RNA Trans-Splicing Targeting Endogenous β -Globin Pre-Messenger RNA in Human Erythroid Cells

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Sickle cell disease results from a point mutation in exon 1 of the β -globin gene (total 3 exons). Replacing sickle β -globin exon 1 (and exon 2) with a normal sequence by trans-splicing is a potential therapeutic strategy. Therefore, this study sought to develop trans-splicing targeting β -globin pre-messenger RNA among human erythroid cells. Binding domains from random β -globin sequences were comprehensively screened. Six candidates had optimal binding, and all targeted intron 2. Next, lentiviral vectors encoding RNA trans-splicing molecules were constructed incorporating a unique binding domain from these candidates, artificial 5' splice site, and γ -globin cDNA, and trans-splicing was evaluated in CD34⁺ cell-derived erythroid cells from healthy individuals. Lentiviral transduction was efficient, with vector copy numbers of 9.7 to 15.3. The intended trans-spliced RNA product, including exon 3 of endogenous β -globin and γ -globin, was detected at the molecular level. Trans-splicing efficiency was improved to 0.07–0.09% by longer binding domains, including the 5' splice site of intron 2. In summary, screening was performed to select efficient binding domains for trans-splicing. Detectable levels of trans-splicing were obtained for endogenous β -globin RNA in human erythroid cells. These methods provide the basis for future trans-splicing directed gene therapy.

Keywords: lentiviral vector, RNA trans-splicing, β -globin gene

INTRODUCTION

SICKLE CELL DISEASE (SCD) results from a point mutation in exon 1 of the β -globin gene (total 3 exons), which leads to vaso-occlusion, severe anemia, organ damage, and early mortality. Hydroxyurea is a commonly used treatment to induce fetal hemoglobin and reduce organ damage in SCD. However, lifelong intake at a sufficient dose is required to maintain the beneficial hematologic effects.^{1,2} In contrast, one-time cures using hematopoietic stem cell (HSC)-based therapies, including allogeneic HSC transplantation and HSC-targeted gene therapy, are increasingly accessible to affected patients. Allogeneic HSC transplantation has been developed in adult patients with SCD. However, a suitable sibling donor is required and is only found for ~10% of SCD patients.^{3,4} HSC-targeted gene therapy is

theoretically applicable for most patients, since autologous HSCs are used for therapeutic gene transfer.⁵ Clinical trials in which autologous HSCs are transduced with lentiviral vectors to produce β -globin (or γ -globin) gene are currently ongoing.^{6,7} Another strategy with iPS cell-based regenerative therapy is very attractive, as it allows for gene correction in autologous iPS cells followed by differentiation into red blood cells or HSCs.^{8,9} However, this is currently only available for *in vitro* cell culture or mouse models.

RNA splicing from two different pieces of pre-messenger RNA, known as trans-splicing, is another method, allowing the mutated exon 1 to be replaced with a normal exon.^{10–12} Spliceosome-mediated RNA trans-splicing would be induced by an RNA trans-splicing molecule (RTM), which

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contains (1) a coding region to replace mutation in target RNA, (2) a 5' or 3' splice site, and (3) a binding domain, which is complementary to target RNA.¹³ A major benefit of the trans-splicing-based gene therapy over the whole gene insertion strategy in SCD is the ability to induce therapeutic gene expression and to reduce pathogenic sickle globin production concurrently. The trans-splicing in β -globin RNA was previously reported in cell line models.^{10–12} However, the clinical application for SCD will require high efficiency of trans-splicing. Therefore, this study sought to improve the efficiency of trans-splicing targeting the β -globin gene, which replaces exon 1 in human erythroid cells.

