OBSTACLES AND CIRCUMVENTION STRATEGIES FOR HEMATOPOIETIC STEM CELL TRANSDUCTION BY RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

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ABSTRACT

Caroline Njeri Maina

OBSTACLES AND CIRCUMVENTION STRATEGIES FOR HEMATOPOIETIC STEM CELL TRANSDUCTION BY RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

High-efficiency transduction of hematopoietic stem cells (HSCs) by recombinant adeno-associated virus serotype 2 (AAV2) vectors is limited by (i) inadequate expression of cellular receptor/co-receptors for AAV2; (ii) impaired intracellular trafficking and uncoating in the nucleus; (iii) failure of the genome to undergo second-strand DNA synthesis; and (iv) use of sub-optimal promoters. Systematic studies were undertaken to develop alternative strategies to achieve high-efficiency transduction of primary murine HSCs and lineage-restricted transgene expression in a bone marrow transplant model *in vivo*. These included the use of: (i) additional AAV serotype (AAV1, AAV7, AAV8, AAV10) vectors; (ii) self-complementary AAV (scAAV) vectors; and (iii) erythroid cell-specific promoters. scAAV1 and scAAV7 vectors containing an enhanced green-fluorescent protein (EGFP) reporter gene under the control of hematopoietic cell-specific enhancers/promoters allowed sustained transgene expression in an erythroid lineage-restricted manner in both primary and secondary transplant recipient mice.

Self complementary AAV vectors containing an anti-sickling human β-globin gene under the control of either the β-globin gene promoter/enhancer, or the human parvovirus B19 promoter at map-unit 6 (B19p6) were tested for their efficacy in a human erythroid cell line (K562), and in primary murine hematopoietic progenitor cells (c-kit^{+}, lin^{-}). These studies revealed that (i) scAAV2-β-globin vectors containing only the HS2 enhancer are more efficient than ssAAV2-β-globin vectors containing the

HS2+HS3+HS4 enhancers; (ii) scAAV-β-globin vectors containing only the B19p6 promoter are more efficient than their counterparts containing the HS2 enhancer/β-globin promoter; and (iii) scAAV2-B19p6-β-globin vectors in K562 cells, and scAAV1-B19p6-β-globin vectors in murine c-kit^{+}, lin^{-} cells, yield efficient expression of the β-globin protein. These studies suggest that the combined use of scAAV serotype vectors and the B19p6 promoter may lead to expression of therapeutic levels of β-globin gene in human erythroid cells, which has implications in the potential gene therapy of β-thalassemia and sickle cell disease.

Arun Srivastava, Ph.D., Chair

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ABBREVIATIONS

AAV	adeno-associated virus
AP	alkaline phosphatase gene
ATP	adenosine triphosphate
B19p6	parvovirus B19 promoter at map 6
BSA	bovine serum albumin
CaCl2	calcium chloride
CaPO4	calcium phosphate
CAR	coxsackie and adenovirus receptor
CBAp	cytomegalovirus enhancer/chicken β-actin promoter
cDNA	complementary DNA
cGy	centigray
CMV	cytomegalovirus
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH2O	dionized, distilled water
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DI	defective interfering particles
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ds	double stranded

dsDNA	double stranded DNA
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EGFR-PTK	epidermal growth factor receptor tyrosine kinase
EIAV	equine infectious anemia virus
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor 1
GFP	green fluorescent protein
HbF	hemoglobin F
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
hrs	hours
HS	DNase I hypersensitive site
HSC	hematopoietic stem cell
HSPG	heparan sulfate proteoglycan
HSV	herpes simplex virus
HU	hydroxyurea
IEF	isoelectric focusing
IL	interleukin

IMDM	Iscove's Modified Dulbecco's Medium
ITR	inverted terminal repeat
kb	kilobase
LCR	locus control region
LDBM	low density bone marrow cells
LE	late endosome
LR	lactated ringers
LTR	long terminal repeat
M	molar
mcs	multiple cloning sites
MgCl2	magnesium chloride
µCi	microcurie
µF	microfarad
µg	microgram
µl	microliter
min	minute
mITR	mutant inverted terminal repeat
ml	milliliter
MOI	multiplicity of infection
MoMLV	moloney murine leukemia virus
mRNA	messenger ribonucleic acid
msec	millisecond
NaCl	sodium chloride

NaOH	sodium hydroxide
NCS	newborn calf serum
ng	nanogram
p	map position
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI3-kinase	phosphatidylinositol 3 kinase
PNRE	perinuclear recycling endosome
PP5	protein phosphatase 5
qPCR	quantitative real time polymerase chain reaction
rAAV	recombinant AAV
RBE	rep-binding element
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
scAAV	self- complementary adeno-associated virus
SCD	sickle cell disease
SCL	stem cell leukemia gene
SDS	sodium dodecyl sulfate
ssAAV	single stranded adeno-associated virus
SV40pA	simian virus 40 polyadenylation signal

TC-PTP	cellular T cell protein tyrosine phosphatase
TC-PTP-TG	TC-PTP transgenic mice
trs	terminal resolution site
U	units
UV	ultraviolet
wt-AAV	wild-type AAV

INTRODUCTION

Adeno-associated virus 2 (AAV2) is a small, non-enveloped, icosahedral virus of approximately 22 nm (Berns,1990; Siegl et al., 1985). AAV2 belongs to the Parvoviridae family and since co-infection with a helper virus is required for productive infection to occur, AAV is assigned to the Dependovirus genus (Siegl et al., 1985). The other two genera within the parvoviridae family are *Parvovirus*; which consists of human parvovirus B19 and minute virus of mice, and *Densovirus*; which consists of Galleria densovirus and Junonia densovirus (Siegl et al., 1985). AAV2 was isolated from rhesus monkey kidney cells in 1965 as a contaminant of adenovirus preparations. (Atchison et al., 1965).

A. AAV2 Genome

AAV2 consist of a single-stranded DNA genome of 4680 nucleotides (Srivastava et al., 1983). The terminal 145 nucleotides forms the inverted terminal repeat (ITR) consisting of the T-shaped hairpin of 125 bases and the D sequence of 20 bases (Koczot et al., 1973). The ITR provides a free 3' hydroxyl group which serves as the origin of replication. In addition, the ITR has sequences that are needed for integration, rescue and encapsidation (Beaton et al., 1989; Labow et al., 1986; Labow et al., 1988; Samulski et al., 1983; Samulski et al., 1989; Senapathy et al., 1984; Tratschin et al., 1984). A cellular protein, later identified as FK506 binding protein, FKBP52, has been shown to bind to the D sequence and regulate viral second strand synthesis (Qing et al., 1997; Qing et al., 1998; Srivastava et al., 1983; Wang et al., 1995a; Wang et al., 1996). Within the ITR are two open reading frames encoding the *rep* (replication) and *cap* (capsid) genes. The *rep* gene, which is under the control of two promoters located at map position 5 (p5) and 19

(p19), encodes four non-structural proteins named Rep78, Rep68, Rep52 and Rep40, which are named based on their molecular weights (Mendelson et al., 1986; Trempe et al., 1987). While all the Rep proteins possess ATPase and helicase activities, (Collaco et al., 2003; Im et al., 1990; Smith et al., 1998), Rep 78 and 68 also exhibit endonuclease and site-specific DNA binding activities, which play a role in AAV2 DNA replication (Berns et al., 1996). In addition, Rep 78 and 68 are also involved in regulating AAV gene transcription during a productive infection (Pereira et al., 1997).

The *cap* gene is under the control of a single promoter at map position 40 (p40) and encodes three structural proteins, VP1, VP2 and VP3 in a ratio of 1:1:10 with molecular weights of 87, 73, and 61 kDa, respectively. Processing of the cap mRNA by alternative splicing and translation initiation from two different sites allows the synthesis of the three cap proteins from a single promoter. VP1 is generated from the larger transcript, while VP2 and VP3 are generated from the smaller transcript at the ACG and AUG initiation codons, respectively (Becerra et al., 1988; Cassinotti et al., 1988; Siegl et al., 1985; Trempe et al., 1988).

B. AAV serotypes

Since the tissue tropism of AAV2 is relatively broad, efforts are currently underway to understand the biology and infection profiles of other AAV serotypes. Having additional serotypes allows for use of less viral load if the alternative serotype has a better transduction profile and also helps evade the immune system if the recipient is seropositive for AAV2. Up to date, there are approximately 11 AAV serotypes and over 100 variants that have been isolated from human/non-human primates and adenovirus stocks (Atchison et al., 1965; Bantel-Schaal et al., 1984; Gao et al., 2004; Gao et al.,

2002; Hoggan et al., 1966; Mori et al., 2004; Parks et al., 1967; Rutledge et al., 1998; Schmidt et al., 2006). Some of the newly isolated serotypes have a different tropism and enhanced transduction over AAV2. AAV1 and AAV7 have a propensity for muscle (Gao et al., 2004; Gao et al., 2002), AAV5 for the central nervous system (Alisky et al., 2000; Burger et al., 2004), and AAV8 for the liver (Gao et al., 2004; Gao et al., 2002).

C. AAV receptor and co-receptors

The primary attachment receptor for AAV2 was identified as heparan sulfate proteoglycan (Summerford et al., 1998), while fibroblast growth factor receptor 1(Qing et al., 1999), α5βV integrin (Summerford et al., 1999) and hepatocyte growth factor receptor, c-Met (Kashiwakura et al., 2005) were identified as coreceptors. Although several other AAV serotypes have also become available, the receptors for some these serotypes have not yet been identified. The binding of AAV3 is inhibited by heparin, implying that it may also use HSPG as an attachment receptor (Handa et al., 2000; Rabinowitz et al., 2002). AAV4 and AAV5 utilize sialic acid as a receptor, with AAV5 and AAV4 utilizing the N-linked sialic acid and O-linked sialic acid moeties, respectively (Kaludov et al., 2001; Walters et al., 2001). In addition, platelet-derived growth factor receptor has been identified as the receptor for AAV5 (Di et al., 2003). To date, the receptors for AAV1, AAV6, AAV7, AAV8, AAV9 and AAV10 remain to be identified.

D. AAV entry

The mechanism AAV entry into cells has been mainly studied in AAV2. Using real-time tracking of fluorescence labeled AAV2, entry of AAV2 has been shown to occur at a very rapid rate of 100 msec (Seisenberger et al., 2001). Following binding to

the primary receptor, AAV enters the cell in dynamin-dependent, clathrin-coated pits (Bartlett et al., 2000; Duan et al., 1999a). Although the mechanism is not fully understood, interaction of the virus with the cell receptor and co-receptor is believed to trigger Rac1/phosphatidylinositol 3-kinase (PI3-kinase)-dependent signal transduction pathway that results in endocytosis of the virus (Sanlioglu et al., 2000).

E. Intracellular trafficking and uncoating of AAV2

In permissive cells, AAV traffics through early and late endosomes, while in less permissive cells, AAV2 fails to enter the late endosome (LE) (Hansen et al., 2001a). Following endocytosis, AAV2 migrates through early and late endosomes in a Rab GTPase dependent manner before entering the nucleus. Early endosomes are routed either to the late endosome or the perinuclear recycling endosome (PNRE). The virus then escapes from the LE or PNRE or gets transported to the golgi prior to its escape into the nucleus (Ding et al., 2005). Several studies have shown that acidication in the late endosome and lysosomal compartments may play a role in activating "competency" of the virus for transduction (Douar et al., 2001; Hansen et al., 2001a; Parker et al., 2000; Xiao et al., 2002). Movement of free virus in the cytoplasm and nucleus may be facilitated by ATP-dependent molecular motors. Nucleolin, a protein that shuttle between the cytoplasm and the nucleus has been shown to interact with AAV2 virions (Qiu et al., 1999). In addition, Zhao and colleagues documented that FKBP52 interacts with intact AAV2 capsid and the retrograde motor protein, dynein, and facilitates intracellular trafficking of AAV2 (Zhao et al., 2006). The ubiquitin-proteosome pathway has also been shown to play a role in nuclear transport and capsid disassembly (Yan et al., 2002b). Inhibition of EGFR-PTK, a kinase that phosphorylates FKBP52 at tyrosine residues, was

recently shown to decrease ubiquitination of AAV2 capsids (Zhong et al., 2007). Since ubiquitination is required for the proteasome-mediated degradation of AAV2 vectors, less ubiquitination improved the nuclear transport of AAV2 vectors. Whether uncoating occurs inside or outside the nucleus is still unknown. Hansen and colleagues documented infection of purified nuclei from both permissive and non-permissive cells, indicating that the nucleus contains all the machinery necessary for uncoating (Hansen et al., 2001b). While uncoating of AAV2 in primary murine hepatocytes is inefficient, uncoating of AAV8 occurred rapidly in these cells (Thomas et al., 2004).

F. Viral second-strand DNA synthesis

Since AAV2 is a single-stranded virus, conversion of it genome to a double-stranded form is required for it to be transcriptionally active. Several studies have indicated that conversion to double stranded genome is a major limiting step in AAV2 transduction (Ferrari et al., 1996; Fisher et al., 1996). Treatment of cells with adenovirus, E4ORF6 gene and genotoxic agents has been shown to improve transduction of cells by AAV2 by improving the conversion of single-stranded DNA genomes to duplex forms (Alexander et al., 1994; Hillgenberg et al., 1999; Sanlioglu et al., 1999; Yalkinoglu et al., 1988). In 1997, Qing et. al identified a cellular protein that interacts with the D-sequence in the AAV2 ITR, and regulates viral second strand DNA synthesis (Qing et al., 1997). The protein was later identified as a cellular protein that binds the immunosuppressant drug FK506, known as FK506-binding protein (FKBP52) (Qing et al., 2001). FKBP52 can be phosphorylated at tyrosine residues by epidermal growth factor receptor tyrosine kinase (EGFR-PTK) and at serine/threonine residues by a yet unidentified kinase. Phosphorylation at tyrosine residues has been shown to inhibit viral second strand

synthesis by approximately 90%, while phosphorylation at serine/threonine residues inhibits transduction by about 40% (Qing et al., 1998). Later, the group reported that FKBP52 was dephosphorylated at tyrosine residues by the cellular T cell protein tyrosine phosphatase (TC-PTP) (Qing et al., 2003; Zhong et al., 2004b), and at serine/threonine residues by protein phosphatase 5 (PP5), (Zhao et al., 2007) which leads to an increase in the transduction efficiency of AAV2 vectors.

G. AAV2 life cycle

AAV2 can follow two pathways in its life cyle that are known as the lytic or lysogenic pathways. The lytic pathway develops when AAV2 is co-infected with a helper virus such as adenovirus, vaccinia and herpes virus (Buller et al., 1981; Schlehofer et al., 1986). In the presence of helper virus, the provirus genome is rescued from host chromosome in a Rep-dependent manner, and then replicated and packaged into virions. Following lysis of host cell, the newly assembled virions are released (Mendelson et al., 1988; Tratschin et al., 1985; Wang et al., 1995a; Wang et al., 1996). Treatment with genotoxic agents can also lead to a productive infection although the viral yield is much lower than that in the presence of a helper virus (Schlehofer et al., 1986; Yakobson et al., 1987; Yalkinoglu et al., 1988). In the absence of helper virus, AAV2 undergoes latency by integrating site-specifically into a region of chromosome 19 (19q13.3) designated AAVS1 (Cheung et al., 1980; Handa et al., 1977; Kotin et al., 1990; Kotin et al., 1991; Kotin et al., 1992; Samulski et al., 1991). The viral components required for site-specific integration are the AAV ITRs, in *cis* and Rep 78/68, in *trans* (Surosky et al., 1997; Weitzman et al., 1994; Young, Jr. et al., 2000; Young, Jr. et al., 2001). Another *cis* – element that harbors the Rep-binding element (RBE) and also overlaps with the p5

promoter has also been shown to be necessary site-specific integration (Philpott et al., 2002b; Philpott et al., 2002a). The AAVS1 locus has recently been shown to be in close proximity with the muscle-specific genes p85 (Tan et al., 2001a), TNNT1 and TNN13 (Dutheil et al., 2000). An insulator within this locus has also been identified (Ogata et al., 2003).

H. Recombinant AAV vectors and gene therapy

Gene therapy offers great promise for the treatment of genetic and acquired human diseases and viral-based vectors have emerged as the most efficient gene delivery systems. Viruses can easily be manipulated to express therapeutic genes since they have evolved to effectively infect and replicate in specific cell types. The commonly used viruses in gene therapy are retrovirus, adenovirus, adeno-associated virus and herpes simplex virus. Although retroviruses were the first vectors used in a human clinical trial for adenosine deaminase deficiency (SCID-ADA) success with these vectors has been hampered by several obstacles (Blaese et al., 1992; Culver et al., 1991b; Culver et al., 1991a). First, retrovirus vectors were initially based on the Moloney murine leukemia virus (MoMLV). These vectors are incapable of crossing the nuclear membrane and consequently, infect only dividing cells (Havenga et al., 1997). In hematopoietic gene transfer, cells have to be induced to divide which also induces differentiation and loss of the stem cells properties (Glimm et al., 1997). Further, although retroviruses integrate into the host genome, prolonged expression has been difficult to achieve. Retroviruses are inactivated by c1-complement and anti-α galactosyl antibodies, both present in human sera (Rollins et al., 1996; Rother et al., 1995). In addition, retroviruses have also been

associated with oncogenesis through insertional mutagenesis upstream of the LMO2 protooncogene in three patients from the X-linked immunodeficiency SCID-X1 clinical trial (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Lentiviruses, based on the human immunodeficiency virus (HIV) or equine infectious anemia (EIAV), have emerged as alternatives to the conventional retroviruses due to their ability to infect non-dividing cells (Uchida et al., 1998; Whittaker et al., 1998). However, a high frequency of liver cancer was reported in mice infected with EIAV-based lentiviruses and the highly pathogenic nature of HIV is still a serious safety concern (Deglon et al., 2000; Schnell et al., 2000; Themis et al., 2005; Wu et al., 2000). Adenoviruses possess several qualities that make them an attractive vector for gene therapy of certain human diseases. First, adenoviruses use the ubiquitously expressed Coxsackie and adenovirus receptor (CAR) as primary receptor and αv integrins for entry, allowing them to have a broad host range (Bergelson et al., 1997; Tomko et al., 1997; Wickham et al., 1993). In addition, adenovirus vectors which can be produced to very high titers, can infect both dividing and non-dividing cells (Hutchins et al., 2000). However, since adenoviruses do not integrate into the host genome, expression is only transient (Engelhardt et al., 1993a; Engelhardt et al., 1993b; Hitt et al., 1997; Young et al., 2006). Further, the highly immunogenic nature of adenovirus, which resulted in the death of a patient undergoing treatment to correct ornithine transcabamylase deficiency, remains a very serious concern (Young et al., 2006). Because herpes simplex virus is a highly neurotropic virus, gene therapy efforts have been mainly focused in the nervous system. Several trials using mutant HSV1 to treat glioma have highlighted the efficacy of this virus to treat tumors that overexpress cellular enzymes such as thymidine kinase and ribonucleotide reductase

(Harrow et al., 2004; Papanastassiou et al., 2002; Rampling et al., 2000). There is still concern that neuropathic effects may develop due to the high tropism of HSV for neuronal cells (Wolfe et al., 2004).

Adeno-associated virus 2, a single-stranded, non-pathogenic human parvovirus, has gained attention as a potentially safe vector for gene therapy and transfer (Conlon et al., 2004; Marshall., 2001). AAV2 infects a wide variety of cells (Flotte et al., 1993; Griffey et al., 2005; Kaplitt et al., 1994; Kessler et al., 1996; McCown et al., 1996; Muzyczka,1992; Snyder et al., 1997; Xiao et al., 1996; Yang et al., 2002), allowing them to be used in gene therapy trials of several human diseases. AAV2-based vectors are currently in phase I/II clinical trials for gene therapy of a number of diseases such as, cystic fibrosis, hemophilia B and α-1 anti-trypsin deficiency (Aitken et al., 2001; Flotte et al., 1996; Flotte et al., 2004; Kay et al., 2000; Manno et al., 2003; Snyder et al., 2005; Wagner et al., 2002). While wt-AAV integrates site specifically on chromosome 19, recombinant AAV integrates randomly into the host genome at the site of double stranded (ds) DNA breaks (McCarty et al., 2004; Miller et al., 2004). On the other hand, in post-mitotic tissues like muscle, brain and retina, majority of AAV vectors fail to integrate and have been shown to persist as head-to-tail concatemers (Chao et al., 1999; Duan et al., 1998; Duan et al., 1999b; Yan et al., 2005). However, the small transgene capacity of approximately 5 kb remains a serious hurdle (Samulski et al., 1989). The advent of split dual vectors, where a gene is split into two vectors that are simultaneously delivered has increased the transgene capacity, although the efficiency of concatemer formation and transgene expression is still low (Duan et al., 2000; Duan et al., 2001; Lai et al., 2006; Nakai et al., 2000; Reich et al., 2003; Sun et al., 2000; Yan et al., 2000).

I. Self-complementary AAV vectors

Transduction of cells by recombinant AAV vectors is limited by the requirement to convert its single stranded genome to a transcriptionally active double-stranded form (Ferrari et al., 1996; Fisher et al., 1996). During packaging of wild-type AAV, atypical genomes consisting of inverted repeats are formed (de la Maza et al., 1980). These particles are called defective interfering particles (DI). Ensuing studies showed that rAAV DNA of half the size of wt AAV could also be packaged as dimers that are similar to a DI particle (Dong et al., 1996; Hirata et al., 2000; Muzyczka,1992). These vectors are now referred to as self-complementary AAV vectors because they by-pass the need for second-strand synthesis (McCarty et al., 2001; Wang et al., 2003). Because the scAAV vector was a by-product of ssAAV vector production, high titer stocks were difficult to achieve. To generate AAV vectors that predominantly packaged dsDNA genome, Wang et al introduced a mutation in one of the AAV ITRs. By deleting the D-sequence and the terminal resolution site (trs) on the left ITR, the dimers fail to resolve into monomers ensuring that only a double stranded genome is packaged by the other intact ITR (Wang et al., 2003). McCarty et al also made a similar scAAV vector by only deleting the trs from one of the ITRs (McCarty et al., 2003). Several studies have confirmed the previous observation that these vectors can mediate rapid and high efficiency transduction in several cell types *in vitro* and *in vivo* (Aldrich et al., 2006; Fu et al., 2003; Gao et al., 2006; Nathwani et al., 2007; Ren et al., 2005; Sipo et al., 2007; Veron et al., 2007; Wu et al., 2007; Yokoi et al., 2007).

J. Transduction of hematopoietic cells by recombinant AAV vectors

Three sets of opinions exist regarding transduction of human hematopoietic cells by recombinant AAV2 vectors. First, primary human hematopoietic cells are impervious to transduction by AAV and that the observed transgene expression was due to "pseudo-transduction" by contaminants in the AAV viral stock (Alexander et al., 1997). The second opinion is that primary human hematopoietic cells can be transduced by AAV vectors but only at a high vector:cell ratio (approximately 10^6 particles/cell) (Hargrove et al., 1997; Malik et al., 1997; Nathwani et al., 2000). Still we and others have achieved successful transduction at a low vector:cell ratio (Chatterjee et al., 1999; Lubovy et al., 1996; Ponnazhagan et al., 1997a; Ponnazhagan et al., 1997b; Santat et al., 2005; Zhou et al., 1994). To resolve this controversy, Ponnazhagan et al (Ponnazhagan et al., 1997a) undertook a systematic study in which they used $CD34^+$ cells from 12 healthy donors, and infected them under identical conditions with 10^5 particles per cell of recombinant AAV2-lacZ vector. Only $CD34^+$ cells from 6 of the 12 donors studied had significant lacZ expression (15-80%), and the rest had no detectable lacZ gene expression. In addition, they documented binding of ^{35}S-labeled AAV to $CD34^+$ cells from a positive donor and not a negative donor which reinforced the group's hypothesis that AAV infection required a cellular receptor. A year later, cell surface heparan sulfate proteoglycan (HSPG) was identified as an attachment receptor for AAV2 (Summerford et al., 1998). Subsequent studies identified fibroblast growth factor receptor 1 (FGFR1) (Qing et al., 1999), $\alpha v\beta 5$ integrin (Summerford et al., 1999) and hepatocyte growth factor receptor (c-met) as coreceptors for AAV2 (Kashiwakura et al., 2005).

