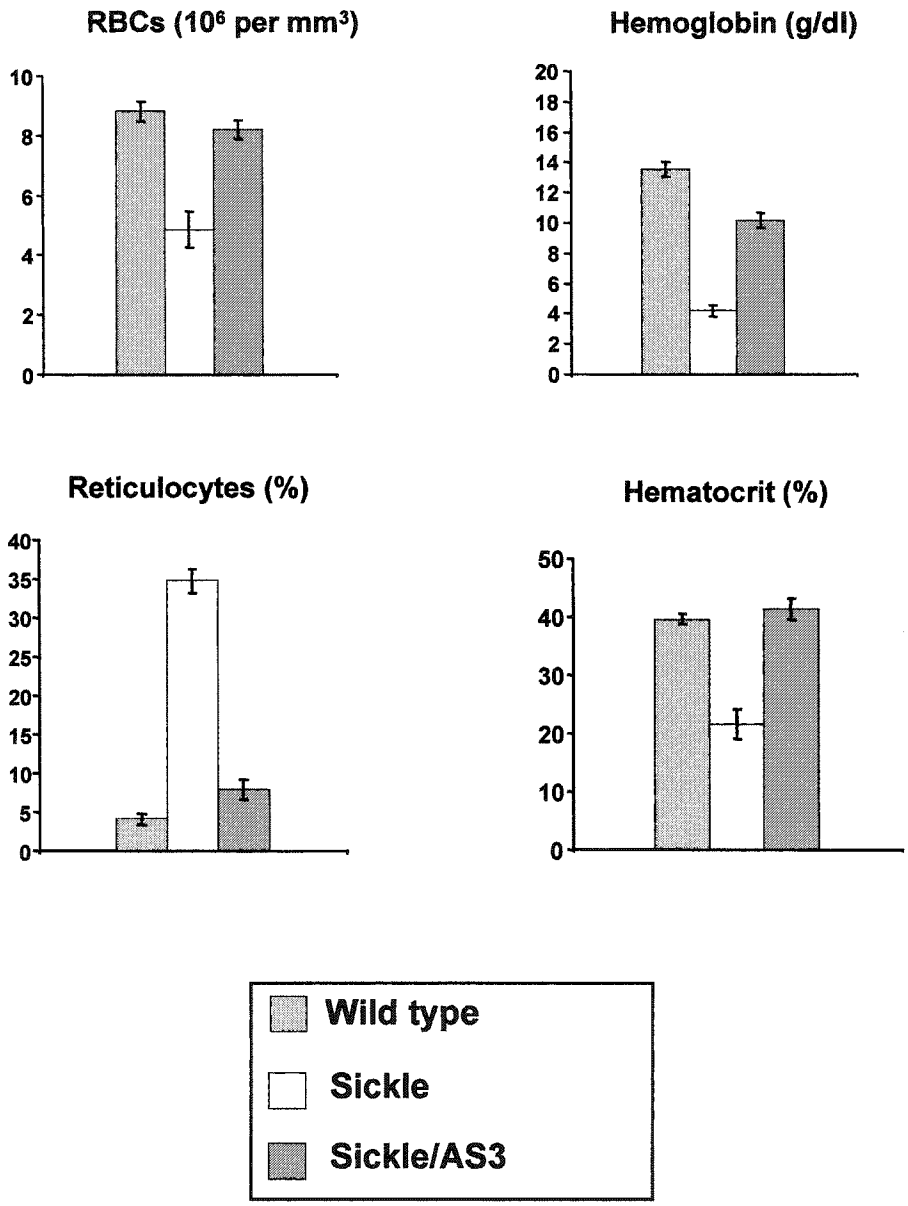


Figure 6. Hematological parameters of sickle-AS3 corrected mice. Shown are hematological indices of control, sickle, and sickle-AS3 mice. RBC counts of sickle-AS3 mice are nearly doubled, hemoglobin levels are increased by 6 g/dL, hematocrit is normalized, and reticulocytosis is reduced to levels approaching those of wild-type mice.



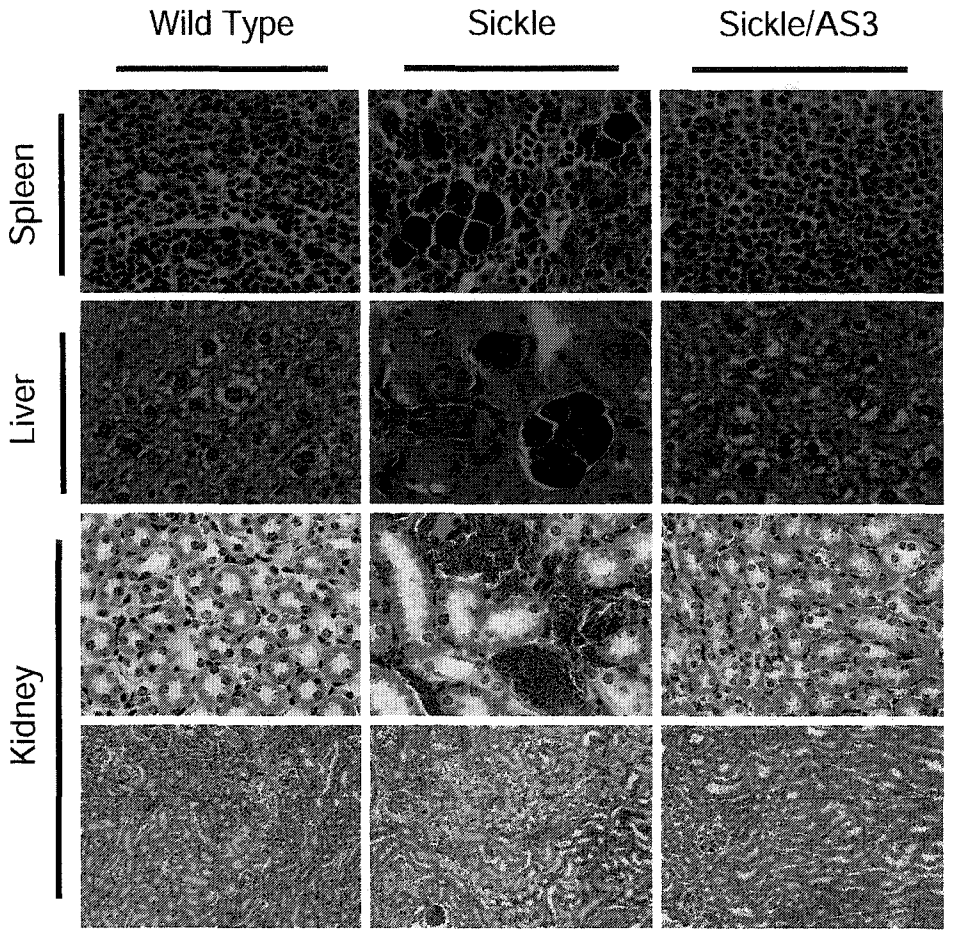
AS3 animals also lack the anisocytosis, poikilocytosis, and polychromasia characteristic of the erythrocyte morphology observed in SCD mice and HbS patients [33, 36]. Figure 6 compares hematological indices of control, sickle, and sickle-AS3 mice. RBC counts are nearly doubled, hemoglobin levels are increased by 6 g/dL in sickle-AS3 animals, hematocrit is normalized, and reticulocytosis is reduced to levels approaching those of wild-type mice. Sickled erythrocytes are not observed in blood smears or in tissues of sickle-AS3 mice. This demonstrates that β^{AS3} results in the formation of a potent anti-sickling hemoglobin in vivo.

