Correction of RNA splicing defect in β^{654}-thalassemia mice using CRISPR/Cas9 gene-editing technology

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Abstract

β^{654}-thalassemia is a prominent Chinese subtype of β-thalassemia, representing 17% of all cases of β-thalassemia in China. The molecular mechanism underlying this subtype involves the IVS-2-654 C→T mutation leading to aberrant β-globin RNA splicing. This results in an additional 73-nucleotide exon between exons 2 and 3 and leads to a severe thalassemia syndrome. Herein, we explored a CRISPR/Cas9 genome editing approach to eliminate the additional 73-nucleotide by targeting both the IVS-2-654 C→T and a cryptic acceptor splice site at IVS-2-579 in order to correct aberrant β-globin RNA splicing and ameliorate the clinical β-thalassemia syndrome in β^{654} mice. Gene-edited mice were generated by microinjection of sgRNA and Cas9 mRNA into one-cell embryos of β^{654} or control mice: 83.3% of live-born mice were gene-edited, 70% of which produced correctly spliced RNA. No off-target events were observed. The clinical symptoms, including hematologic parameters and tissue pathology of all of the edited β^{654} founders and their offspring were significantly improved compared to those of the non-edited β^{654} mice, consistent with the restoration of wild-type β-globin RNA expression. Notably, the survival rate of gene-edited heterozygous β^{654} mice increased significantly, and live-born homozygous β^{654} mice were observed. Our study demonstrated a new and effective gene-editing approach that may provide groundwork for the exploration of β^{654}-thalassemia therapy in the future.

Introduction

β-thalassemia is one of the most common inherited hematopoietic disorders resulting from the absence or deficient synthesis of the β-globin chain of hemoglobin. It poses an important public health problem, and there have been over 200 different mutations in the β-globin locus reported worldwide.[1] The absence or reduction of β-globin chains results in an imbalanced ratio of synthesis of α/β-globin chains. The excess free α chains precipitate in red cells and reduce erythrocyte membrane elasticity, thus causing hemolytic anemia. Patients with severe β-thalassemia depend on lifelong blood transfusion to sustain their life and suffer the risk of tissue damage in multiple organs stemming from iron accumulation.[2-4]

The β^{654} mutation is one of the most common mutations in the Chinese population, accounting for 17% of all cases of β-thalassemia in China.[5] It is a C→T substitution at IVS-2, position 654, which produces a new 5' donor splice site and activates a cryptic 3' acceptor splice site at position 579, leading to insertion in the RNA message of an extra 73-nucleotide (nt) exon between exons 2 and 3 which contains a premature termination codon.[6] This produces a severe reduction in the amount of normal processed β-globin mRNA and β-globin chains, ultimately resulting in severe β-thalassemia symptoms in β^{654} patients.[5] Our interests have been to pursue various gene therapy treatment options in an effort to cure β^{654}-thalassemia by increasing the synthesis of normal β-globin chains. We have previously explored an antisense RNA strategy to reduce abnormal β^{654}-globin mRNA, combination therapy of RNA interference and antisense RNA to balance α/β-globin gene expression, as well as adding a functional β-globin gene using a lentiviral vector.[7-9] These approaches yielded gratifying results, ameliorating symptoms of β-thalasse-

mia in β^{654} mice. Other recent studies aimed to restore normal β-globin expression by targeting the IVS-2-645 mutation site and also yielded promising results.[10,11]

The highly clustered, regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), derived from an element of the bacterial immune system,[12,13] has proven to be a more efficient gene-editing tool compared with the other newly developed editing technologies such as transcription activator-like effector nucleases (TALEN). CRISPR/Cas9 has the advantages of high gene-targeting efficiency, low cost, and easy operability. This makes it a very attractive technology for gene therapeutic applications to correct genetic disorders.[14-16] It would be highly desirable to improve the targeting efficiency by using CRISPR/Cas9 to disrupt the aberrant splicing site directly. Unfortunately, for the β^{654} point-mutation site, no simple and appropriate single guide (sg)RNA is available for such a purpose. We hypothesized that targeting both sites (IVS-2-654 C→T and IVS-2-579) by CRISPR/Cas9 would effectively correct the aberrant splicing.