In addition to the lack of receptor and/or coreceptor in some donor $CD34^+$ cells, conversion of AAV2 single stranded genome to a transcriptionally active double stranded genome is still a rate-limiting step (Ferrari et al., 1996; Fisher et al., 1996). A more comprehensive study of recombinant AAV2 mediated transduction of primary murine hematopoietic stem cells (HSCs) has revealed more obstacles to AAV2 mediated transduction of these cells. Similar, to the primary human hematopoietic cells, the lack of co-expression of the receptor (HSPG) and co-receptor (FGFR1, $\alpha v\beta 5$ integrin) of AAV2 remains a rate limiting step in the transduction of primary murine HSCs. While approximately 50% of primary murine HSCs express HSPG and 40-60% express FGFR1, only 10% of these cells co-express both receptors (Zhong et al., 2004b). Based on the previous observation that the phosphorylated forms of FKBP52 inhibits second strand synthesis, Qing and colleagues made FKBP52 knock-out mice (FKBP52-KO) based on the premise that complete removal of the inhibitory tyrosine and serine/threonine phosphorylated forms, would improve AAV mediated transduction of primary murine HSCs. The group also made TC-PTP transgenic mice (TC-PTP-TG) which would only have the dephosphorylated forms of FKBP52, and most likely have efficient transduction by AAV2 vectors. Following transduction of normal control, FKBP52-KO and TC-PTP-TG mice, little transduction was observed in normal control mice, while transduction was improved by 3- and 10-fold in the FKBP52-KO and TC-PTP-TG mice, respectively. There results confirmed that conversion of ssAAV2 to a double stranded form was a rate limiting step in the transduction of these cells. The group has also recently shown that co-infection of AAV1 with self-complementary AAV2 vectors containing the TC-PTP gene improves transduction of murine HSCs in long term transduction studies, confirming that

conversion of single stranded AAV2 genome to transcriptionally active double stranded genome is a rate limiting step in AAV2 mediated transduction of murine HSCs (Zhong et al., 2006a). To determine whether intracellular trafficking was a rate limiting step in the transduction of murine HSCS by AAV2 vectors, murine HSCs were transplanted into lethally-irradiated syngeneic recipient mice and the nuclear and cytoplasmic fractions isolated from spleen colonies 12 days post-transplantation (Zhong et al., 2006a). The group observed that the majority of the viral genome was present in the cytoplasmic fractions of the control, FKBP52-KO and TC-PTP-TG mice, but treatment with hydroxyurea (HU) augmented nuclear transport of the viral genomes. HU treatment increased the nuclear fraction from 20% to 60% in the control mice, from 45% to 85% in the TC-PTP-TG mice and from 13% to 55% in the FKBP52-KO mice. The reason behind the low viral genomes in the nuclear fraction of FKBP52-KO mice was not evident at the time. However, in recent studies, FKBP52 has been shown to be a cellular chaperone and may have an additional role of trafficking AAV into the nucleus (Zhao et al., 2006). Despite the improved trafficking in mice overexpressing TC-PTP in the TC-PTP-TG or in all groups following HU treatment, the group found that majority of the viral genome remained single stranded. In addition, a significant number of viral genomes in the cytoplasmic and the nuclear fraction was resistant to DNase I treatment, indicating that the virus had failed to undergo uncoating. Thus, although HU treatment improved trafficking and second strand synthesis in these cells, the effect was minimal since the majority of the virus remained uncoated in the murine HSCs for at least 48 hrs post-transduction.

K. Strategies to improve transduction of HSCs

(i) Self-complementary AAV vectors

While transduction from single stranded AAV vectors is low in murine HSCs (Qing et al., 2003; Zhong et al., 2004b), transduction from double stranded self-complementary AAV vectors is readily detectable (Zhong et al., 2006a, Maina., 2008a) In a comparative study of scAAV1-, scAAV2-, scAAV3- and scAAV5-mediated transduction of murine HSCs in 7-day liquid cultures, scAAV1 mediated transgene expression in 5% of these cells. Expression from scAAV2 was 2%, while expression from scAAV3 and scAAV5 was insignificant. These results confirm that second strand synthesis is a rate limiting step in the transduction of these cells. Although scAAV vectors have been shown to overcome the limitation of second strand synthesis, their use is restricted to small transgene cassettes. However, Wu et al, have recently shown that the packaging capacity of scAAV vectors can be increased from 2.5 kb to 3.3 kb (Wu et al., 2007). For transgene cassettes larger than 3.3 kb, conversion of single stranded AAV vectors can be facilitated through the use of scAAV-TC-PTP and scAAV-PP5 as helper viruses (Zhao et al., 2007; Zhong et al., 2006a). In a long-term bone marrow transduction study, Zhong and colleagues showed that co-infection of single stranded AAV1 vectors with scAAV2 vectors containing the TC-PTP gene improved transduction of murine HSCs, by presumably increasing conversion of single stranded AAV genome to double stranded forms (Zhong et al., 2006a). In a recent study, Zhao and colleagues have also shown that dephosphorylation of FKBP52 at serine/threonine residues by PP5 can also be exploited to increase the transduction efficiency of ssAAV vectors (Zhao et al., 2007). Another strategy to increase the packaging capacity of self-complementary vectors is to employ the split dual vector strategy in which a gene is split into two scAAV vectors

(Chao et al., 2002; Duan et al., 2000; Duan et al., 2001; Ghosh et al., 2006; Ghosh et al., 2007b; Lai et al., 2006; Nakai et al., 2000; Reich et al., 2003; Sun et al., 2000; Xu et al., 2004; Yan et al., 2000; Yan et al., 2002a; Yan et al., 2005, Maina, 2008b). In this strategy, a single gene is split into two separate scAAV vectors, which then undergo intermolecular recombination following cellular entry and viral uncoating. Using a split GFP vector, Choi and colleagues provided evidence that the closed-to-closed ITRs were more efficient than the open-to-open ITRs at undergoing recombination (Choi et al., 2005). The efficiency of recombination can also be improved through the inclusion of a 600bp fragment of the alkaline phosphatase gene (AP) in the two split vectors (Ghosh et al., 2008).

(ii) Use of serotypes other than AAV2

In short and long term transduction studies using AAV serotypes 1, 2, 3 and 5, Zhong and colleagues documented improved transduction with AAV serotype 1 in murine HSCs (Zhong et al., 2006a). Approximately 9% of progenitor cell assay colonies established from $Sca1^+$, $c\text{-}kit^+$, lin^- cells, infected with AAV1, expressed the transgene. In bone marrow transplantation studies, 2% and 7% of peripheral blood (PB) cells from mice infected with AAV1 or co-infected with AAV1 and scAAV-TC-PTP vectors, expressed the transgene 6 months post transplant, respectively. In addition, 21% of bone marrow derived colonies contained the proviral genome in integrated forms. These studies document that AAV1 is by far the most efficient serotype in transducing murine HSCs. In a separate study involving transduction of human and murine HSCs *in vitro* and *in vivo* , Li and colleagues demonstrated that AAV7 and AAV8 were the most efficient in transducing murine HSCs, while AAV7 followed by AAV8 and AAV1, were the most

efficient in transducing human $CD34^+$ cells (Li et al., 2005). Thus, AAV7, AAV8 and AAV1 are by far the most efficient serotypes for gene transfer of HSCs. Further studies with hematopoietic stem cell promoters and scAAV genomes should further augment the transduction efficiency of HSCs by AAV1/7/8 serotypes.

(iii) Use of hematopoietic stem/progenitor cell-specific promoters

In all recent studies with murine hematopoietic stem/progenitor cells, investigators have used AAV vectors containing a transgene under the control of the CMV promoter. Several studies have shown the inability of CMV promoter to sustain long-term expression in various tissue types *in vivo*. In 1998, Nakai *et al*. reported high and sustained expression (6 months) of FIX using EF1-α promoter, whereas expression from the CMV promoter was lower and transient (5 weeks) despite the persistence of the vector genome in the mouse liver (Nakai et al., 1998) Similar results were reported by Cordier *et al*., using AAV vectors to deliver γ-sarcoglycan intramuscularly in a mouse model of muscular dystrophy. While the muscle creatine kinase promoter allowed expression of therapeutic levels of γ-sarcoglycan for 11 months, the CMV-driven vector expression was sub-therapeutic and declined significantly in 3 months (Cordier et al., 2001). In the hematopoietic lineage, Kurpad *et al*. reported transient, ubiquitous, CMV promoter-driven expression of a reporter gene using AAV vectors in primary human hematopoietic cells *in vitro*, while expression from the human β-globin promoter and the parvovirus B19 promoter at map unit 6 (B19p6) was sustained and restricted to the erythroid lineage (Kurpad et al., 1999). Similar results were reported by Gardner *et al*. in T cells where they reported robust but transient expression from the CMV promoter

(Gardner et al., 1997). Several reports have indicated that the relatively weak expression from the CMV promoter is very sensitive to silencing mechanisms in hematopoietic cells (Choi et al., 2001; Kung et al., 2000; O'Rourke et al., 2005). Fortunately, other studies have explored the potential of eukaryotic and viral promoters to allow high level, tissue specific expression in the hematopoietic lineage (Papadakis et al., 2004). A eukaryotic promoter that has been studied extensively is the β-globin promoter with its enhancer elements. The β-globin enhancer consists of five DNase I hypersensitive sites (HS1, HS2, HS3, HS4 and HS5), collectively known as the locus control region (LCR), which is required for optimal activity of the β-globin promoter (Grosveld et al., 1987; Leboulch et al., 1994). Using AAV vectors containing the human β-globin gene under the control of the β-globin promoter with a mini-LCR cassette consisting of HS2, HS3 and HS4, Tan *et al.* reported long-term, erythroid specific expression of a human β-globin gene in primary murine HSCs from β-thalassemic mice (Tan et al., 2001b). May *et al*. reported phenotypic correction of murine β-thalassemia using a lentiviral vector expressing β-globin gene under the control of β-globin promoter linked to the core elements of the LCR. Tetramers of two human β-globin and two murine α-globin molecules accounted for 13% and 17%-24% of the total hemoglobin in mature red cells of normal mice and β-thalassemic heterozygous mice, respectively (May et al., 2000). In 2003, Persons and colleagues reported similar results albeit at a higher vector number, using a lentiviral vector containing the γ-globin gene under the control of the β-globin promoter and enhancer elements (Persons et al., 2003). A second promoter that has been extensively studied is the promoter for the cell membrane protein, ankyrin, which has been shown to direct erythroid-specific expression of γ-globin in transgenic mice (Sabatino et al.,

2000b). The same group demonstrated long-term expression of γ-globin mRNA in mouse erythrocytes from retrovirus vectors containing the human γ-globin gene under the control of the ankyrin promoter. They were able to achieve ~8% of the level of the mouse α-globin mRNA, a level that was 50% of the level required to treat severe β-thalassemia (Sabatino et al., 2000a) Richard *et al*. demonstrated long-term *in vivo* erythroid-specific expression of ferrochelatase and correction of protoporphyria in a mouse model using a self-inactivating lentiviral vector containing ferrochelatase cDNA driven by the human ankyrin-1/α-globin HS-40 chimeric promoter/enhancer. They were able to achieve transduction levels that were greater or equal to those achieved by a CMV construct and expression was restricted to the erythroid lineage (Richard et al., 2001).

Another erythrocyte membrane protein promoter that has been evaluated for erythroid-specific expression is the β-spectrin promoter, which is responsible for erythrocyte shape and membrane stability and is defective in many patients with abnormalities of red blood cell shape including hereditary spherocytosis and elliptocytosis (Winkelmann et al., 1993). A viral promoter of significant interest to us is the parvovirus B19p6 promoter. Although parvovirus B19 is an autonomously replicating virus, its replication is mainly limited to human hematopoietic progenitor cells undergoing erythroid differentiation (Ozawa et al., 1986; Ozawa et al., 1987; Srivastava et al., 1988). In human progenitor assays *in vitro*, activity of B19p6 has been shown to be restricted to erythroid lineage (Kurpad et al., 1999; Wang et al., 1995b), but it remains to be determined whether expression from this promoter will occur in murine hematopoietic cells, presumably because of the absence of a putative transcription factor that is required for expression from the B19p6 in human erythroid progenitor cells

(Ponnazhagan et al., 1995). Li *et al.* (Li et al., 2005) have also demonstrated that CMV enhancer/chicken β-actin promoter (CBAp) is active in primitive human hematopoietic cells. The stem cell leukemia (SCL) gene is critical for the development of HSCs in the embryo (Curtis et al., 2004) and the ability of the SCL promoter to drive gene expression in HSCs remains to be explored. Thus, long-term transgene expression in human and murine HSC can be expected to be achieved by using the hematopoietic stem/progenitor cell-specific promoters in the context of AAV vectors.

(iv) Use of Engineered hybrid vectors

(a) Mosaic AAV vectors

To form an AAV capsid, all AAV serotypes utilize 60 monomers of VP1, VP2 and VP3. In mosaic AAV vectors, monomers from different serotypes are combined and the input ratio of different helper constructs determines the ratio of capsid subunits in the mosaic virion (Wu et al., 2006). The benefit of this technique is the ability to combine unique features from different serotypes to synergistically enhance transgene expression. Rabinowitz and colleagues generated mosaic AAV vectors by pairwise combination of AAV serotypes 1 to 5 at different ratios (Rabinowitz et al., 2004). These vectors had dual binding characteristics of parent serotypes or new properties different from parental viruses. While neither AAV1 nor AAV2 infect C2C12 muscle cells efficiently, mosaic AAV1/2 virions demonstrated enhanced transduction of these cells. As demonstrated above, this procedure can be utilized to generate cell type specific vectors with enhanced transduction than either of the parent vectors.

(b) Chimeric AAV vectors

Chimeric AAV virions are vectors containing capsids that are modified by amino acid or domain swapping. This method relies on marker rescue which requires cotransfection of an AAV clone carrying a mutation in the capsid gene with wild type DNA sequences from a different serotype (Bowles et al., 2003; Hauck et al., 2003; Wu et al., 2006). Homologous recombination usually occurs due to the significant degree of homology between the AAV serotypes. The chimeric virions can either be amplified by PCR and cloned into a shuttle vector, or transfected onto specific cell types for analysis of biological properties. Bowles and colleagues were able to rescue three non-infectious clones of AAV2, that could not bind heparin sulfate, by contransfecting the helper plasmids with DNA sequences from AAV3 (Bowles et al., 2003). The group demonstrated correlation between infectivity and the restoration of heparin binding.

(c) DNA Shuffling

DNA shuffling is an *in vitro* evolution technique that can be used to generate proteins with new phenotypes. In this technique, different alleles are digested and the fragments allowed to randomly hybridize, before being amplified in a self-priming PCR reaction (Bacher et al., 2002). Soong and colleagues applied this technique to generate Moloney Leukemia viruses (MLV) with a different tropism than the parental vectors (Soong et al., 2000). A number of groups have used various combinations of AAV serotype vector 1 through 9 to generate vectors with improved transduction ability for skeletal muscle, liver, brain and lung (Grimm et al., 2006; Grimm et al., 2008; Li et al., 2008).

(d) AAV-Parvovirus B19 hybrid vectors

As mentioned above, AAV2 vectors infect a wide variety of cells while parvovirus B19 vectors have a narrow tropism for human erythroid progenitors (Ozawa et al., 1986; Ozawa et al., 1987; Srivastava et al., 1988; Yaegashi et al., 1989). Our group has combined the advantageous features of these two viruses to generate hybrid AAV-B19 vectors with remarkable tropism for erythroid progenitors (Ponnazhagan et al., 1998; Wang et al., 1995b; Weigel-Kelley et al., 2001; Weigel-Kelley et al., 2002; Weigel-Kelley et al., 2003). AAV2-B19 virus contains the parvovirus B19p6 promoter encapsidated in AAV2 capsid while B19-AAV2 contains the parvovirus B19p6 promoter-driven vector encapsidated in B19 capsid. As a result, AAV2-B19 vectors infect a wide variety of cells but expression is limited to the erythroid cells. On the other hand, infection and expression of B19-AAV2 vectors is limited to erythroid cells. Recently, we have generated hybrid B19-AAV serotypes 1, 7, 8, and 10 vectors containing the B19p6 promoter encapsidated in the respective serotype, with efficient transduction of both murine and human HSCs as shown in the Results (Maina et al., 2008a).

L. Gene Therapy for Sickle Cell Disease

Sickle cell disease (SCD) is an autosomal recessive disorder affecting millions of people worldwide. The causative mutation is an A-T transversion in the sixth codon of the β-globin gene which leads to the substitution of a valine for a glutamic acid, resulting in the formation of abnormal hemoglobin, known as hemoglobin S (HbS) (Ingram, 1957). Following deoxygenation in red blood cells (RBCs), HbS forms polymers causing the

RBCs to become deformed (sickled) and adherent, leading to vaso-occlusive events, resulting in splenic infarct, kidney failure, stroke, painful crises, and chronic anemia (Pauling et al., 1949; Bunn et al., 1994; Steinberg et al., 2001). Treatment modalities for SCD involves allogeneic bone marrow transplantation, induction of fetal hemoglobin (Swank et al., 1998), control of infections, and pain management (Buchanan et al., 2004; de Franceschi et al., 2004). Allogeneic transplantation usually requires myeloablative conditioning with cyclophosphamide, busulfan, antithymocyte globulin or total lymphoid radiation. In addition, cyclosporine and methotrexate are used post-transplant to induce immunosuppression (Walters,2005). Hydroxyurea (HU) is the only drug approved by the US Food and Drug Administration (FDA) to treat SCD. HU increases HbF in sickle cell anemia by inducing erythroid regeneration and augmenting γ-globin gene expression in a nitric oxide-dependent pathway (Buchanan et al., 2004; Steinberg, 1999). Other drugs that increase HbF and that are currently in Phase I/II clinical trials for SCD are described below. Decitibine, an analog of 5-azacytidine induces HbF by causing hypomethylation of the γ-globin genes (Saunthararajah et al., 2003). Histone deacetylase (HDAC) inhibitors have also been shown to enhance γ-globin gene expression through histone hyperacetylation and alteration of chromatin structures (Atweh et al., 1999; Cao et al., 2004). Autologous bone marrow transplantation with genetically modified cells would be a great treatment modality, but has been limited by the inability to achieve therapeutic levels of β-globin expression in the HSC's progeny (Sadelain, 1997; Sadelain, 2006). Previously, gene transfer of β-globin gene and its regulatory elements relied on oncoretroviruses. However, oncoretroviruses have the disadvantage of requiring cell division for nuclear entry, which necessitates the use of cytokines. In addition, these

vectors have the problems of low titers, variable expression, vector instability and insertional mutagenesis. To overcome these limitations, 'self-inactivating' lentiviruses have emerged as an alternative to oncoretroviruses. A deletion of the promoters in the U3 region of both the 3' and 5' LTR, results in transcriptional inactivation of the LTR in integrated provirus. And as already mentioned above, lentiviruses can infect non-dividing cells. May and colleagues were the first to show stable transduction of a lentiviral vector carrying the globin gene and a large LCR fragement (3.2kb) and therapeutic correction of β-thalassemia intermedia. These studies were followed by others which showed therapeutic correction of several β-thalassemia phenotypes, using SIN lentiviral vectors expressing β/γ globin, in various mouse models (Hanawa et al., 2004; Imren et al., 2002; May et al., 2000; Persons et al., 2003; Puthenveetil et al., 2004; Rivella et al., 2003). Therapeutic levels of β/γ globin expression has also been achieved by retroviral and lentiviral vectors expressing β-globin (Imren et al., 2004; Oh et al., 2004; Pawliuk et al., 1998) and phenotypic correction of SCD has been reported in mouse models (Levasseur et al., 2003; Pawliuk et al., 2001).

Since lentiviral vectors are relatively new, the risks of insertional mutagenesis has not been studied in humans. Preliminary results from a a phase I clinical trial with a lentiviral vector for HIV gene therapy has so far shown no adverse effects. However, several studies have reported the propensity of lentiviral vectors to integrate into genes (Imren et al., 2004; Schroder et al., 2002; Wu et al., 2003). Genomic sequencing of vector containing fragments from $CD34^+$ cells transduced with a lentiviral vector expressing anti-sickling β-globin showed that 86% of proviral integration occurred in genes (Imren

et al., 2004). Thus the potential for insertional mutagenesis and safety issues with lentiviral vectors remains to be addressed.

Adeno-associated viral vectors are currently in Phase I/II clinical trials for several human diseases and AAV virus has not been implicated in any human disease. As a result, AAV vectors offer a safer alternative to the commonly used lentiviral vectors for gene therapy of hemoglobinopathies. While several groups had demonstrated erythroid restricted expression of globin from an AAV vector *in vitro* (Walsh et al., 1992) (Ponnazhagan et al., 1994) (Einerhand et al., 1995; Miller et al., 1994) (Zhou et al., 1996), Ponnazaghan and colleagues were the first to demonstrate AAV mediated expression of globin *in vivo* (Ponnazhagan et al., 1997b). Using a γ-globin gene, under the control of the β-globin gene and the HS2 enhancer, they documented a transduction efficiency of 8% and transgene expression of approximately 4% at an MOI of 1 and 6% at an MOI of 10. However, the level of expression was not very high because these experiments were carried out using low density bone marrow cells (LDBM) and not enriched hematopoietic stem/progenitor cells. Using an AAV vector containing the human β-globin gene under the control of the β-globin promoter with a mini-LCR cassette consisting of HS2, HS3 and HS4, and enriched murine HSCs, Tan *et al.* reported long-term, erythroid specific expression of a human β-globin gene in hematopoietic cells from β-thalassemic mice (Tan et al., 2001b). The group showed that the transduced globin gene sequences were present at one copy number per cell. While the proviral sequences were present in all lineages as determined by PCR analysis, expression was limited to the erythroid cells as determined by RT-PCR. In addition, expression level was up to 35% of the endogenous murine globin.

M. Specific Aims

The specific aims of the work proposed herein are:

1) Determine whether transduction of murine hematopoietic/progenitor cells can be improved by: (a) Use of additional AAV serotypes 1, 7, 8 and 10; (b) Self-complementary, double-stranded AAV-EGFP vectors; and (c) Erythroid cell-restricted promoters such the eukaryotic β-globin- and parvovirus B19p6-promoters

To test this hypothesis, hematopoietic stem/progenitor cells were infected with self-complementary AAV serotype vectors *ex vivo*, and either cultured in liquid cultures *in vitro* or transplanted into lethally-irradiated congeneic recipients *in vivo*. EGFP transgene expression was determined by flow cytometry 7-days post-transduction for *in vitro*, or 6-months post-transduction for *in vivo*.

2) Determine whether (a) AAV integrates in bone marrow cells and (b) whether AAV-mediated transduction results in any overt toxicity

(a) DNA from mice transplanted with mock-infected or vector-infected hematopoietic stem/progenitor cells were isolated and digested by restriction enzymes that either do not cleave, or cleave 1 or 2 times within the expression cassette. Southern blot analysis using an EGFP-specific DNA probe was performed to confirm the presence of vector DNA. (b) Peripheral blood and bone marrow cells were analyzed morphologically.

3) Determine whether AAV vectors can mediate expression of β-globin in K562 cells and mouse hematopoietic progenitor cells *in vitro* and in sickle cell disease mice *in vivo*

RNA isolated from mock-infected or vector-infected K562 cells was subjected to RT-PCR analysis with primers specific to vector transcripts. Southern blot analysis with a β-globin-specific probe was performed to quantify the level of expression. AAV-mediated β-globin protein expression in K562 cells and primary murine hematopoietic cells was quantified by flow cytometry. Blood and bone marrow cells drawn from mice transplanted with hematopoietic stem/progenitor cells that were mock-transduced or vector-transduced were analyzed by high performance liquid chromatography (HPLC) and isoelectric focusing (IEF) for globin gene expression. The results were compared to the endogenous β-globin gene expression.

MATERIALS AND METHODS

A. Cells and plasmids

The human erythroleukemia cell line K562 was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cultures were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% newborn calf serum (NCS) and 1% penicillin/ streptomycin (Sigma, St. Louis, MO).

B. AAV plasmids and scAAV serotype vectors

Plasmids pB19p6 (Zhou et al., 1994) and scAAV-CBAp-EGFP (Wang et al., 2003) have been previously described. Plasmid pB19p6EGFP was constructed by inserting the EGFP gene and the SV40pA from plasmid pEFGP-C1 (AfeI-SphI) into plasmid pB19p6 (HincII-SphI). The overall strategy to construct the recombinant AAV genomes containing the enhanced green fluorescent protein (EGFP) under the control of the parvovirus B19p6 promoter (pB19p6) and the β-globin promoter with the hypersensitive site 2 (HS2) of the locus control region (LCR), (HS2-βp), is depicted in Figure 1. Briefly, the plasmid scAAV-CBAp-EGFP was used as the vector for all the double-stranded constructs. scAAV-CBAp-EGFP plasmid was first linealized with Acc65I followed by filling in of the 5'-overhang with Klenow fragment of *Eschelichia coli* DNA polymerase I. The vector was then digested with AgeI to remove the chicken β-actin promoter. The AgeI site was filled in for construction of scAAV-HS2-βp-EGFP and dephosphorylated using Antarctic Phosphatase (New England Biolabs, Ipswich, MA). The promoter inserts were obtained as follows: plasmid pB19p6EGFP was digested with BsrBI and AgeI to isolate the pB19p6 promoter and plasmid ssHS23-βp-

β87^{+}-globin (DB3) was digested with XhoI and NcoI and both ends blunted to obtain the β-globin promoter with the HS2 enhancer. All digested fragments were run on a 0.7% agarose gel and purified using the QIAquick ^{TM} gel extraction kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. The respective plasmids were ligated using T4 ligase (New England Biolabs, Ipswich, MA) at 16ºC overnight. The ligation mixture was purified by butanol precipitation and used to transform Sure cells (Stratagene, La Jolla, CA) by electroporation at 2500 Volts, 25μF, 200 Ohms and then streaked on agar plates containing ampicillin. Several plasmids containing the respective promoters were identified rescued as described below. These plasmids were used to generate recombinant enhanced green fluorescent protein (EGFP)-AAV virions, scAAV-B19p6-EGFP and scAAV-HS2- βp-EGFP at the Vector Core, Powell Gene Therapy Center, University of Florida, Gainesville, FL.

