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VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

Statement Concerning Government Funding

This application was supported by funds provided under NHLBI grant No. HL57612. The United States government may have certain rights in the invention.

Statement Concerning Related Applications

This application claims the benefit of US Provisional Application No. 60/301,861 filed June 29, 2001 and US Provisional Application No. 60/302,852 filed July 2, 2001, both of which are incorporated herein by reference.

Background of the Invention

This application relates to a vector comprising a mammalian, and particularly a human globin gene and to the use thereof in treatment of hemoglobinopathies, including α- and β-thalessemia and sickle-cell disease.

Current treatment modalities for β-thalassemias consist of either red blood cell transfusion plus iron chelation (which extends survival but is cumbersome, expensive and an imperfect therapy), or allogeneic bone marrow transplant (which carries a lethal risk and is not available to the majority of patients). Thus, there is a substantial need for improved therapeutic approaches. The present invention provides a genetic correction in autologous hematopoietic stem cells, thus using gene therapy to provide a less-risky and more effective long-term treatment.

While gene therapy has been proposed for many years, a significant challenge facing efforts to develop gene therapy vectors is the ability to produce therapeutically useful levels of a desired protein or peptide. The present invention provides a vector which is capable of providing therapeutically meaningful levels of human globin for sustained periods of time. This ability arises from the ability to transmit large genomic regulatory sequences that control expression of the therapeutic gene.

Summary of the Invention

In accordance with the invention, a recombinant lentiviral vector is provided comprising:

(a) a region comprising a functional globin gene; and

(b) large portions of the β-globin locus control regions which include large portions of DNase I hypersensitive sites HS2, HS3 and HS4. The regions may be the complete site or some lesser site which provides the same functionality as the specific sequences set forth below. This vector provides expression of β-globin when introduced into a mammal, for example a human, *in vivo*. Optionally, the vector further comprises a region encoding a dihydrofolate reductase.

By incorporation of different globin genes, the vector of the invention may be used in treatment of hemoglobinopathies, including α- and β-thalessemia and sickle-cell disease. For example, hematopoietic progenitor or stem cells may be transformed *ex vivo* and then restored to the patient. Selection processes may be used to increase the percentage of transformed cells in the returned population. For example, a selection marker which makes transformed cells more drug resistant than un-transformed cells allows selection by treatment of the cells with the corresponding drug. Selection and/or enrichment may also be carried out *in vivo*, for example using methotrexate or similar antifolates to select for cells rendered resistant by the expression from the vector of a dihydrofolate reductase (DHFR).

Brief Description of the Drawings

Fig. 1 shows the genomic structure of a recombinant onco-retroviral vector in accordance with the invention.

Fig. 2 shows the genomic structure of recombinant onco-retroviral vector within the scope of the invention.

Fig. 3 shows experimental results demonstrating increased mean β-globin expression in transduced MEL cells.

Fig. 4 shows the average vector copy number in peripheral blood cells, measured periodically for 24 weeks, which confirms showed highly efficient gene transfer in cells transduced with the vector of the invention.

Figs. 5A and B show human β-globin expression per endogenous allele 12 days and 22 weeks after introduction of cells transduced with the vector of the invention.

Fig. 6 shows haematocrit level, red blood cell count, reticulocyte count and haemoglobin level fifteen weeks after transplantation with unselected TNS9-transduced $Hbb^{th3/+}$ bone marrow.

Detailed Description of the Invention

In a first aspect of the present invention, a recombinant lentirviral vector is provided comprising:

(a) a region comprising a functional globin gene; and

(b) large portions of the β-globin locus control regions, which include DNase I hypersensitive sites HS2, HS3 and HS4.

As used in the specification and claims hereof, the term "recombinant lentiviral vector" refers to an artificially created polynucleotide vector assembled from a lentiviral-vector and a plurality of additional segments as a result of human intervention and manipulation.

The term "functional globin gene" refers to a nucleotide sequence the expression of which leads to a globin that does not produce a hemoglobinopathy phenotype, and which is effective to provide therapeutic benefits to an individual with a defective globin gene. The functional globin gene may encode a wild-type globin appropriate for a mammalian individual to be treated, or it may be a mutant form of globin, preferably one which provides for superior properties, for example superior oxygen transport properties. The functional globin gene includes both exons and introns, as well as globin promoters and splice doners/acceptors. Suitably, the globin gene may encode α-globin, β-globin, or γ-globin. β-globin promoters may be sued with each of the globin genes.