Amelioration of Spleen, Liver, and Kidney Pathology and Restoration of Kidney Function in Sickle-AS3 Mice

SCD in mice and humans is characterized by a futile attempt to replace the rapidly turned over population of sickled progenitors and erythrocytes that result from premature destruction. Sickled cells produced in response to this extramedullary hematopoiesis result in organ sequestration that leads to damage and eventual failure of the afflicted tissues. Sickle-AS3 mice developed little spleen, liver, and kidney pathology compared to sickle controls. Histological sections of wild-type, mock-transduced and sickle-AS3 animals are shown in Figure 7 (panel A). The spleens of sickle mice are characterized by a massive expansion of red pulp, dramatic pooling of sinusoidal erythrocytes, vaso-occlusion, and a complete loss of lymphoid follicular structure. In sickle-AS3 mice, normal splenic red and white pulp is observed, and virtually no pools of sickle erythrocytes or infarcts are evident. In addition, splenomegaly is substantially diminished in sickle-AS3 mice (Fig. 7C); sickle-AS3 spleens are approximately 0.6% of total body weight compared to sickle spleens that are almost 4% of body weight.

Figure 7. Normalization of spleen, liver and kidney pathology in sickle-AS3 mice. (A) Spleen, liver, and kidney sections were analyzed at high magnification (100X), and kidney was examined at low (10X, bottom 3 panels) magnification. In sickle-AS3 mice, normal splenic red and white pulp is observed, and no pools of sickle erythrocytes or infarcts are evident. In livers of sickle-AS3 animals, focal areas of necrosis and aggregation of sickled erythrocytes are not observed; also, extramedullary hematopoiesis and hemosiderin deposition are absent. Kidneys of sickle-AS3 mice appear normal and free of the disruptive vascular RBC pooling and hemosiderin deposits observed in mock-treated animals. (B) Urine concentrating ability is restored to wild-type levels. (C) Correction of splenomegaly in sickle-AS3 transduced mice.

A



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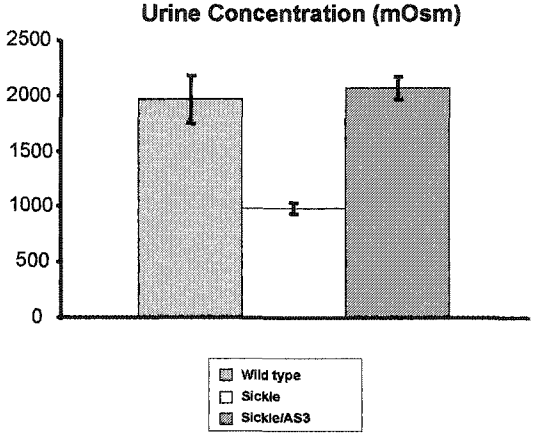

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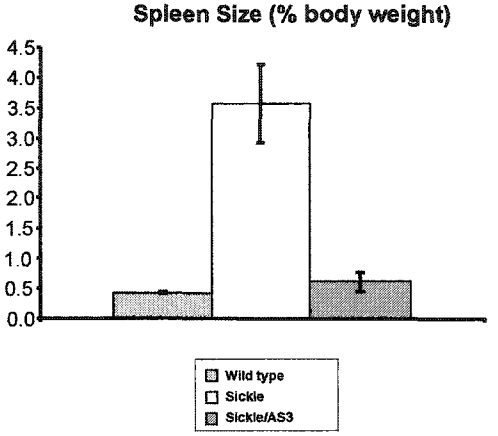

Livers of mock-transduced animals are characterized by focal areas of necrosis and pronounced congestion of the intrahepatic vasculature with pooling of sickled RBCs. Large aggregates of erythroid progenitors are evident in the sinusoids and this extramedullary hematopoiesis is indicative of severe anemia. There is also abundant hemosiderin deposition subsequent to Kupffer cell erythrophagocytosis. In sickle-AS3 animals, focal areas of necrosis and aggregation of sickled erythrocytes are not observed; also, extramedullary hematopoiesis and hemosiderin deposition are absent.

In the kidneys of mock-transduced mice, engorgement and occlusion of blood vessels results in vascular, tubular, and glomerular changes. Sequestration and occlusion are most obvious at the corticomedullary junction where dilated capillaries are easily observed in this region of reduced oxygen tension. Reduced medullary blood flow in HbSS patients causes extensive tubular damage that results in hyposthenuria, and this same loss of urine concentrating ability is observed in the sickle mice. Kidneys of sickle animals also accumulate abundant hemosiderin in the cortical region, and these aggregates are easily visualized with Gomori iron staining (data not shown). In contrast, kidneys of sickle-AS3 mice appear normal and free of the disruptive vascular RBC pooling and hemosiderin deposits observed in mock-treated animals. Most importantly, urine concentrating ability is completely restored in sickle-AS3 mice (Fig. 7, panel B).

The results described above demonstrate that the new β^{AS3}-globin has a strong competitive advantage over the β^{S}-subunit for dimerization with α-globin polypeptides. The β^{AS3}-subunit is more negatively charged than the γ-globin chain of HbF and, therefore, should dimerize more efficiently with the α-subunit. Expression of β^{AS3}-globin should effectively lower the concentration of HbS in erythrocytes of patients with SCD,

especially in the 30% percent of these individuals who coinherit α-thalassemia [37]. As discussed above, the β-globin LCR enhances β-globin gene expression much more efficiently than γ-globin gene expression in adult erythroid cells. Therefore, β^{AS3}-globin genes may be more suitable than γ-globin genes for future genetic therapy of SCD. We recently corrected our mouse model of SCD using lentiviral transduction of the β^{AS3}-antisickling gene into purified hematopoietic stem cells (*Levasseur et al.* Blood-in press). These results suggest that stem cell and genetic-based therapies using recombinant β^{AS3}-globin may be translatable to human sickle patients.

