Decrease of α-chains in β-thalassemia

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Abstract

In the pathophysiology of β-thalassemia, globin chain imbalance plays a central role in predicting red blood cell (RBC) life span and disease severity. Strategies to improve globin chain imbalance are therefore a legitimate target in the management of this incurable genetic disorder. Classical gene addition with the available retroviral vectors can alter one of the two variables while combined reduction of α-chains could provide a more potent therapeutic effect. We developed foamy virus (FV) vectors for the production of β-globin and vectors targeting the α-globin transcript using the shRNA technology. Using FV-derived vectors, we expressed human anti-α-globin short hairpin RNAs, off a potent PolIII promoter (H1); of the 4 different shRNAs tested, α-globin mRNA reduction varied from 6.3 to 54% of the control CD34+ cells. Similarly, vectors developed for the mouse α-globin, resulted in a significant reduction (range 15-28% of the control) of α-globin in erythroid colonies of Lin- cells. To assay vector performance *in vivo*, we investigated the hematological parameters in thal3+/- mice transpalnted with FV-transduced Lin- cells, transduced with anti-α-globin shRNA. Despite low chimerism and low vector copy numbers (<0.5 per cell), we observed a 10% reduction in red cell distribution width, a marker for distorted erythropoiesis. We finally developed a combination FV vector expressing β-globin off a HS40 enhancer and anti-α-globin shRNA and tested its performance in human CD34+ cells from a thalassemic patient. Globin chain imbalance was ameliorated from a β/α ratio of 0.12 to the level of 0.5, clearly indicating a therapeutic benefit.. Overall, shRNA control of α-globin excess is a feasible target but requires improvements since the RNAi effect is tough to predict and should ideally be combined with controllable elements.

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Thalassemia Reports 2013; 3(s1):e40
doi:10.4081/thal.2013.s1.e40

Parts of this work were presented at the "3rd Pan-European Conference on Haemoglobinopathies and Rare Anaemias", Limassol (Cyprus), 24-26 October 2012.

Introduction

β-Thalassemia syndromes are a group of hereditary blood disorder-scharacterized by deficient synthesis of the hemoglobin β-chain and-chronic non-compensated hemolysis. Thalassemias are considered as the most commonmonogenic disorders worldwide and are inherited in an autosomalrecessive manner. The defective synthesis of hemoglobin β-chains inthe erythroid progenitors results in the accumulation and precipitationof excess α-chains, causing damage to the developing red bloodcell (RBC) membrane. As a result of globin chain imbalance, a significant number of erythroblasts are lost prematurely in the bone marrow while mature RBCs with defective membranes are eliminated in the spleen. Thus, both ineffective hematopoiesis and extravascular hemolysis contribute equally to the chronic anemia that characterizes the course of the disease. Patients with thalassemiamajor, the most severe form, require regular blood transfusions thatnot only affect the quality of life but also are often accompanied by life-threatening side effects such as iron overload.Allogeneic bone marrow transplantation, when available, is the onlytherapeutic approach, which, however, is restricted by donor availability and could be complicated with graft *versus* host disease.(1) As a result, alternative treatment options for β-thalassemia have been developed, and therapeutic β-globin vectors have been constructed and tested for over two decades. Accomplishments in globin gene regulation and vector technology has allowed the development of therapeutic vectors and the first clinical trial in β-thalassemia.(2)

In the gene therapy setting, beyond provision of the missing β-globin chains, control of the excess α-chains would allow a more physiological approach in tackling globin chain imbalance. Such technology is offered by RNAi. Since its discovery in C. elegans (3) and the subsequent successful experiments in mammalian cells (4), RNA interference (RNAi) has emerged as a powerful tool for the sequence-specific posttranscriptional silencing of target genes, leading to the expansion of the relative technology into a variety of biological and therapeutic applications. (5) One of the most important parameters for an effective RNAi experiment is a suitable delivery method. For a transient RNAi effect in mammalian cells, chemically synthesized short interfering RNA oligos (siRNAs) or short hairpin RNAs (shRNAs) from plasmid vectors are widely used. For long-term silencing of disease-related genes and a durable therapeutic effect, the expression of shRNA molecules from integrated viral vectors systems is an obligatory step.(6) However, when it comes to stable RNAi expressionfrom viral vectors, several critical parameters could compromise the system's efficiency such as the length and sequence of the encoded shRNA, the number of integrated vector copies, and the choice of the promoter.(7,8)The most commonly used promoters for shRNA expressionfrom vector systems are the human H1 and themouse/human U6 RNA polymerase III (Pol III) promoters. For the purpose of our study, we tested both promoters in the relative species (mouse and human) for their efficiency in reducing α-globin transcripts using foamy virus (FV) derived retroviral vectors as gene transfer vehicles.