The recombinant vectors of the invention also include large portions of the locus control region (LCR) which include DNase I hypersensitive sites HS2, HS3 and HS4. In prior studies, smaller nucleotide fragments spanning the core portions of HS2, HS3 and HS4 have been utilized. Sadelain et al. *Proc. Nat'l Acad. Sci. (USA)*92: 6728-6732 (1995); Lebouich et al., *EMBO J.* 13: 3065-3076 (1994). The term "large portions"

refers to portions of the locus control region which encompass larger portions of the hypersensitive sites as opposed to previously tested fragments including only the core elements. The regions may be the complete site or some lesser site which provides the same functionality as the specific sequences set forth below. In preferred embodiments of the invention, the large portions of the locus control regions are assembled from multiple fragments, each spanning one of the DNase I hypersensitive sites. In addition, the locus control region has two introduced GATA-1 binding sites at the junction between HS3 and HS4. While not intending to be bound by any specific mechanism, it is believed that the incorporation of these transcription factor binding sites enhances the effectiveness of the vector.

The genomic structure of one embodiment of the vector of the invention (TNS9) is shown in Fig. 1. TNS9 incorporates human β-globin gene (from position -618 to +2484) that includes an extended promoter sequence and a 3'-enhancer element. Optionally, a portion of 3' U3 region of the lentiviral backbone can be deleted for increased safety. In Fig. 1, the exons and introns of the human β-globin gene are represented by filled and open boxes, The locations are indicated for the splice donor (SD), splice acceptor (SA), packaging region (ψ), rev-response element (RRE), human β-globin promoter (P) and 3'-β-globin enhancer (E). Thus, in the vector TNS9, a functional β-globin gene, which includes both the exons and introns of the gene and the relevant control sequences from the human β-globin locus. These are combined with the large fragments of the locus control region. The 3.2 kb LCR assembled into dTNS9 consists of an 840 bp HS2 fragment (*Sna*BI-*Bst*XI), a 1308 bp HS3 fragment (*Hind*III-*Bam*HI) and a 1069 bp HS4 fragment (*Bam*HI-*Ban*II).

In a further aspect of the invention, the β-globin gene coding sequence can be exchanged and replaced with either the gamma globin gene (for sickle cell disease) or the alpha globin gene (for alpha-thalassemias). In one strategy, a NcoI-Pst I fragment of the β-globin gene is replaced with the corresponding NcoI-HindIII fragment of the gamma globin gene or the NcoI-PstI fragment of the human alpha globin gene. These fragments start at the translational start of each globin gene (spanning the NcoI site) and end past their respective polyadenylation signals. In the second strategy, chimeric genes can be generated by only swapping the coding sequence of each one of the three exons of

these genes. Thus, for the gamma globin gene, the result is a vector that comprises the beta globin promoter, the beta globin 5' untranslated region, the gamma exon 1 coding region, the gamma intron 1 the gamma exon 2, the beta intron 2, the gamma exon 3, and the beta 3' untranslated region. Thus all the elements of the TNS9 vector remain in place (promoter, enhancers, 5' and 3' untranslated regions, the LCR elements, the 2 additional GATA-1 binding sites and the introns of the beta globin gene (at least intron 2, which is most important). In a third strategy, the codon usage within exon 3 of the gamma globin gene can be modified so that its sequence will resemble as much as possible that of the beta globin gene. The reason for testing this is that the beta globin gene is always the best expressed.

Additional elements may be included in the vectors of the invention to facilitate utilization of the vector in therapy. For example, the vector may include selectable markers, to confirm the expression of the vector or to provide a basis for selection of transformed cells over untransformed cells, or control markers which allow targeted disruption of transformed cells, and thus the selective removal of such cells should termination of therapy become necessary.

In a further specific embodiment, the vector of the invention includes the mouse PGK promotor and human dihydrofolate reductase (DHFR) cDNA as a transcriptional unit. Mutant forms of DHFR which increase the capacity of the DHFR to confer resistance to drugs such as methotrexate are suitably used. For example, single and double mutants of DHFR with mutations at amino acids 22 and 31 as described in commonly assigned PCT Publication No. WO 97/33988, which is incorporated herein by reference, may be advantageously utilized.