METHODS

Comprehensive selection for optimal binding domains for RTMs to target β -globin RNA

To make random binding domain libraries to target the β -globin gene, DNA fragments were produced by sonicating the β -globin sequence (from 5' untranslated region [UTR] to intron 2) or synthesizing

random primer-mediated cDNA using β -globin RNA converted from genomic DNA, and analyzed by electrophoresis (around 20–600 bp of DNA smear). The β -globin DNA libraries were inserted into the binding domain region of an RTM plasmid. The prototype RTM plasmid contained a red fluorescent protein (RFP) mCherry gene, an internal ribosome entry site (IRES), the 5' half of an enhanced green fluorescent protein (GFP) gene, an artificial 5' splice site (underlined) with a linker sequence (CAG GTA AGT GTC GAC AAG AGA GCT CGT TGC GAT ATT ATT ACA GA), and a unique binding domain, which was created from the β -globin gene libraries (Fig. 1a). Due to the randomness of cloning, half of the binding domains were in the 3' to 5' orientation (complementary to the target sites), while the other half were in the 5' to 3' orientation (same as the target sites), which should not facilitate RNA trans-splicing. In addition, a target plasmid encoding the β -globin gene was designed (from 5'UTR to intron 2) and an artificial 3' splice site (TGC AGG T) connected to the 3' other half of the GFP gene. In this screening system, successful trans-splicing would result in concomitant

Figure 1. Comprehensive selection of optimal binding domains for RNA trans-splicing molecules (RTMs) to target β -globin gene. **(a)** To optimize the binding domain targeting the β -globin gene, the randomized binding domains were generated by sonicating the β -globin gene or random primer-mediated β -globin cDNA synthesis, and these were inserted into RTM-expressing plasmids downstream of the 5' half of GFP and a 5' splice site. In addition, a target plasmid was designed that encodes the β -globin gene and an artificial 3' splice site connected to the other half of GFP. **(b)** Following co-transfection of both target and RTM plasmids into 293T cells, six candidates were selected from a library of RTMs, which produced the brightest GFP-positive cells (maximal trans-splicing). Interestingly, all six binding domains targeted the β -globin intron 2. RFP, red fluorescent protein mCherry; GFP, enhanced green fluorescent protein; P, promoter; ss, splicing site; pA, polyadenylation signal; IRES, internal ribosome entry site; Ex, exon.

GFP and RFP expression to allow for selection of optimal binding sites for the β -globin gene.

We performed co-transfection of both target plasmid and RTM plasmids in 293T cells (American Type Culture Collection [ATCC], Manassas, VA). One to three days later, the transfected cells were sorted by flow cytometry (BD FACSAria III cell sorter; BD Biosciences, Franklin Lakes, NJ) to select a small population of high GFP and high RFP cells ($\sim 1\%$) that had a proportionate GFP-to-RFP intensity ratio, similar to a control plasmid containing full sizes of *GFP* and *RFP* genes. The RTM plasmids were purified from the selected cells, amplified, and used to re-transform 293T cells with the target plasmid. Following three cycles of selection, single clones of RTM plasmids were isolated and tested individually.

Trans-splicing for targeting β -globin RNA with co-transfection of both target and RTM plasmids

The six candidate (1E1, 2D10, 13-1, 1G6, 1D12, and 1C12; Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/hgtb) binding domains were inserted into an RTM plasmid that contained γ -globin cDNA (from exon 1 to exon 3) connected to an artificial 5' splice site with a linker sequence (CAG GTA AGT GTC GAC AAG AGA GCT CGT TGC GAT ATT ATT ACA GA; Fig. 2b). Successful trans-splicing for β -globin RNA would result in the joining of the γ -globin coding sequence to the exon 3 of β -globin, which allows for γ -globin expression instead of β -globin expression. 293T cells were transfected with both a β -globin expressing target plasmid (under the control of a human phosphoglycerate kinase [hPGK] promoter) and the RTM plasmids, which contain the six candidate binding domains (under the control of the hPGK promoter). Total RNA was extracted (RNeasy Mini Kit; Qiagen, Hilden, Germany), and the trans-splicing was analyzed by reverse transcription polymerase chain reaction (RT-PCR) (SuperScript III First-Strand Synthesis System for RT-PCR; Life Technologies, Grand Island, NY) using a forward primer targeting γ -globin exon 2 (GG Ex2 F2; 5'-GAC TTC CTT GGG AGA TGC CAT AAA G-3') and a reverse primer targeting β -globin 3'UTR (BG 3'UTR R2; 5'-AAT TGG ACA GCA AGA AAG CG-3').