Retroviruses are divided into two subfamilies: orthoretroviruses (OV) that include gammaretrovirus (GV) and lentivirus (LV) and foamy viruses(FV). In common with OV, FVs have the principal retroviral structure of LTR (long terminal repeat), gag-pol-env-accessory genes-LTR, use reverse transcription for genome replication and integrate stably into the host cell genome.(9) A distinct feature of FVs is that the viral particles are made of DNA (instead of RNA), a property that provides stability in the infected cell and is believed to be one of the reasons for the excellent ability of FV vectors to transduce rarely dividing cells, such as HSCs.(10) FV vectors have evolved over the last 15 years to a level that makesthem an attractive gene transfervehicle for clinical applications. The vectors have deletions in the U3 promoter region, deletions in most of their genome, and in effect have room toaccommodate about 9 kb of foreignDNA. In addition they are true self-inactivating (SIN) vectors asboth the viral promoter and itstransactivator are deleted. FV vectors are produced in 293T cellsby transient transfection of vectoralong with three different helperplasmids coding for gag, pol andenv, thereby further minimizing thepossibility of generating a replication-competent recombinant retrovirus. By ultracentrifugation of the crude supernatant, vector titers can be enhanced close to 100-fold with a yield of about 60%.(11) However, vector titersare less of an issue for FV applications since efficient gene transfer rates can be obtained with low multiplicities of infection (MOI) as it has been shown with human and canine HSC gene marking experiments.(12,13) Regarding their integration pattern, compared to GV, FV vectors integrate less often in promoter regions while, compared to LV, they integrate less often within genes, in effect displaying a relatively superior integration profile.(14) In addition, in assays where long term expression is a prerequisite, FV vectors have shown persistent expression with no evidence of silencing.(15) Thus, FV vectors derived from the non-pathogenic foamy virus, are suited for clinical gene therapy applications.

The aim of our study was to develop FV vectors expressing shRNAs againstα-globin chain transcripts in order to improve globin chain imbalance in β-thalassemia. We also generated a combination vector expressing β-globin and the anti-α-globin shRNA in order to test whether such a strategy could have an added benefit in improving the thalassemic phenotype.

Materials and Methods

Cell lines

All adherent cell lines were grown in DMEM and suspension lines in RPMI, both supplemented with 10% fetal bovine serum and 1% penicillin- streptomycin (all from Invitrogen, San Diego, CA).

Vector production and titration

Vector stocks were produced by CaP cotransfection of 293T cells with the vector plasmid and three different packaging plasmids (coding for the gag, pol, and env genes) as described.(11) Vector supernatants were collected 72 hr post transfection, centrifuged at 200 g for 10 min, filtered through 0.45-mm Durapore filters (Millipore, Bedford, MA) and further concentrated by centrifugation at 20,000 g for 4 hr at 20C. Vector stock titers were determined on HeLa or HT1080 human cells and were calculated by flow cytometry (FCM) as GFP transducing units per milliliter of supernatant (TU/ml).

The pDF.Lb vector (GV, unpublished) that codes for the human b-globin gene was used as a backbone. A sequence containing the HS40 element (425 bp) along with the 127 bp β-globin promoter was isolated as a NotI-NcoI fragment from another cassette (16) andinserted into the pDF.Lb backbone, replacing the LCR cassette and generatingthe pDF.HS40.b vector. The HS2 (756 bp) and HS3 (1334 bp) sequences wereisolated as a single PCR fragment from the pDF.Lb LCR cassette using primerswith XbaI and NotI restriction sites.

F(forward): 5'-GCCGGGTCTAGACATTTGATTCACAAT-3' and R(reverse): 5'-CTAAGCGGCCGCCTAGTGCTTAGATTC-3'). The HS2/HS3 sequence replaced the HS40element in the pDF.HS40.b vector, generating the pDF.HS2.HS3.b vector. Theentire human b-globin gene expression cassette was integrated in reverse orientation to avoid splicing during vector production.