ACKNOWLEDGMENTS

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CORRECTION OF β-THALASSEMIA BY LENTIVIRAL TRANSDUCTION OF EMBRYONIC STEM CELLS

by

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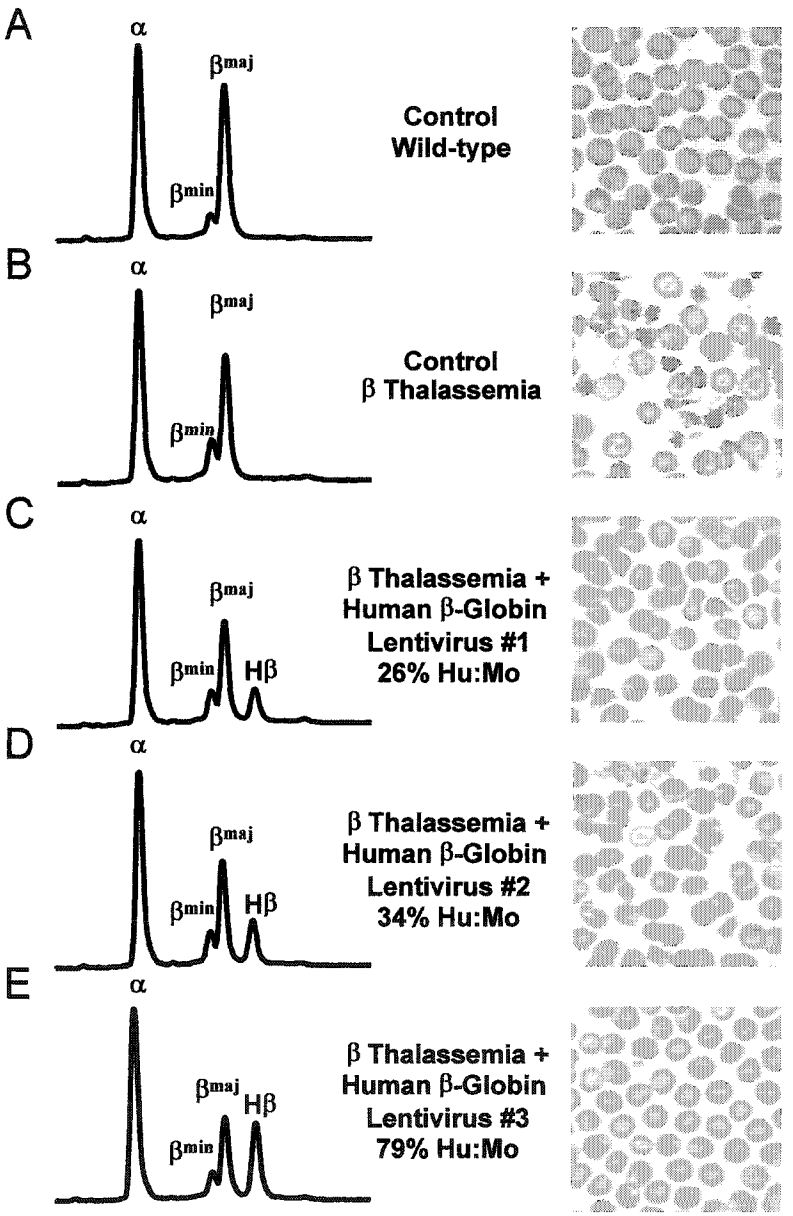
Genetic correction of patient-derived embryonic stem cells (ES) cells is a powerful strategy for treatment of hemoglobinapathies such as β-thalassemia and sickle cell disease. In this study, we used lentiviral gene therapy of murine ES cells to cure a mouse model of β thalassemia. Mice derived from thalassemic ES cells are severely anemic; however, mice derived from the identical ES cells after human β-globin gene transduction are normal.

Beta thalassemia is one of the most common hereditary diseases in the human population. The disease results from an imbalance of the α- and β-globin chains of hemoglobin inside red blood cells. Excess α-globin chains in erythroid progenitor cells of the bone marrow form intracellular aggregates causing ineffective erythropoiesis and anemia. Knockout mice mimicking this disorder were generated by targeted gene deletion of the adult murine β-globin genes [1, 2]. Embryonic stems cells derived from β thalassemic mice were injected into tetraploid blastocysts [3, 4] to generate genetically identical mutant mice. These cloned β thalassemic mice have a severe microcytic anemia characterized by a marked reduction of the erythrocyte mean corpuscular volume (MCV), hemoglobin level, hematocrit, and red blood cell count. In contrast to the normocytic erythrocytes of wild-type clones (Fig. 1a), peripheral blood smears from β thalassemic mice (Fig. 1b) exhibit extreme variation in size and shape. Spiculated microcytes, hypochromic macrocytes, and numerous target cells are prevalent.

The β thalassemic ES cells were transduced with a self-inactivating (SIN) lentiviral vector containing a human β -globin gene and Locus Control Region hypersensitive

Figure 1. Rescue of β thalassemia by lentiviral transduction of embryonic stem cells. Shown are high performance liquid chromatography chromatograms of blood hemolysates and peripheral blood smears of B6/129 F1 hybrid mice cloned by tetraploid embryo complementation from wild-type and β thalassemic embryonic stem (ES) cells before or after lentiviral globin gene transduction. The top set of panels are from a wild-type control. The second set are from control β thalassemic clones. The lower three sets of panels are from mice cloned from control β thalassemic ES cells that were infected with a recombinant human β-globin lentivirus. The ratio of human (Hb) to mouse β-globin (β^{maj}, β^{min}) for each clone is indicated.