Trans-splicing for targeting endogenous β -globin RNA with RTM-encoding lentiviral vectors

Human CD34⁺ cells from healthy individuals were selected from granulocyte colony-stimulating

factor-mobilized peripheral blood stem cells under a study (03-H-0015) that was approved by the Institutional Review Board of the NHLBI and under another study (08-H-0156) that was approved by the Institutional Review Board of the NIDDK.¹⁴ All individuals gave written informed consent for the sample donation, and consent documents are kept in the donor's medical records. The consent document was approved by the Institutional Review Board prior to study initiation and is reviewed and updated yearly.

To replace endogenous β -globin exons 1 and 2 with the γ -globin coding region (Fig. 2a), lentiviral vectors expressing RTMs were prepared, which include the γ -globin cDNA connected to a 5' splice site and three of the candidate β -globin binding domains (1E1, 2D10, and 13-1, selected from the six candidates), as previously described.¹⁵⁻¹⁷ The RTM-encoding vectors include γ -globin cDNA (a full length of three exons without introns) under the control of a constitutive murine stem cell virus (MSCV) promoter, and the γ -globin cDNA was connected to artificial 5' splice site with a linker sequence (CAG GTA AGT GTC GAC AAG AGA GCT CGT TGC GAT ATT ATT ACA GA) and β -globin binding domain (complementary to the target site). The RTM-encoding lentiviral vectors were prepared in 293T cells by co-transfection of gag/pol, rev/tat, vesicular stomatitis virus glycoprotein envelope, and vector plasmids, and the viral titers were evaluated in transduced HeLa cells (ATCC), as previously described.^{17,18} Human CD34⁺ cells were transduced with these three candidate RTM-encoding lentiviral vectors and a GFP-encoding control vector (as well as γ -globin cDNA-encoding control vector) at multiplicity of infection (MOI) of 50 (at 2 days after initiating culture), and the transduced cells were differentiated to erythroid cells *in vitro* based on the human erythroid massive amplification protocol, in which both stem cell factor (R&D systems, Minneapolis, MN) and erythropoietin (EPO; AMGEN, Thousand Oaks, CA) were used for the first week and EPO was used for the following week.^{19,20} After 2 weeks of differentiation, vector copy number per cell was evaluated by quantitative PCR (qPCR; Mx3000P; Agilent Technologies, Santa Clara, CA) with the SIN-LTR probe/primers using total DNA (QIAamp DNA Blood Mini Kit; Qiagen), compared to a control cell line containing one copy of vector, as previously described.²¹ The trans-splicing products were evaluated by RT-PCR and SYBR Green RT-qPCR (Brilliant II SYBR® Green; Agilent Technologies) using the same primers (GG Ex2 F2 and BG 3'UTR R2). The RTM expression from lentiviral

Figure 2. Detection of trans-splicing for targeting β -globin RNA with co-transfection of both target and RTM plasmids. **(a)** An RTM was designed to replace β -globin exon 1 (containing the sickle mutation and exon 2) with the γ -globin coding region. The RTM contains (1) the γ -globin coding region to replace the sickle cell disease mutation in the β -globin exon 1, (2) an artificial 5' splice site, and (3) a binding domain, which is complementary to the β -globin gene. **(b)** The six candidates of binding domains were inserted into an RTM plasmid that contains γ -globin cDNA connected to artificial 5' splice site. Successful trans-splicing for β -globin RNA would result in γ -globin expression instead of β -globin expression. 293T cells were transfected with both a β -globin expressing target plasmid and the RTM plasmids that contain the six candidate binding domains. **(c)** In all six binding domains, trans-splicing events were detected between γ -globin RNA and β -globin exon 3, which were evaluated by reverse transcription polymerase chain reaction (RT-PCR). A tendency of higher band density was observed when longer binding domains were contained in RTM plasmids (13-1, 0.8 kb; 1D12, 0.8 kb). γ -globin, co-transfection of target plasmid and mock plasmid, including γ -globin cDNA without artificial 5' splice site and binding domain; RTM only, RTM 13-1 plasmid transfection without target plasmid; Target only, target plasmid transfection without RTM plasmid; No plasmid, no plasmid transfection; H₂O, no addition of cDNA.