Flow cytometry and fluorescence microscopy

Transduction efficiency and mean fluorescence intensities (MFIs) were determined on a Becton Dickinson FC500 flow cytometer. Exclusion of dead cells was based on propidium iodide or 7-aminoactinomycinD staining (Sigma) and subsequent acquisition of live cells. For detection of apoptosis, cells were washed two times in PBS and stained using the Annexin V-PE reagent (BD Pharmingen) as per the manufacturer's instructions.

Bone marrow transplantation studies

All C57Bl/6J and thal3 animals were kept at the Biomedical Research Foundation of the Academy of Athens (BRFAA) Animal Facility. The study was fully approved by Institutional and Regional Ethical Review Boards. For bone marrow transplantation (BMT) studies, we performed partial myeloablativeconditioning using intraperitoneal injections with Busilvex at 20 mg/kg for 4 consecutivedays prior to BMT. Sex-mismatched BMTs were performedusing lineage-depleted (Lin-) BM cells (StemSep MouseHematopoietic Progenitor Cell Enrichment Kit; Stem CellTechnologies, Vancouver, BC, Canada) according to themanufacturer's protocol. Transduction was performed for 18-20 hr on RetroNectin-coated plates (TakaraBiomedicals, Otsu, Japan) in StemSpan serum-free medium(Stem Cell Technologies) supplemented with 3% fetal calfserum, 100 ng/ml Flt-3 ligand, 100 ng/ml murine stem cellfactor, and 50 ng/ml human interleukin-6 (all from Peprotech,London, UK). Following transduction, the cells were eitherplated in methylcellulose colony assays (Methocult H 4230;Stem Cell Technologies) or administered intravenously for BMT studies (0.5-1.5E105 per mouse). Donor cell engraftmentwas determined on animal BM upon termination of theexperiments by real-time PCR for the mouse single-copytestis-specific Y pseudogene (TSPY).

Isolation, transduction and ex vivo erythroid differentiation of human primary hematopoietic progenitor cells

PB samples from human β-thalassemia major (β0) patients were collected and used to isolate CD34-positive cells (CD34 Microbead kit, Miltenyi Biotec). The cells were transduced with concentrated FV stocks for 18-20 h with the addition of huFlt3L, huSCF, huTPO, huIL-6 and huIL-3 (all from PeproTech EC Ltd). After 16-20 h, transduced cells were washed with PBS and cultured inStemSpan medium supplemented with 1% PenStrep, 5% fetal calf serum and: EPO,huSCF, huIL-3 and either dexamethasone and b-estradiol (Sigma-Aldrich) or huTPOand huFlt3L.(17)

Construction of shRNA-expressing foamy vectors

We have designed all shRNA molecules according to currently accepted rules for siRNA design.(18) Theoligonucleotides contained a sense target sequence, thepublished loop sequence TTCAAGAGA,(19) the antisense sequence, a termination sequence offive thymidines, and appropriate sequences for restrictionenzymes at both ends, to allow cloning into the human H1 ormU6 promoters.

All pDF (deleted foamy) are safety-enhanced third-generation FV vectors that do not produce replication-competent viruses. The FV vec-

tor plasmid pDFmU6F, harboring the mU6 promoter, was a gift from D.W. Russell (U. of Washington, Seattle, WA). The H1 promoter (NR_002312, nt 145-486) was amplified by PCR from the commercially available pSUPER vectors (OligoEngine, Seattle, WA) and inserted as an NcoI fragment in the exact same position as the mU6. For H1 amplification, the sense primer was ATCCCATGGAATTCGAACGCTGACGTC and the reverse ATCCCATGGACGCGTGTCGACGGTATCGA. All shRNA oligos were cloned as BglII-MluI fragments at the H1 promoter.(20)

Results

β-Globin expression from FV vectors in cell lines and primary hematopoietic stem cells

To assay FV vector performance, we first tested β-globin expression in cell lines and primary hematopoietic stem cells. We constructed and compared two FV vectors combining elements from both α- and β-globin loci; in the pDF.HS40.β vector, expression of the β-globin gene was driven by the native β-globin promoter and regulated by the HS40 element of the human α-globin locus (Figure 1), whereas in the pDF.HS2.HS3.β vector the core HS2 and HS3 regulatory elements from the β-globin LCR were used. The titers were similar indicating no detrimental effects from any of the elements on vector production.