Here, we report the efficacy of CRISPR/Cas9 genome editing to create targeted elimination of the extra 73-nt exon in β^{654} mice. The resulting gene-edited mice showed improvement of normal β-globin gene expression, and their clinical thalassemia symptoms were significantly alleviated, illustrating the potential of this strategy for translation into a clinical therapy for β^{654} thalassemia in the future.

Methods

β^{654}-thalassemia mice

The β^{654}-thalassemia mice (β^{654} mice, also known as *Hbb^{th-4}/Hbb^{+}*, JAX #003250) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). These mice are heterozygous for a human gene with a βIVS-2-654 splicing mutation and the normal mouse β-globin allele. Clinically, β^{654} mice mimic the symptoms of moderate to severe forms of β-thalassemia, for example, low red blood cell counts, inefficient erythropoiesis, increased red blood cell destruction, and splenomegaly.[17]

Mouse lines developed in this study are designated as follows: $\beta^{654\text{-Ctrl}}$ (β^{654} mice with the same genotype as those purchased from the Jackson Laboratory); $\beta^{654\text{-E}}$ (β^{654} Edited: mice exposed to CRISPR/Cas9 editing at the one-cell stage); $\beta^{654\text{-ER}}$ (β^{654} Edited Responsive: mice edited which subsequently showed corrected RNA splicing); $\beta^{654\text{-ENR}}$ (β^{654} Edited Non-Responsive: mice edited but in which the resulting alterations were ineffective on RNA splicing); $\beta^{654\text{-EF}}$ (β^{654} Edited Failed: mice exposed to CRISPR/Cas9 but with failure of alteration of any target sequences).

All the animal procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Animal Care and Use Committee of Shanghai Children's Hospital.

Generation and screening of gene-edited $\beta654^{-E}$ mice

Male $\beta^{654\text{-Ctrl}}$ mice were mated with wild-type (WT) female mice, and the resulting fertilized eggs were collected at the late one-cell stage and subjected to microinjection with the sgRNA (50 ng/μL) and Cas9 mRNA (50 ng/μL) (Invitrogen) and transferred into the Fallopian tubes of pseudopregnant mice. WT and $\beta^{654\text{-E}}$ mice were subject to the following screening at about 3 weeks of age.

Quantitative polymerase chain reaction (PCR) was used to measure the copy numbers of normal mouse β-globin gene and to differentiate WT (with two copies) and β^{654} (that carry only one normal mouse β-globin gene) offspring mice. Additionally, human sequence PCR primers were designed to amplify the IVS-2 region to identify mice carrying the human allele after CRISPR/Cas9 treatment. The resulting PCR products were sequenced to determine the effects of gene editing.

Expression of the human β^{654}-globin gene

Expression of the human β^{654}-globin gene in mice was examined at the transcription level by reverse transcriptase PCR and at the translational level by western blot.

For reverse transcriptase PCR, cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions and subjected to PCR analysis with IVS-2-654 specific primers HBG-L and HBG-R, which produce an amplicon of 399 bp for the normal cDNA, or 472 bp for the β^{654}-globin cDNA. The mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene with a 530 bp amplified fragment was used as a control.

Western blot analysis was performed as described previously,[18] with primary human β-globin monoclonal antibody (Abcam, Cambridge, USA; 1:2000 diluted) and horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody (Dako, Denmark; 1:2000). Polyclonal rabbit anti-human α-globin antibody (Abcam, Cambridge, USA; 1:2000) was used to detect human and mouse α-globin as an internal control.

Details of plasmid constructions, off-target analysis, *in vitro* transcription of sgRNA, cell culture and transfection, hematologic analysis, histopathology analysis, genomic sequencing, and statistical analysis are available in the *Online Supplementary Methods*.