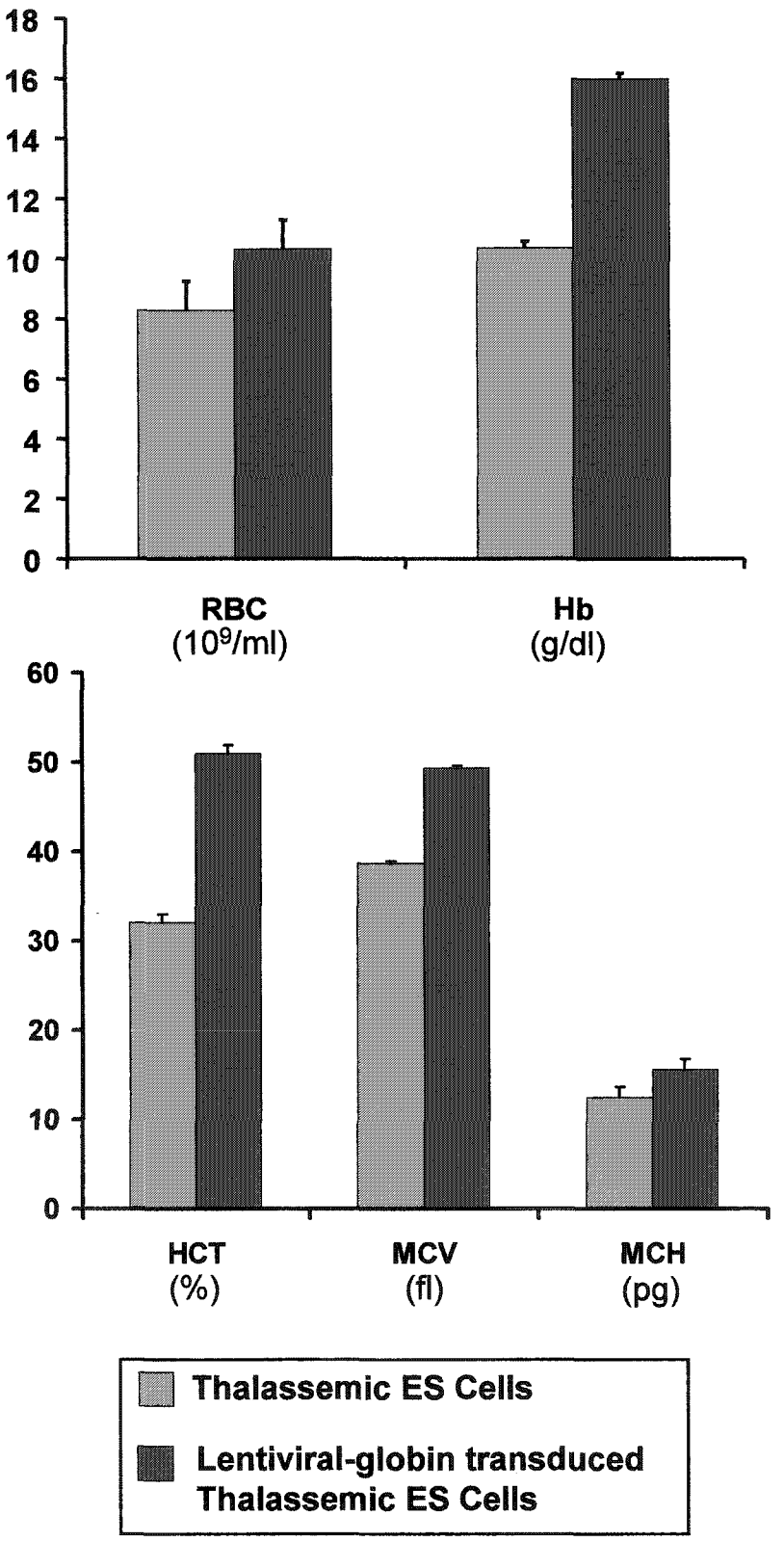


sites 4, 3, and 2 [5]. After a 1 h infection, ES cells were plated to isolate subclones arising from individually infected cells. Analysis of DNA purified from individual ES cell colonies demonstrated the presence of the human β-globin gene in 69% of the subclones.

Human β-globin DNA positive ES cell lines were subsequently injected into tetraploid blastocysts to produce mice that are derived solely from transfected β thalassemic ES cells. Hemolysates from adult mice produced from control, uninfected, and lentivirus infected β thalassemic ES cell lines were analyzed by high performance liquid chromatography (Fig. 1A-E). The quantity of human β-globin polypeptide measured in two animals produced from the same lentivirus transfected cells was 78% and 79% of the endogenous mouse β-globin chains. This increased production of human β -globin polypeptide corrects the α- to β-globin chain imbalance, thereby eliminating the thalassemic erythrocyte morphology (compare Fig. 1B to Fig. 1C, D, and E). Measurement of the mean corpuscular volume, hemoglobin, hematocrit, red blood cell, and reticulocyte levels in the blood of mice produced from this infected cell line are normal (Fig. 2). Human β-globin lentiviral gene therapy of mutant ES cells has corrected the anemia in this mouse model of β thalassemia.

Corrected ES cells can now be in vitro differentiated to produce hematopoietic stem cells for autologous cell therapy of β thalassemic mice. This study provides insight on the possible utility of this experimental approach for human cell therapy. Once diseased ES cell lines are established, their genomes can be modified and carefully analyzed in vitro or in in vivo animal models to determine their therapeutic benefit and safety, prior to reintroduction back into the original diseased host. The results described here

Figure 2. Correction of hematological defects in ES cell-derived tetraploid embryos rescued by self-inactivating lentiviral β-globin transduction. β thalassemic mouse clones are anemic. They have a marked reduction in red blood cells (RBC), mean corpuscular volume (MCV), hematocrit (Hct), hemoglobin (Hb), and mean corpuscular hemoglobin (MCH) values and have increased reticulocytosis and red cell distribution widths. After infection of β thalassemic ES cells with a human β-globin lentivirus, clones were produced and analyzed for anemia. The hematological values of peripheral blood of mice generated from lentiviral transduced ES cell line #3 (Fig. 1) have all returned to normal. Therefore, infection of β thalassemic ES cells with a self-inactivating lentiviral vector containing a human β-globin gene has corrected the anemia in this mouse model of β thalassemia.



demonstrate that lentiviral transduction of ES cells can result in high level β-globin expression levels. This work provides an important step toward translating autologous cell therapy to patients with hemoglobinopathies.

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DISCUSSION

Hematopoietic Stem Cells: Can They Reconstitute Lineages Other Than Blood?

Experiments described above by our group and by others document that hematopoietic stem cells (HSCs) have enormous potential for correction of blood disorders. Recent data suggest other potential therapeutic benefits of HSCs. A large body of evidence is accumulating to indicate that HSCs may be endowed with powers of regeneration that enable reconstitution of tissues outside the hematopoietic compartment. The term "stem cell plasticity" has recently been invoked to describe the potential ability of HSCs to form other tissue types, resulting in a contribution to germ layers other than mesoderm, including the ectoderm of neurons [240] or lung epithelium [241] or the endoderm of liver [242]. These results challenge the long-held developmental dogma that "transdifferentiation," or conversion of one cell type to another, is not possible for cell types other than embryonic stem cells or somatic cells reprogrammed through nuclear transfer (see below). Certain groups have demonstrated elegantly that hematopoietic stem cell plasticity appears to be a real phenomenon [241, 243], while others argue against the ability of HSCs to contribute to other tissue types [244, 245]. A central tenet underlying the argument may be how HSCs are defined and the experimental systems employed to test their function.