Fig. 2 shows the genomic structure of specific vector within the scope of the invention. The vector includes a deleted LTR, from -456 to -9 of HIV LTR and the PGK promoter (530 bp) from the murine phosphoglycerate kinase 1 gene. It also includes a DHFR-encoding region encoding human DHFR with s/f mutation at amino acid 22. The locus control region and the β-globin region are the same as in TSN9. This vector is designated dTNS9-PD. This incorporation of DHFR into this vector provides transformed cells with a methotrexate-resistant phenotype. As a result, methotrexate, and other antifolates can be used, both *in vitro* and *in vivo* as a selection to tool to

enhance levels of the functional hemoglobin. When hematopoietic stem cells were transformed using dTNS9-PD and reintroduced to mice that were then treated with NMBPR-P (0.5 mg/dose) and TMTX (0.5 mg dose) for five days, observed levels of expressed human β-globin were much higher in mice transduced with dTNS9-PD vectors after treatment with TMTX and NMBPR-P for selection of transduced cells.

The vectors of the invention are used in therapy for treatment of individuals suffering from hemoglobinopathies. In one embodiment of the invention, hematopoietic progenitor or stem cells are transformed *ex vivo* and then restored to the patient. As usedin the specification and claims hereof, the term "hematopoietic progenitor sand stem cells" encompasses hematopoietic cells and non-hematopoietic stem cells, e.g., embryonic stem cells, hematopoietic stem cell precursors, or any of the latter generated by nuclear transfer from a somatic cell. It is know in the art that efficient genes transfer into human embryonic stem cells can be achieved using lentiviral vectors.

Selection processes may be used to increase the percentage of transformed cells in the returned population. For example, a selection marker which makes transformed cells more drug resistant than un-transformed cells allows selection by treatment of the cells with the corresponding drug. When DHFR is used as a selection marker, it can be used for enrichment of transduced cells *in vitro,* or for *in vivo* selection to maintain the effectiveness of the vector.

The invention will now be further described with reference to the following non-limiting examples.

Example 1

To produce vector TNS9, the human β-globin gene was subcloned from Mβ6L (Sadelain et al. *Proc. Nat'l Acad. Sci. (USA)*92: 6728-6732 (1995)) into lentiviral vector pHR'LacZ (Zuffery et al., Nature 15: 871-875 (1997)) replacing the CMV-LacZ sequence. pHR'eGFP was constructed by replacing LacZ with the eGFP sequence (Clontech). Viral stocks were generated by triple transfection of the recombinant vectors pCMVΔR8.9 (Zuffrey et al.) and pMD.G in 293T cells as previously described in Dull, et al., *J. Virol.* 72: 8463-8471 (1998). The pseudotyped virions were concentrated by ultracentrifugationm resuspended and titrated as described in Gallardo et al., *Blood* 90:

952-957 (1997). For comparison, RSN1 was used which has a similar structure, except that the LCR contains only the core portion of HS2, HS3 and HS4. Northern blot analysis showed full length RNA transcripts, indicating that the recombinant lentiviral genomoes are stable. Southern blot analysis on genomic DNA from transduced cells, digested once in each long terminal repeat (LTR) results in a single band corresponding to the expected size for the vector, indicating that the proviral structure is not rearranged.

Example 2

To investigate the tissue specificity, stage specificity and expression level of the vector-encoded human B-globin gene, we transduced RNS1 and TNS9 into MEL cells, lymphoid Jurkat cells and myeloid HL-60 cells. Cell-free viral supernatant was used to infect C88 MEL cells in the presence of polybrene (8 $\mu g\ ml^{-1}$). Transduced MEL cells were subcloned by limiting dilution, and screened by PCR for transduction[30] using primers that anneal in the human β-globin promoter sequence (βPS, 5' - GTCTAAGTGATGACAGCCGTACCTG-3') and in HS2 (C2A, 5'- TCAGCCTAGAGT GATGACTCC TATCTG-3'). Vector copy number and integration site analysis was determined by Southern blot analysis[9]. Transduced MEL cells were induced to maturation by 5-day culture in 5 mM N,N'- hexamethylene bisacetamide (HMBA, Sigma).