Results

Designing and screening of sgRNA targeting the β^{654}-globin splicing mutation

Based on predicted high on-target efficiency and low off-

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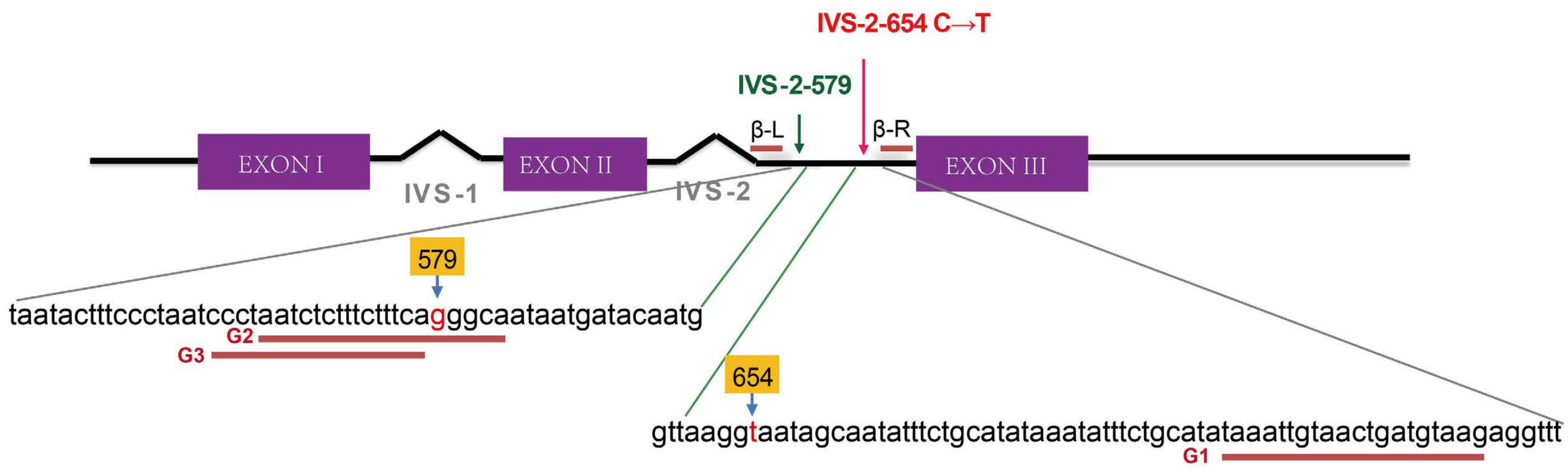

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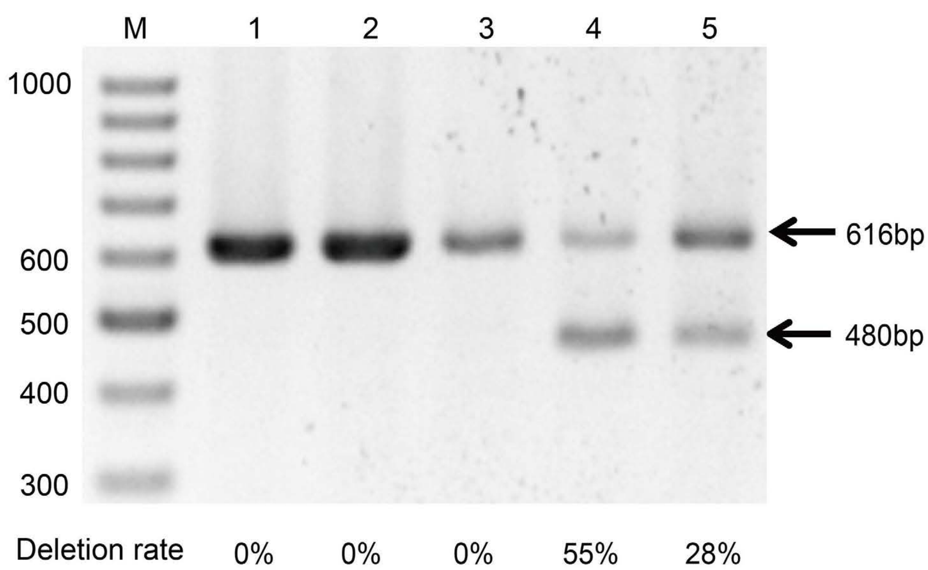