Hematopoietic stem cells are broadly characterized by the long-term, high-level repopulation of all hematopoietic lineages and the ability to self-renew. As mentioned

above, bone marrow cells bearing a surface marker profile of Sca-1^{+}, c-Kit^{+}, and low expression of mature lineage markers (lineage$^{-/low}$) are a highly enriched population for long-term repopulating HSC activity [121, 246]. These cells comprise approximately 0.1% of nucleated bone marrow cells and occur at a similar frequency as human stem cells bearing the phenotype CD34^{+}, CD38^{-}, lineage$^{-/low}$ [247]. However, this is one of only many different phenotypes used to describe HSCs resident in the bone marrow. Methods to purify HSCs have been based on size, density, counterflow centrifugal [248, 249], surface marker expression [121, 246, 250-252], resistance to 5-fluorouracil [253], vital dye uptake and the ability to exclude the mitochondrial dye rhodamine-123 [254] or the DNA binding dye Hoechst [255] (reviewed in [256]). It will be important for those employing future studies dissecting the function of HSCs to remember that each of these HSC phenotypes may exhibit different potentials for plasticity.

Although many groups claim to have demonstrated stem cell plasticity, most studies lack rigorous proof to conclusively support this assertion. A functional definition of stem cell plasticity should include the following four criteria: (1) self-renewal ability; (2) differentiation of a single cell into cells of the original tissue and at least one cell type of a different tissue; (3) cellular function in vivo of cells from the original tissue and at least one other cell type of a tissue other than the tissue of origin; and (4) demonstration that stem cell plasticity is not due to cellular fusion [257, 258] resulting in a nuclear "reprogramming" event rather than true plasticity [259, 260]. Of the more than 50 studies published over the last 5 years documenting stem cell plasticity, only a few have met these criteria.

Approximately 80 % of studies reporting adult stem cell plasticity have utilized whole bone marrow, or bone marrow or peripheral blood enriched for HSCs. These reports have documented differentiation into cells with characteristics of skeletal muscle [243, 261, 262], cardiac muscle [243, 263, 264], neuroectoderm [243, 265, 266], skin [241, 243], endothelium [243, 267, 268], endodermal cells, including lung and gastrointestinal epithelium [241, 243], hepatocytes [242, 243, 269], pancreatic β cells [270], and glomerular mesangial and tubular epithelial cells of the kidney [243, 271, 272]. These studies are all extraordinary and suggest that bone marrow stem or progenitor cells may repopulate across tissue boundaries; however, only the studies by *Jiang et al.* [243], *Krause et al.* [241], and *Grant et al.* [268] meet the first three criteria of stem cell plasticity outlined above, and only the study by *Masuya et al.* [271] meets all the criteria of stem cell plasticity. It must be noted that the remarkable plasticity demonstrated by the cells used by *Jiang et al.* likely was not due to nuclear fusion since reconstitution of other tissues by these cells occurred too quickly to be due to a reprogramming event, suggesting these cells may be more pluripotent than any other bone marrow stem cells analyzed to date.

Claims for stem cell plasticity from tissue types outside the hematopoietic compartment have also been reported. Several notable studies purported the production of blood cells from single neural spheres, which are thought to be derived from a single neural stem cell (NSC) [273, 274]. Unfortunately, neither of these studies confirmed clonality using retroviral marking or transplantation of single cells by flow cytometric sorting or limit dilution cloning. The study by *Morshead et al* [274]indicated that the frequency of lineage switching for murine NSCs is at best extremely low. Finally, neither study

tested the functionality of generated hematopoietic cells in vivo by competitive repopulation in irradiated hosts. *Clarke et al* [275] assessed the plasticity of brain-derived neural spheres by injection of these cells into chick embryos or murine blastocysts. Although clonality of the presumptive NSCs was not established, the NSC progeny were able to contribute to multiple tissues. Interestingly, NSCs failed to contribute to hematopoietic tissue, casting doubt on whether these cells can contribute to hematopoiesis. Finally, none of the chimeric mice were born, precluding assessment of functional differentiation of the cells in vivo. The question of clonality is important because it is necessary to confirm the identity of the donor cell tissue. It is easy to imagine that an NSC or other tissue-specific stem cell could actually be an itinerant HSC that has traveled from the bone marrow. Several groups recently reported that muscle-derived cells could give rise to muscle and hematopietic cells following transplantation into lethally irradiated recipients or into a mouse model of muscular dystrophy [262, 263]. However, subsequent work demonstrated that apparent plasticity was caused by HSCs present in muscle tissue [276, 277].

Stem cells with plasticity have been revealed in unexpected niches within the human body. Monocytes are differentiated cells of the hematopoietic system. Huberman and colleagues have studied a subset of monocytes that adhere to plastic when cultured in vitro. These cells express the hematopoietic marker CD45, the monocyte-specific marker CD14, and the CD34 marker expressed on most hematopoietic stem cells. These cells can be induced to mature into phagocytic macrophages, which is the typical differentiation program for this cell type. The more interesting result of this study is the unexpected ability of these monocytes to differentiate into cells expressing markers of the lympho-

cytic, epithelial, endothelial, neuronal, and hepatocyte lineages following cytokine treatment in vitro [278]. The function of these cells was not tested in vivo following transplantation; however, their apparent plasticity, coupled with ease of availability and expansion, would make them a very attractive source for cell therapy.

Another surprise has emerged with the discovery of potential stem cell plasticity in human teeth. It appears that deciduous teeth, commonly known as "baby teeth," contain stem cells in the lower pulp region that can form new dental tissue. Shi and colleagues have termed this population stem cells from human exfoliated deciduous teeth or SHED. There are 20 deciduous teeth that are shed over a seven year period of human development. Work in this study demonstrates that each deciduous tooth contains 12 to 20 SHED that can individually reconstitute dental pulp, including connective tissue, blood vessels, and tooth-forming odontoblasts [279]. The more notable discovery of this work is that these cells also recapitulate dental and bone formation when transplanted subcutaneously and neural cell development when introduced into the brains of mice.