To induce β-globin transcription, transduced MEL cell pools were differentiated using hexamethylene bisacetamide HMBA). Human β-globin (β^A) and mouse β-globin transcripts were measured by quantitative primer extension. After normalization to vector copy number and to endogenous β-globin expression per allele, human β-globin levels were 14.2 ± 4.7% for RNS1 and 71.3 ± 2.3% for TNS9 in pooled MEL cells (Fig. 2a). MEL, Jurkat and HL-60 cells were transduced with RNS1, TNS9 or control GFP recombinant lentivirus. Human β-globin RNA expression in HMBA induced MEL cells (grey bars) was measured by quantitative primer extension and normalized to mouse β-globin RNA expression per locus. Expression was then normalized to the vector copy number determined by Southern blot. No human β-globin RNA expression was detected in non-induced MEL (black bars), Jurkat (white bars) or HL-60 cells (hatched bars), indicating that globin expression was erythroid- and differentiation-specific. No human

β-globin expression was detected in non-induced MEL, Jurkat and HL-60 cells (Fig. 3), indicating that human β-globin expression was appropriately regulated in terms of tissue specificity and state of differentiation. We generated a panel of MEL cell clones that carried a single copy of either vector to distinguish whether the increased expression obtained in HMBA-treated Mel cells transduced with TNS9 rather than RNS1 was the result of an increase in β^A expression per cell or of an increase in the fraction of cells expressing human β-globin. Transduced MEL cells were subcloned by limiting dilution immediately after transduction, avoiding any bias towards favourable chromosomal integration sites as produced by drug selection[5]. The proportion of clones expressing human β-globin varied significantly between the two vectors. One out of ten RNS1 positive clones yielded measurable human β-globin transcripts, in contrast to 12 out of 12 for TNS9 also expressed higher levels of human β-globin than did those bearing RNS1 ($P < 0.01$, Fisher's exact test). Cells bearing TNS9 also expressed higher levels of human β-globin than did those bearing RNS1 ($P < 0.01$, Wilcoxon rank sum test). These findings established that both the level and probability of expression at random integration sites was increased with the TNS9 vector.

Example 3

Quantification of human β-globin mRNA

Total RNA was extracted from MEL, Jurkat and HL-60 cells, or mouse spleen and blood using TRIzol. Quantitative primer extension assays were done using the Primer Extension System-AMV Reverse Transcriptase kit (Promega) with [^{32}P] dATP end-labelled primers specific for retroviral-derived human β-globin (5' - CAGTAACGGCAGACTTCTCCTC -3') and mouse β-globin (5' - TGATGTCTGTTTCTGGGGTT GTG -3'), with predicted extension products of 90 bp and 53 bp, respectively. The probes yield products of identical length for β^{maj}, β^{min}, β^s and β^t. Primers were annealed to 4μg of RNA and reactions were run according to manufacturer's protocols. Radioactive bands were quantitated by phosphorimager analysis (BioRad). RNA isolated from A85.68 mice[20] was used as positive control. After correction for primer labelling, the human to mouse RNA signal was 29 ± 1% per gene copy in repeated experiments ($n > 8$), in agreement with previous findings based on

RT-PCR[20]. Values measured in bone marrow chimaeras that were obtained in separate runs were standardized to the value obtained in the A85.68 RNA sample. In Figs. 2 and 3c, d, human β-globin expression is expressed per vector copy and normalized to the endogenous transcripts (accounting for two endogenous alleles). In Fig. 3b, human transcripts are reported as the fraction of total β-globin RNA (Huβ / Huβ + Muβ) to reflect absolute contribution of vector-encoded transcripts.

Example 4

To investigate the function of the vectors *in vivo*, we transduced and transplanted murine bone marrow cells without any selection in syngeneic, lethally irradiated recipient mice. Donor bone marrow was flushed from the femurs of 8- to 16-week-old male C57BL/6 or $Hbb^{th3/+\ mice}$ (Jackson Laboratories) that had been injected intravenously (i.v.) 6 days earlier with 5-flurouracil (5-FU, Pharmacia; 150 mg kg^{-1} body weight). Bone marrow cells were resuspended in serum-free medium, and supplemented with IL-1α (10 ng ml^{-1}), IL-3 (100 U ml^{-1}), IL-6 (150 U ml^{-1}), Kit ligand (10 ng ml^{-1}) (Genzyme), β-mercaptoethanol (0.5 mM; Sigma), $_L$-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg m^{-1}), and cultured for 18 h. Recipient mice (11- to 14-week-old C57/BL6 or $Hbb^{th3/+}$ mice) were irradiated with 10.5 Gy (split dose 2 x 5.25 Gy) on the day of transplantation. Bone marrow cells were pelleted and resuspended in serum-free medium containing concentrated lentiviral supernatant, and supplemented with polybrene (8μg ml^{-1}), $_L$-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}), and cultured for 6 h. Transduced bone marrow cells (1 x 10^5 or 5 x 10^5) were then i.v. injected into each of the irradiated female recipients to establish short-term and long-term bone marrow chimaeras, respectively.