C

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Reference                                                                                                        % of clones
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCT   30.5%(68/223)

Deletions                                                                                                        43.0%(96/223)
TAATACTTTCCCTAAT------------------------------------------//---------------------------TAAGAGGTTTCATATTGCT   22.4%(50/223)
TAATACTTTCCCTAAT------------------------------------------//----------------------------AAGAGGTTTCATATTGCT   5.8%(5/223)
TAATACTTTCCCTAA-------------------------------------------//----------------------------AAGAGGTTTCATATTGCT   2.2%(5/223)
TAATACTTTCCCTAATC-----------------------------------------//---------------------------TAAGAGGTTTCATATTGCT   2.2%(5/223)
TAATACTTTCCCTAATCTCTT-------------------------------------//-------------------AACTGATGTAAGAGGTTTCATATTGCT   1.3%(3/223)
TAATACTTTCCCTA--------------------------------------------//--------------------------GTAAGAGGTTTCATATTGCT   1.3%(3/223)
TAATACTTT-------------------------------------------------//--------------------------------GGTTTCATATTGCT   1.3%(3/223)
TAATACTTTCCCTAATC-----------------------------------------//----------------------------AAGAGGTTTCATATTGCT   1.3%(3/223)
TAATACTTTCCCTAATCTCTTTCTTT--------------------------------//-------------------------TGTAAGAGGTTTCATATTGCT   0.9%(2/223)
TAATACTTTCCCTAATCTCTTTCTTTC-------------------------------//-----------------------------AGAGGTTTCATATTGCT   0.9%(2/223)
TAATACTTTCCCTAATCTCTTTC-----------------------------------//----------------------------AAGAGGTTTCATATTGCT   0.9%(2/223)
TAATACTTTCCCTAAT------------------------------------------//-----------------------------AGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTA--------------------------------------------//------------------------ATGTAAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTAATC-----------------------------------------//---------------------------TAAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTA--------------------------------------------//---------------------------TTTAAGAGGTTTCATATTG   0.4%(1/223)
TAATACTTTCCCTAAT------------------------------------------//---------------------------ATAAGAGGTTTCATATTGC   0.4%(1/223)

Indels                                                                                                           23.3%(52/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATG-AAGAGGTTTCATATTGCT   9.9%(22/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGAT-------GTTTCATATTGCT   2.7%(6/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGAT------GGTTTCATATTGCT   1.8%(4/223)
TAATACTTTCCCTAATCTCTT--TTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGAT-------GTTTCATATTGCT   1.3%(3/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATAAGATAAGAGGTTCCATATT   1.3%(3/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTTTAAGAGGTTTCATATTGC   1.3%(3/223)
TAATACTTTCCCTA-------------AGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTTTAAGAGGTTTCATATTGC   0.9%(2/223)
TAATACTTTCCCTA------------------AATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTAATC------------------------ACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATG-AAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATG-------------------   0.4%(1/223)
TAATACTTTCCCTAATC---------------AATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACT----TAAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTAATACTTTTCCTCTTTCTTTCAGGGCAATAATGATACAATGTATC//ATTTCTGCATATAAATTGTAACTGATG-AAGAGGTTTCATATTGCT   0.4%(1/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGCTTTCATATTGC   0.4%(1/223)
TAATACTTTCCCTAATCTCTT--TTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGAT-------AGTTTCATATTGC   0.4%(1/223)
TAATACTTTCCCTAATC----------------------------TGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTTTAAGAGGTTTCATATTGC   0.4%(1/223)
TAATACTTTCC---------------CAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTTTAAGAGGTTTCATATTGC   0.4%(1/223)