The overall strategy for the construction of the self-complementary AAV genomes containing the β-globin gene under the control of the β-globin promoter [scAAV2-βp-β87^{+}-globin (Figure 7)], β-globin promoter with the hypersensitivity site 2 (HS2) of the locus control region [scAAV2-HS2-βp-β87^{+}-globin (Figure 8A)], and the parvovirus B19p6 promoter [scAAV2-B19p6-β87^{+}-globin (Figure 8B)] is as follows. Plasmid HPV 436 was kindly provided by Philippe Leboulch (Harvard University). Plasmid DB3 (Figure 2) was constructed by inserting the globin gene and the HS2 and HS3 enhancer elements from HPV 436 (XbaI-XhoI) into pXS65/D10 (EcoRV). A two-step cloning procedure was employed when cloning plasmid ds-βp-β87^{+}-globin. First, plasmid PBSK^{+}-β87^{+}-globin-I (Figure 3A) was generated by cloning the first ½ of globin gene (globin-1) from DB3 into plasmid pBluescript SK+ (Stratagene, La Jolla, CA)

following digestion of both plasmid with restriction enzymes EcoRI and BamHI. Secondly, plasmid PBSK+βp-β87$^+$-globin I+2 (Figure 3B), which contained the β-globin promoter and the proximal ½ of the globin gene was cloned by PCR amplification from DB3 using the following pair of primers: forward primer; 5'-GCACTGGTGGGGTGAATTCTTTGCCAA- 3' and reverse primer; 5'-CCATCGATACCACCTGACTAAAACTCC- 3'. A ClaI site was created in the PCR product using the reverse primer. The PCR product was cloned into plasmid PBSK+-β87$^+$-globin-I following digestion of both plasmids with restriction enzymes ClaI and EcoRI. Plasmid scAAV-βp-β87$^+$-globin which contained the entire the β-globin promoter and β-globin gene was generated by cloning the Acc65I and XbaI fragments from plasmid PBSK+-βp-β87$^+$-globin I+2 into plasmid scAAV-CBAp-EGFP (Acc65I+ BbsI). The XbaI and BbsI sites were filled in with Klenow prior to digestion with Acc65I, allowing for a sticky end ligation at this site. For the construction of ds-HS2-βp-β87$^+$-globin, the HS2 enhancer and the β-globin promoter (HS2βp) were cloned from DB3 into scAAV-βp-β87$^+$-globin following digestion of both plasmids with restriction enzymes XhoI and NcoI. Plasmid scAAV-B19p6-β87$^+$-globin (Figure 8B) was constructed following ligation of the B19p6 promoter (EcoRI [filled in] + NcoI) from pB19p6EGFP into scAAV-βp-β87$^+$-globin (HincII + NcoI). Plasmid PBSK+ HS234-βp-β87$^+$-globin (Figure 4) was constructed by inserting the entire LCR cassette and βp (HS234βp) into plasmid PBSK+-βp-β87$^+$-globin I+2 following digestion of both plasmids with restriction enzymes Acc65I and NcoI. Plasmid ssAAV-HS234-βp-β87$^+$-globin (Figure 5) was constructed by inserting the Acc65I and XbaI fragment of plasmid PBSK+-HS234-βp-β87$^+$-globin into the ITR containing XbaI fragment of plasmid

psub201. Plasmids scAAV-HS234 and scAAV-HS432 (Figure 6) were constructed by inserting the HS234 cassette (Acc65I and AvrII) from plasmid HPV 436 into scAAV-CBAp-EGFP (Acc65I+ BbsI). All restriction sites were filled in which allowed the construction of scAAV-LCR into 2 different orientations. scAAV-HS234 is in closed-to-open orientation while scAAV-HS432 is in open-to-closed orientation. These plasmids were rescued and used to generate self-complementary and conventional AAV $\beta 87^{+}$-globin virions - scAAV-βp-$\beta 87^{+}$-globin, scAAV-HS2-βp-$\beta 87^{+}$-globin, scAAV-B19p6-$\beta 87^{+}$-globin, ssAAV-HS234-βp-$\beta 87^{+}$-globin, scAAV-HS234 and scAAV-HS432 in several serotypes (AAV1, AAV2 and AAV7) at the University of Florida vector core, Gainesville, FL. Plasmid phelper (Stratagene, La Jolla, CA) containing adenovirus genes (E2A, E4 and VA RNAs) and AAV serotype plasmids ACG2 (Li et al., 1997), AAV1, AAV7, AAV8 and AAV10 containing AAV2 Rep and the respective serotype Cap protein were used along with the recombinant AAV plasmids to produce rAAV virions.

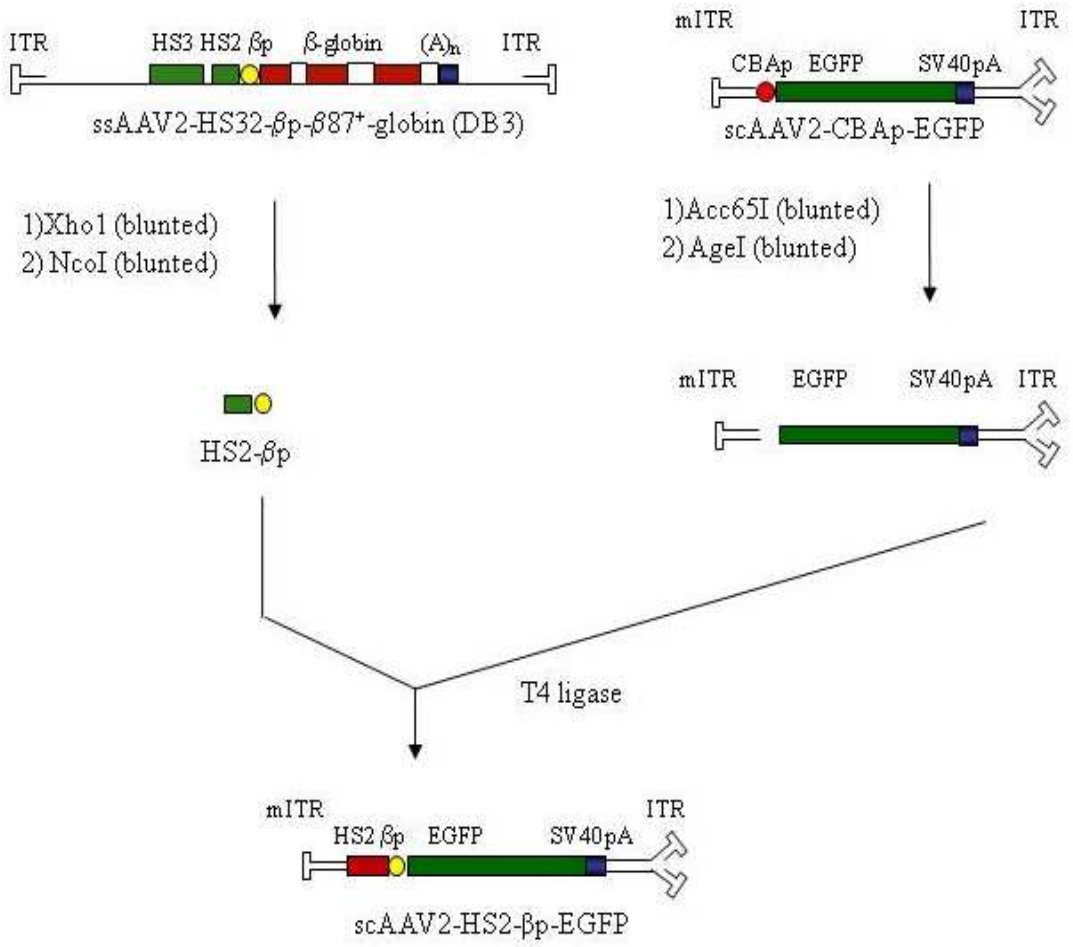

Figure 1A: Strategy for the construction of scAAV2-HS2-βp-EGFP. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; EGFP, enhanced green fluorescent protein; SV40pA, simian virus 40 polyadenylation signal; HS32, DNA-hypersensitive sites 3 and 2 enhancer, HS2, DNA-hypersensitive site 2 enhancer; βp, β-globin promoter; CBAp, chicken beta-actin promoter; scAAV, self complementary AAV vector.

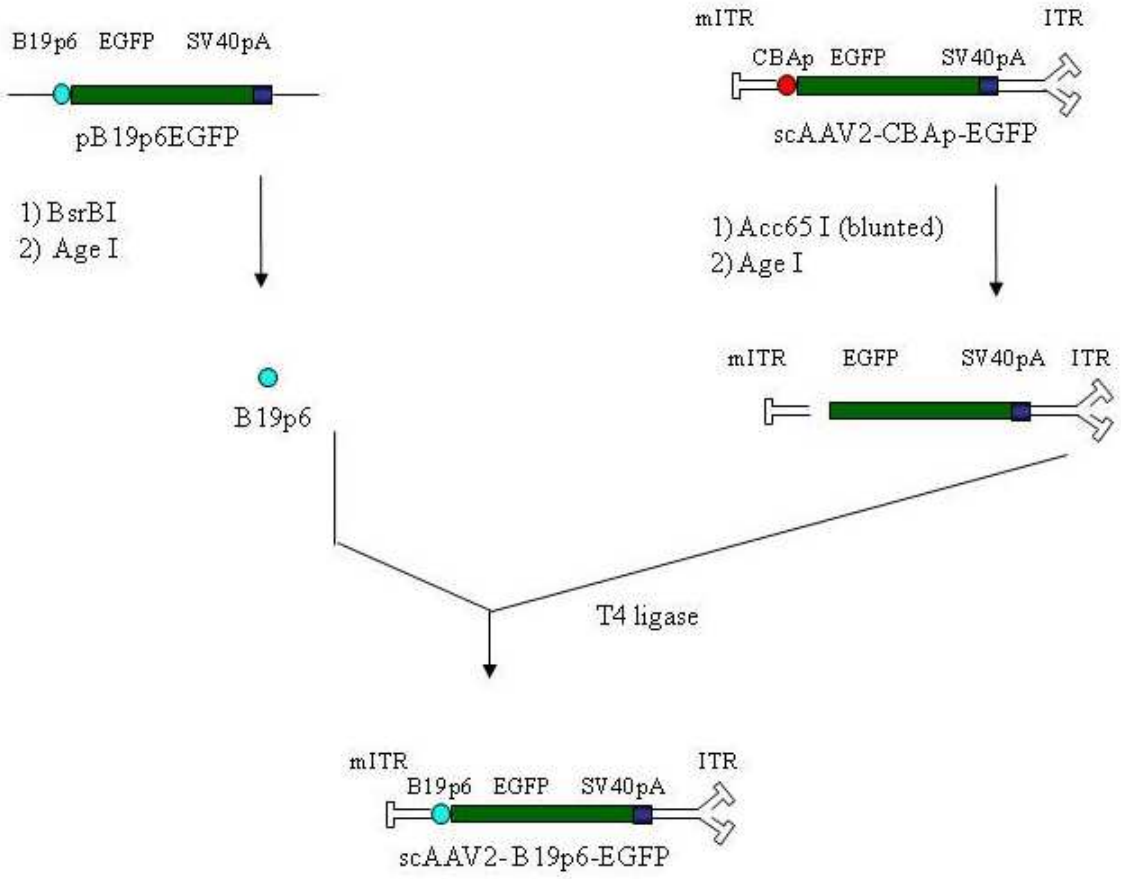

Figure 1B: Strategy for the construction of scAAV2-B19p6-EGFP. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; EGFP, enhanced green fluorescent protein; SV40pA, simian virus 40 polyadenylation signal; pB19p6, parvovirus B19 p6 promoter; CBAp, chicken beta-actin promoter; scAAV, self complementary AAV vector.

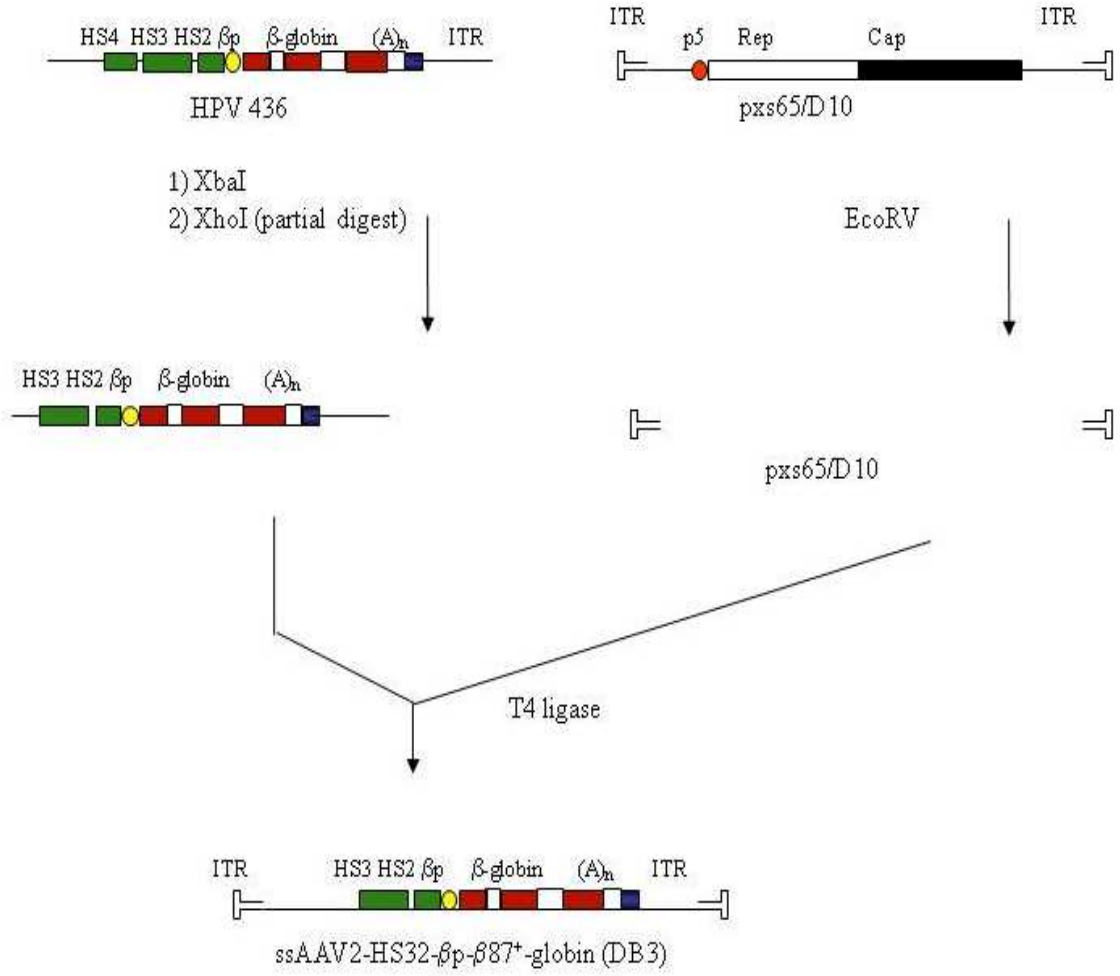

Figure 2: Strategy for the construction of ssAAV2-HS32-βp-β87^{+}-globin vector (DB3). ITR, inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; HS4, DNA-hypersensitive site 4 enhancer; HS3, DNA-hypersensitive site 3 enhancer; HS2, DNA-hypersensitive site 2 enhancer; βp, β-globin promoter; p5, wt-AAV2 promoter.

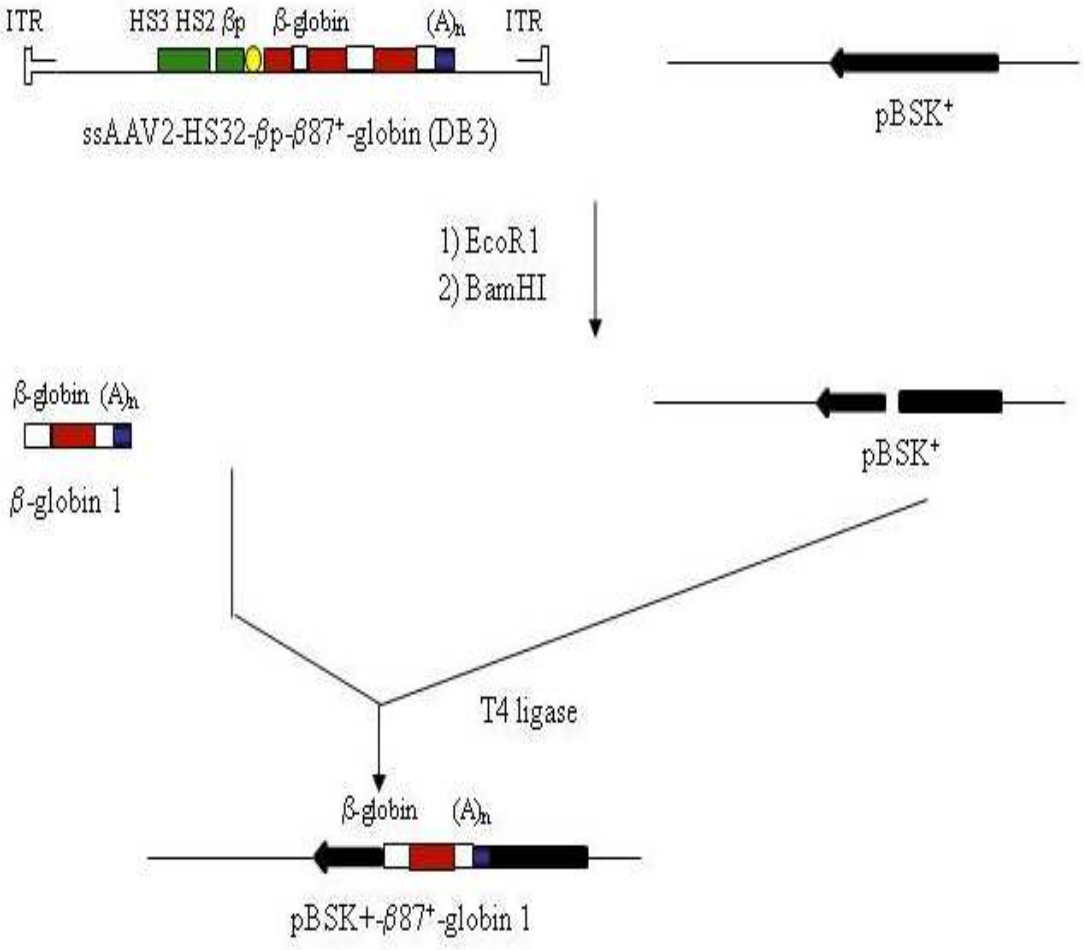

Figure 3A: Strategy for the construction of pBluescript SK^+-β87^+-globin 1 vector. ITR, inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; HS32, DNA-hypersensitive site 3 and 2 enhancers; βp, β-globin promoter; $pBSK^+$, pBluescript SK^+ plasmid; mcs, multiple cloning sites.

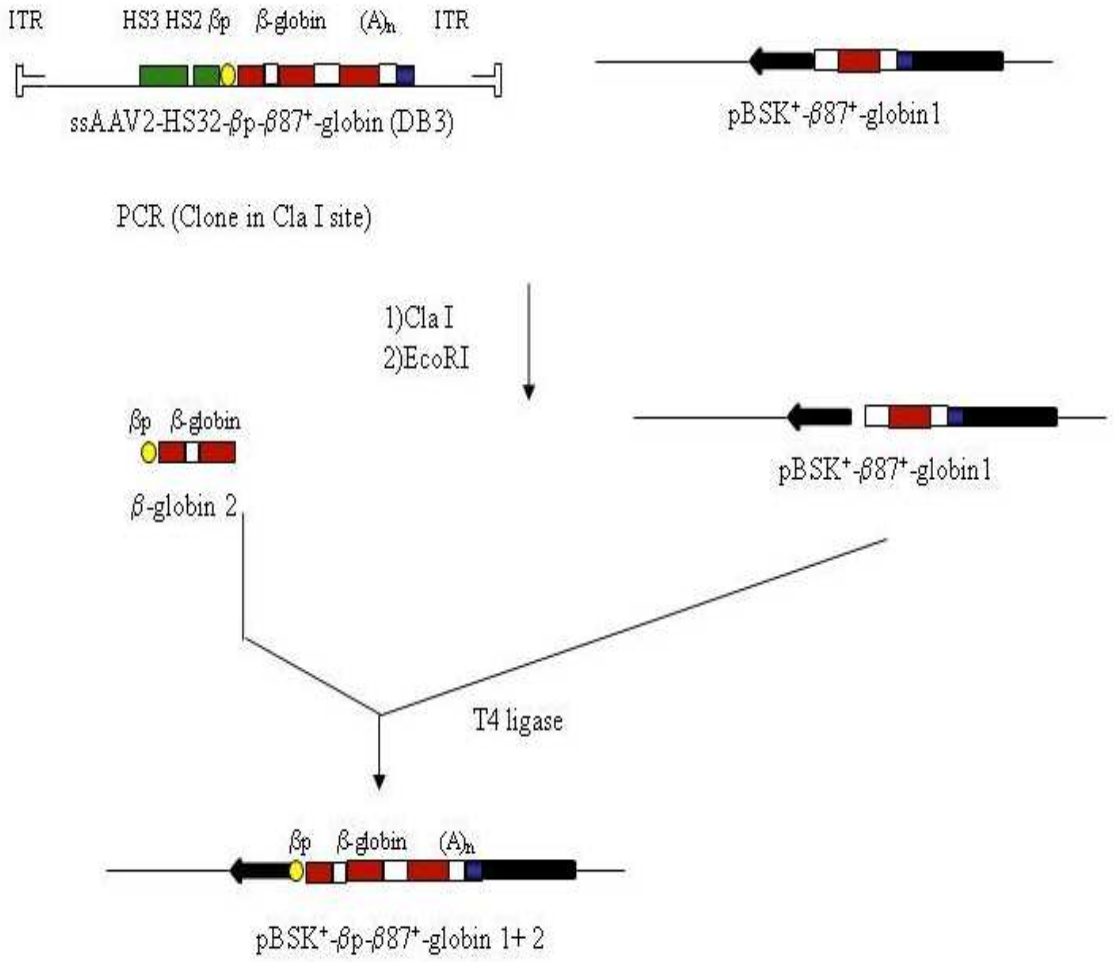

Figure 3B: Strategy for the construction of pBluescript SK^+-βp-$β87^+$-globin 1+2 vector. ITR, inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; HS32, DNA-hypersensitive site 3 and 2 enhancers; βp, β-globin promoter; $pBSK^+$, pBluescript SK^+ plasmid; mcs, multiple cloning sites.

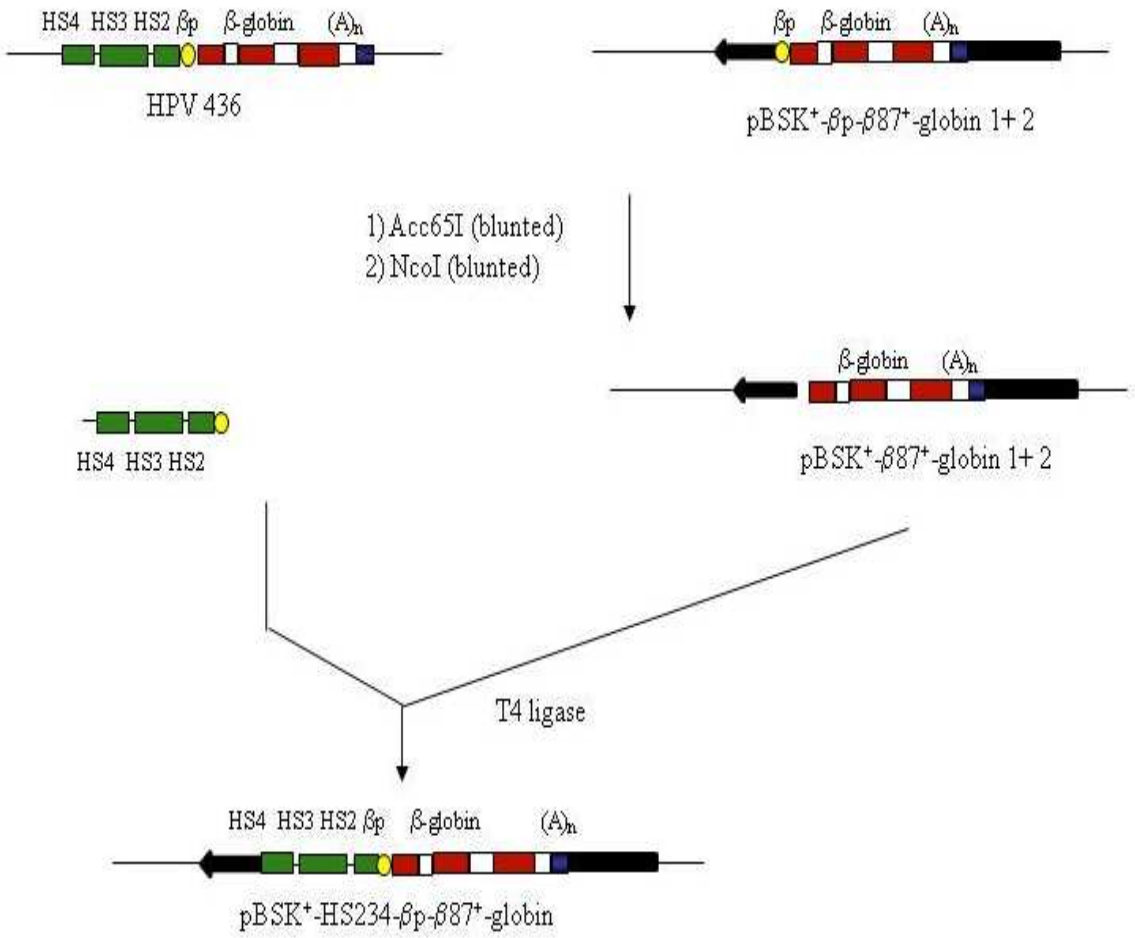

Figure 4: Strategy for the construction of pBluescript SK^+- HS432-βp-$β87^+$-globin vector. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; scAAV, self-complementary AAV; HS2, DNA-hypersensitive site 2 enhancer; HS3, DNA-hypersensitive site 3 enhancer; HS4, DNA-hypersensitive site 4 enhancer.