vectors was detected by RT-PCR using a forward primer targeting γ -globin cDNA (GG Ex1-2 F; 5'-AGG AGA AAC CCT GGG AAG GC-3') and a reverse primer targeting an RTM-specific sequence upstream of the binding domain region (TRM R; 5'-TCG CAA CGA GCT CTC TTG TC-3'). The β -globin RNA amounts (including spliced and un-spliced RNA) were evaluated by an hBG forward primer (5'-GTG CCT TTA GTG ATG GCC TGG-3'), reverse primer (5'-CCT GAA GTT CTC AGG ATC CAC

G-3'), and probe (5'-ACC TCA AGG GCA CCT TTG CCA CA-3'), which target the β -globin exon 2. The trans-splicing efficiency was calculated by comparing trans-splicing RNA amounts to β -globin RNA amounts using a 1:1 molecular ratio of control plasmid (containing one copy of trans-splicing sequence and one copy of β -globin gene). The hemoglobin production was evaluated by hemoglobin electrophoresis (Helena Laboratories, Beaumont, TX), as previously described.²²

Statistical analysis

Statistical analyses were performed with the JMP 12 software (SAS Institute, Inc., Cary, NC). The averages were analyzed in various conditions by Dunnett's test (one-way analysis for a control). A p -value of <0.01 or 0.05 was deemed significant. Standard error is shown as error bars in all figures.

RESULTS

Comprehensive selection of optimal binding domains for RTMs to target β -globin gene

To optimize the binding domain of RTM targeting the β -globin gene, screening experiments were performed in which randomized binding domains were produced by sonicating the β -globin gene sequence or random primer-mediated cDNA synthesis using β -globin RNA converted from genomic DNA (Fig. 1a). The random binding domains were inserted into RTM-encoding plasmids downstream of the 5' half of a *GFP* gene and an artificial 5' splice site. In addition, a target plasmid was designed that contains the β -globin gene and an artificial 3' splice site connected to the other half of the *GFP* gene. In this screening system, successful trans-splicing would result in concomitant GFP and RFP expression to allow for selection of optimal binding domains for the β -globin gene. In co-transfection of both target and RTM plasmids, approximately 20 candidates for the β -globin gene binding domain were found from a library of RTMs, and six candidates resulted in the brightest GFP-positive cells (more trans-splicing). Interestingly, all six binding domains targeted the β -globin intron 2 in the 3' to 5' orientation (complementary to the target sites; Fig. 1b and Supplementary Table S1).

These six candidate binding domains were then inserted into an RTM that contained γ -globin cDNA connected to the artificial 5' splice site (Fig. 2b). Successful trans-splicing would replace exons 1 and 2 of β -globin (including the sickle mutation in exon 1), inserting the γ -globin coding sequence on β -globin exon 3, leading to γ -globin expression (Fig. 2a). Co-transfection of 293T cells was performed with both the β -globin target plasmid and the RTM plasmids containing these six binding domains, and several negative controls were included (co-transfection of target plasmid and mock plasmid containing γ -globin cDNA without binding domain, transfection of RTM plasmid only [RTM 13-1], transfection of target plasmid only, and no plasmid transfection). The chimeric γ -globin and β -globin exon 3 RNAs were detected in samples containing all six binding domains by RT-PCR (Fig. 2c). A tendency of higher band density was observed when longer binding

domains were contained in RTM plasmids (13-1, 0.8 kb; 1D12, 0.8 kb).

Detection of trans-splicing for targeting endogenous β -globin RNA with RTM-encoding lentiviral vectors

The trans-splicing efficiency for human endogenous β -globin RNA was evaluated, in which lentiviral vectors expressing RTMs were constructed, which include the γ -globin cDNA, connected to a 5' splice site, and three (1E1, 0.6 kb; 2D10, 0.7 kb; and 13-1, 0.8 kb) β -globin binding domains, which do not contain the 5' splice site in the β -globin intron 2 (Fig. 3a). Human CD34⁺ cells were