It is possible that most or all tissues of the body have specialized stem cells that can be mobilized to regenerate their respective tissues following injury, either directly by asymmetric cell division or indirectly by contributing support to the damaged cells and their niche. An equally likely possibility is that the bone marrow is an obvious choice for residence of a pluripotential stem cell that can be readily mobilized into the circulation to assist in regeneration of damaged tissues within and outside the hematopoietic compartment. A final possibility suggests that a pluripotential stem cell with high plasticity and proliferative potential exists in all tissues. This cell type could potentially be a relic from

embryonic development that confers additional proliferative potential during times of injury.

Biological systems often have redundance built into them to afford assistance or backup in the event that one component fails. In response to injury or regeneration, "terminally differentiated" cells have also been known to dedifferentiate into a more primitive cell type followed by redifferentiation into the required cell type. This underlies the poorly understood basis of limb regeneration in amphibians and fish. There is little evidence for this phenomenon in mammals; however, the homeobox gene *Msx1* can induce dedifferentiation when introduced into mammalian muscle cell lines [280]. The majority of these cells fail to show expression of muscle markers and can be induced to redifferentiate into cells expressing chondrogenic, adipogenic, myogenic, and osteogenic markers. There are few known factors responsible for maintaining pluripotence in cells, and their role is not well characterized. There is evidence to suggest that cells with pluripotent characteristics persist even after embryological development. Pluripotency of embryonic stem (ES) cells is maintained at the molecular level in part by the leukemia inhibitory factor (LIF)/STAT3 signaling pathway and by the transcription factor octamer-binding protein-4 (Oct-4). Oct-4 is required for maintaining the undifferentiated state of ES cells and contributes a role in determining early steps during embryogenesis and differentiation [281, 282]. Very recently, two groups using differential display or expression cloning discovered a new contributor to ES cell pluripotence with the characterization of the novel homeobox protein Nanog [283, 284]. Another classical characteristic of pluripotent cell types is the presence of high levels of telomerase, which allows these cells to undergo unlimited cell divisions [285, 286]. Multipotent adult progenitor cells

(MAPC) in the bone marrow express low levels of oct-4 and stage-specific embryonic antigen-1 and are the only known adult stem cells to exhibit any of these characteristics [243]. It is not clear whether MAPCs exist as pluripotential cells in vivo or if they are a result of dedifferentiation into a cell with greater potential during their extensive culturing ex vivo. Because of the remarkable characteristics of MAPCs and other putative pluripotential bone marrow stem cells, it may not matter whether transdifferentiation is taking place. The most salient question for purposes of cell therapy is whether these cells can regenerate tissues damaged by injury or disease.

Hematopoietic Stem Cells and Cell Therapy Across Lineage Boundaries

Initial studies purporting stem cell plasticity indicated transdifferentiation might be very rare and therefore not therapeutically useful [262, 265, 266]. During the last 3 years, a number of studies have refuted this assertion. As mentioned above, experimental differences employed by different laboratories and the cell types studied can often explain the varying experimental outcomes. If partially purified bone marrow hematopoietic stem and progenitor are injected into an animal models of myocardial infarction, the donor cells enable a remarkable phenotypic recovery in the recipients [264, 287]. Using a surgical ligation of the left coronary artery to model myocardial infarction in mice, *Orlic et al.* showed that 54 % of cardiac tissue at the site of injury was of donor origin post-transplantation [264]. A band of regenerating myocardium was seen 9 days after surgery, and blood pressure improved 30-40% in hearts reconstituted with partially purified bone marrow stem and progenitor cells (c-kit$^+$, lineage$^-$, or Lin$^-$) compared to negative control mice. Bone marrow stem cells can be mobilized into peripheral blood by intravenous

administration of the cytokines granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), increasing the incidence of c-kit$^+$ Lin$^-$ stem cells 250-fold. The authors asked if mobilization with these cytokines for 5 days prior to experimental induction of myocardial infarction and subsequently for 3 additional days could rescue the animals from cardiac failure. Remarkably, cytokine-induced cardiac repair reduced mortality 68%, infarct size by 40%, and diastolic stress by 70% and resulted in the formation of approximately 15 x 10^6 new myocytes 27 days after the injury [287]. Although the mobilization regimen used in this study would be highly toxic and not translatable to human patients, purified autologous bone marrow stem cells or minimally mobilized peripheral blood stem cells may be highly effective at ameliorating acute or chronic cardiac failure. Work by Kocher and colleagues using intravenously injected human CD34$^+$ cells (enriched for hematopoietic and endothelial progenitors) resulted in the sustained rescue of a rat model of myocardial infarction for 15 weeks following experimentally induced injury [267]. Early stage clinical testing is currently underway to determine if HSC transplantation will be a viable supplement or alternative to current cardiac treatment options [288].

Hematopoietic stem cells have also shown promising therapeutic potential for the treatment of liver disease. As mentioned above, it was once thought impossible for cells of mesodermal origin to alter their lineage specification to enable differentiation into cells of an endodermal fate. Lagasse, Grompe and colleagues achieved such an outcome treating a hereditary mouse model of tyrosinemia that mimics a lethal human liver disorder caused by a defective fumarylacetoacetate hydrolase (FAH) gene. Using whole bone marrow or 50-1,000 c-kit$^+$Thy-1.1^{lo}Lin$^{-/lo}$Sca-1$^+$ HSCs from wild-type FAH$^+$ donors, the authors were able to regenerate large numbers of hepatocytes, restore liver function, and

correct the disorder [242]. It must be noted that the greater than 30% liver repopulation was a result of extraordinary selection since the transdifferentiation event occurred only at a frequency of between 10^{-4} and 10^{-6}, with a total of 50-500 repopulating events dividing and seeding regenerated liver tissue. Further analysis of chromosomal ploidy from regenerated FAH^+ hepatocytes showed that these cells originated from a product of cellular fusion between FAH^+ donor HSCs and FAH^- hepatocytes, with the hematopoietic donor genome adapting a more hepatocyte-specific expression profile after cell fusion[259, 260]. These results were not entirely surprising, however, since liver and other tissue types such as muscle and giant cell-forming mononuclear phagocytes are tolerant of polyploidy and may occur as a result of normal mammalian development. Although these studies demonstrated that regeneration was not taking place because of hematopoietic stem cell plasticity, they may still be therapeutically useful for correction of liver disease using genetically corrected autologous HSCs. Using genetic marking or examination of the Y chromosome in sex mismatched transplantations, cellular fusion has been ruled out as the cause of HSC transdifferentiation into other tissue types [241, 243, 271, 272, 289]. Further study of this phenomenon will be important for the understanding of stem cell biology and translation toward regenerative medicine.

Embryonic Stem Cells and Their Lineage Specific Differentiation: Targets for Cell Therapy?