Substitutions                                                                                                    2.2%(5/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAGGAGGTTTCATATTAAC   0.4%(1/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTCCATATTAAC   0.4%(1/223)
TAATACTTTCCCTAACCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTAAC   0.4%(1/223)
TAATACTTTCCCTAATCTCTTTCTTTCGGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTAAC   0.4%(1/223)
TAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTC//ATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTTATATTAAC   0.4%(1/223)

Inversions                                                                                                       0.9%(2/223)
TAATACTTTCCCTAATACATCAGTTACAATTTATATGCAGAAATATTTATATGCAGAG//TGTATCATTATTGCCCTGAAAGAAAGAGAAGAGGTTTCATATTAAC   0.4%(1/223)
TAATACTTTCCCTAATACATCAGTTACAATTTATATGCAGAAATATTTATATGCAGAG//ATCATTATTGCCCTGAAAGAAA--------TAGGTTTCATATTAAC   0.4%(1/223)
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Figure 1. Single-guide RNA screening and gene-editing profiles in 293T cells. (A) Schematic of the single-guide (sg)RNA design within the human β-globin chain. The red arrow indicates the position of IVS-2-654 C→T, and the green arrow is IVS-2-579. G1 is the sgRNA designed to target the downstream IVS-2-654. G2 and G3 are sgRNA designed to target the upstream IVS-2-579. β-L/β-R are polymerase chain reaction (PCR) primer pairs. (B) Gel electrophoresis of PCR amplicons from 293T cells transfected with different sgRNA pairs. Lane M, 100 bp DNA ladder; lane 1, 293T cell control; lanes 2-5, 293T cells transfected with water (2), Cas9 (3), G1+G2 (4), and G1+G3 (5). Deletion rates, determined by the ratio of 480 bp band intensity *vs*. 616 bp band intensity, are shown below each lane. (C) Representative G1+G2 gene-editing DNA profiles. The underlined sequences are G1 and G2. A total of 223 clones were sequenced. The percentage of each alteration observed is shown on the right side. The yellow highlighted sequences represent the protospacer adjacent motifs. The red sequences represent inserted bases. The bordered sequences represent substitution nucleotides.

target effects determined by the CRISPOR program,[19] three sgRNA (G1, G2 and G3) that target the aberrant donor or acceptor site (Figure 1A and *Online Supplementary Table S1*) were selected to be tested in pairs. After cloning sgRNA into the pX459 plasmid vector, two pools containing CRISPR plasmid pairs (G1+G2 or G1+G3) were used to create two double-strand breaks targeted deletion in 293T cells. The dual sgRNA-mediated deletions were verified by PCR using the primer pairs β-L/β-R depicted in Figure 1A. The G1+G2 pool produced a higher deletion efficiency, up to 55% (Figure 1B), and was thus selected for the experiments that follow.

PCR products were subcloned and used for Sanger sequencing (Figure 1C). A total of 223 clones were examined. Large deletions (>100 bp) occurred in 96 clones (43.0%), and two inversion clones (0.9%), five substitution clones (2.2%), 68 WT sequence clones (30.5%) and 52 short insertion or deletion clones (23.3%) were identified. The 140 bp deletion was most frequent among the large deletion clones and accounted for one quarter of all the deletions noted. In addition, single cuts repaired by non-homologous end joining (NHEJ) were observed at just the 5' or 3' target sites, with frequencies of 17.9% and 0.4%, respectively.

Off-target sites were predicted using the CRISPOR program. The top ten sites ranked by the cut-frequency determination off-target score for each sgRNA were subjected to targeted deep sequencing (*Online Supplementary Table S2* and *Online Supplementary Figure S1*). There was no evidence of off-target events at these sites.