In short-term studies, spleens were removed 12 d after transplantation to extract total RNA and genomic DNA. To monitor long-term chimaeras, two or three capillary tubes of blood were collected every 4-6 weeks, from which genomic DNA, total RNA and haemoglobin were extracted. To examine vector function reliably in long-term animals, erythroid cell populations were purified from spleen. Single-cell suspensions were incubated with rat anti-mouse TER-119 monoclonal antibody (PharMingen). Sheep anti-Rat IgG dynabeads (M-450, Dynal Inc.) Were added to the antibody-coated spleen

cells and purified as recommended by the manufacturer. Vector copy number, integration pattern and chimaerism were determined by Southern blot analysis. The fraction of donor DNA relative to recipient was determined by stripping and reprobing the blot with a [^{32}P] dCTP-labelled probe specific for the Y chromosome and normalizing to an endogenous mouse band. Radioactive bands were quantitated by phosphorimager analysis. Sera from five randomly selected long-term bone marrow chimaeras (30 weeks after transplantation) tested negative for HIV-1 *gag* by RT-PCR using the Amplicor HIV-1 monitor kit (Roche).

Vector copy number and human β-globin RNA transcripts were measured during a 24-week period in mice transplanted with RNS1 (n = 8) or TNS9 (n = 10) transduced bone marrow. a, Vector copy number was assessed by southern blot analysis of genomic DNA isolated from peripheral blood at weeks 6, 10, 16 and 24. The average vector copy number in peripheral blood cells, measured periodically for 24 weeks (Fig. 4), showed highly efficient gene transfer with both vectors (1.8 ± 0.6 and 0.8 ± 0.6 average vector copies per cell for β-globin transcript levels in the 10-20% range during the same time period. To assess transcriptional activity per vector copy, steady-state RNA transcripts and vector copy number were quantified in pooled CFU-S_{12} and in erythroid TER-119+ spleen cells. Twelve days after transplantation, human β-globin expression per endogenous allele, (Fig. 5a). Twenty weeks later these values were 0.5 ± 0.1% (significantly lower than on day 12, P = 0.02) and 15.8 ± 0.9% respectively (Fig. 5b). These findings established that the larger LCR fragments increased globin expression *in vivo* and, furthermore, suggested that TNS9 is more resistant to transcriptional silencing than is RNS1.

The levels of TNS9-encoded human β-globin could be produced. Haemoglobin tetramers incorporating vector-encoded human β^A and endogenous murine α-globin (designated Hbb^{hu}) were quantitated in peripheral blood red cell lysates after cellulose acetate gel fractionation. Hbb^{hu} levels accounting for up to 13% of total haemoglobin were found 24 weeks after transplantation (Fig. 3e, Table 1 in Supplementary Information). In the same assays, transgenic mice bearing one copy of a 230-kb yeast artificial chromosome (YAC) encompassing the entire human β-globin like gene cluster[20] showed 14% of their total haemoglobin incorporating human β^A. No haemoglobin

tetramers containing human β^A were measurble in any of the mice bearing RNS1 (table 1 in Supplementary Information). The proportion of mature peripheral blood red cells expressing human β^A was elevate in most TNS9 bone marrow chimaeras, as shown by dual staining of human β^A and TER-119. In contrast, chimaeras engrafted with RNS1-transduced bone marrow showed highly variable fractions of weakly staining β^A -positive erythrocytes. Normalized to the fraction of circulating β^A -positive mature red cells, the levels of haemoglobin containing lentivirus-encoded β^A were on average 64% of those obtained in the YAC transgenic mice.

Example 5

To ascertain that true HSCs were transduced, we carried out secondary transplants using marrow from primary recipients collected 24 weeks after transplantation. The TNS9 and RNS1 vectors were readily detected in all secondary recipients 13 weeks after transplantation. Human β-globin expression was maintained in all recipients of TNS9-transduced marrow. The successful transduction of HSCs was confirmed by integration site analyses. Southern blot analysis was performed on genomic DNA isolated from bone marrow, spleen and thymus of secondary bone marrow transplant recipients collected 13 weeks after transplant (one representative RNS1, and two representative TNS9 secondary transplant recipients are shown). Two endogenous bands are found in the genomic DNA of C57BL/6 (B6) mice.