More than 20 years has passed since Martin [290]and Evans and Kaufman [291] first isolated embryonic stem (ES) cells from mouse blastocysts. Recently, ES cells have been derived from non-human primate [292] and human blastocysts [293]. These cells have the ability to differentiate into all somatic cell types after injection into a mammal-

ian host [290, 291] or a developing blastocyst [294]. Further proof of the pluripotentiality of ES cells comes from the observation that all cells are ES cell-derived in animals derived by tetraploid embryo complementation [295, 296]. The electrically fused blastocysts obtained from this method are tetraploid and not viable; however, they can complement development of microinjected ES cells and contribute the extraembryonic trophectoderm tissue necessary for full development in utero. Murine ES cells have been induced to differentiate in vitro into cardiomyocytes [297], insulin-secreting cells [298, 299], neurons [300], and glial cells of the brain and spinal cord [301, 302] that function in vivo. In a rodent model of Parkinson's disease employed by Bjorklund *et al.* [303], undifferentiated ES cells directly injected into the striatum of rats resulted in the spontaneous production of dopaminergic cells that ameliorated the disease. This result corroborates previous work suggesting neural tissue specification represents a default pathway during development [304]. For this reason, neural degenerative disorders or other injuries to the central nervous system, such as spinal cord damage, may be the most straightforward to treat using neural tissue differentiated from ES cells. However, appropriate caution must be exercised using undifferentiated ES cells for correction of neural disorders because 20% of the animals succumbed to teratomas. This is not surprising since ES cells are known to form teratomas if directly injected into recipients. For this reason, McKay and colleagues introduced the nuclear receptor related-1 or Nurr1 transcription factor into ES cells and used a neural cell specific cocktail of growth factors [305] to obtain dopaminergic neurons in vitro. Using this defined differentiation protocol, the investigators were able to yield high levels of midbrain precursors that functioned properly after injection into the brain striatum and corrected a mouse model of Parkinson's disease

[300]. Encouraging data have also been obtained for differentiation of ES cells into pancreatic cells able to produce insulin and regulate glucose properly. Using a mouse model of streptozotocin-induced diabetes mellitus, one study was able to increase glucose-stimulated insulin production and reverse the course of disease following transplantation of in vitro differentiated insulin-producing cell clusters [299]. Investigators estimated that insulin-producing cell clusters released insulin at levels 13% of wild-type pancreatic islets. Although complete normalization of blood glucose levels was not achieved, the authors were able to show survival of all transplanted mice using this fatal experimental form of diabetes.

Differentiation of ES cells into hematopoietic cells able to reconstitute blood cell development *in vivo* has been more challenging. Though blood formation from *in vitro* differentiated ES cells was reported nearly two decades ago [306], stable engraftment of irradiated mice was only shown recently. Delivering the homeobox transcription factor HoxB4 by GFP-tagged retroviral transduction, Daley and colleagues [307] were able to differentiate embryoid body disaggregated ES cells on an OP9 stromal cell layer in the presence of a cocktail containing stem cell factor (SCF), vascular endothelial growth factor (VEGF), thrombopoietin, and flt-3 ligand FL. Two million in vitro differentiated hematopoietic cells were able to repopulate lethally irradiated recipients; however, the donor cell population decreased with time to only 20% by 15 weeks posttransplantation. Despite this reconstitution decline, the authors did demonstrate production of long-term renewing HSCs because secondary transplants yielded donor derived cells 5 months post-transplantation. It may be that the particular combination of forced HoxB4 expression and cytokines used may predominantly yield a population of hematopoietic progenitors

and stem cells that have altered homing or repopulating abilities. Using this differentiation protocol, the Jaenisch and Daley laboratories achieved the first example of cell therapy by "therapeutic cloning" in a mouse model of SCID. The authors obtained ES cells by nuclear transfer of tail fibroblasts from Rag-2 knockout mice and in vitro differentiated these cells into hematopoietic progenitor and stem cells that were then transplanted back into Rag-$2^{-/-}$ mice [308]. Reconstitution of irradiated recipients was very low: 0.1-0.4% of cells stained with markers against CD4 and CD8 T cells and 2% of cells stained with B220, which is a marker for B cells. To achieve this modest result, the Rag-$2^{-/-}$ transplant recipients required breeding onto a background null for the interleukin-2 common cytokine receptor γ chain (γC) to remove all natural killer T cells. This requirement was necessary because the authors observed during the course of this work that ES cell derived hematopoietic cells express low levels of MHC class I antigens, and this elicits a potent natural killer cell-mediated graft rejection [309]. Such a strategy will not be possible during clinical translation to patients. Finally, a much higher level of donor reconstitution will be necessary to correct most blood disorders, including SCD and β-thalassemia.

Adult or Embryonic Stem Cells: Which is the Best Choice for Cell Therapy?

At this point, the reader may inevitably be drawn to ask a difficult question. What is the best source of stem cells to be used for cell replacement therapy? Many indications seem to suggest that adult bone marrow-derived MAPCs and HSCs, as well as other adult stem cells, have a remarkable capacity to reconstitute injured tissues. Interestingly, MAPCs and different HSC populations may even exhibit "stem cell plasticity," allowing

them to cross embryonic germ layer boundaries and reconstitute tissues other than blood. Every time a potentially promising cell therapy using ES cell differentiated tissues is demonstrated, it seems that a companion study is published showing equal potential for adult stem cells. Part of the quandary is the source and derivation of the cell type used for therapy. The destruction of non viable embryos is necessary for the establishment of ES cell lines, and this makes adult MAPCs or HSCs a more attractive alternative. However, in the case of a recent correction of a mouse model of multiple sclerosis, neural stem cells harvested from the brains of fetal mice were used to transplant recipients intravenously and correct the disease [310]. These data provided a remarkable demonstration of an easily non invasive correction of a neurological disorder, but an obvious issue arises once again: how do you obtain the necessary neural stem cells? Recent clinical trials for Parkinson's disease used neural tissue aborted fetuses to transplant the brains of patients [311]. Transplant recipients exhibited a modest improvement in motor coordination; however, 15% of the patients sustained persistent dyskinesia and dystonia, likely due to the unregulated excess of dopamine produced by the fetal grafts. Fetal tissue is difficult to obtain, and considerable amounts of tissue must be transplanted to achieve results. Alternatively, ES cell-derived neural cells are abundant and easily obtainable. Further preclinical work will likely be necessary to tailor ES cell differentiation protocols for human neural cell transplantation, but it appears that ES cells may be a more palatable option than adult neural stem cells.