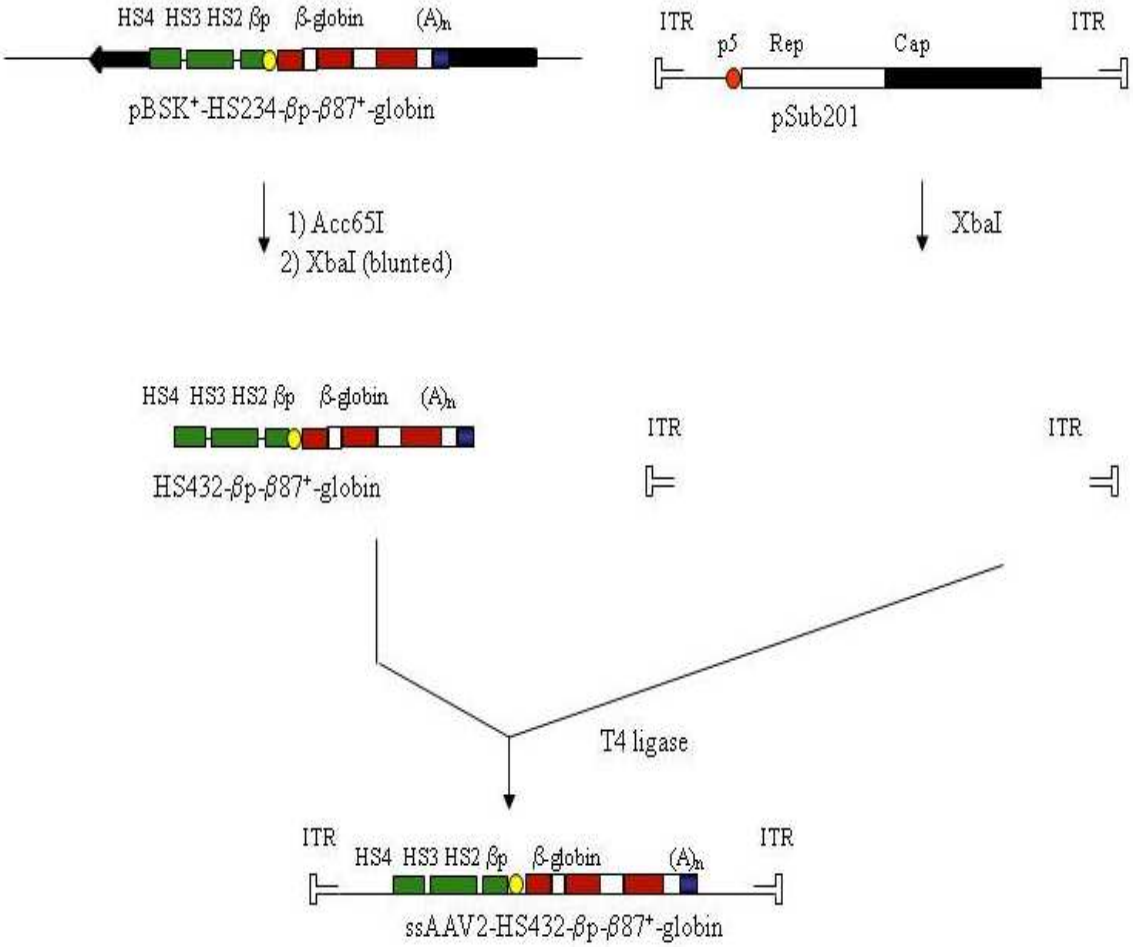

Figure 5: Strategy for the construction of ssAAV-HS234-βp-β87^{+}-globin vector. ITR, inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; HS2, DNA-hypersensitive site 2 enhancer; HS3, DNA-hypersensitive site 3 enhancer; HS4, DNA-hypersensitive site 4 enhancer; p5, wt-AAV2 promoter.

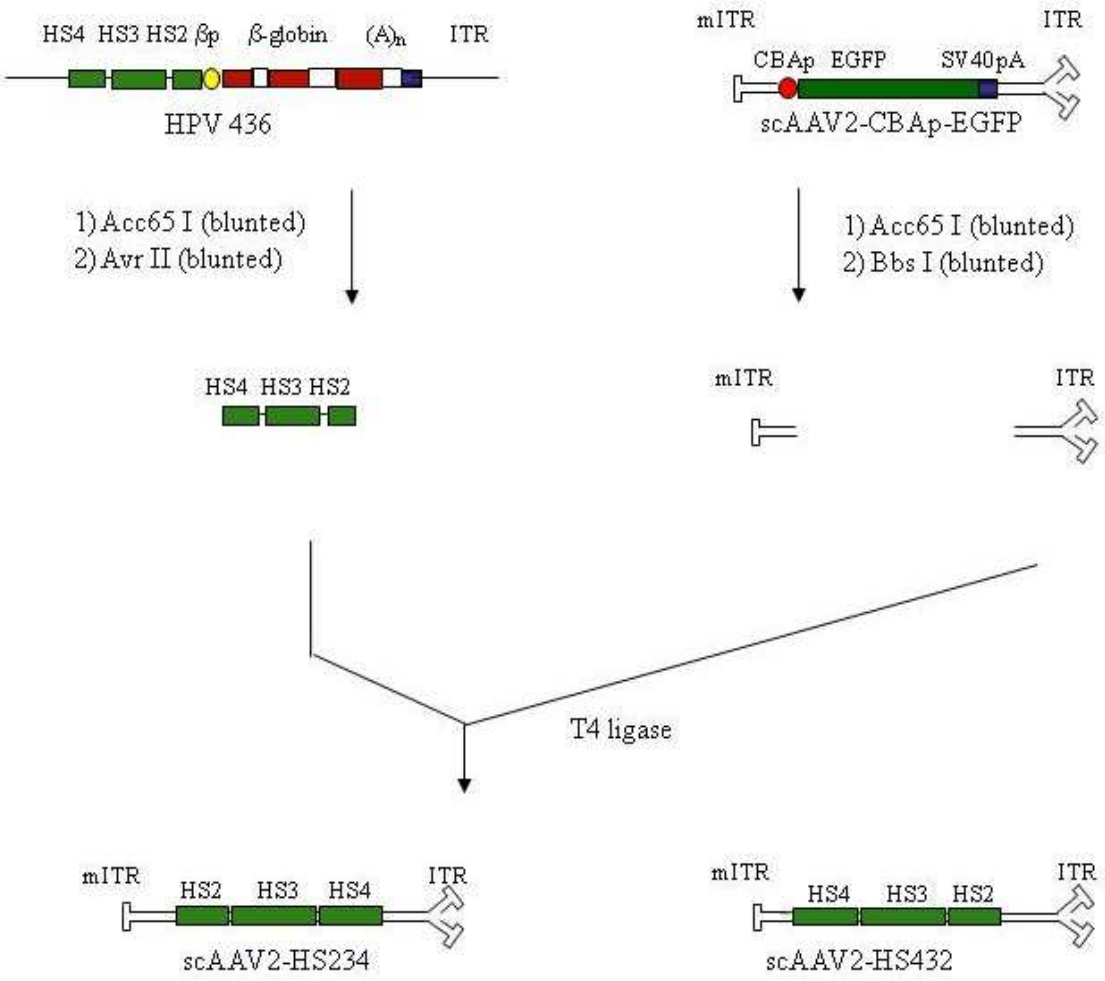

Figure 6: Strategy for the construction of scAAV2-HS234 and scAAV2-HS432 vectors. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; CBAp, chicken beta-actin promoter; EGFP, enhanced green fluorescent protein; SV40pA, simian virus 40 polyadenylation signal; scAAV2, self-complementary AAV2; HS2, DNA-hypersensitive site 2 enhancer; HS3, DNA-hypersensitive site 3 enhancer; HS4, DNA-hypersensitive site 4 enhancer.

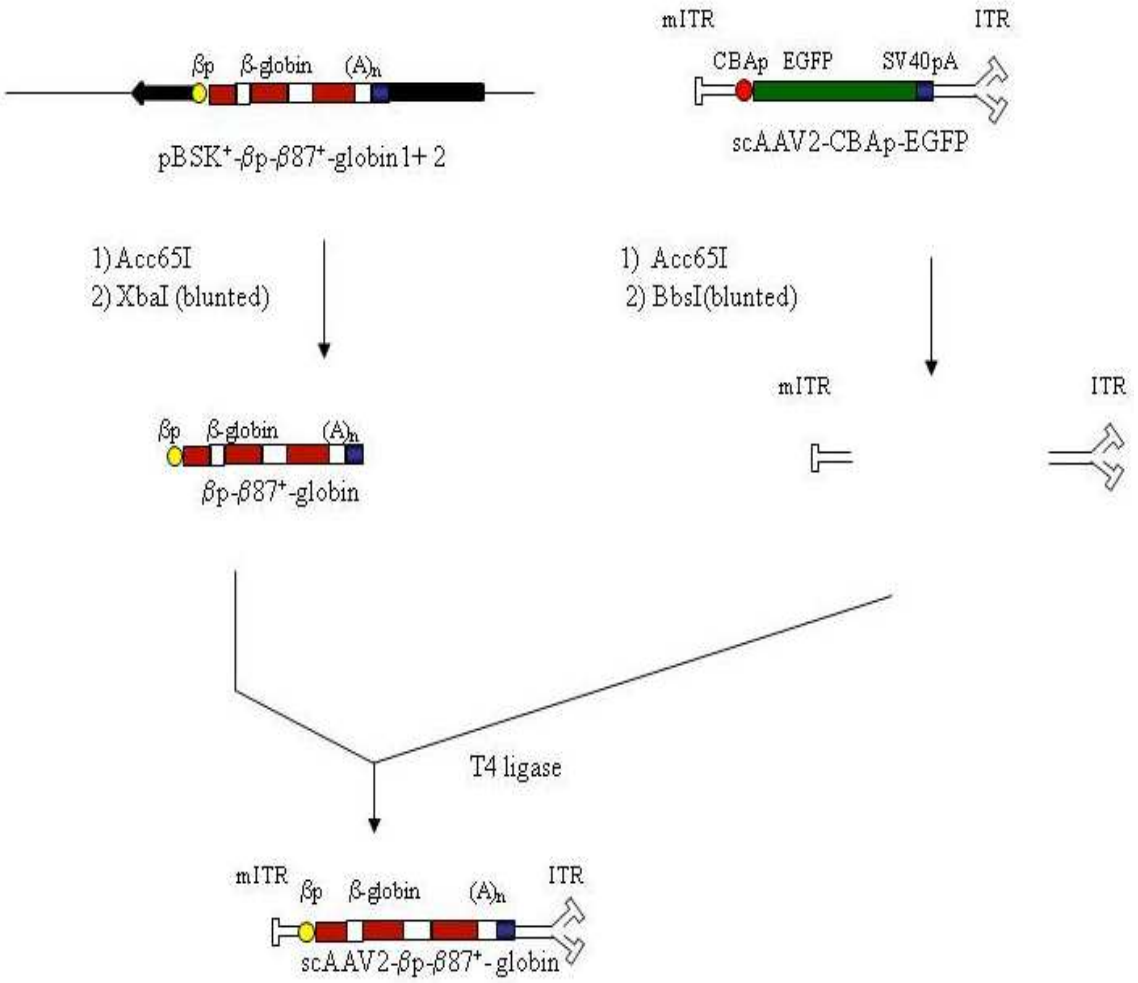

Figure 7: Strategy for the construction of scAAV-βp-β87^{+}-globin vector. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; CBAp, chicken beta-actin promoter; SV40pA, simian virus 40 polyadenylation signal; scAAV, self-complementary AAV vector.

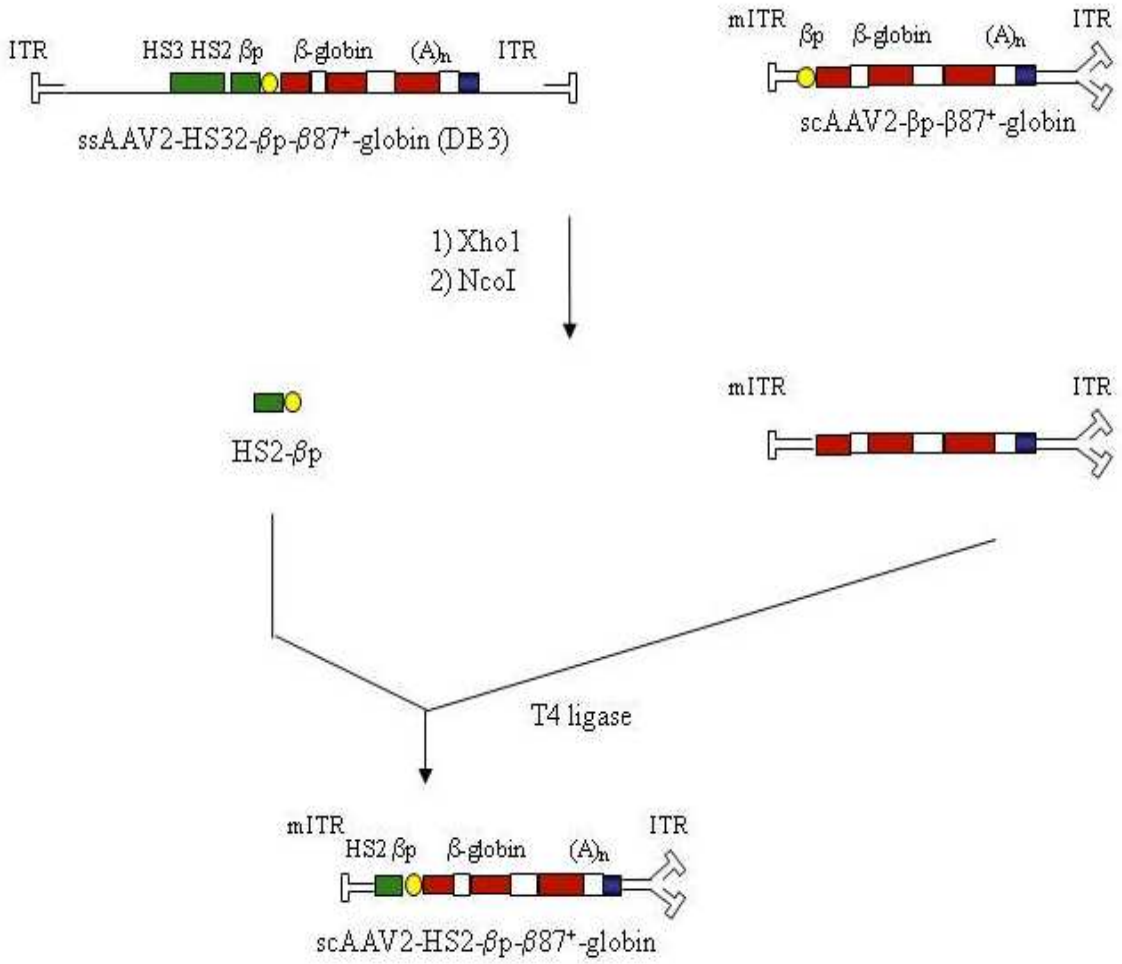

Figure 8A: Strategy for the construction of scAAV-HS2-βp-β87^{+}-globin vector. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; SV40pA, simian virus 40 polyadenylation signal; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; scAAV, self complementary AAV vector; HS32, DNA-hypersensitive site 3 and 2 enhancers; HS2, DNA-hypersensitive site 2 enhancer.

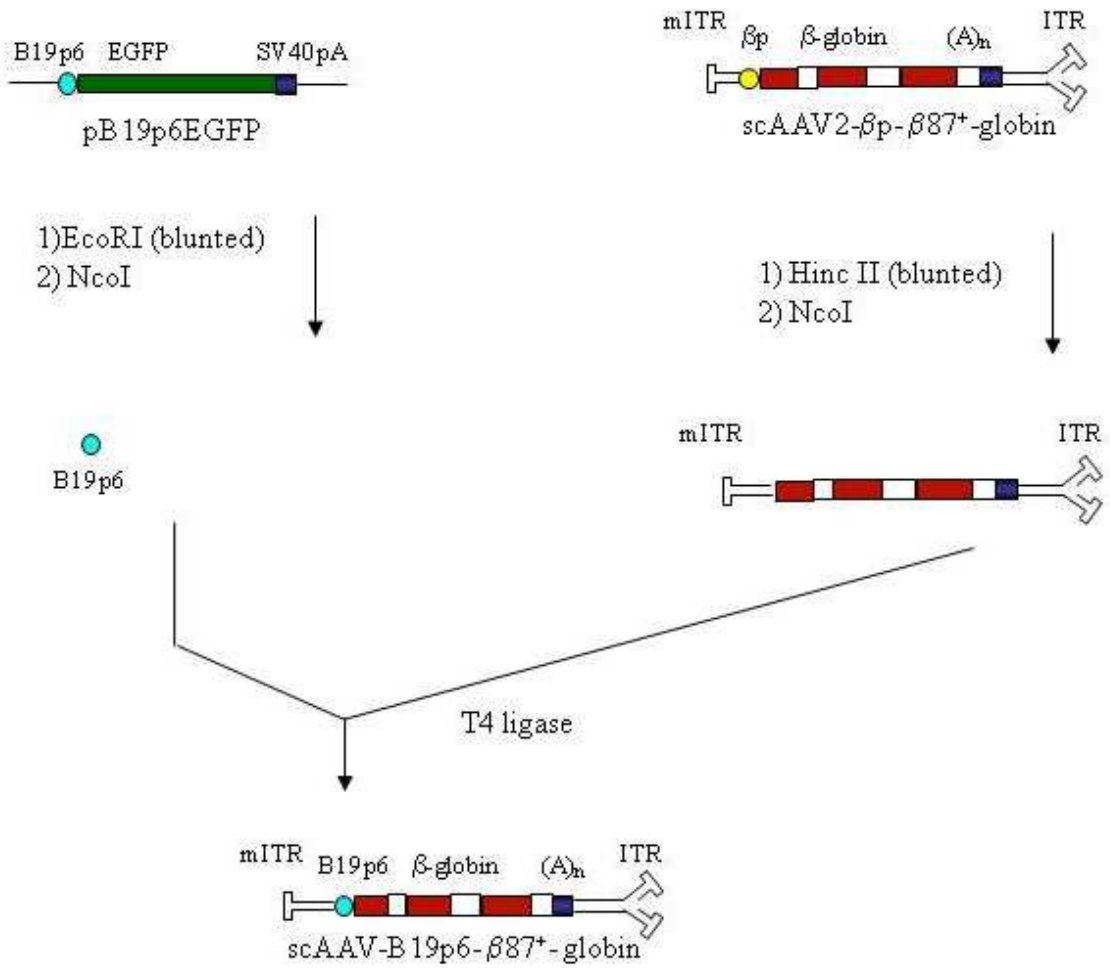

Figure 8B: Strategy for the construction of scAAV-B19p6-β87^{+}-globin vector. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; SV40pA, simian virus 40 polyadenylation signal; $(A)_n$, β-globin polyadenylation signal; βp, β-globin promoter; scAAV, self complementary AAV vector; HS32, DNA-hypersensitive site 3 and 2 enhancers; HS2, DNA-hypersensitive site 2 enhancer.

C. Large scale plasmid DNA preparations

Large scale DNA was prepared by alkaline lysis method and purified using CsCl-ethidium bromide density gradients (Sambrook J et al., 2001).

D. Preparation and purification of recombinant AAV vectors

AAV serotype vectors were packaged as previously described (Zolotukhin et al., 2002). One day before transfection, confluent 293 cells in a Nunc cell factory system [Cat # 164327] (Nunc, Rochester, NY) were washed once with 500 ml of sterile phosphate buffered saline (PBS) and treated with 500 ml of PBS containing 5mM EDTA for about 3-5 minutes at room temperature with gentle shaking to detach the cells from the plate surface. The dissociated cells were poured into a sterile 1L bottle containing approximately 500 ml of DMEM supplemented with 5% fetal calf serum and antibiotics (complete-DMEM). 5 x 10^8-1 x 10^9 cells were seeded in a Nunc cell factory. The DNA/$CaCl_2$ precipitate was formed by addition of the molar amounts of the rAAV plasmid, phelper and the respective serotype plasmid (in a total plasmid concentration of 2.5 mg/ cell factory) to 50 ml of 0.25M $CaCl_2$. 50 ml of 2x HBS buffer (1.5 mM Na_2HPO_4, 280mM NaCl, 50mM HEPES, pH 7.05) was added to the DNA/$CaCl_2$ precipitate and incubated at RT for 1-2 minutes followed by addition of 1100 ml of complete-DMEM. The conditioned medium in the cell factory was exchanged with the $CaPO_4$ precipitate-containing medium followed by incubation of the cells for 60-72 hrs at 37ºC in 5% CO_2. At the end of the incubation, the cells were harvested as follows. The conditioned media was discarded and the cells washed in 500 ml of PBS. The cells were dissociated from the plate by addition of 500 ml of PBS containing 5 mM EDTA for about 3-5 minutes at room temperature with gentle shaking, to detach the cells from the plate surface. The dissociated cells were poured into a sterile 500 ml centrifuge tube (Corning, Cat # 431123) and centrifuged at 2000 rpm for 10 min in a Beckman GPKR centrifuge (GH.7 rotor). The cell pellet was resuspended in 60 ml of lysis buffer (150

mM NaCl, 50 mM Tris, pH 8.5) and aliquoted into 4 sterile 50 ml conical polypropylene centrifuge tubes (Sarstedt) and subjected to 3 cycles of freezing in dry-ice-ethanol bath for 10 min and thawing in a 37^{o}C water bath for 10 min, with vortexing after each thaw. The cell lysate was subjected to benzonase digestion using 1.16 µl of benzonase (Sigma, St.Louis, and MO) and 3 µl of 4.82 M $MgCl_2$ per 15 ml of cell lysate and incubated for 30 min at 37^{o}C. The cell lysate was cleared by centrifugation at 3700 rpm for 20 min at 4^{o}C and transferred into Beckman ultracentrifuge tubes (Cat # 342414). The virus was purified by discontinuous iodixanol step gradients prepared as described here. Iodixanol gradients of 40%, 25% and 15% were made from the 60% (w/v) OptiPrep (Nycomed) stock solution diluted in PBS-MK buffer (1X PBS, 1 mM $MgCl_2$, 2.5 mM KCl). 1M NaCl was also added to the 15% density solution. The discontinuous iodixanol step gradients was made from the bottom to top with 5 ml of 60%, 7.5 ml of 40%, 5 ml of 25%, 7.5 ml of 15% iodixanol and 15 ml of cell lysate and centrifuged in a Beckman 70 Ti rotor at 69,000 rpm for 1hr at 18^{o}C. The virus-containing 40-60% interface was collected using an 18-gauge needle connected to a syringe and subjected to further purification by column chromatography. For AAV2 purification, 5 ml HiTrap SP column (Amersham Biosciences) was equilibrated using a Pharmacia P-1 peristalsis pump (Pharmacia) with 12 ml buffer A (0.3X PBS), 25 ml buffer B (0.3X PBS + 1 M NaCl) and 50 ml buffer A at a flow rate of 5 ml/min. The virus/iodixanol fraction was diluted in buffer A at 1:1 ratio, loaded onto the column, and washed with 50 ml of buffer A. The virus was then eluted in 20 ml of Buffer C (0.3X PBS, 350 mM NaCl) and buffer exchanged with 10 ml of Lactated Ringers (Abbott Laboratories) in a 20 ml Apollo centrifugal spin concentrator (Cat # AP2015010) and centrifuged at 3000 rpm at 6 min. The sample was

buffer exchanged a second time using 25 ml of Lactated Ringers and concentrated to a final volume of 0.5 ml. For AAV serotype 1, 7, 8 and 10, a 5 ml HiTrap Q column (Amersham Biosciences) was equilibrated in buffer A (20 mM Tris, 15 mM NaCl, pH 8.5), buffer B (20 mM Tris, 1M NaCl, pH 8.5) and buffer A. The sample was eluted in buffer C (20 mM Tris, 350 mM NaCl, pH 8.5) buffer exchanged in LR and concentrated to 0.5 ml as described for AAV2 above. 100 µl virus-containing aliquots were distributed into siliconized/low retention microcentrifuge tubes (Fisher Scientific) and stored in -80^{o}C.

E. Quantitative DNA slot blot analysis

Viral titers were quantified by DNA slot blot analysis as previously described (Kube et al., 1997; Samulski et al., 1989; Srivastava et al., 1989). Briefly, 1µl and 10 µl of AAV particles and a 1 µg and 10 µg of a plasmid standard, containing the packaged transgene were disrupted and denatured in 100mM NaOH at 65ºC for 30-60 min in a total volume of 100 µl. The samples and standards were then chilled on ice for 5 min and pulsed down prior to the addition of 100µl of 20X SSC (3M NaCl, 0.3 M sodium citrate, pH 7.0) and 2µl of loading dye. The sample was mixed and loaded onto Immobilon NY+^{TM} membranes (Millipore, Bedford, MA) using a Bio Dot SF vacuum manifold (Bio-Rad, Hercules, CA) in a two-fold serial dilution. The membranes were cross-linked with UV Stratalinker (Stratagene, La Jolla, CA) and prehybridized for 2hrs in a roller bottle containing 20 ml hybridization solution containing 6x SSC, 0.5% sodium dodecyl sulphate (SDS), 100 µg/ml freshly boiled herring sperm DNA and 5x Denhardt's reagent [1% ficoll, type 400 (Pharmacia), 1% polyvinypyrrolidone (Kodak), 1% bovine serum albumin (BSA) (Sigma)]. To hybridize the membrane, a ^{32}P-labeled DNA probe was

boiled for 5 min, and chilled for 5 min before being added to the hybridization solution, and incubated for 16-20 hrs at 68ºC. The membrane was then washed in 2x SSC, 0.1% SDS for 5 min at room temperature followed by three 15 min wash cycles in 2L of 0.1x SSC, 0.1% SDS at 65ºC prior to being exposed to BIOMAX MR ™ X-ray films (Kodak, Rochester, NY) at -70ºC. The films were developed and viral titers computed.