Example 6

In view of the high levels of expression, we tested the extent to which the TNS9 vector could correct the phenotype of thalassaemic cells using β^0 -thalassaemic heterozygote mice that lack a copy of their b1 and b2 β-globin genes ($Hbb^{th3/+}$)[21]. These heterozygotes have a clinical phenotype similar to human thalassaemia intermedia and exhibit chronic anaemia (haematocrit 28-30%, haemoglobin 8-9 g dl^{-1}) and anomalies in red cell size (anisocytosis) and shape (poikilocytosis). Fifteen weeks after transplantation with unselected TNS9-transduced $Hbb^{th3/+}$ bone marrow, the haematocrit level, red blood cell count, reticulocyte count and haemoglobin level were markedly improved in five out of five recipient mice (Fig. 6). Anisocytosis and poikilocytosis

were markedly reduced in the peripheral blood smears of chimaeras bearing the TNS9 vector. Control mice transplanted with $Hbb^{th3/+}$ bone marrow cells transduced with a vector encoding enhanced green fluorescent protein (eGFP) all remained severely anaemic (n = 5, Fig. 6) and maintained their abnormal red cell morphology. These results establish that levels of circulating haemoglobin obtained with TNS9 were indeed therapeutically relevant.

The combined effect of the high efficiency of gene transfer and the absence of vector rearrangements afforded by the recombinant lentivirus carrying the β-globin gene and LCR configuration adopted in TNS9 yielded levels of human β^A expression in the therapeutic range. The higher expression obtained with TNS9 compared with RNS1 was associated with a higher fraction of permissive integration sites in MEL cells and a higher fraction of human β^A -containing red blood cells in bone marrow chimaeras. RNS1, which carries a weaker enhancer, silenced over time whereas TNS9 retained undiminished transcriptional activity over the same time period and in secondary transplant recipients.

Higher levels of murine α_2: human $\beta^A{}_2$ tetramers were obtained in peripheral blood samples from recipients of TNS9-transduced $Hbb^{th3/+}$ bone marrow (21 ± 3% of total haemoglobin, n = 5, than with $Hbb^{+/+}$ bone marrow (6 ± 4%, n + 10). The two groups showed comparable peripheral blood vector copy numbers and levels of human β-globin RNA (0.8 ± 0.2 compared with 0.8 ± 0.6, and 16.8 ± 6% compared with 10.8 ± 7%, respectively). This observation is consistent with a competitive advantage of murine β-globin over human β-globin in associating with murine α-globin[22]. In thalassaemic patients, added human β-chain synthesis would improve the α:β chain imbalance and thus increase red cell survival and ameliorate the ineffective erythropiesis in these patients. In patients with sickle cell disease, transduced β^A chains are expected to have an advantage over the β^S chains produced by both endogenous genes in competing for the available α-chains[23]. Given that patients with S/β-thalassaemia whose HbA represents 10-30% of their total haemoglobin are very mildly affected[1,24], the clinical benefit of such an intervention would be highly significant.

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Example 7

To investigate long-term expression of the transduced human *β-globin* genes and its therapeutic efficacy, we generated bone marrow chimeras engrafted with TNS9-transduced $Hbb^{th3/+}$ bone marrow cells (n =5) and studied them over a 40-week period.

Donor bone marrow was flushed from the temurs of 8-to 16- week old male c57/BL6 or $Hbb^{th3/+}$ mice [23] obtain from Jackson Laboratories (Bar Harbor , ME) that had been injected intravenously (IV) 6 days earlier with 5-flurouracil (5-FU) 150 mg/kg body weight obtained from Pharmacia (Piscataway, NJ). Bone marrow cells were resuspended in X-VIVO-15 serum-free medium and supplemented with 10 ng/mL interleukin-1 α (IL-1α) 100 U/mL IL-3, 150 U/mL IL-6, 10ng/mL Kit ligand obtained from Genzyme (Cambridge, MA), 0.5mM β-mercaptoethanol obtained from Sigma (St. Louis, MO), 200-mM $_L$-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Bone marrow cells were ten pelleted and resuspended in serrum-free medium containing concentrated lentiviral supernatant and supplemented with 8 mg/mL polybrene (Sigma), 200mM $_L$-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and cytokines as above, and cultureed for 8 hours. Transduced bone marrow cells (5×10^5) were ten injected IV into each of the irradiated female recipients to establish bone marrow chimeras. Recipients mice (11- to 14-week-old C57/BL6 or $Hbb^{th3/+}$ mice) were irradiated with 10.5 Gy (Split dose 2 × 5.25 Gy) on the day of transplantation.