As mentioned above, there are other attractive reasons to use ES cells obtained by nuclear transfer for autologous transplantation of patients. ES cells are expandable in vitro, can be grown at high density, and are able to contribute to all tissue types in vivo.

Additionally, human ES cells are available, and mutations in these cells can be potentially corrected since conditions for homologous recombination in these cells have recently been perfected [312]. We and others have demonstrated that murine and human ES cells can be easily transduced with viral vectors (paper 1 and 2 and [313]); and this forced expression can deliver a therapeutic protein to ES cells and correct a mouse model of the hemoglobinopathy β-thalassemia (discussed in paper 4). Unfortunately, great care must be taken to purify cell grafts differentiated from ES cells since transplanted murine and human ES cells can form malignant teratomas in vivo [290, 291, 293].

Recent work highlights another unexpected quality of ES cells and suggests that they may actually be totipotent. This is a designation reserved only for the mammalian oocyte, which is able to contribute the developing embryo following fertilized and to supply the extraembryonic tissues necessary to maintain viability. Scholer and colleagues have shown that ES cells can even differentiate into oocytes in vitro and that these cells undergo parthenogenesis, proceeding through the first few stages of embryonic development [314]. This remarkable discovery will have enormous implications for the future of regenerative medicine using ES cells. If oocytes can be derived from human ES cells, this would obviate the ethically problematic need for egg donation to enable research and clinical development of therapeutic cloning.

A less ethically contentious cell type can be obtained from adult bone marrow and may be pluripotent. As mentioned above, the MAPC is multipotential in vitro and appears to contribute to all somatic cell types in vivo. Although MAPCs must be plated at low density (400-800 cells/cm^2), they can be expanded in vitro and are not tumorigenic. Further studies will be required to determine whether MAPCs can be modified by ho-

mologous recombination for cell and genetic-based therapies in the autologous transplantation setting. If MAPCs are able to readily differentiate into erythroid cells in vivo, adult stem cells may be the most appealing cell source for correction of hemoglobinopathies and other blood disorders in the future.

Future Directions

My current work is focused on differentiating our corrected β-thalassemic embryonic stem cells into hematopoietic stem cells for transplantation. Recent work suggests that overexpression of the transcription factor HoxB4 directs ES cells to differentiate into hematopoietic progenitors that can rescue lethally irradiated mice [307]. We recently transduced our corrected β-thalassemic ES cell line with a retroviral vector containing the human HoxB4 cDNA with a downstream internal ribosomal entry site (IRES) and GFP. Transduced ES cell colonies were picked and are being assayed for expression of the HoxB4 gene. Clones expressing varying levels of HoxB4 will be differentiated on a stromal cell line and assayed for their ability to reconstitute lethally irradiated β-thalassemic mice. We are also collaborating with Catherine Verfaillie to learn how to purify and propogate bone marrow MAPCs from our β-thalassemic mice.

Other work will focus on demonstrating the safety and efficacy of lentiviral transduction of human hematopoietic stem cells in a nonhuman primate model. $CD34^+$, $CD38^-$, Lin^- HSCs from patients with SCD will be transduced using the same protocol we have used to correct our sickle mouse model. In collaboration with Alice Tarantal, we will transplant sickle patient HSCs into second term preimmune fetal rhesus macaques. Human cells persist in the blood of transplanted preimmune macaques at levels of ap-

proximately 1%. We will analyze the ability of our lenti/β^{AS3} vector to transduce sickle HSCs by plating out bone marrow from these xeno-transplanted animals into semisolid medium containing cytokines that direct growth of only human progenitors. Messenger RNA will be isolated from progenitor colonies and assayed for β^{AS3} expression by the allele-specific assay described above. Finally, using a monoclonal antibody that specifically binds only human red blood cells, we will purify human red blood cells from rhesus transplants and determine if lenti/β^{AS3} inhibits sickling under deoxygenating conditions in vitro.

Conclusion

We have corrected our mouse model of SCD utilizing minimal amounts of a SIN lentiviral-based vector designed to deliver a novel, anti-sickling human beta globin gene into purified hematopoietic stem cells. The correction was accomplished with a transduction protocol that should be translatable to human patients. The protocol does not include mobilization of bone marrow or cytokine stimulation of HSCs that would result in loss of stem cell function or potential harm to sickle cell patients. Our results with a lenti/CMV-GFP vector demonstrated that efficient lentiviral transduction of purified HSCs does not require cytokine induction of cell cycle progression; therefore, we used the same protocol to correct our mouse model of SCD. Anticipating embryonic stem cell-derived tissue stem cells will be available for regenerative medicine in the future, we chose to correct our mouse model of β-thalassemia by lentiviral transduction of ES cells derived from these animals. However, a more attractive alternative for correction of blood disorders may already reside in the bone marrow of adults. MAPCs are pluripotent mesenchymal

stem cells that can repopulate the hematopoietic compartment of lethally irradiated mice like their HSC counterparts, but, MAPCs are pluripotent and can contribute to all somatic cell types when injected into fertilized blastocysts [243]. More importantly, MAPCs behave like embryonic stem cells in culture, and preliminary experiments suggest that they can be targeted by homologous recombination (C. Verfaillie, personal communication). Correction of MAPCs from patients with SCD or β-thalassemia would provide an effective approach for treatment of hemoglobinopathies and other blood disorders.

Recently, several groups have focused on determining the genetic expression profile of long-term renewing adult stem cells using gene arrays [315, 316]. These studies seek to elucidate common transcriptional programs linking adult hematopoietic and neural stem cells. The groups of Melton and Lemischka have taken these studies one step further. Their gene array studies attempt to determine genomewide transcriptional changes shared among fetal and adult hematopoietic stem cells (long- and short-term renewing), neural stem cells, and embryonic stem cells [317, 318]. The defined "molecular signature" gleaned from these studies should provide further insight toward understanding stem cell pluripotentiality and the specific genes responsible for its endowment. This information will likely also afford valuable data necessary for improving ES cell differentiation protocols or for bestowing greater pluripotentiality on adult stem cells.

The recent corrections of mouse models of SCD and β-thalassemia using combined stem cell and gene-based therapies are very encouraging. These pre-clinical results attract speculation about the translation of these therapies to human patients. Several barriers remain before correction of β-thalassemia and SCD will be routinely employed in the clinic; however, extraordinary progress has been made in the last few years toward

the realization of this goal. There is a rapidly growing body of literature that suggests adult bone marrow-derived stem cells and embryonic stem cells will be used with improved gene delivery systems as part of a potent arsenal for curing human hemoglobinopathies and other blood disorders some time during this decade.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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