As estimated by RealTime-PCR, the average titer was 3.8x10E6 IU/ml and 2.5x10E6 IU/ml for HS40.β and HS2.HS3.β respectively. Efficient production of human β-globin was observed with both vectors even when compared with the MEL-YAC-βGLO control, an MEL clone that carries 150 kb of the β-globin locus.(21) In the vast majority of the clones, this expression was achieved with a vector copy number (VCN) of 1-3, as evaluated by Taqman real-time PCR. When we calculated the β-globin mean fluorescence intensity levels per integrated vector copy, we observed a 65±16% increase in β-globin expression with the HS40.β vector over the untransduced cells and 55±12% with the HS2.HS3.β vector (Figure 1d). These findings indicated that both vectors can support the expression of human β-globin gene in the MEL cellular environment. Since however the HS40.β vector had stably higher titers and was superior in expression (though it did not achieve statistical significance) we decided it to use it as the backbone for further experimentation. When Lin- mouse HSC were tested for globin expression using the DF vectors, a clear advantage of the HS40 backbone was evident (Figure 2). The assay was performed after overnight trasnduction of Lin- and plating under condition for BFUe expansion. mRNA from individual BFUe was quantitated and expressed as fraction of the endogenous α-globin mRNA. To assay vector performance *in vivo*, we transplanted transduced thal3 Lin- cells in myeloablated wt C57Bl6/J mice and followed β-globin expression over an observation period of maximum 25 weeks (Figure 3). Animals that received cells transduced with the HS40.β vector expressed human β-globin at an average of 30% at 7-12 weeks following B and 43% at termination. verall, vector performance in the mouse thalassemia model was superior for the HS40 vector indicating a species specific effect as this finding was not verified in subsequent experiments where globin expression was tested in human CD34+ cells.

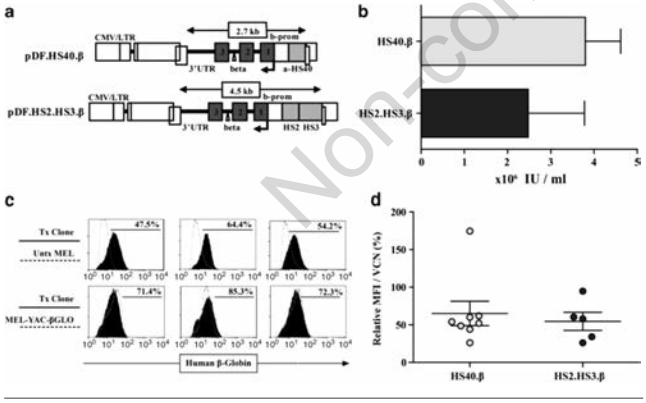

Figure 1. FV vector performance in the Murine Erythroleukemia cell line.(a) Diagram of pDF.HS40.b and pDF.HS2.HS3.b FV vectors. (b) Vector titers expressed as HT1080 integrating units per ml calculated by Taqman real-time PCR. Error bars indicate s.e.m. (c) Flow cytometry analysis for the expression of human β-globin in representative FV-transduced MEL clones. In the upper panel, filled histograms show transduced MEL cell clones over untrasduced control MEL cells (dotted line), whereas in the lower panel, filled histograms show individual clones layed over the MEL-YAC-bGLO cell line that is used as a positive control (dotted line). (d) Human β-globin levels in single HS40.b- or HS2.HS3.b-transduced MEL clones presented as antibody mean fluorescence intensity increase over the negative control per integrated vector.

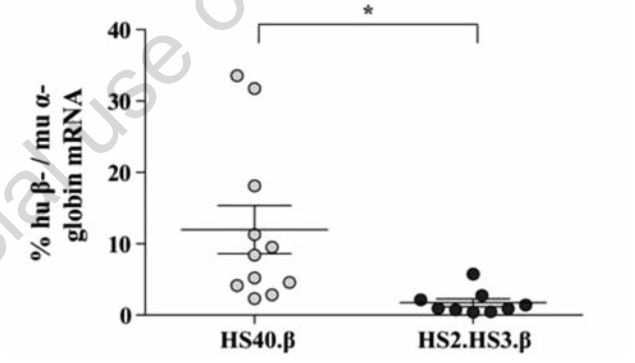

Figure 2. Foamy VirusVector performance in murine Lin- HSCs. Relative quantitation of human β-globin expression in individual mouse BFUe. Total mRNA levels were analyzed by Taqman real-time PCR for the expression of human β-globin and endogenous murine α-globin. The levels of human β-globin gene were calculated as percentage of the endogenous mouse α-globin gene. Each symbol represents a single BFUe.