Age-matched chimeras engrafted with eGFP-transduced $Hbb^{th3/+}$ (n=5) and $Hbb^{+/+}$ (n=5) bone marrow cells served as controls. Vector copy number was monitored in peripheral blood by quantitive Southern blot analysis, and found to remain stable, between 0.5 and 1.0 copy/cell on average (data not shown). Protein expression was assessed by quantitive hemoglobin analysis, to measure the proportion of hemoglobin tetramers that incorporate human β^A (Hbb^{hu}, mu α_2: hu$\beta^A{}_2$) or murine β-globin (Hbb^{mu}, muα_2:muβ_2), and immunofluorescence, to determine the fraction of mature RBCs that contain human β^A protein. Transgenic mice bearing one copy of a 230-kb yeast artificial chromosome encompassing the entire human β-globin-like gene cluster[28] served as reference, showing 14% of their total hemoglobin incorporating human β^A and 100% β^A+RBCs[19,28] Hbb^{hu} accounted for 19% to 22% of the totalhemoglobim in TNS9 chimeras. These levels remained stable up to 40 weeks after transplantation. Over this

same time period, the proportion of mature peripheral RBCs expressing human β^A also remained elevated and stable (about 70% to 80%), as shown by dual staining of human β^A and TER-119.

Example 8

Long-Term amelioration of anemia

The stability of TNS9-encoded β^A expression detected in peripheral blood suggested that long-trem hematologic and systemic therapeutic benefits could be obtained. To investigate whether Hbb^{hu} production would suffice to teart the anemia, we closely monitored hemoglobin parameters over 40 weeks. The marked increase in hemoglobin concentration, RBC counts, and hematocrit was sustained throughout this time period. Control mice that received transplants of eGFP-transduced $Hbb^{th3/+}$ bone marrow cells remained severely anemic, indicating that the transplantation procedure itself did not alter the anemic state. The reticulocyte counts decreased to 5% to 8% in TNS9 treated-chimeras, compared to 19% to 21% in control eGFP-treated $Hbb^{th3/+}$ chimeras and age-matched $Hbb^{th3/+}$ mice, suggesting an increase in RBC life span and a decreasein erythropoietic activity.

Example 9

To determine the impact of sustained human β-globin gene expression on hematopoiesis, we studied the degree of splenomegaly (enlargement of the spleen) and EMH in 1-year-old chimeras and age-mateched control mice. Spleen weights measured in Tns9-treated $Hbb^{th3/+}$ chimeraas were indistinguishable from recipients of eGFP-transduced normal bone marrow, as were the total number of cells per spleen. In contrast, mice engrafted with eGFP-transduced $Hbb^{th3/+}$ bone marrow cells showed spleen weights and total cell numbers that were about 3-fold greater. The correction of spleen weight in TNS9 bone marrow chimeras corresponded to a concomitant normalization in total hematopoietic progenitor cell content. Spleen CFU-Es, BFUEs, andCFUs-GM were reduced to levelsmeasured in recipients of eGFP-transduced $Hbb^{th+/+}$ bone marrow, whereas they remained elevated in control chimeras engrafted with eGFP-trasduced

$Hbb^{th3/+}$ bone marrow cells and in age-matched $Hbb^{th3/+}$ mice, as previously observed in another murine modelof β-thalassemis.[29]

The regression of EMH was corroborated by morphologic examination of spleen and liver in long-term chimeras and age-match controls. Histopathology of spleens of mice that received transplants of eGFP-tranduced $Hbb^{th3/+}$ marrow was virtually identical to that of slpeen from control $Hbb^{th3/+}$ mice. Specifically, the red pulp was significantly expanded, accounting for 80% to 90% of the cross-sectional area, and densely occupied by nucleated erythroid precursors. The white pulp, based on cross-sectional area, wsa relatively decreased and the marginal zones were obscured by the large number of nucleated RBCs, reflecting major expansion of the red pulp and erythroid precursors. In TNS9-treated chimeras, the amount of red pulp was considerably decreased, accounting for olny about 50% to 60% of the cross-sectional area. In addition, the number of nucleated erythroid precursors in the red pulp was decreased. Other immature hematopoietic cells were present in the red pulp, but much less frequently than in the spleens of control $Hbb^{th3/+}$ thalassemic mice. The livers from TNS9-treated chimeras were similar to those of the normal control mice in that no EMH was detected. In contrast, livers from mice engrafted with eGFP-trasduced $Hbb^{th3/+}$ bone marrow cells showed seral small foci of intrasinusoidal EMH.