Figure 3. Trans-splicing of endogenous β -globin RNA with RTM-encoding lentiviral vectors. **(a)** To evaluate efficiency of trans-splicing for human endogenous β -globin RNA, lentiviral vectors encoding RTMs were constructed that contain the γ -globin cDNA connected to a 5' splice site and three candidates of β -globin binding domains (1E1, 0.6 kb; 2D10, 0.7 kb; 13-1, 0.8 kb). Human CD34⁺ cells were transduced, and differentiated to erythroid cells *in vitro*. **(b)** RTM RNA expression was detected by RT-PCR in all RTM-transduced cells. **(c)** Trans-splicing was detected by RT-PCR in RTMs 2D10 and 13-1. **(d)** The trans-splicing was confirmed by sequencing. LTR, long terminal repeats; MSCV, murine stem cell virus; MOI, multiplicity of infection; Primer F, forward primer; Primer R, reverse primer.

transduced with these three RTM-encoding lentiviral vectors or a GFP-encoding control vector at an MOI of 50, and the transduced cells were differentiated to erythroid cells *in vitro* for 2 weeks. To investigate whether the artificial 5' splice site in the RTM interfered with lentiviral vector function, the vector copy number per cell was evaluated in the transduced cells by qPCR as well as RTM expression by RT-PCR using primers upstream and downstream of the 5' splice site (Fig. 3b). Vector copy numbers (11.1–15.3) were observed in all RTM-transduced cells, comparable to the vector copy number (9.7) in *GFP*-transduced control cells. The RTM expression was detected by RT-PCR in all RTM-transduced cells (Fig. 3b), suggesting that the lentiviral vectors could express RTMs (including the artificial 5' splice site) in the transduced cells. Trans-splicing was detected in RTMs 2D10 and 13-1, but not in RTM 1E1 and controls (*GFP* transduction and no transduction; Fig. 3c). In sequencing of the PCR products for γ - and β -globin chimeric cDNA, the fixed junction sequence was observed between the γ -globin stop codon with a remaining 5' splice site (TAA CAG) and the head of β -globin exon 3 (CTC CTG), suggesting that the γ - and β -globin chimeric DNA depends on trans-splicing (Fig. 3d). Then RT-qPCR was performed, which demonstrated up to 0.01–0.05% trans-splicing efficiency compared with endogenous β -globin RNA (Fig. 4a), suggesting that longer binding domains are associated with more efficient trans-splicing. An increase of fetal hemoglobin (γ -globin) production was not detected by hemoglobin electrophoresis in RTM-transduced cells due to the overall level of trans-splicing observed (Supplementary Fig. S1).

The RTM BGin2 (including 5' splice site of β -globin intron 2; 0.9 kb) and BGex1-in2 (including the entire β -globin sequence except exon 3; 1.3 kb) were designed to elongate the binding domain in RTM 13-1 (Fig. 4b1 and b2). Efficient transduction was observed in all CD34⁺ cell-derived erythroid cells (1.0–13.4 VCNs). In both RTM BGin2 and BGex1-in2, two- to fourfold higher trans-splicing was detected by RT-qPCR than by RTM 13-1 ($p < 0.05$), which resulted in 0.07–0.09% trans-splicing efficiency compared with endogenous human β -globin RNA. No increase of trans-splicing signals was observed in *GFP*-transduced erythroid cells or mock (γ -globin cDNA without binding domain)-transduced erythroid cells. These data suggest that for more efficient trans-splicing, the 5' splice site of intron 2 should be contained as a binding domain.

Figure 4. More efficient trans-splicing by longer binding domains that include 5' splice site of intron 2. **(a)** Quantitative RT-PCR was performed in RTM-transduced erythroid cells (1E1, 0.6 kb; 2D10, 0.7 kb; 13-1, 0.8 kb), which demonstrated up to 0.01–0.05% trans-splicing efficiency compared with endogenous β -globin RNA, suggesting that longer binding domains improve trans-splicing efficiency. **(b1 and b2)** To elongate the binding domain in RTM 13-1, RTM BGin2 (containing 5' splice site of β -globin intron 2; 0.9 kb) and BGex1-in2 (containing whole β -globin sequence except exon 3; 1.3 kb) was designed. In both RTM BGin2 and BGex1-in2, two- to fourfold higher trans-splicing was detected by quantitative RT-PCR than RTM 13-1 ($p < 0.05$), which resulted in 0.07–0.09% trans-splicing efficiency compared with endogenous human β -globin RNA. No increase of trans-splicing signals was observed in *GFP*-transduced erythroid cells or mock (γ -globin cDNA without binding domain)-transduced erythroid cells. These data suggest that the 5' splice site of intron 2 should be included as a binding domain for efficient trans-splicing.