F. ^{32}P-labeling of DNA probes

DNA probes were synthesized by random priming as described by Feinberg and Vogelstein (Feinberg et al., 1983). The 50-100 ng of DNA fragment was purified from a 0.7% agarose gel using the QIAquick™ gel extraction kit (Qiagen, Valencia, CA) as per manufacturer's instructions and resuspended in 30 µl of ddH2O. To denature the template, approximately 10 µl of oligonucleotide buffer [1M HEPES,pH 6.6 250 mM Tris-HCl, pH 8.0, 52 mM β-mercaptoethanol, 0.1mM dATP, 0.1mM dTTP, 0.1 mM dGTP, 27 U/ml of random hexanucleotides] (Roche, Indianapolis, IN) was added to the DNA, and the mixture was heated for 5 min at 100°C and then rapidly chilled on ice for 5 min. 1 µl of BSA, 8 units of klenow (Promega, Madison, Wi) and 5 µl of α ^{32}P-dCTP (10 µCi/ µl) (Amersham, Piscataway, NJ) were added to the reaction mixture and then incubated at 37ºC for 2 hr. Unincorporated α ^{32}P-dCTP was removed from the radiolabeled-DNA fragments by spun-column chromatography through a Microspin™ G-50 Columns (Amersham, Piscataway, NJ). Cerenkov radiation was detected in 2 µl of probe using a liquid scintillation spectrometer.

G. Animal handling

All animal experiments were performed according to the guidelines for animal care at the University of Florida Animal Care Services (ACS). Six weeks old C57BL/6J female recipient mice (stock number 000664), 6-weeks old B6.SJL-Ptprc (a) Pep3 (b)/BoyJ donor male mice (stock number 002014) and 6-week *Hba*^{tm1Paz} *Hbb*^{tm1Tow} Tg(HBA-HBBs) 41Paz/J (stock number 003342) donor female mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility of the University of Florida College of Medicine, Gainesville, FL. The Institutional Animal Care and Use Committee approved all protocols for the care and handling of mice.

H. Isolation of murine Scal^{+}, c^{-}Kit^{+}, lin^{-} cells

Following inhalation of anesthesia, donor mice were sacrificed by cervical dislocation and the femur and tibia removed using a pair of scissors. Muscles were removed from the bones using a pair of scissors and paper towels. A small cut was made at the distal aspects of the femur and tibia and bone marrow collected into sterile 15 ml conical tubes (Sarstedt) by flushing with 1 ml of PBS using a ½ cc U-100 insulin syringe (Becton Dickinson). The cells were pelleted at 1100 rpm for 5 min at 4ºC, followed by lyses of red blood cells (RBC) using 0.5 ml of ACK lysis buffer (0.15 M NH4Cl, 10 mM $KHCO_3$, 0.1mM Na2EDTA, pH 7.2-7.4) per animal at room temp for 5 min. PBS was immediately added to a final volume of 14 ml, and cells were centrifuged at 1,100 rpm for 5 min at 4ºC and counted. Cells were resuspended in PBS containing 10% fetal bovine serum (FBS) at a cell count of 5 x 10^7 cells/ml. Lineage antibody cocktail (specific for antigens expressed by erythroid, myeloid, and lymphoid lineages) was added at a dilution of 1:10 and cells incubated on ice for 20 min. Cells were washed using 10

ml PBS+10% FBS and centrifuged at 1,100 rpm for 5 min at 4ºC. Cells were resuspended in 1 ml PBS+10%FBS. Lineage-positive cells were separated from lineage-negative by immuno-magnetic beads (sheep anti-rat specific; Dynal, Great Neck, NY) columns. The depletion process was repeated, and lineage-negative cells were resuspended at a cell count of 2 x 10^6 cells/100µl of PBS+10% FBS. Phycoerythrin-conjugated anti-Sca-1 (Ly-6A/E), and allophycocyanin-conjugate (APC) anti-c-kit (BD Bioscience) was added at 1:200 dilutions and incubated on ice for 20 min. Cells were washed and suspended in 2 ml PBS+10% FBS, filtered through nylon filter, and sorted using a Becton Dickinson Vantage Fluorescence-Activated Cell Sorter (FACS). Sca-1^+, c-kit^+, lin^- cells were collected in PBS+10% FBS.

I. Analysis of AAV2 receptor and co-receptor expression

Primary murine HSCs were prepared as described above and sent for sorting by FACS at the Indiana University Flow Cytometry core. $Sca1^+$, c-kit^+, lin^- cells were then stained with HSPG-phycoerythrin conjugated antibody (HSPG-PE) and Fc-FGF antibody for 20 min and washed 3x with PBS/10% FBS. Secondary staining with an anti-Fc-APC antibody was then performed. Cells were washed 3x in PBS/10% FBS and analyzed by flow cytometry using a FACS caliber machine. 10^5 events were recorded.

J. Recombinant AAV transduction assays

Equivalent numbers of K562 cells were washed once in serum-free IMDM and either mock infected or infected with rAAV vectors containing the EGFP gene or the anti-sickling β-globin gene ($\beta87^+$) with 2 x 10^4 particles/cell at 37ºC for 2 hrs (Ponnazhagan et al., 1994). The cells were incubated for 72 hrs following the addition of

complete-IMDM. Transgene expression was evaluated by flow cytometry and reverse-transcription PCR (RT-PCR) as described below.

For 7-day transduction assays, mouse bone marrow c-kit^{+}, lin^{-} hematopoietic cells were obtained as described above and infected with 5 x 10^4 particles/cell at 37ºC for 2 hrs. The cells were incubated for 7 days following the addition of complete IMDM supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml), stem cell factor (SCF) (1ng/ml) and erythropoietin (EPO) (5U/ml) and transgene expression was evaluated by flow cytometry using a FACS caliber machine.

For long term transduction assays, approximately 10,000 double positive (Sca1^{+}, c-kit^{+}, lin^{-}) or 20,000- 50,000 single positive (c-kit^{+}, lin^{-}) cells per mouse were infected with the recombinant AAV vector at 5 x 10^4 -1 x 10^5 particles/cell in serum-free IMDM at 37ºC for 2 hrs with mixing every 15 min. The cells were washed in PBS and injected into the retro-orbital sinus of lethally irradiated C57/BL/6J (CD45.2) mice. 5-6 mice were used for each virus and transgene expression evaluated 3-6 months post transduction.

K. Bone marrow transplantations

Six weeks-old recipient mice were irradiated with 950 cGy from a cesium^{137} source and transferred to clean cages. The recipient mice were anesthetized with isofluorane and injected retro-orbitally with mock-transduced or vector-transduced cells suspended in 100 µl PBS. The mice were maintained on water treated with trimethoprim/sulfasoxazole for 2 weeks after transplantation.

L. Fluorescence-activated cell sorter (FACS) analysis

Mock and EGFP- vector transduced K562 cells were washed in PBS and analyzed on a FACsort machine (BD Biosciences, San Jose, CA) at the University of Florida Flow Cytometry Core. 10^5 events were recorded. For analysis of β-globin expression, mock and ($\beta 87^+$) vector-transduced K562 cells and mouse bone marrow c-kit$^+$, lin$^-$ hematopoietic cells were fixed with 4% paraformaldehyde, washed in PBS and permeabilized with 100% ice-cold methanol as per manufacturer's instructions (Santa Cruz Biotechnology, Inc, CA). The cells were washed in PBS and stained intracellularly with PE-conjugated mouse anti-human β-globin antibody (Santa Cruz Biotechnology, Inc, CA) at 1:100 dilution for 10^6 cells in 100 µl of FCM wash buffer. Human β-globin protein was detected by flow cytometry on a FACsort machine (BD Biosciences, San Jose, CA) at the University of Florida Flow Cytometry Core. 10^5 events were recorded.

For multilineage analysis of scAAV mediated EGFP expression, bone marrow cells from mice 3-6 months post-transplantation were harvested as described above and equal aliquots stained separately using phycoerythrin (PE)-conjugated lineage specific rat anti-mouse mAbs. Rat anti-mouse B220 was used for B lymphocytes; anti-CD4 &anti-CD8a for T lymphocytes and anti-Gr-1 for myeloid cells. For analysis of erythroid cell lineage, dual staining with rat anti-mouse c-kit$^+$-PE and CD71- PE-Cy5 for early erythroblasts, and CD71- PE-Cy5 +Ter119-PE for late erythroblasts and mature RBCs was performed. Cells were incubated on ice for 20 min, washed in PBS/10% FBS and analyzed on a FACsort machine. 1-3 x 10^5 events were recorded.

M. Southern blot analysis for AAV integration

Total genomic DNA was isolated from bone marrow cells from secondary transplant recipient mice 3-months post- transplantation. Briefly, red blood cells (RBC) were lysed using 0.5 ml/mouse of ACK lysis buffer at room temperature for 5 min. PBS was added immediately and cells were centrifuged at 1,100 rpm for 5 min at 4ºC. Cells were lysed using a genomic DNA lysis buffer (10 mM Tris [pH 8], 1 mM EDTA, 200 mM NaCl, 0.5% sodium dodecyl sulfate, 200 μg of proteinase K per ml) at 37ºC overnight. The samples were extracted with phenol and chloroform, and precipitated with 2 volumes of ethanol overnight at -20ºC. The DNA was pelleted at 4ºC for 30 min, washed with 70% ethanol, and air-dried briefly. The DNA was resuspended in 500 μl of TE (10 mM Tris [pH 8], 1 mM EDTA), digested with 10 μg of RNase A (Sigma, St. Louis, Mo.) at 37ºC for 3 hrs, extracted with phenol and chloroform, and precipitated with ethanol at -20ºC. Each pellet was resuspended in 200 μl of TE. Twenty micrograms of DNA samples were digested with restriction endonucleases that cleave the proviral genome once (NcoI), or cleave twice (NcoI + HindIII), and electrophoresed on 1% agarose gels followed by Southern blot hybridization with a ^{32}P-labeled EGFP-specific DNA probe.

N. Morphological analysis of peripheral blood and bone marrow cells

Peripheral blood and bone marrow samples from mice transplanted with mock-transduced or scAAV vector-transduced HSCs were reviewed by the Molecular Pathology Core in the Department of Pathology at the University of Florida. These cells

were stained with hematoxylin–eosin (H&E), and visualized under a light microscope at a magnification of x400.

O. RT-PCR for β-globin transgene expression

RNA was isolated using Invitrogen RNA isolation kit as per manufacturer's instructions (Invitrogen, Carlsbad, CA) and subjected to a two-step RT-PCR procedure (MyiQ Single-Color Real-Time PCR Detection System, BIO-RAD, Hercules, CA). First the RNA was run on a 1.2% agarose gel made using DEPC treated 1X TBE buffer to determine its quality. The RNA was reverse-transcribed using iScript ^{TM} cDNA Synthesis kit and the cDNA was then amplified using iQ ^{TM} SYBR® Green supermix as per manufacturer's instructions (both kits were purchased from BIO-RAD, Hercules, CA). A three step PCR procedure was performed as follows: Step 1 (95^{o}C for 5 min, 95^{o}C for 30 min), step 2 (55^{o}C for 1 hr), Step 3 (72^{o}C for 30 min). Primer sets common to both endogenous and vector-derived β-globin transcripts (Common A: 5'-CGAGGAGAAGTCCGCCGTTACTG-3', and Common B: 5'CACTTTCTGATAGGCAGCCTGCACT-3') and unique to vector-derived β-globin transcript (Mutant A: 5'-GAGAAGTCCGCCGTTACTGTT-3, and Mutant B: 5'-GAAGTTCTCAGGATCCACGT-3') were used as previously described (Oh et al., 2004). Half of the PCR product was digested with PvuII and electrophoresed on 1.5% agarose gel followed by southern blot analysis using a ^{32}P-labeled β-globin DNA probe.

P. Analysis of globin expression in sickle cell disease mice

Peripheral blood was drawn from the retroorbital sinus of mock and vector treated donor recipient mice 3 months post-transplant using heparin treated glass tubes and place in EDTA treated Eppendorf tubes and then shipped on ice to the laboratory of Dr. Ching-Nan Ou at Texas Children's Hospital, Texas who performed the HPLC, globin chain, and isoelectric focusing (IEF) analyses.

RESULTS

A. Expression of AAV2 receptor and co-receptor

AAV2 uses cell surface heparan sulfate proteoglycan (HSPG) as a receptor, and fibroblast growth factor receptor 1 (FGFR1) or αvβ5 integrin as co-receptors. Previous studies in our laboratory have shown donor variability in the ability of AAV2 vectors to transduce human $CD34^+$ cells due to the inability of the virus to bind cells that do not express the receptor for AAV2 (Ponnazhagan et al., 1997a). To determine whether the barrier to transduction of murine HSCs was due to lack of binding and entry of AAV2 into these cells, we studied the level of expression of HSPG and FGFR1 in primary murine $Sca1^+$, lin^- cells by FACS. These results are shown in Figure 9. Although approximately 50% of these cells expressed HSPG and 40-60% expressed FGFR, only 10-20% of these cells co-express the receptor and co-receptor for AAV2. These results indicate that viral binding and entry limits transduction of murine HSCs by AAV2 vectors. Moreover, AAV2 failed to transduce this subset (10-20%), indicating that other barriers to transduction of murine HSCs do exist.

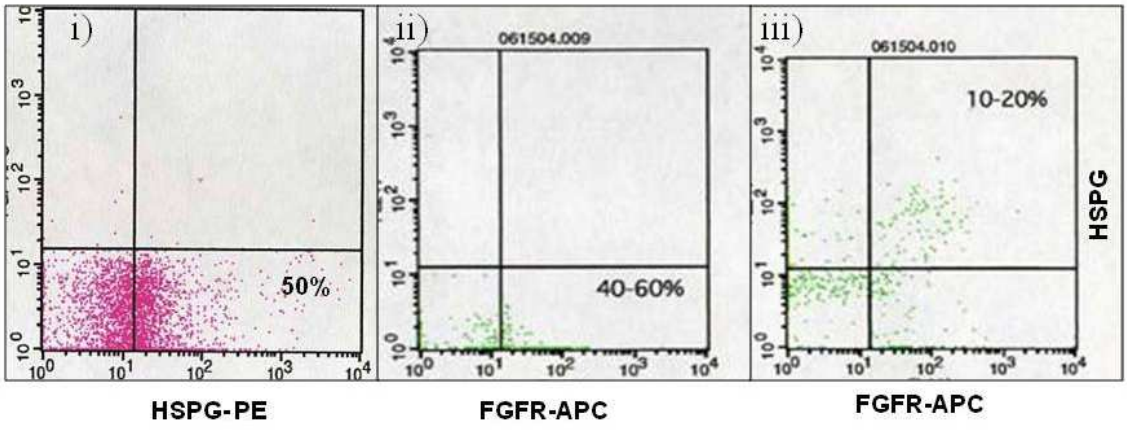

Figure 9: Analysis of cell surface heparan sulfate proteoglycan (HSPG) and fibroblast growth factor receptor (FGFR) receptor expression in primary murine Sca1+, lin- cells by FACS. 5 x 10^6 low density bone marrow cells (LDBM) from C57Bl6 mice were isolated and stained with 1µg/10^6 of Sca-1-PECy5, lineage-specific-FITC antibodies and i)HSPG-PE (BD) ii) Fc-FGF fusion protein for 1hr, washed twice in PBS and stained with secondary anti-human Fc-APC [Fc-FGF+Fc-APC] (Leinco) and iii) both HSPG-PE and [Fc-FGF+Fc-APC] antibodies. Cells were washed in PBS and analyzed with a FACStar instrument. 10^5 events were recorded. Experiment was repeated three times. Data shown is from one representative experiment.

B. AAV1 serotype vector is the most efficient in murine HSCs in liquid cultures *in vitro*

Since CMV-driven ssAAV2 vectors failed to efficiently transduce primary murine HSCs, we generated HS2-β-globin and B19p6-promoter- driven scAAV1 and scAAV7 EGFP-vectors (Figure 10) and used then to transduce $Sca1^+$, $c\text{-}kit^+$, lin^- murine hematopoietic cells in 7-day liquid cultures *in vitro.* These results are shown in Figure 11. Whereas scAAV1-B19p6-EGFP transduced murine HSCs efficiently, transduction from all other vectors was insignificant. These results are consistent with our previous observation of AAV1 vector's ability to transduce murine HSCs in short-term liquid cultures efficiently (Zhong et al., 2006a).

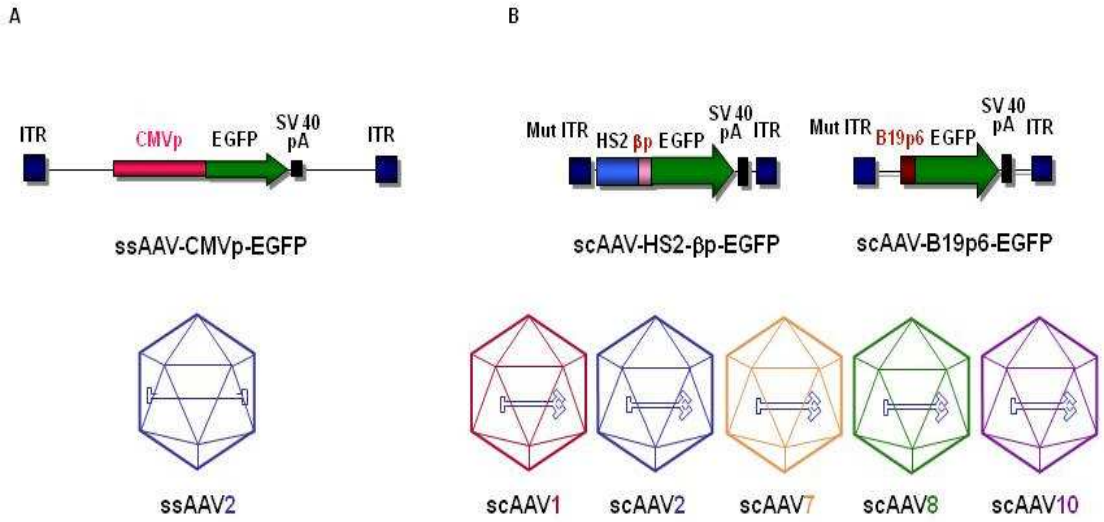

Figure 10: Schematic representation of conventional, single-stranded AAV2 and double-stranded, self-complementary AAV serotype vectors. ITR, inverted terminal repeat; mITR, mutant inverted terminal repeat; CMVp, cytomegalovirus immediate-early promoter; EGFP, enhanced green fluorescent protein; sv40pA, simian virus 40 polyadenylation signal; HS2, DNA-hypersensitive site 2 enhancer; βp, β-globin promoter; ssAAV, single-stranded AAV; scAAV, self-complementary AAV.

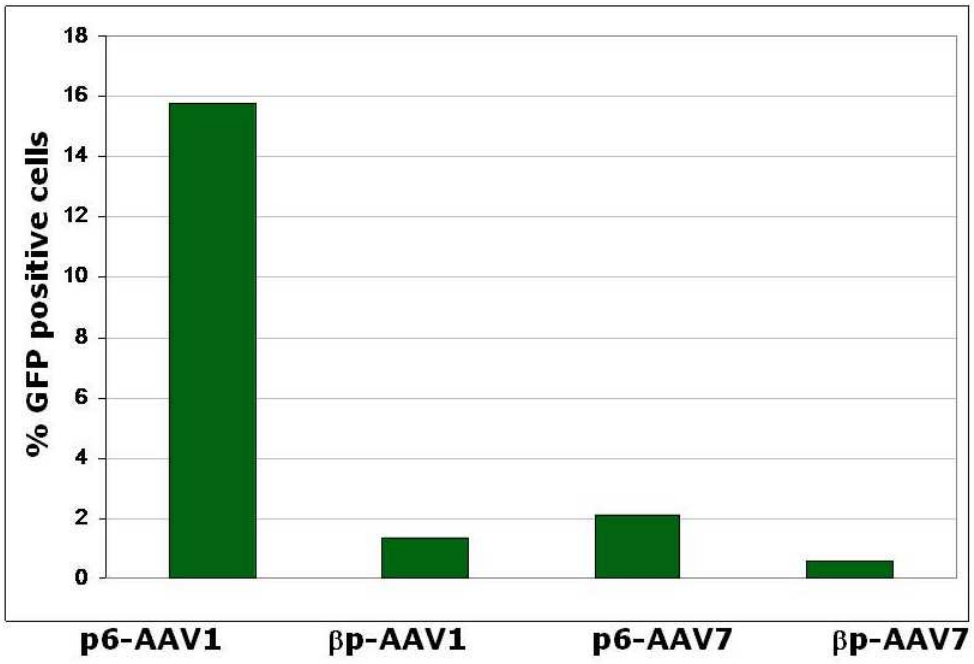

Figure 11: Comparative analysis of AAV1 & AAV7 vectors containing B19p6 or HS2-β-globin promoter-driven EGFP expression in Sca1+, c-kit+, lin- murine hematopoietic cells *in vitro.* Equal numbers of bone marrow-derived Sca1$^+$, c-kit$^+$, lin$^-$ cells were mock-infected or infected with scAAV vectors at 37^{o}C for 2 hrs and maintained in liquid culture for 7 days. Transgene expression was evaluated by flow cytometry using a FACStar instrument.

C. Comparative analyses of EGFP gene expression from HS2-β-globin promoter or the B19p6 promoter using AAV serotype vectors in primary murine hematopoietic progenitor cells *in vivo*

Equal numbers of primary murine bone marrow-derived Sca1$^+$, c-kit$^+$, lin$^-$ cells were mock-infected or infected with scAAV serotype 1, 2, 7, 8 and 10 vectors at 37^{o}C for 2 hrs, followed by transplantation into lethally-irradiated syngeneic recipient mice as described in Materials and Methods. Erythroid-specific transgene expression was evaluated in bone marrow mononuclear cells 6 months post-transplantation after co-staining with the c-kit$^+$/CD71 antibodies. Since expression of c-kit$^+$ is down-regulated,

while expression of CD71 is upregulated during erythroid development, EGFP expression was analyzed based on the co-expression of the above pair of receptors (Figure 12 and Table 1) as previously described (Socolovsky et al., 2001; Spangrude et al., 2006). These results are shown in Figure 13 and 14. Consistent with the published data (Spangrude et al., 2006), the highest level of EGFP expression was observed in the early intermediate erythroblast stages R2 (c-kit$^{+\ high}$, CD 71 ^{low}), followed by R3 (c-kit^{+} ^{high}, CD71 ^{high}) and R4 (c-kit$^{+\ low}$, CD71 ^{high}). EGFP expression in R5 was insignificant. In contrast to previously published data, expression in R1 (c-kit$^{+\ high}$, CD71 ^{negative}) and R6 (c-kit$^{+\ low}$, CD71 ^{low}) was insignificant. This observation can be explained by the use of erythroid-specific promoters in the AAV constructs, which are inactive in the multipotent progenitors and lymphocyte lineage. Analysis of EGFP expression showed progressive loss of fluorescent during erythroid development. The loss of EGFP fluorescence could be due to i) global repression of non-globin genes during development, ii) degradation of the EGFP protein in the erythroblasts, or iii) fluorescent quenching by hemoglobin (Spangrude et al., 2006).

Consistent with our previously published studies, the transduction efficiency of AAV1 vectors was higher than that of AAV2 vectors in the intermediate erythroblast stage (c-kit$^{+\ high}$, CD 71 ^{low}) as shown again in Figure 14. The transduction efficiency of AAV7 vectors was similar to that of AAV1, while that of AAV8 and AAV10 was similar to that AAV2. It is noteworthy that up to 20% transduction by AAV2 vectors could also be achieved, which was due to the combined use of the double-stranded vector genomes and the hematopoietic cell-specific promoters.

In addition, EGFP expression from the B19p6 promoter was more efficient than that from the HS2 enhancer/ β-globin promoter in AAV1 and AAV7 serotypes even in the absence of HS2 enhancer. In order to be active, the β-globin promoter requires the locus control enhancer element (LCR), consisting of hypersensitivity regions 1 through 5 (HS1, HS2, HS3, HS4 and HS5), with HS2 having the highest enhancer activity. Only the HS2 enhancer was included in the AAV constructs due to the size limitation of self-complementary AAV vectors, which could have a limiting effect on the strength of the HS2-β-globin promoter containing vector. Although viral promoters are smaller in size, several studies have documented their stronger promoter activity compared to eukaryotic promoters (Zhong et al., 2006b, Maina et al., 2008a and b).

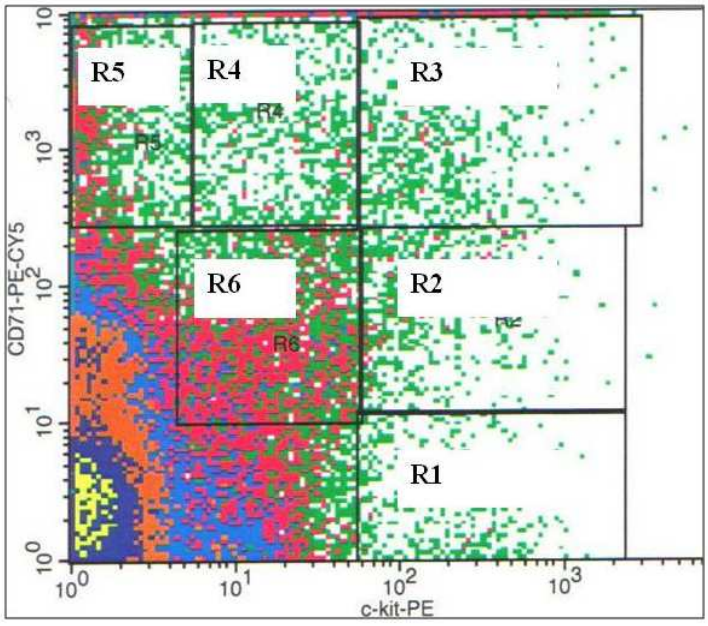

Figure 12: Analysis of erythroid-restricted EGFP expression in primary murine hematopoietic progenitor cells. Gate R1 (c-kit$^{+\ high}$, CD71 ^{negative}), R2 (c-kit$^{+\ high}$, CD71 ^{low}), R3 (c-kit$^{+\ high}$, CD71 ^{high}), R4 (c-kit$^{+\ low}$, CD71 ^{high}) R5 (c-kit$^{+\ negative}$, CD71 ^{high}) and R6 (c-kit$^{+\ low}$, CD71 ^{low}).