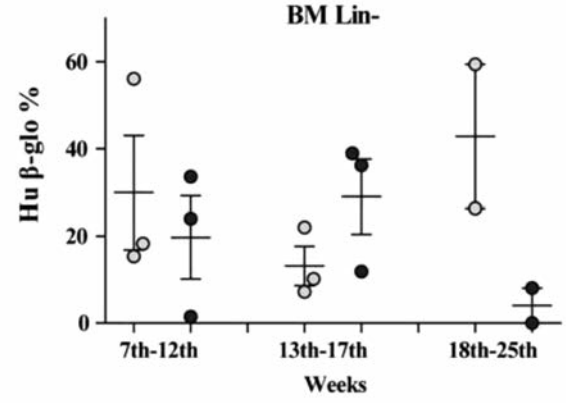

Figure 3. Human β-globin expression (%) in the PB of animals transplanted with BM Lin- cells transduced with the HS40.β or the HS2HS3. β vectors. Human β-globin expression is presented in terms of antibody mean fluorescence intensity (MFI) fold increase over untransduced control mice and in three time periods after transplantation. Symbols represent individual animals.

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Control of murine and humana-globin expression using anti-a shRNAs

For α-globin mRNA targeting, we designed 4 different shRNA molecules based on standard rules for oligonucleotide structure that are available from internet based software. Basically, we targeted sequences 25 nt downstream of the ATG codon where regions of 21 nt with the structure AA(N19) or NAR(N17)YNN were located (N is any nt, R is a purine and Y is pyrimidine). The four different oligos (B9, C7, D3 E1, Figure 4) were synthesized as 52-63 nt long complementary DNA stretched with the TTCAAGAGA loop and were cloned in the FV backbone.(20)All plasmids were sequenced verified before cloning into the FV vectors.A maximum of 84% reduction in α-globin transcripts was observed in the BFUe as compared to the non-specific control (range from 84 to 72%) indicating, a robust effect in the control of α-globin mRNA availability. To test whether α-globin mRNA reduction could improve erythropoiesis in the thal3 HSCs, we transduced Lin- from thal3 mice with two shRNAs and measured BFUe numbers; distorted erythropoiesis *in vitro* is manifested as increased erythroid potential.(22) Compared to the untransduced control, we observed 1.5x and 4x fold decrease of the BFUe numbers for the B9 and C7 oligos respectively. Our next step was to design shRNAs for the human α-globin transcripts and assay mRNA expression in BFUe colonies from transduced CD34+ cells (Figure 5). A total of 4 different hairpins was assayed (H1 to H4) and mRNA reduction reached a maximum of 94% (range from 46 to 94%), indicating the potential of the technology.

Combination vectors

To test our original hypothesis on whether we could improve thalassemic erythropoiesis by ameliorating globin chain imbalance, we generated a combination vector expressing β-globin off the HS40 promoter and the potent H4 shRNA. A schematic diagram of the vector design is shown in Figure 6. Vector titers were in the order of 2x10E5 which is within normal range for DF vectors. Vector performance was assayed in CD34+ cells from a thalassemic patient; shown in Figure 6, lower panel, is the ratio of β/α globin in liquid erythroid cultures from the thalassemic sample. Transduction with the globin vector increased the ratio from 0.1 in the untransduced population to 0.21 with the HS40.β vector and to 0.53 with the combination vector. It is clear that control of the excess α-chains had a significant effect in converting the erythroid cells from a thalassemic to the disease carrier state.

Discussion

The aim of our study was to test whether improving β/α globin chain balance in the erythroid cells could affect erythropoiesis. We developed FV vectors that had the potential to express significant amounts of β-globin both *in vitro* and *in vivo*; in addition, we constructed FV vectors with shRNA against α-globin and showed that we could reduce substantially the amount of available α-globin mRNA. Finally, we combined elements from both of the above constructs to generate a combination vector that could correct the globin chain imbalance of a thalassemic cell and bring to a state of carriership.