DISCUSSION

In the current strategy in HSC-targeted gene therapy for SCD, a normal β -globin (or γ -globin) gene is added to autologous HSCs, and the β -globin (or γ -globin) is produced in red blood cells to prevent sickling.⁵ On the other hand, the trans-splicing-based gene therapy has a potential major advantage in that it can produce normal β -globin (or γ -globin), as well as reduce sickle globin production by interfering with normal cis-splicing of sickle globin pre-messenger RNA. The ribozyme-mediated trans-splicing ($\sim 10\%$ efficiency) was previously reported to be detected by RT-PCR in 293 cells co-transfected with a target plasmid (β -globin) and γ -globin ribozyme plasmid.^{10,12} A later report indicated that ribozyme-mediated trans-splicing ($\sim 36\%$) was detected in 293T cells with co-transfection of a target plasmid (mutated β -globin intron 1 3' splice site) and ribozyme plasmid targeting the intron 1 (binding domain and β -globin).¹¹ Trans-splicing is less efficient than normal cis-splicing, and reasonably high levels of trans-splicing efficiency are needed for clinical application.

This study employed a screening system to find the optimal binding domain for the β -globin gene. The RTM plasmid can produce a full-size RFP protein and a partial GFP protein (low intensity) without trans-splicing, while both a full-size RFP protein and full-size GFP protein (high intensity) can be produced with trans-splicing. The GFP-to-RFP intensity ratio with trans-splicing should be higher than that without trans-splicing. Since a single promoter expresses both RFP and GFP genes in the RTM plasmid, the GFP-to-RFP intensity ratios should not depend on gene expression levels and RNA stability. Therefore, high GFP and high RFP cells were selected, which have a proportionate GFP-to-RFP intensity ratio, similar to a control plasmid containing full sizes of GFP and RFP genes. It was found that the intron 2 of β -globin gene is targeted by all six optimal binding domain RTMs (Fig. 1). This is consistent with the previous data in which the intron 2 is important for stability of β -globin RNA, since the RTM binding to the intron 2 might reduce cis-splicing as well as RNA stability in β -globin gene.²³ The splicing procedure of the intron 2 might improve gene expression of β -globin gene. In addition, it was found that the 5' splice site of the β -globin intron 2 should be contained in the binding domain for more efficient trans-splicing (Fig. 4b1 and b2). Inclusion of the binding domain and the 5' splice site may reduce the normal cis-splicing and enhance the trans-splicing of intron 2.

This study used γ -globin cDNA in RTM plasmids to convert β -globin (sickle globin) into γ -globin, which is similar to previous ribozyme-mediated

trans-splicing experiments,^{10,12} because γ -globin was reported to interfere more strongly with polymerization of sickle hemoglobin than β -globin did.²⁴ In addition, this strategy allowed trans-splicing for targeting endogenous β -globin RNA in human CD34⁺ cell-derived erythroid cells to be evaluated from not only SCD patients but also healthy volunteers, and it allowed trans-splicing events to be detected more reliably due to numerous nucleotide differences between γ -globin and β -globin (sickle globin).