Gate	c-kit$^+$	CD71	Stage
R1	high	negative	multipotent progenitor
R2	high	low	intermediate
R3	high	high	intermediate
R4	low	high	intermediate
R5	neg	high	basophilic erythroblast
R6	low	low	lymphoid lineage

Table 1: Gate R1 represents multipotent progenitor cells, R2-R4 represents progressive intermediate erythroblast stages, R5 is the basophilic erythroblast stage while R6 represents the lymphoid lineage.

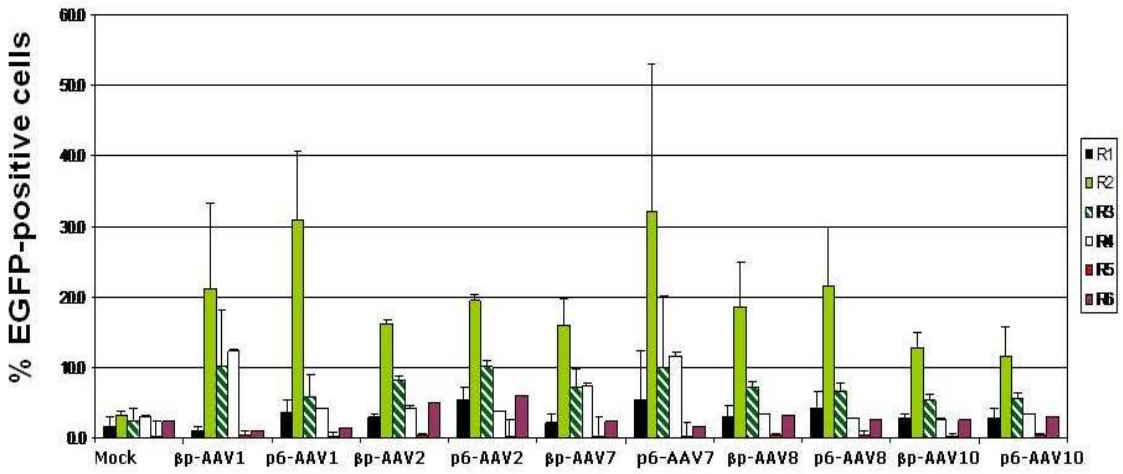

Figure 13: Comparative analyses of EGFP gene expression from HS2-β-globin promoter or the B19p6 promoter using AAV serotype vectors in primary murine hematopoietic progenitor cells. Equal numbers of bone marrow-derived $Sca1^{+}$, c-kit^{+}, lin^{-} cells were mock-infected or infected with scAAV vectors at $37^{o}C$ for 2 hrs followed by transplantation into lethally- irradiated syngeneic recipient mice. Transgene expression was evaluated 6 months post-transplantation by flow cytometry using a FACStar instrument after staining with c-kit and CD71 antibodies. Results are shown for all the stages of erythroid differentiation (Gate R1-R6).

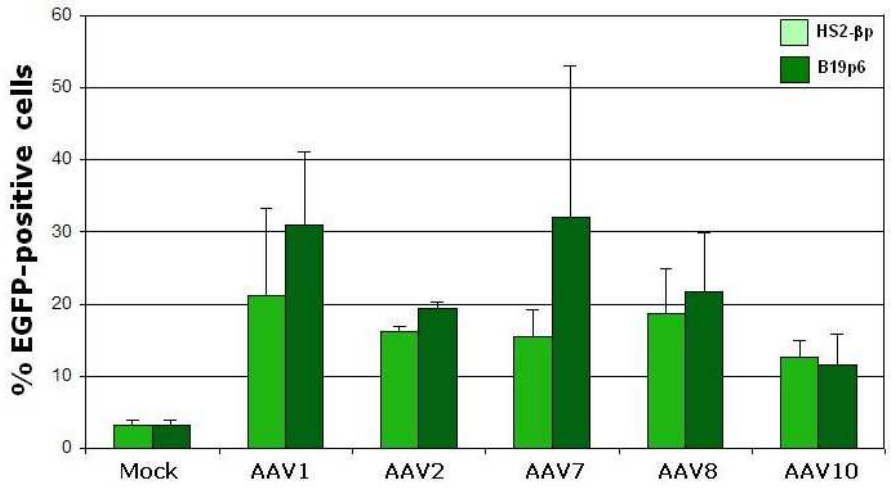

Figure 14: Comparative analyses of EGFP gene expression from HS2-β-globin promoter or the B19p6 promoter using AAV serotype vectors in primary murine hematopoietic progenitor cells. Equal numbers of bone marrow-derived $Sca1^{+}$, c-kit^{+}, lin^{-} cells were mock-infected or infected with scAAV vectors at $37^{o}C$ for 2 hrs followed by transplantation into lethally- irradiated syngeneic recipient mice. Transgene expression was evaluated 6 months post-transplantation by flow cytometry using a FACStar instrument after staining with anti-c-kit and anti-CD71 antibodies. Results are shown only for the intermediate erythroblast stage (Gate 2).

D. Lineage analysis of transgene expression mediated by scAAV serotype vectors in murine hematopoietic progenitor cells 6 months post-primary transplantation and 3-months post-secondary transplantation

To confirm the erythroid lineage restricted activity of the HS2-β-globin and the B19p6 promoters, we also performed lineage analysis of transgene expression mediated by scAAV serotype vectors in murine hematopoietic progenitor cells 6-months post-primary transplantation using lineage specific antibodies as described in Materials and Methods. These results are shown in Table 2. Whereas EGFP expression was clearly observed in the erythroid lineage (9-35%), no significant EGFP expression was present in any of the other lineages tested (T lymphocytes, B lymphocytes, myeloid cells, and

mature RBCs). These results further confirm that expression from the HS2-β-globin and B19p6 promoters was erythroid restricted regardless of the serotype used. The lack of EGFP expression in the terminally differentiated Ter119 positive erythroid population is likely due to the quenching of EGFP fluorescence by globin which indicates that EGFP is not the ideal reporter for erythroid engraftment as previously shown (Spangrude et al., 2006). While several previously published studies have shown the activity of B19p6 promoter to be mainly limited to human erythroid cells undergoing differentiation (Kurpad et al., 1999; Wang et al., 1995b), this is the first study demonstrating that the activity of B19p6 promoter is also erythroid restricted in murine hematopoietic cells.

The presence of EGFP-positive cells in the bone marrow mononuclear cells implied but did not confirm that a hematopoietic stem cell(s), capable of long-term bone marrow reconstitution, had been transduced by the scAAV vectors. To address this issue directly, bone marrow mononuclear cells from a scAAV7-B19p6-EGFP vector-transduced or a mock-transduced primary recipient mouse were used to transplant four lethally-irradiated syngeneic mice. Three months post-secondary transplantation, bone marrow mononuclear cells were harvested and analyzed for transgene expression in lymphoid, myeloid, and erythroid lineages as previously described. These results are shown in Table 3. Consistent with the primary transplantation results, erythroid lineage-restricted expression in up to 30% of erythroid cells was readily detectable, 9-months post-transplantation, suggesting that a primitive stem cell(s), capable of long-term reconstitution, had indeed been transduced.

	T lymphocytes	B lymphocytes	Myeloid	Erythroblasts	Mature RBCs
Mock	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	3 ± 0.7	0.3 ± 0.01
scAAV1-HS2-βp-EGFP	0.1 ± 0.05	0.3 ± 0.07	0.2 ± 0.11	21 ± 12	0.3 ± 0.06
scAAV1-B19p6-EGFP	0.2 ± 0.13	0.5 ± 0.28	0.3 ± 0.21	38 ± 10	0.1 ± 0.08
scAAV2-HS2-βp-EGFP	0.2 ± 0.06	0.3 ± 0.02	0.2 ± 0.07	16 ± 0.7	0.3 ± 0.07
scAAV2-B19p6-EGFP	0.2 ± 0.03	0.3 ± 0.03	0.3 ± 0.03	19 ± 0.9	0.3 ± 0.07
scAAV7-HS2-βp-EGFP	0.2 ± 0.02	0.2 ± 0.03	0.1 ± 0.02	16 ± 4	0.9 ± 0.03
scAAV7-B19p6-EGFP	0.1 ± 0.06	0.2 ± 0.01	0.2 ± 0.10	32 ± 21	0.2 ± 0.04
scAAV8-HS2-βp-EGFP	0. 3 ± 0.07	0.3 ± 0.05	0.1 ± 0.04	19 ± 6	0.2 ± 0.01
scAAV8-B19p6-EGFP	0.3 ± 0.08	0.3 ± 0.04	0.1 ± 0.02	22 ± 8	0.4 ± 0.1
scAAV10-HS2-βp-EGFP	0.2 ± 0.08	0.2 ± 0.08	0.2 ± 0.04	13 ± 2	0.1 ± 0.02
scAAV10-B19p6-EGFP	0.2 ± 0.08	0.1± 0.14	0.1± 0.04	12 ± 4	0.3 ± 0.07

Table 2: Bone marrow cells were incubated with phycoerythrin (PE)-conjugated lineage specific rat anti-mouse mAbs (anti-B220 for B lymphocytes; anti-CD4 & anti-CD8a for T lymphocytes; anti-Gr-1 for myeloid cells; and anti-Ter119 for mature RBCs) separately and analyzed for GFP expression by flow cytometry using a FACsort machine. Erythroblasts were stained with anti-c-kit and anti-CD71.

	T lymphocytes	B lymphocytes	Myeloid	Erythroblasts	Mature RBCs
Mock	0.02	0.01	0.03	3.0	0.04
#1	0.05	0.05	0.04	22.0	0.08
#2	0.06	0.05	0.04	19.0	0.07
#3	0.04	0.07	0.05	32.0	0.08
#4	0.02	0.04	0.03	23.0	0.08

Table 3: Bone marrow cells from a scAAV7-B19p6-EGFP vector-transduced primary recipient mouse were used to transplant 4 lethally-irradiated syngeneic mice. Three months post-secondary transplantation, bone marrow cells were harvested and incubated with phycoerythrin (PE)-conjugated lineage specific rat anti-mouse mAbs (anti-B220 for B lymphocytes; anti-CD4 & anti-CD8a for T lymphocytes; anti-Gr-1 for myeloid cells; and anti-Ter119 for mature RBCs) separately and analyzed for EGFP expression by flow cytometry using a FACsort machine. Erythroblasts were stained with anti-c-kit-PE and anti-CD71-PECy5.

E. Southern blot analysis for integration of the proviral AAV-EGFP genome in murine hematopoietic progenitor cells 3-months post-secondary transplantation

In order to determine whether the proviral AAV genomes were stably integrated within the host chromosomal DNA, total genomic DNA was isolated from bone marrow mononuclear cells from mock-transduced and three secondary transplant recipient mice. Equivalent amounts of total genomic DNA were digested with restriction enzymes that cleave within the recombinant genome once (NcoI), or twice (NcoI+HindIII) and analyzed by southern blot analysis using an EGFP DNA as a probe. These results are shown in Figure 15. Following digestion with NcoI, no hybridization signal was detected in the mock transduced mice, while unique bands were detected in the three vector transduced mice. In addition, following digestion with NcoI + HindIII, the predicted 0.9 kb band was detected from DNA samples from each transplanted mouse. The distinct band pattern following digestion with NcoI and NcoI+ HindIII suggest random integration. These data are consistent with the interpretation that the AAV proviral genomes are stably integrated into the host cell chromosomal DNA, and further corroborate our previously published studies (Han et al., 2008; Tan et al., 2001b; Zhong et al., 2006a).

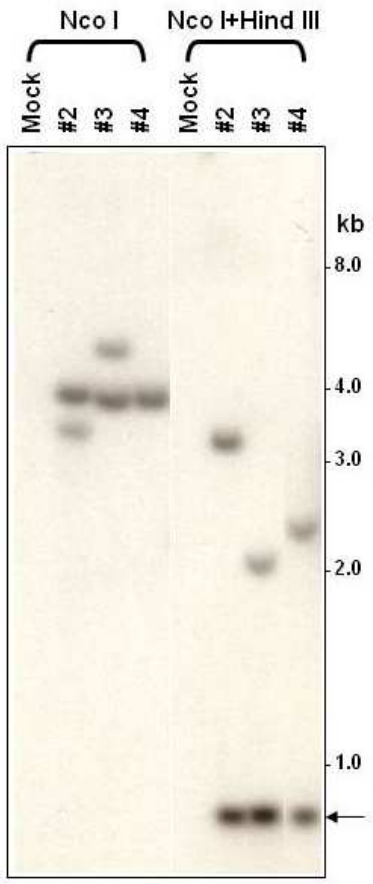

Figure 15: Southern blot analysis for integration of the proviral AAV2-EGFP genome in murine hematopoietic progenitor cells 3 months post-secondary transplantation. Equivalent amounts of total genomic DNA from bone marrow nuclear cells from secondary recipient mice following bone marrow transplantation with mock-transduced or scAAV7-B19p6-EGFP vector-transduced primary murine HSCs were digested with NcoI (one site in the vector genome), or with NcoI+HindIII (two sites in the vector genome), electrophoresed on agarose gels, and probed with an EGFP-specific DNA probe. The arrow indicates the 743-bp expected size fragment from the EGFP transgene.

F. Morphological analysis of peripheral blood and bone marrow cells following transplantation with scAAV serotype vector-transduced primary murine hematopoietic stem cells

A recently published report of the development of hepatocellular carcinoma in mice injected as neonates with AAV vectors has raised safely concerns (Donsante et al.,

2007). To determine whether stable integration of AAV sequences led to any toxicity in hematopoietic tissues, peripheral blood and bone marrow samples from primary recipients 6 months post-transplantation were reviewed by the Molecular Pathology Core in the Department of Pathology at the University of Florida and the results are shown in Figure 16. Analysis of the hematopoietic tissues from mock-, scAAV-HS2-βp-EGFP- and scAAV-B19p6-EGFP- vector transduced mice showed no deleterious effects of AAV integration in this study or in a previous study from our lab with a larger cohort (Han et al., 2008).

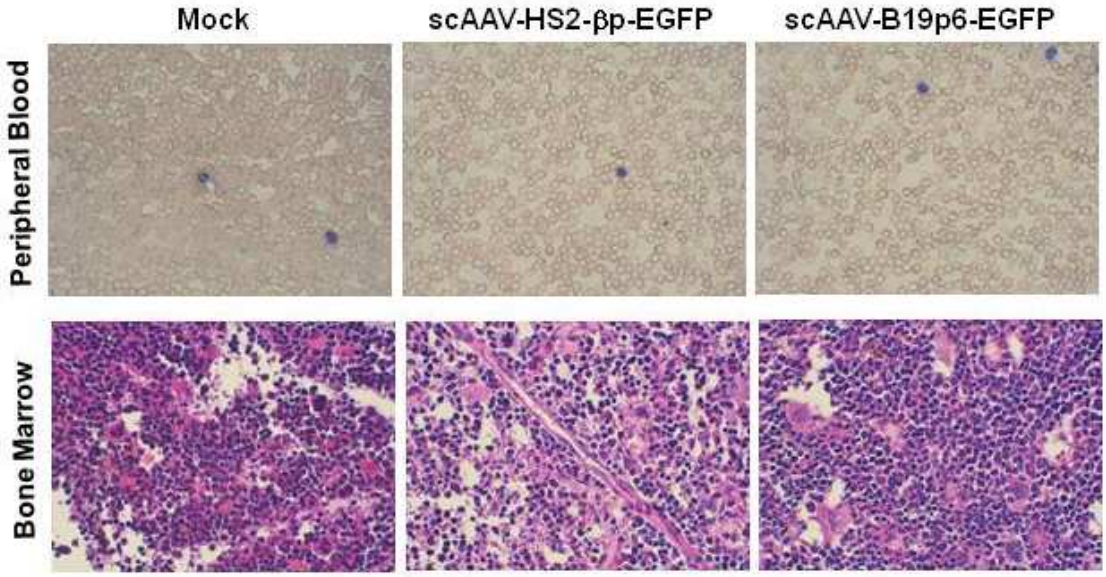

Figure 16: Morphological analysis of peripheral blood and bone marrow cells following transplantation with scAAV serotype vector-transduced primary murine hematopoietic stem cells. Peripheral blood and bone marrow samples from mice transplanted with mock-transduced or the indicated scAAV vector-transduced HSCs were stained with hematoxylin-eosin (HE), and visualized under a light microscope. X400.

G. Self-complementary AAV-β87$^+$-globin vectors containing only the HS2 enhancer element are more efficient than their single-stranded counterpart containing HS2 HS3 HS4 enhancers

We first wished to determine whether scAAV vectors, which bypass the rate-limiting step of viral second-strand synthesis, are more efficient in transducing a human erythroleukemia cell line, K562, which does not express the endogenous β-globin gene (Lozzio et al., 1979). Equivalent numbers of K562 cells were infected with a conventional, single-stranded AAV vector containing the entire locus control region cassette (ssAAV2-HS234-βp-β87$^+$-globin; Figure 17A, i) and an scAAV vector containing only the HS2 enhancer (scAAV2-HS2-βp- β87$^+$-globin; Figure 17A, ii) at 2 x 10^4 vector particles/cell at 37ºC for 2 hr. Transgene expression analyses were performed by RT-PCR 72 hr post-infection, using primers that were common to endogenous and vector derived-globin transcripts (Pr. comA and Pr. comB; Figure 17B) as previously described (Oh et al., 2004). Restriction digestion with Pvu II was also used to discriminate between endogenous and vector-derived β87$^+$-globin transcripts. These results are shown in Figure 17C. As can be seen, when the common set of primers was used, the expected 384-bp band could be cleaved with Pvu II, yielding the expected 244- and 140-bp products only in vector-transduced, not in mock-transduced cells. Although K562 cells do not express the endogenous β-globin gene (Lozzio et al., 1979), they do express 13% of the endogenous δ-globin gene (Poddie et al., 2003). The 384-bp fragment in mock-transduced cells was generated only when common primers were used, which amplify the endogenous β-globin gene, but not when mutant primers specific for the β87$^+$ -globin gene were used, which do not amplify the endogenous β-globin gene (see below).

No product was obtained after amplification in the absence of reverse transcriptase (RT), or in the negative control sample. Densitometric scanning and quantitation of these data (Figure 17D) indicated that although the scAAV vector contained only one enhancer element (HS2), expression from this vector was 3-fold higher than that from the ssAAV vector, which contained all three enhancers (HS2+HS3+HS4). These results corroborate that viral second-strand DNA synthesis is a rate-limiting step in the transduction of K562 cells with conventional ssAAV2 vectors. Previous studies in our laboratory have documented that the phosphorylated form of FKBP52 is predominant in K562 cells, and is responsible for the low recombinant AAV2-mediated transgene expression in this cell type (Qing et al., 1998). Thus, although the scAAV2 vector contained only one enhancer element, it was able to lead to efficient transduction of K562 cells.

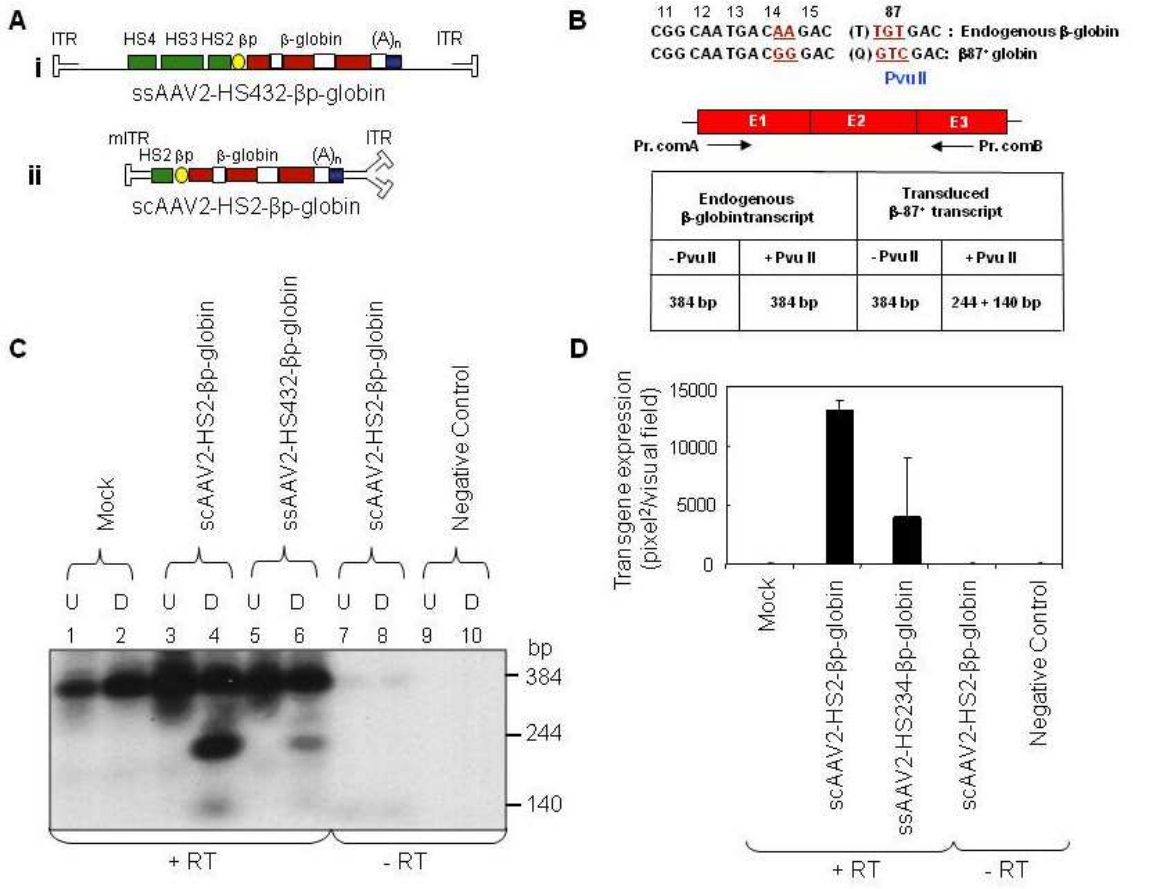

Figure 17: A) Schematic structures of the single-stranded (ssAAV2-HS432-βp-β87$^+$-globin, i), and self-complementary (scAAV2-HS2-βp-β87$^+$-globin, ii) vectors. **B)** Nucleotide sequence alignment of relevant codons of the endogenous β-globin and the anti-sickling β87$^+$-globin genes. The schematic structure of the β-globin cDNA, relative positions of the common primers (Pr. comA and Pr. comB), and the expected sizes of DNA products, with and without Pvu II digestions, are also indicated. **C)** Comparative analysis of scAAV2-HS2-βp-β87$^+$-globin and ssAAV2-HS432-βpβ87$^+$-globin vector-mediated β-globin gene expression in K562 cells. Equivalent numbers of cells were either mock-infected (Mock) or infected with recombinant vectors under identical conditions. Total RNA samples were subjected to a two-step RT-PCR procedure using the common primers. Half of the samples were digested with Pvu II and electrophoresed on agarose gels followed by Southern blotting and hybridization using a β-globin-specific DNA probe. U: undigested, D: digested with Pvu II, RT: reverse transcriptase. **D)** Densitometric scanning of autoradiographs for the quantification of relative amounts of the PCR products.

H. scAAV-β87^{+}-globin vectors and scAAV-HS234 vectors recombine and lead to β-globin expression after dual vector transduction.

Because optimal expression of the β-globin gene requires the entire enhancer element (HS2+HS3+HS4), and because the packaging capacity of scAAV vector precludes such a possibility, we sought to take advantage of the dual-vector approach, which has been shown by a number of investigators to enhance AAV-mediated gene expression through intermolecular recombination (Duan et al., 2000) (Chao et al., 2002) (Duan et al., 2001; Nakai et al., 2000; Sun et al., 2000; Yan et al., 2000) (Yan et al., 2002a) (Reich et al., 2003; Xu et al., 2004) (Ghosh et al., 2006; Ghosh et al., 2008; Lai et al., 2006; Yan et al., 2005). In this strategy, a single gene is split into two separate scAAV vectors, which then undergo intermolecular recombination after cellular entry and viral uncoating. We therefore constructed the following three vectors: scAAV2- βp-β87^{+}-globin (Figure 18A, i); scAAV2-HS2+HS3+HS4 Figure 21A, ii), in a closed-to-closed (C-C) orientation of the AAV inverted terminal repeats (ITRs) relative to the scAAV2-βp-β87^{+}-globin vector; and scAAV2-HS4+HS3+HS2 (Figure 18A, iii) in a closed-to-open (C-O) orientation. Each scAAV genome contains a closed (C) and an open (O) end structure of the ITR, and these orientations refer to the two possible alignments, as described previously (Choi et al., 2005). These vectors were used to infect K562 cells and transgene expression was evaluated by RT-PCR analyses as described above. These results are shown in Figure 18B. Whereas no transgene expression occurred after transduction with the scAAV2-HS234 vector or the scAAV2- βp-β87^{+}-globin vector alone, as expected, significant expression was detected after co-infection when the two vectors were in a C-C orientation, which was 6-fold higher than when the two vectors were in an C-O orientation (Figure 18C). A similar study, using split enhanced green

fluorescent protein (EGFP) vectors, provided evidence that the C–C orientation is more efficient than the C-O or O-O orientation for intermolecular recombination between scAAV genomes (Choi et al., 2005).