The use of the RNAi technology as an adjuvant to gene therapy of β-thalassemia has not yet been widely explored.(23,24) Although one can speculate on the reasons, from our experience, the major shortcoming of this technology is its unpredictability. Beyond issues related to the design of the shRNA per se, common limitations include the induction of innate immune responses, saturation of the intracellular RNAi processing machinery and off-target silencing effects.(25,26,27) Considering also that retroviral vectors integrate randomly in the host cell genome and expression can be influenced from the surrounding

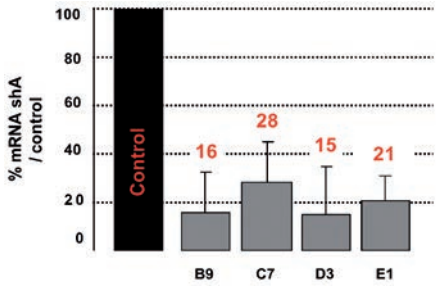


Figure 4. α-globin mRNA expression in mouse BFUe after transduction of Lin- cells with FV vectors expressing the relative anti-α-globin shRNA.

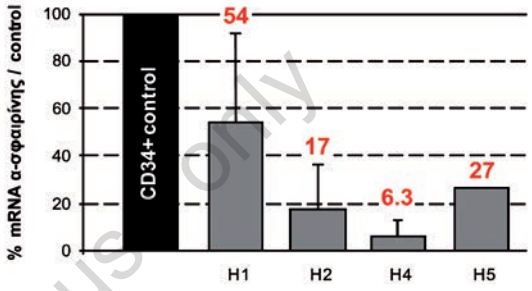
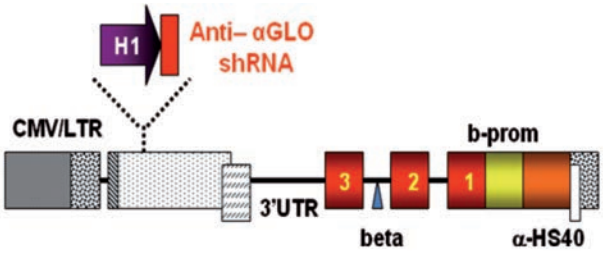


Figure 5. Human-globin mRNA reduction in BFUe colonies derived from CD34+ cells using 4 different shRNA expressed from foamy virus vectors.



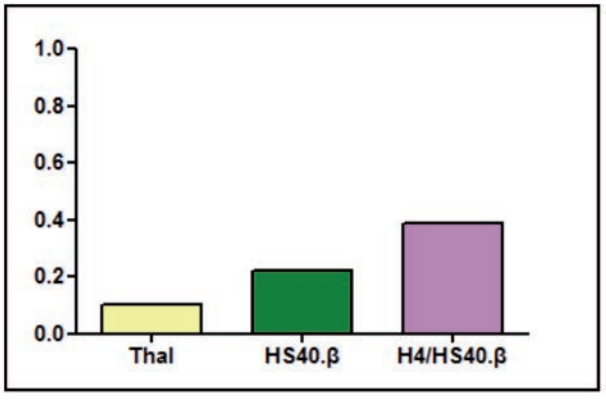


Figure 6. Diagram of the combination vector. Shown in the lower panelare the β/α globin ratios obtained in untransduced (thal), HS40. β and H4.HS40. β vector transduced CD34+ cells grown under liquid erythroid culture conditions.

chromatin, one could contemplate why such a much promising technology has not been widely deployed. It is the feeling of the authors that RNAi technology could assist in globin gene regulation but in a package different from the integrated hairpin form. Delivery as siRNA with a transient effect or within a miR that is physiologically expressed in a certain developmental stage of a cell could be part of technologies that will emerge. Till then, amelioration of globin chain imbalance will rely on the provision of the missing β-globin gene.

Conclusions

Control of excess α-globin chains can be obtained with FV vectors and has a clear phenotypic effect in thalassemic erythropoiesis. However, lack of proper shRNA regulation and unpredicted off target effects have limited its use. Whether control of α-globin excess will be part of future vector constructs remains to be seen.

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Oral presentation

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