In a cell line model with co-transfection of a target plasmid (β -globin gene) and an RTM plasmid (binding domain and γ -globin cDNA), initially both trans-splicing events and β -globin expression were detected by the same RT-PCR reaction (data not shown), similar to previous reports using a ribozyme.^{10–12} However, the ratios of DNA band densities between β -globin cDNA (target) and trans-splicing products were strongly affected by PCR annealing temperatures. To calculate the efficiency of trans-splicing precisely (trans-splicing events per β -globin RNA), a more reliable RT-qPCR method was established by using a 1:1 molecular ratio of control plasmid containing both the trans-splicing sequence and the β -globin gene. Differences in trans-splicing signals among RTMs were evaluated by using the same conditions (same master mixture and same reaction plate) of RT-qPCR (as well as RT-PCR), and similar results were observed (higher trans-splicing efficiency when using a longer binding domain of RTM 13-1) by not only RT-qPCR (Fig. 4a) but also RT-PCR in plasmid co-transfection (Fig. 2c) and lentiviral transduction (Fig. 3c). Similar trans-splicing efficiency was obtained in RTM 13-1 (0.05%, 0.02%, and 0.05%) among three different transduction experiments by RT-qPCR evaluation. However, variability was still observed in the trans-splicing efficiency data, which was maybe caused by lentiviral transduction, erythroid differentiation, timing of RNA extraction, and/or conditions of RT-qPCR (different master mixture and different reaction plate). The RTMs delivered by lentiviral vectors generated up to 0.07–0.09% trans-splicing efficiency compared with human endogenous β -globin RNA (Fig. 4).

If the PCR reaction amplified γ - and β -globin chimeric DNA (mimic trans-splicing events) by skipping between γ -globin cDNA (without introns) and intron-containing β -globin gene (contaminated genomic DNA or cDNA from pre-messenger RNA), the PCR products should contain some intron sequence (independent of splicing). On the other hand, in the γ - and β -globin chimeric cDNA of trans-splicing products, the γ -globin cDNA should

be connected to the β -globin exon 3 by losing the exact site between 5' splice site in the RTM and 3' splice site in the β -globin intron 2. After sequencing of the PCR products for γ - and β -globin chimeric cDNA, the fixed junction sequence was observed between the γ -globin stop codon with a remaining 5' splice site (TAA CAG) and the head of β -globin exon 3 (CTC CTG; Fig. 3d), suggesting that the γ - and β -globin chimeric DNA depends on trans-splicing.

To deliver the RTMs into human hematopoietic stem/progenitor cells, a lentiviral vector system was used in which efficient transduction for hematopoietic repopulating cells was previously demonstrated in both rhesus HSC transplantation and humanized xenograft mouse models.^{14,17,25} However, there was a concern that the artificial 5' splice site in the RTMs might interfere lentiviral gene delivery due to unpredictable splicing during vector production. The present study obtained highly efficient transduction (up to 15.3 of vector copy number; Fig. 4) and RTM expression in transduced human CD34⁺ cell-derived erythroid cells (Fig. 3b). These data suggest that a lentiviral vector system allows for the delivery of RTMs (including the artificial 5' splice site) in human cells. Following the delivery of the RTMs containing optimal binding domains for β -globin RNA, the trans-splicing events were detected in human endogenous β -globin RNA (Fig. 3), whereas previous ribozyme studies were unable to detect trans-splicing into wild-type endogenously expressed β -globin. This work could provide for improved tools to develop therapeutic strategies in the future. However, the rate of trans-splicing in differentiated CD34⁺ cells remains low (0.07–0.09%) compared with β -globin expression (Fig. 4). Furthermore, an increase of fetal hemoglobin (γ -globin) protein production could not be detected by hemoglobin electrophoresis (Supplementary Fig. S1).

In this study, a wide spectrum constitutive viral promoter (MSCV promoter) was used to express RTMs in lentiviral vectors. However, it is not optimal for high-level transgene expression in erythroid cells.¹⁹ An optimal promoter in erythroid cells might improve trans-splicing efficiency with higher expression of RTMs. As another possibility, lentiviral vectors might partially lose the RTM expression cassette due to alternative splicing during vector production, resulting in vector integration without RTM expression. In addition, trans-splicing efficiency might be affected by specific cell types. Further development of the trans-splicing technology is required for eventual clinical application in SCD therapy.

In summary, screening was performed to select better binding domains for trans-splicing. For the first time, detectable levels of spliceosome-mediated trans-splicing (0.07–0.09%) were obtained for endogenous β -globin RNA in erythroid cells from healthy individuals, which were transduced with RTM-encoding lentiviral vectors. These methods provide the basis for future β -globin targeted trans-splicing-based gene therapy.

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AUTHOR DISCLOSURE

B.M. was employed by RetroTherapy. L.M. has a financial interest in RetroTherapy and is also an employee of RetroTherapy. No competing financial interests exist for the remaining authors.

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