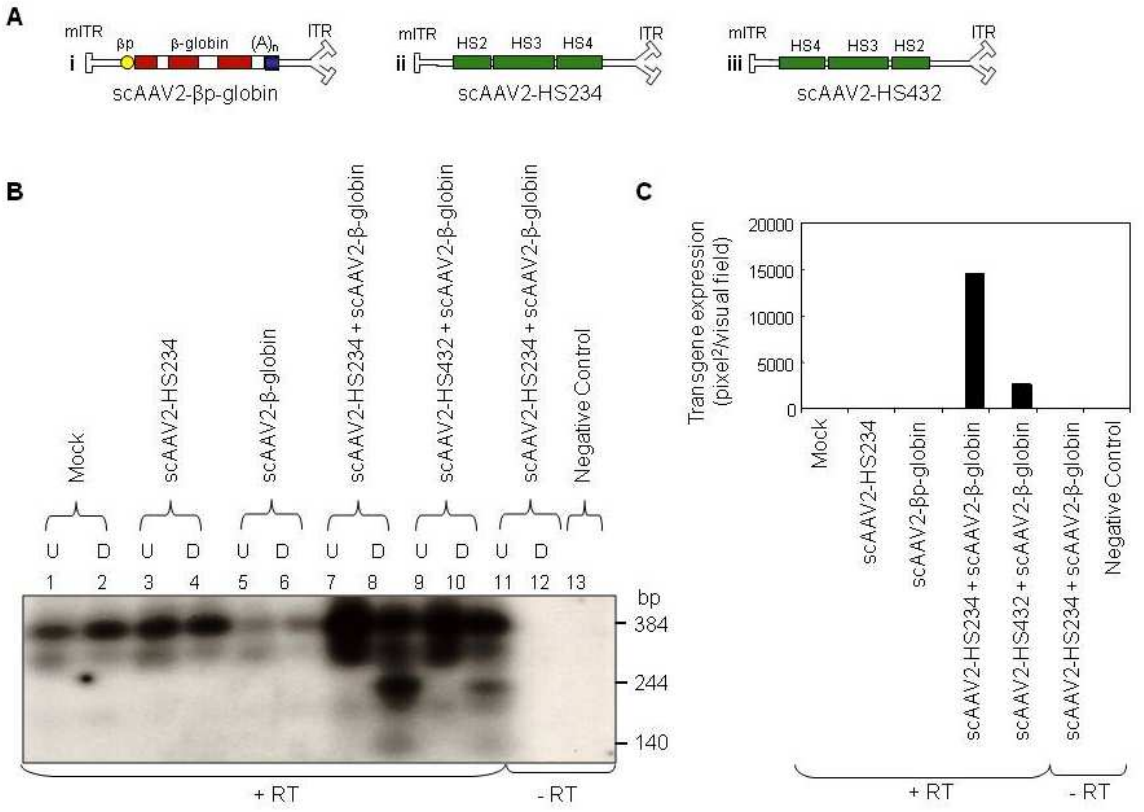

Figure 18: A) Schematic structures of scAAV2-βp-β87^{+}-globin (i), scAAV2-HS234 (ii), and scAAV2-HS432 (iii) vectors. **B)** Comparative analysis of dual vectors-mediated β-globin gene expression in K562 cells. Equivalent numbers of cells were either mock-infected (Mock) or infected with single or dual vectors under identical conditions. The rest of the steps were the same as described in the legend to Figure 17. **C)** Densitometric scanning of autoradiographs in **(A)**.

I. AAV2 and AAV1 serotypes are the most efficient in transducing K562 cells *in vitro*

In order to determine the most efficient serotype for the transduction of K562 cells, equal number of cells were infected with scAAV 1, 2, 7, 8 and 10 vectors under the control of HS2-β-globin and B19p6 promoters and transgene expression evaluated by FACS 72 hrs post- infection. These results are shown in Figure 19. Whereas scAAV1-B19p6-EGFP and scAAV2-B19p6-EGFP vectors mediated high efficiency transduction of K562 cells (40-67%), expression from scAAV10-B19p6-EGFP was moderate (10%), while expression from scAAV1-HS2-βp-EGFP and scAAV2-HS2-βp-EGFP was 5-6%.

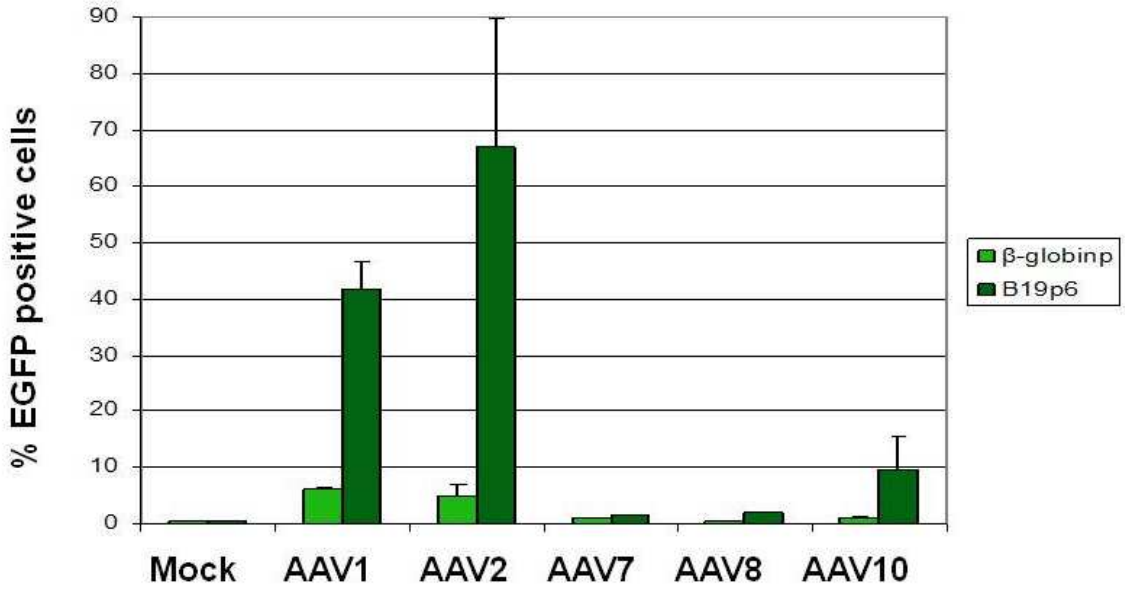

Figure 19: Comparative analysis of AAV serotype vector 1,2,7,8 & 10 containing B19p6 or HS2-β-globin promoter-driven EGFP expression in K562 cells. Approximately, 50,000 cells were mock-infected or infected with scAAV vectors at 37^{o}C for 2 hr. Transgene expression was evaluated 72 hr post-infection by flow cytometry on a FACStar instrument. Data shown represents the average from two different experiments.

J. Efficient expression of β87$^+$-globin gene occurs from human parvovirus B19 promoter in the absence of β-globin gene enhancer elements

Although the dual-vector approach worked, the extent of transgene expression was not significantly enhanced compared with that from a single scAAV2-HS2-βp-β87$^+$ -globin vector. Because the human parvovirus B19 promoter at map unit 6 (B19p6) is a strong viral promoter, expression from which is restricted to differentiating hematopoietic erythroid progenitors (Ozawa et al., 1986; Ozawa et al., 1987)and because it is small (200 bp),we sought to determine whether expression of the β87$^+$-globin gene could be obtained from the B19p6 promoter alone, and whether expression from the scAAV2-HS2-βp-β87$^+$-globin vector Figure 20A, i) could be superseded by that from the scAAV2-B19p6-β87$^+$-globin vector (Figure 20A, ii). In these studies we also included AAV serotype 1 (AAV1), on the basis of our preliminary studies with the EGFP reporter gene that showed that scAAV1 vectors mediate significant transduction of K562 cells (Figure 19). ScAAV2 and scAAV1 vectors containing either the HS2-βp-β87$^+$-globin or B19p6-β87$^+$ -globin genome were used to infect K562 cells and transgene expression analyses were performed as described above. These results are shown in Figure 20B. It is evident that expression from the scAAV-B19p6-β87$^+$-globin vector, even in the absence of the HS2 enhancer element, was 4-fold higher than expression from the scAAV-HS2-βp-β87$^+$-globin vector in both AAV2 and AAV1 serotypes (Figure 20C). The expression levels were further corroborated when using mutant primers (Pr. mutA and Pr. mutB; Figure 21A). The use of this primer pair leads to amplification of only the transduced β87$^+$-globin transcripts, and yields a 290-bp product, which can be cleaved by Pvu II to generate 240- and 50-bp fragments. This primer set also amplifies a 384-bp DNA product

from the input vector genomes, which is cleaved by Pvu II to generate 334- and 50-bp fragments. The 384-bp DNA fragment generated in the absence of RT can also be cleaved with Pvu II, which indicates that the PCR product was derived from the recombinant AAV vector genome. As can be seen in Figure 21B, expression from the scAAV-B19p6-β87^{+}-globin vector was 3- and 13-fold higher than expression from scAAV-HS2-βp-β87^{+}-globin vectors in serotypes 2 and 1, respectively (Figure 21C). Although the vector copy number was higher in scAAV- HS2-βp-β87^{+}-globin vector-transduced cells, expression levels were still lower than in scAAV-B19p6-β87^{+}-globin vector-transduced cells. Densitometric scanning and quantitation of the signals in Figure 21B revealed the ratios of the vector genome copies and the β87^{+}-globin transcripts to be 0.15–0.5 for scAAV-HS2-βp-β87^{+}-globin vectors, and 3.0 for scAAV-B19p6-β87^{+}-globin vectors. These results corroborate the ability of the B19p6 promoter to mediate high-efficiency β-globin gene expression in the complete absence of β-globin gene enhancer elements, and suggest that high-level expression of the β-globin gene from the B19p6 promoter might be achievable in primary human erythroid progenitor cells.

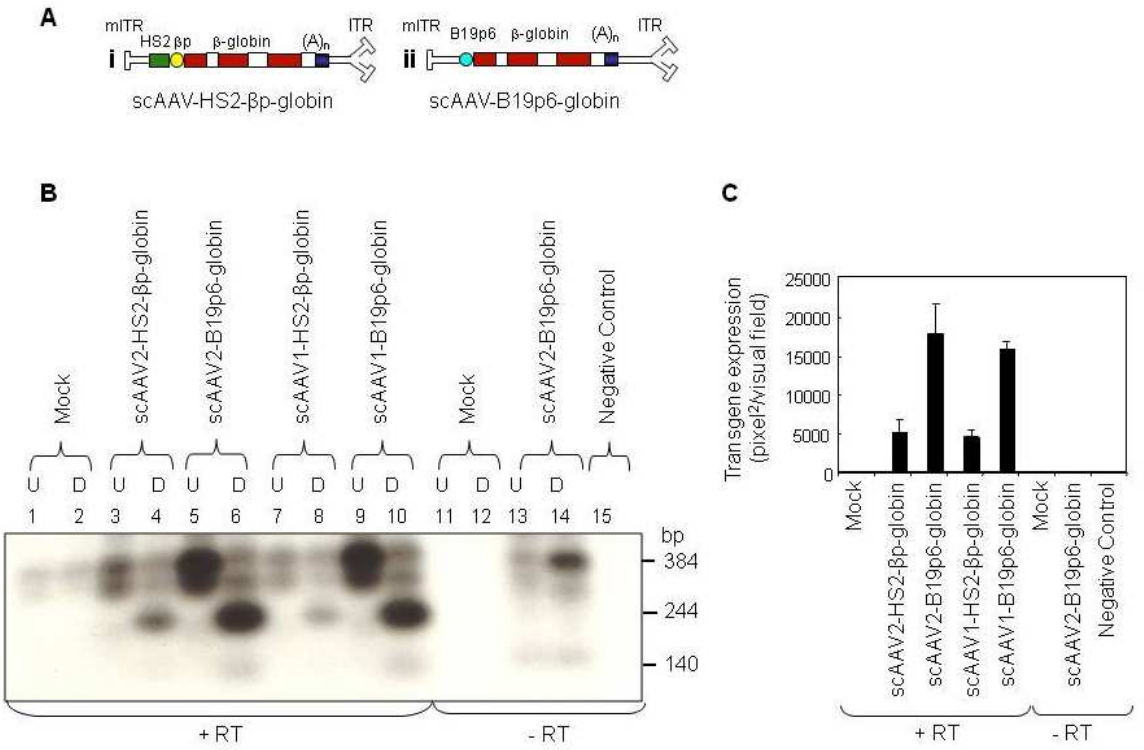

Figure 20: A) Schematic structures of scAAV-HS2-βp-β87^{+}-globin (i), and scAAV-B19p6-β87^{+}-globin (ii) vectors. **B)** Comparative analysis β-globin gene expression in K562 cells. Equivalent numbers of cells were either mock-infected (Mock) or infected with each vector genome encapsidated in AAV2 or AAV1 serotype under identical conditions. The rest of the steps were the same as described in the legend to Figure 17. **C)** Densitometric scanning of autoradiographs in **B)**.

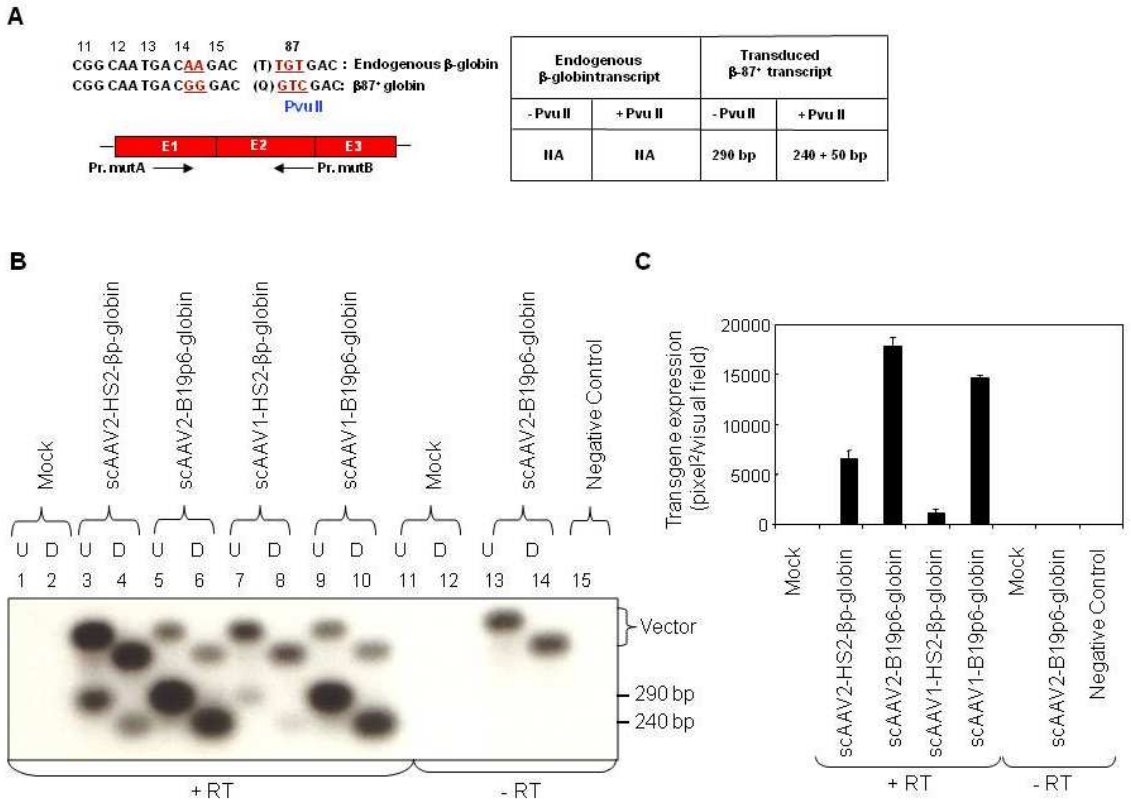

Figure 21: A) Nucleotide sequence alignment of relevant codons of the endogenous β-globin and the anti-sickling $\beta 87^{+}$-globin genes. The schematic structure of the β-globin cDNA, relative positions of the mutant primers (Pr. mutA and Pr. mutB), and the expected sizes of DNA products, with and without Pvu II digestions, from both the cDNA and the vector genome are indicated. **(B)** Comparative analysis of β-globin gene expression in K562 cells. Equivalent numbers of cells were either mock-infected (Mock) or infected with each vector genome encapsidated in AAV2 or AAV1 serotype under identical conditions. The rest of the steps were the same as described in the legend to Figure 17. **(C)** Densitometric scanning of autoradiographs in **B)**.

K. Efficient expression of β-globin protein occurs after transduction of human erythroid cells with AAV2-β-globin, and primary murine hematopoietic stem/progenitor cells with AAV1-β-globin, serotype vectors

Because all previous studies were limited to expression from the B19p6 promoter at the transcriptional level, we next evaluated whether the β-globin gene was also being

expressed at the protein level. To this end, we infected K562 cells and primary murine hematopoietic progenitor (c-kit$^+$, lin$^-$) cells with scAAV2-B19p6-β87$^+$-globin and scAAV1-B19p6-β87$^+$-globin vectors, respectively. Mock-infected and vector-infected cells were analyzed for β-globin protein expression 3 days post-transduction for K562 cells, and 7 days post-transduction for primary murine c-kit$^+$ lin$^-$ cells. Briefly, mock- and vector-transduced cells were fixed, permeabilized, and stained intracellularly with PE-conjugated mouse anti-human β-globin antibody, and β-globin protein was detected by flow cytometry. These results are shown in Figure 22. In K562 cells transduced with scAAV2-B19p6-β87$^+$-globin vector, 30% of cells expressed vector-derived β-globin protein (Figure 22A). Approximately 13% of globin protein expression observed in mock-transduced K562 cells stained with Hb-PE antibody was due to the fact that the antibody also cross-reacted with the endogenous δ-globin protein, which is expressed in K562 cells. Thus, ~17% of scAAV2-B19p6-β87$^+$-globin vector-transduced K562 cells expressed the β87$^+$-globin protein (Figure 22B). Similarly, primary murine c-kit$^+$, lin$^-$ cells were either mock-infected or infected with scAAV1-B19p6-β87$^+$-globin vectors under identical conditions, and half of the cells were cultured in stem cell medium (complete IMDM containing IL-3 [10 ng/ml], IL-6 [10 ng/ml] and stem cell factor (SCF) [1 ng/ml]) supplemented with erythropoietin (scAAV+EPO), and the other half were cultured in stem cell medium lacking EPO (scAAV-EPO). β-globin protein expression was determined 7 days post-transduction essentially as described above. As can be seen in Figure 22C and D, ~16% of primary murine c-kit$^+$, lin$^-$ cells expressed vector-derived β87$^+$-globin protein in the absence of EPO-treatment, and ~24% of these cells expressed vector-derived β87$^+$-globin protein following EPO-treatment, which is known to induce

erythroid-differentiation. The β-globin protein-expressing cells were also CD71 (transferrin receptor)-positive, which suggests, but does not prove, that the transgene expression was restricted largely to the erythroid lineage. Approximately 5% of β-globin signal in mock-transduced cells stained with Hb-PE antibody was considered background since mouse anti-human β-globin antibody does not cross-react with the mouse endogenous β-globin protein.

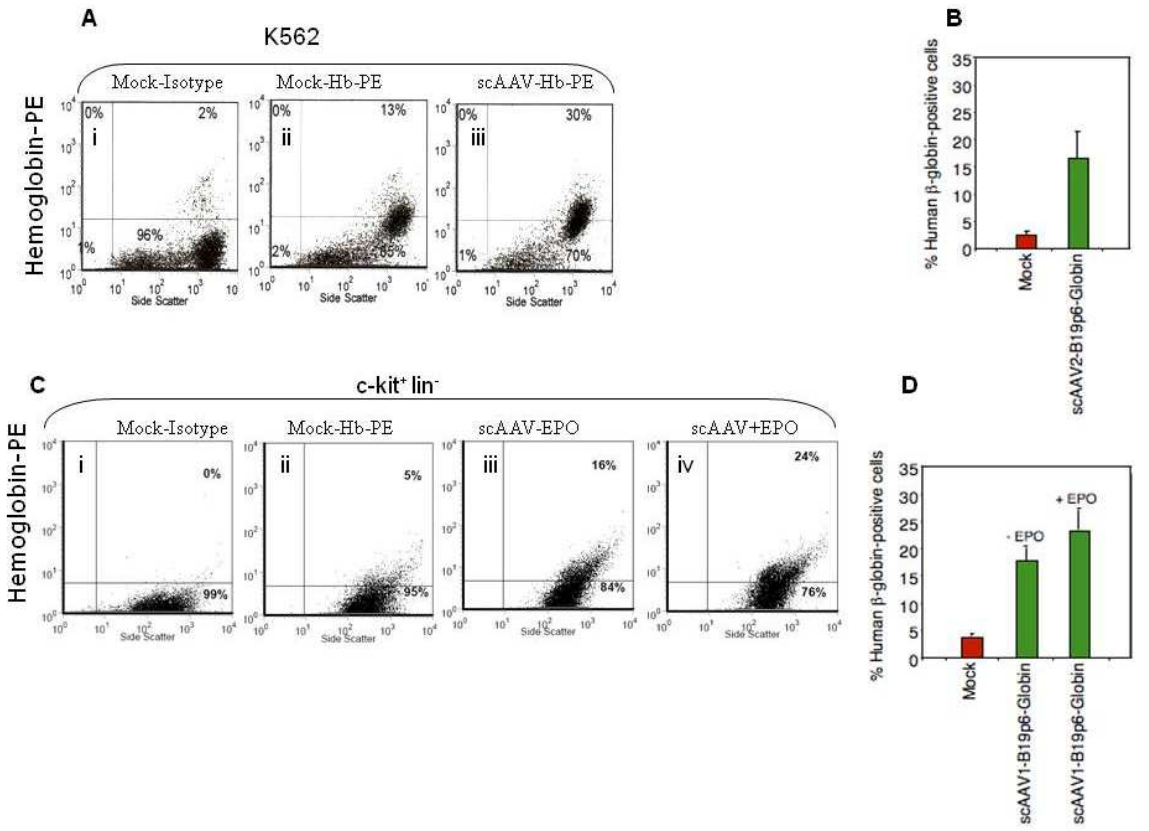

Figure 22: A) Fluorescence-activated cell sorting (FACS) analyses of expression of the β-globin protein in human K562 cells. Cells were either mock-transduced (Mock) or transduced with scAAV2-B19p6-β87^{+}-globin vectors and β-globin protein expression was determined 3 days post-transduction. Representative dot-plots are shown. Mock-transduced K562 cells were stained with isotype control [Mock-Isotype; (i)], and Hb-PE antibody [Mock-Hb-PE; (ii)], and vector-transduced cells were stained with Hb-PE antibody [scAAV-Hb-PE; (iii)]. **B)** Quantitation of the data in **A)**, corrected for the human δ-globin protein expression, represent the average expression levels from two experiments. **C)** FACS analyses of expression of the β-globin protein in primary murine c-kit^{+}, lin^{-} cells. Equivalent numbers of cells were either mock-transduced (Mock) or transduced with scAAV1-B19p6-β87^{+}-globin vectors under identical conditions. Half of the c-kit^{+}, lin^{-} cells were cultured in stem cell medium supplemented with erythropoietin (scAAV+EPO), and the other half were cultured in stem cell medium lacking EPO (scAAV-EPO). β-globin protein expression was determined 7-days post-transduction essentially as described above. Mock-transduced c-kit^{+}, lin^{-} cells were stained with isotype control [Mock-Isotype; (i)], and Hb-PE antibody [Mock-Hb-PE; (ii)], and vector-transduced cells grown either in the absence [scAAV-EPO; (iii)] or presence of EPO [scAAV+EPO; (iv)] were stained with Hb-PE antibody. **D)** Quantitation of the data from **C)** represents the average expression levels from two experiments.

L. Self-complementary AAV-β87$^+$-globin vectors fail to mediate efficient transduction of hematopoietic stem cells from sickle cell disease mouse model *in vivo*

We next wished to determine whether scAAV-B87$^+$-globin vectors could also mediate efficient transduction and globin gene expression in a sickle cell disease (SCD) mouse model. Sca1$^+$, c-kit$^+$, lin$^-$ cells from BERK mice were transduced as described in Material and Methods. A total of fifty mice were transplanted with the SCD cells, but only 5 survived for more than 2 weeks indicating that the SCD HSCs failed to rescue the irradiated animals, which would have allowed survival. Unfortunately, however, none of the mice that survived showed any globin expression following HPLC, globin chain and IEF analysis, as shown in Figure 23 A, B and C respectively. All mice expressed the endogenous mouse globin likely due to inefficient total body irradiation and recovery by a few mouse stem cells. Two of the mice (BERK-AAV1-βp-globin and BERK-AAV7-p6-globin) also expressed sickled globin, indicating that while engraftment did occur, these cells failed to be transduced by the scAAV-globin vectors.