Example 10

Toxic iron accumulation in the organs of thalassemic patients is a consequence of RBC destruction and increased gastrointestinal iron uptake. To determine whether sustained expression from the TNS9 vector reduced iron overload, we stuied tissue sections of liver and heart, stained using Gomori iron stain. No iron deposition was seen in the livers of normal $Hbb^{+/+}$ control mice, whereas $Hbb^{th3/+}$ mice showed variable amounts of iron, including some large aggregates. TNS9-trasduced treated chimeras demonstrated low to undetectable levels of iron in the livers, whereas iron was readily detected in the livers of all mice that received transplants of eGFP-transduced $Hbb^{th3/+}$ bone marrow cells. No iron accumulation was found in the heart of treated or control mice, as previously observed in another murine model of β-thalassemia,[30] in contrast to what is found in the human disease. [1-3]

Example 11

To assess to efficacy of *in vivo* selection for cells transduced with globin and DHFR-encoding vectors in accordance with the invention, using antifolates the following alternative protocols are used. In protocol 1, the recipient mice are treated daily for 5 days with MTX (25mg/Kg) and NBMPR-P (20mg/Kg), starting 6 weeks after administration of transduced bone marrow cells. In protocol 2, the recipient mice are treated daily for 5 days with TMTX (40mg/Kg) and NBMPR-P (20mg/Kg), starting 6 weeks after administration of transduced bone marrow cells. In protocol 3, the recipient mice, conditioned with busulphan rather than with gamma-irradiation, are treated daily for 5 days with TMTX (40mg/Kg) and NBMPR-P (20mg/Kg), starting 4 weeks after administration of transduced bone marrow cells. (TMTX (Neutrexin; US Bioscience); >MTX (Methotrexate LPF Sodium; Immunex); NBMPR-P (Nitrobenzylthioinosine 5'-monophpsphate disodium salt; Alberta nucleoside therapeutics). Protocol 3 is in principle the most attractive protocol as the recipients are not irradiated and furthermore not treated with a "myeloablative conditioning regimen". They are treated with a relatively milder conditioning regimen consisting of a "non-myeloablative" dose of busulphan. It is hoped that, in combination with "in vivo selection" mediated by DHFR/TMTX, the recipients could be satisfactorily engrafted without receiving a harsh pre-transplant treatment. This would be the way to go for treating subjects with severe hemoglobinopathies.

CLAIMS

1. A recombinant vector comprising:
(a) a region encoding a functional β-globin gene; and
(b) portions of the β-globin locus control region, which-consist essentially of an 840 bp fragment of HS2 extending between SnaBI and BstXI restriction sites, a 1309 bp fragment of HS3 extending between HindIII and BamHI restriction sites and a 1069 bp fragment of HS4 extending between BAMHI and BanII restriction sites, said vector providing expression of β-globin when introduced into a mammal *in vivo*. ;

2. The vector of claim 1, further comprising a mouse PGK promoter, wherein the mouse PGK promoter controls the expression of the region encoding a dihydroflate reductase.

3. The vector of claim 1 or 2, wherein the globin gene encodes human β-globin.

4. The vector of any of claims 1-3, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

5. The vector of claim 4, wherein the human dihydrofolate reductase is a mutant form having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase, said mutant form differing in amino acid sequence from wild-type human dihydrofolate reductase as a result of a set of mutations.

6. The vector of claim 5, wherein the set of mutations comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

7. Use of a vector according to any of claims 1-6 to transduce hematopoietic progenitor or stem cells for the treatment of hemoglobinopathy in a mammalian individual suffering from a hemoglobinopathy.

8. The use of claim 7, wherein a selection step using an antifolate is performed *ex vivo* to produce a population of cells with increased numbers of transduced cells.

9. The use of claim 8, wherein the antiifolate is methotrexate.

10. A mammalian hematopoietic progenitor or stem cell transduced with a recombinant vector according to any of claims 1-6.

11. A method for making a therapeutic composition for treatment of hemoglobinopathy in a mammalian individual, comprising the steps of preparing a recombinant vector in accordance with any of claim 1-6, obtaining hematopoietic progenitor or stem cells

from the mammalian individual, and transducing the cells with the recombinant vector.

12. The method of claim 11, further comprising the step of performing an *ex vivo* selection using an antifolate.