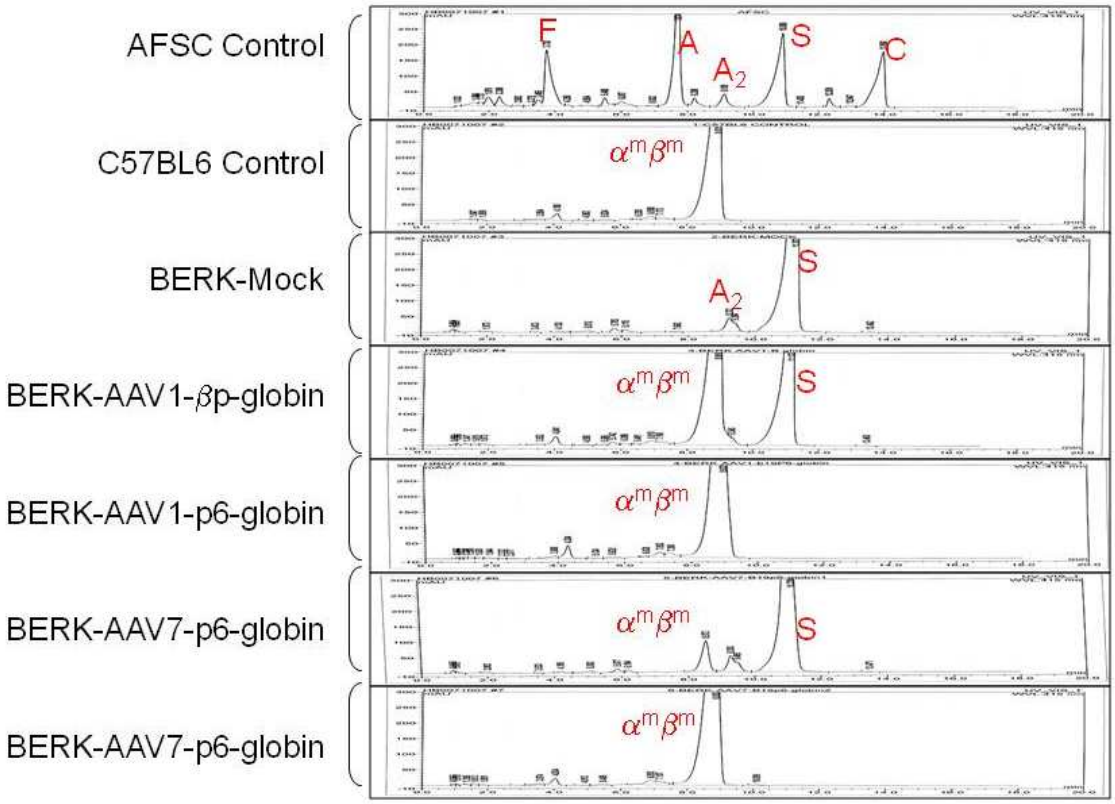

Figure 23A: Analysis of human β-globin expression in SCD mice. Peripheral blood was drawn from the retroorbital sinus of mock and vector treated donor recipient mice 3 months post-transplant and analysed using HPLC.

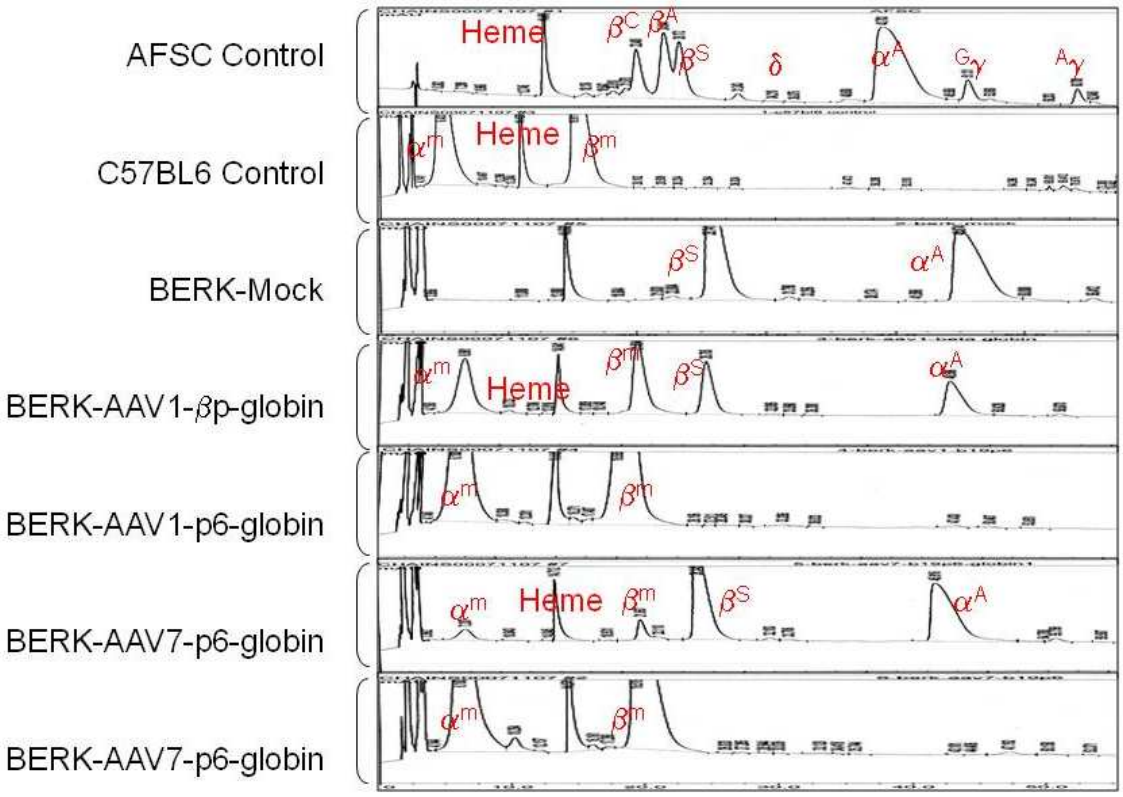

Figure 23B: Analysis of human β-globin expression in SCD mice. Peripheral blood was drawn from the retroorbital sinus of mock and vector treated donor recipient mice 3 months post-transplant and analysed using globin chain analysis.

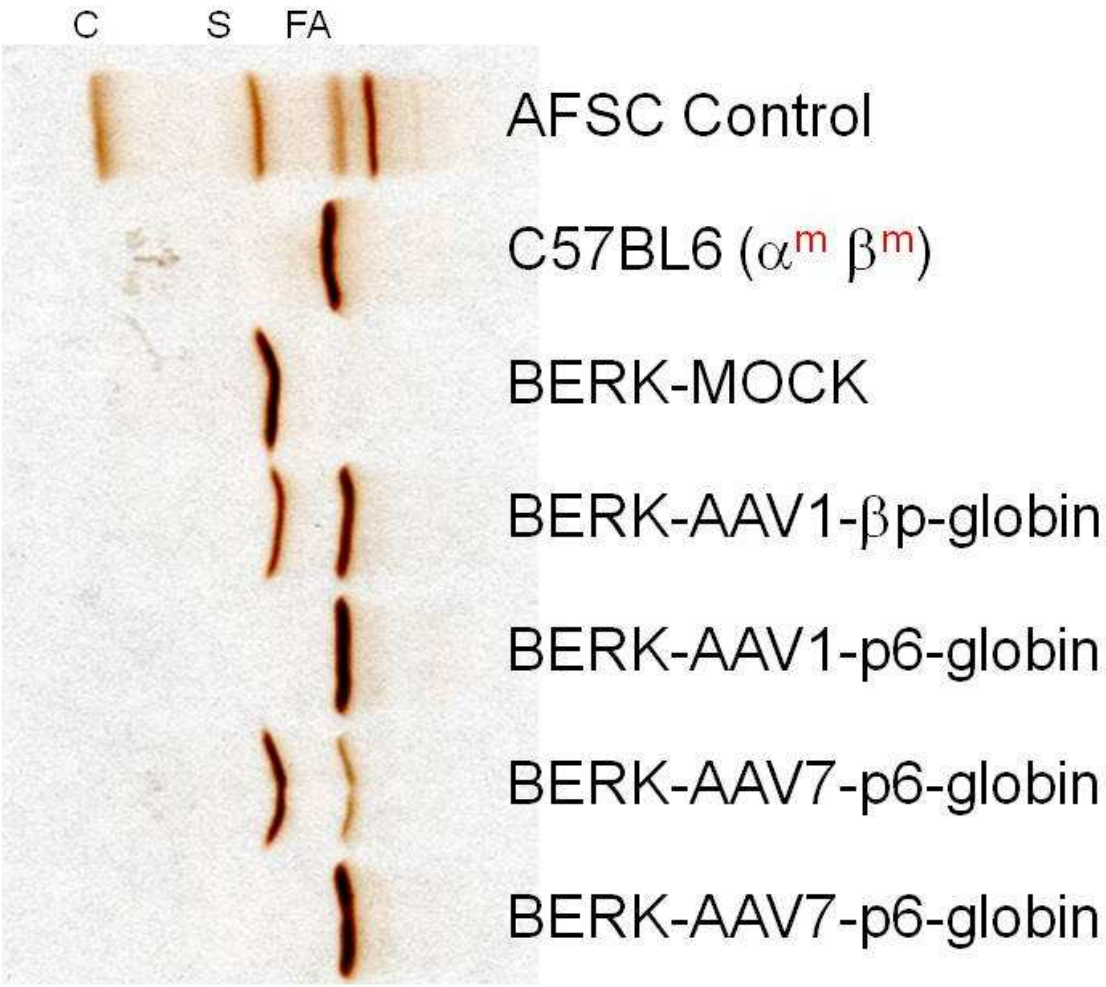

Figure 23C: Analysis of human β-globin expression in SCD mice. Peripheral blood was drawn from the retroorbital sinus of mock and vector treated donor recipient mice 3 months post-transplant and analysed using isoelectric focusing analysis.

This conclusion was further corroborated by qPCR analyses in which HSCs from SCD mice were documented to be non-permissive for entry of recombinant AAV1 and AAV7 serotype vectors. These results are shown in Table 4.

AAV vector	Copy No./cell
Mock	0.2
AAV1	24
AAV2	198
AAV3	108
AAV4	17
AAV5	762
AAV6	67
AAV7	264
AAV8	4928
AAV9	1506
AAV10	39

Table 4: AAV1 and AAV7 fail to enter HSCs from SCD mice. HSCs from SCD mice were mock or vector transduced using AAV1 through AAV10 serotype vectors for 2hrs as described in Material and Methods. Vector copy number per cell was analyzed by qPCR.

DISCUSSION

Controversies exist regarding the ability of adeno-associated virus 2 to transduce human hematopoietic stem cells. While some groups believe that AAV2, does not transduce human HSCs (Alexander et al., 1997), and that the observed transduction was a result of viral contaminants, others believe that AAV2 vectors transduce human HSCs at high vector to cell ratio (Hargrove et al., 1997; Malik et al., 1997; Nathwani et al., 2000). Still, we and others have shown efficient and sustained transduction of human and murine HSCs by AAV2 vectors at a low vector to cell ratio (Fisher-Adams et al., 1996) (Chatterjee et al., 1999; Li et al., 2005; Ponnazhagan et al., 1997a; Ponnazhagan et al., 1997b; Santat et al., 2005; Tan et al., 2001b; Zhong et al., 2004b; Zhong et al., 2004a; Zhong et al., 2006a; Maina et al., 2008a and b). These controversies are beginning to be resolved through a thorough assessment of the factors that limit transduction of murine and human HSCs and these observations have previously been reviewed. (Srivastava., 2002; Srivastava., 2005; Zhong et al., 2006a). Briefly, transduction of HSCs is limited by i) Suboptimal levels of the receptor/co-receptor for AAV2 which limits viral binding and entry, ii) Impaired intracellular trafficking and viral uncoating, iii) Inefficient conversion of AAV genome to transcriptionally active genomes through viral second strand synthesis, iv) Low CMV-promoter mediated transgene expression (Zhong et al., 2004b; Zhong et al., 2006a).

Since only a small percentage of murine HSCs express the receptor and co-receptor for AAV2, and conventional, single-stranded AAV2 vectors do not infect this population, we generated modified, self-complementary AAV vectors encapsidated in alternative AAV serotypes 1, 7, 8 and 10. The rationale was that murine HSCs may express the receptor/co-receptor for at least some of these novel AAV serotypes and

would allow efficient binding and entry. In consistence with our previously published results, AAV1 serotype vector was the most efficient in transducing primary murine HSCs in liquid cultures *in vitro* and *in vivo* (Li et al., 2005). In addition, expression from AAV7 serotype was also efficient, at 16% and 32% with the HS2-β-globin- and parvovirus B19p6- promoters, respectively. We also observed transduction by scAAV2 vector indicating that conversion to transcriptionally active genomes and suboptimal promoters were the main limiting factors in transduction by ssAAV2 vectors. This assessment is based on the assumption that self-complementary AAV vectors do traffic and uncoat in a manner similar to that of conventional, single stranded vectors. Expression from scAAV8 and scAAV10 was efficient and similar to that of scAAV2, indicating that murine HSCs express the receptors for serotype 8 and 10. AAV8 serotype has been shown to undergo more rapid uncoating in murine hepatocytes compared to AAV2 (Thomas et al., 2004) and it remains to be tested whether this occurs in murine HSCs.

Since the CMV promoter is weak and sensitive to silencing in hematopoietic cells (Choi et al., 2001; Kung et al., 2000; O'Rourke et al., 2005), we employed the use of erythroid-specific eukaryotic β-globin- and parvovirus B19p6- promoters. In this study, we were able to demonstrate erythroid lineage restricted expression using the eukaryotic HS2-β-globin and parvovirus B19p6 promoters. Higher level erythroid restricted, transgene expression was detected from the parvovirus B19p6 promoter when compared to HS2-β-globin promoter despite the absence of HS2 in these vectors. No significant expression was detected in any other lineage in both primary and secondary transplant experiments in all serotypes tested. Although HSCs give rise to cells in several lineages,

expression of the HS2-β-globin- and parvovirus B19p6- promoters was restricted to the erythroid lineage. EGFP expression was readily detected in the erythroid lineage 9 months after primary transplantation, indicating that a stem cell with long term repopulation ability had been transduced. While previous reports have shown efficient transgene expression from the B19p6 promoter in human erythroid progenitors (Kurpad et al., 1999; Wang et al., 1995b), this is the first study to show efficient, erythroid restricted expression in murine erythroid progenitors.

Extensive integration analysis of AAV2 vectors has been performed in various murine tissues and it is believed that AAV2 does not integrate in murine cells. It is generally assumed that recombinant AAV vector genomes remain episomal, but those studies have been carried out in tissues that are post-mitotic, where there is no pressure for AAV to integrate (Duan et al., 1998; Inagaki et al., 2007; Nakai et al., 2003; Nakai et al., 2005). However, in an actively dividing tissue like the bone marrow, it is likely that AAV integrates to avoid loss of vector genomes. Using southern blot analysis, we were able to show evidence of vector genomes in mouse bone marrow cells 9 months post primary transplant, suggesting that AAV integrates in dividing cells. However, further studies are needed to determine the site(s) of integration as well as the exact AAV chromosomal junctions. Some of this work, involving a larger cohort of mice has been performed in our laboratory and initial assessment suggests that AAV integrates randomly in the murine bone marrow (Han et al., 2008).

It is only recently that AAV has been reported to lead to insertional mutagenesis in the murine liver, resulting in hepatocellular carcinoma (Donsante et al., 2007). However, in these studies, AAV was injected in neonatal mice using very high viral

titers. Several other studies have not found any toxicity associated with AAV vectors even in p53-deficient mice (Bell et al., 2005; Bell et al., 2006; Schuettrumpf, 2007). To determine whether AAV vectors were associated with any toxicity in the bone marrow, we performed morphological analysis of peripheral blood and bone marrow cells since only bone marrow cells had been infected. As reported in the results, no explicit deleterious effects were seen in the tissues tested; indicating that integration of AAV did not result in any obvious toxicity. Since not all mutations are associated with overt pathologic findings, extensive integration studies are warranted.

Autologous bone marrow transplantation with genetically modified cells would be a great treatment modality for sickle cells disease (SCD), but has been limited by the inability to achieve therapeutic levels of β-globin expression in the HSC's progeny (Sadelain, 1997; Sadelain, 2006). Recently, lentiviral vectors have been used to obtain high-level expression of globin gene cassettes leading to therapeutic correction of several murine models of sickle cell disease (Levasseur et al., 2003; Pawliuk et al., 2001). Recombinant AAV2 vectors are a safer alternative to retroviral and lentiviral vectors for successful gene therapy of human disorders. In this study, we sought to improve recombinant AAV2 mediated β-globin gene expression, by employing the use of scAAV vectors expressing anti-sickling β-globin gene under the control of erythroid cell-specific promoters (HS2-β-globin and parvovirus B19p6 promoters).

In the first set of studies involving comparative analysis of conventional single stranded AAV2 vectors to double stranded, self complementary AAV2 vectors, it is apparent that double stranded AAV2 vectors were more efficient in transducing K562 cells *in vitro*. Although the scAAV2 vector contained only one enhancer element, it was

able to bypass the second strand synthesis step, leading to efficient transduction of K562 cells.

Since the transduction efficiency of scAAV2 vectors was better than that of ssAAV2 vectors, we hypothesized that a vector containing β-globin under the control of the entire locus control cassette (HS2+HS3+HS4) in a double stranded genome would have better transgene expression. Given that the size limitation of scAAV2 vectors is 3.3 kb (Wu et al., 2007), we employed the dual-vector approach. In this strategy, a single gene is split into two separate scAAV vectors, which then undergo intermolecular recombination following cellular entry and viral uncoating. Following co-infection of either scAAV2-HS234/scAAV2-HS432 vector with the ds-βp-globin vector, transgene expression was observed from both groups. However, expression from the scAAV2-HS234 (C-C) vector was 5.6-fold greater than that from the scAAV2-HS432 vector (C-O). Using a split GFP vector, Choi et al. (Choi et al., 2005) demonstrated that the closed-to-closed ITRs were more efficient than the closed-to-open or the open-to-open ITRs at undergoing recombination. These results indicate that the dual vector approach can be applied to globin gene transfer although the transduction efficiency is still low. Ghosh et al has shown that the efficiency of dual vector recombination can be improved by including a portion of the alkaline phosphatase gene in the scAAV vectors (Ghosh et al., 2008).

Our studies also demonstrate that the parvovirus B19p6 promoter can be used to mediate efficient expression of the β-globin gene at the protein level, even in the absence of the β-globin gene enhancer elements in human as well as primary murine hematopoietic stem/progenitor cells. To our knowledge, expression of the β-globin gene

has never been achieved without the use of the β-globin enhancer elements. The implications of this observation are two-fold. First, the small size of the B19p6 promoter not only allows the generation of scAAV-β-globin vectors, but also overcomes the stringent regulations of transcriptional control of the β-globin gene. And second, since expression from the B19p6 promoter leads to production of potentially therapeutic levels of the β-globin protein in primary murine hematopoietic progenitor cells, this bodes well for its potential utility presumably in primary human erythroid progenitor cells.

Although we demonstrated efficient transduction and erythroid lineage-restricted expression using AAV serotype vectors 1 and 7 in HSCs in C57BL/6 mice, we were not successful in demonstrating efficient transduction or transgene expression of anti-sickling globin gene is a SCD mouse model. The failure to demonstrate this was likely due to: i) failure of AAV vectors to transduce SCD mouse HSC or, ii) failure of SCD HSCs to engraft in C57BL/6 strain. SCD mice were generated using FVB/N mouse eggs and consist of a mixture of FVB/N, 129, DBA/2, Black Swiss and C57BL/6. One attempt to correct this entails backcrossing of the SCD mice into C57BL/6, which would generate a mouse model more similar to C57BL/6. With this strain, we would likely be able to demonstrate not only efficient transduction but also expression of therapeutic levels of β-globin and phenotypic correction of the SCD phenotype. Another likely explanation for the failure of these experiments was the failure of engraftment of SCD HSCs in C57BL/6. Although fifty animals were transplanted with the SCD cells, only a small number of animals survived for more than 2 weeks indicating that the SCD HSCs failed to rescue the irradiated animals, which would have allowed survival. All animals that survived had mouse globin chains in addition to the sickling globin. One likely

explanation is that the SCD HSCs are less viable that those from C57BL/6 or the SCD HSC induced a graft versus host reaction in the C57BL/6 recipients resulting in grafting failure.

FUTURE RESEARCH DIRECTIONS

In this work, I have demonstrated efficient transduction and erythroid lineage-restricted expression using AAV serotype vectors in HSCs from C57BL/6, but not those from SCD mice. Thus, AAV vector-mediated transduction of HSCs from other mice strains still needs to be thoroughly studied. One attempt to correct this would entail backcrossing of the SCD mice into C57BL/6 that would generate a mouse model more similar to C57BL/6. With this strain, it should be possible to demonstrate not only efficient transduction but also expression of therapeutic levels of β-globin and phenotypic correction of the SCD phenotype mediated by scAAV1 and scAAV7 serotype vectors.

An additional approach to improve transduction and erythroid lineage-restricted expression of globin gene in mouse and human HSCs would be to extend these studies to include additional novel AAV serotypes vectors such as AAV6, AAV9, AAV11, and AAV12. It is likely that such studies would help identify a novel serotype(s) with which better transduction of murine and/or human HSCs would become feasible. It is also likely that one or more of these novel AAV serotypes would exhibit tropism for SCD HSCs, and would thus mediate not only efficient transduction of these cells but also allow phenotypic correction of the SCD in this mouse model.

Li and colleagues demonstrated that AAV7 followed by AAV8 and AAV1, were the most efficient in transducing human $CD34^+$ cells (Li et al., 2005). However, these studies were performed using conventional single-stranded vectors under the control of a non-erythroid promoter. Thus further studies should be carried out to compare the transduction efficiencies of AAV serotype 1, 7, 8 and 10 vectors containing the B19p6 promoter in these cells *in vitro* and in NOD/SCID mice *in vivo*. However, since human

$CD34^+$ cells do not efficiently reconstitute the erythroid lineage in NOD/SCID mice, CFU assays could be performed using cells from spleen, PB and BM from these mice. Globin gene expression analysis could also be performed on CFU-erythroid-enriched population to increase the chances of detecting vector-derived globin gene expression. Since endogenous AAV sequences are the third most prevalent in the human marrow, an alternative strategy would be to isolate a novel AAV serotype(s) from pooled human $CD34^+$ cells. It is likely that one of these serotypes possesses tropism for $CD34^+$ cells, which, in turn, should allow the development of a novel AAV serotype vector(s) capable of high-efficiency transduction of human $CD34^+$ cells.

With the recent development of novel tyrosine mutant AAV vectors that allow high-efficiency transduction and expression of therapeutic levels of human factor IX protein in murine hepatocytes from BALB/c, C3H/HeJ and C57BL/6 *in vivo* (Zhong et al., 2008), because these vectors bypass ubiquitination and proteasome-mediated degradation, it is likely that one of these mutant vectors would mediate efficient transduction of HSCs from SCD mice and/or human $CD34^+$ cells given their ability to mediate transduction in several mouse strains. Although initial preliminary studies have demonstrated efficient transduction of human $CD34^+$ cells by several of these tyrosine mutant vectors *in vitro* and *in vivo*, further studies should be pursued to identify the optimal tyrosine mutant vector(s) for efficient transduction of murine and human HSCs for globin gene transfer. In addition, tyrosine mutants of AAV serotypes 1, 7, 8, 9 and 10 vectors could also be generated and tested to examine whether improvement in transduction of these cells at low vector to cell ratio would be achieved *in vitro* and *in vivo*.

The ultimate objective is to perform clinical trials using SCD HSCs from patients. However, prior to that, safety and efficacy of stable transduction and erythroid lineage-restricted expression of a transgene must be rigorously established in a non-human primate model. Although successful transduction of $CD34^+$ cells from Rhesus monkeys by conventional single-stranded AAV2 vectors has been demonstrated in the past (Schimmenti et al., 1998), studies should be contemplated to determine these parameters in cynomolgus monkeys since these monkeys can be infected by parvovirus B19, and abundant expression from the B19p6 promoter occurs in erythroid progenitor cells from these monkeys (Gallinella et al., 1995). Thus, it can be anticipated that recombinant scAAV-B19 hybrid vectors or novel tyrosine mutant AAV serotype vectors will mediate high-efficiency transduction and erythroid lineage-restricted expression in these animals. Additional studies on stable transduction of HSCs, long-term expression of therapeutic levels of globin gene in erythroid cells by AAV vectors, and determination of possible deleterious effects, if any, should also be performed. And finally, attempts should be made to isolate novel AAV serotypes from $CD34^+$ cells from cynomolgus monkeys as well as from humans since several novel AAV serotypes have indeed been isolated from various tissues from non-human primates and humans (Chen et al., 2005; Gao et al., 2004), and the successful development of these novel AAV serotypes into recombinant vectors should prove useful in the potential gene therapy of hemoglobinopathies in general, and β-thalassemia and sickle cell disease in particular.

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Publications

A. Research Reports

1. Khalaf W, **Maina C**, Byers J, Harvey W. (2002). Interferon-alpha 2b and vesnarinone influence levels of tumor necrosis factor-alpha, apoptosis, or interleukin 6 in ESKOL, a hairy cell leukemic cell line. A potential cytokine and oncogene relationship regulating apoptosis is suggested. Leukemia Research, **26:** 407-8.

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B. Presentations and abstracts

1. **Maina N**, Zhong L, Zhao W, Aslanidi G, Zolotukhin S, and Srivastava, A . AAV-mediated β-globin gene transfer: The combined use of self-complementary vectors and the parvovirus B19p6 promoter significantly augments transgene expression. Annual Gene Therapy Symposium for Heart, Lung, and Blood Diseases, November 2006, Sonoma, California.

2. **Maina N**, Han Z, Li X, Zhao W, Zhong L, Hu Z, Ma W, Slayton W, Srivastava A . Strategies for improving adeno-associated virus vector-mediated gene transfer in primary murine hematopoietic stem cells *in vivo*. American Society of Gene Therapy, May 2007, Seattle, Washington.

3. Han Z, Berendzen K, Zhong L, Chouthai N, Zhao W, **Maina N**, Srivastava A, Stacpoole P. Optimization of delivery and expression of the pyruvate dehydrogenase E1 alpha subunit gene using self-complementary adeno-associated virus serotype vectors. American Society of Gene Therapy, May 2007, Seattle, Washington.

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