Generation and characterization of $\beta^{654\text{-}E}$ mice

The $\beta^{654\text{-}Ctrl}$ mice are heterozygous for a human gene with a βIVS-2-654 splice mutation and the normal mouse β-globin allele. They typically display moderate to severe clinical symptoms of β-thalassemia[17] and a reduced survival rate (26.8%).[18] Normally, no homozygous $\beta^{654\text{-}Ctrl}$ mice survive postnatally.[17]

By mating $\beta^{654\text{-}Ctrl}$ male mice with WT female mice, a total of 142 fertilized eggs were obtained. After microinjecting the G1+G2 sgRNA and Cas9 mRNA into the zygotes, 123 embryos were transplanted into pseudopregnant mice. A total of 37 offspring were born, and tail vein blood samples were collected 3 weeks after birth (*Online Supplementary Table S3*). Quantitative PCR with mouse-specific β-globin primers was used to measure mouse allele copy number. Twelve of the 37 mice had only one copy of the mouse β-major globin gene and thus were $\beta^{654\text{-}E}$ mice. The remaining mice had two copies of the β-major globin gene and were therefore WT mice (*Online Supplementary Figure S2*).

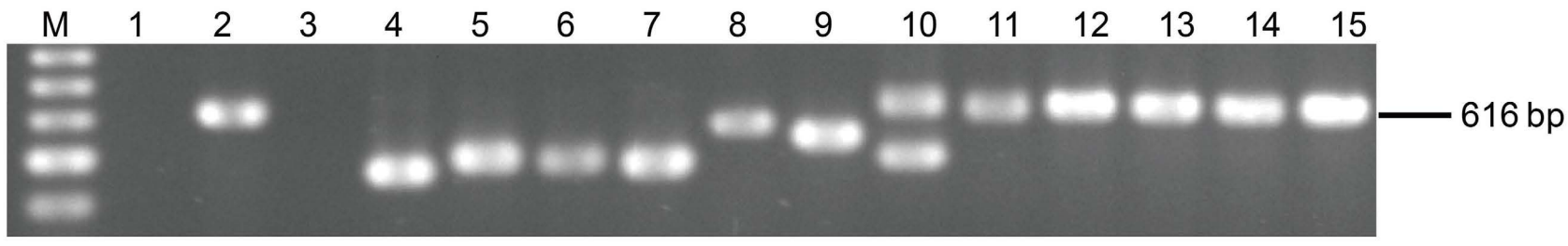
PCR analysis of the 12 mice with β^{654}-specific primers was consistent with the copy number measurements (Figure 2A and *Online Supplementary Figure S2*). The PCR products were subjected to Sanger sequencing. Ten of the 12 mice (83%) had alterations at the targeted loci (*Online Supplementary Table S3*). Multiple PCR subclones from each mouse were sequenced, and six out of the ten mice showed a single deletion while the remaining four mice were mosaic with multiple alterations (Figure 2B).

RNA expression of edited genes

To investigate whether genetic deletion of the extra exon is sufficient to restore normal β-globin splicing and expression, we analyzed RNA from peripheral blood cells of the 12 $\beta^{654\text{-}E}$ offspring mice. Reverese transcriptase PCR showed that the correct β-globin transcript (399 bp) was present in seven gene-edited mice, among which six showed correctly spliced mRNA exclusively, and one (mouse ID 59) showed DNA mosaicism that produced about 60% expression of correctly spliced mRNA (i.e., $\beta^{654\text{-}ER}$ mice). The remaining three mice also displayed genetic mosaicism, but all mRNA examined showed the incorrect splicing message with the 73-nt additional exon, and these mice were, therefore, $\beta^{654\text{-}ENR}$ (Figure 2C). Of the six mice with effective, non-mosaic gene-editing, four have successful deletion of both the IVS-2-654 aberrant splicing acceptor site and the IVS-2-579 splicing donor site, while the other two had deletion of either the IVS-2-654 or IVS-2-579 site but not both (Figure 2B).

Western blot analysis using a β-globin monoclonal antibody that detects only WT human β-globin showed the presence of human β-globin in the seven $\beta^{654\text{-}ER}$ mice. No human β-globin expression was observed in the three $\beta^{654\text{-}ENR}$ mice. These data are consistent with the reverse transcriptase PCR results. The corrected human β-globin protein levels in the seven $\beta^{654\text{-}ER}$ mice were comparable (less than 20% difference) (Figure 2D). Reverse-phase high-performance liquid chromatography was then used to quantify the amounts of mouse and normal human β-globin chains (for

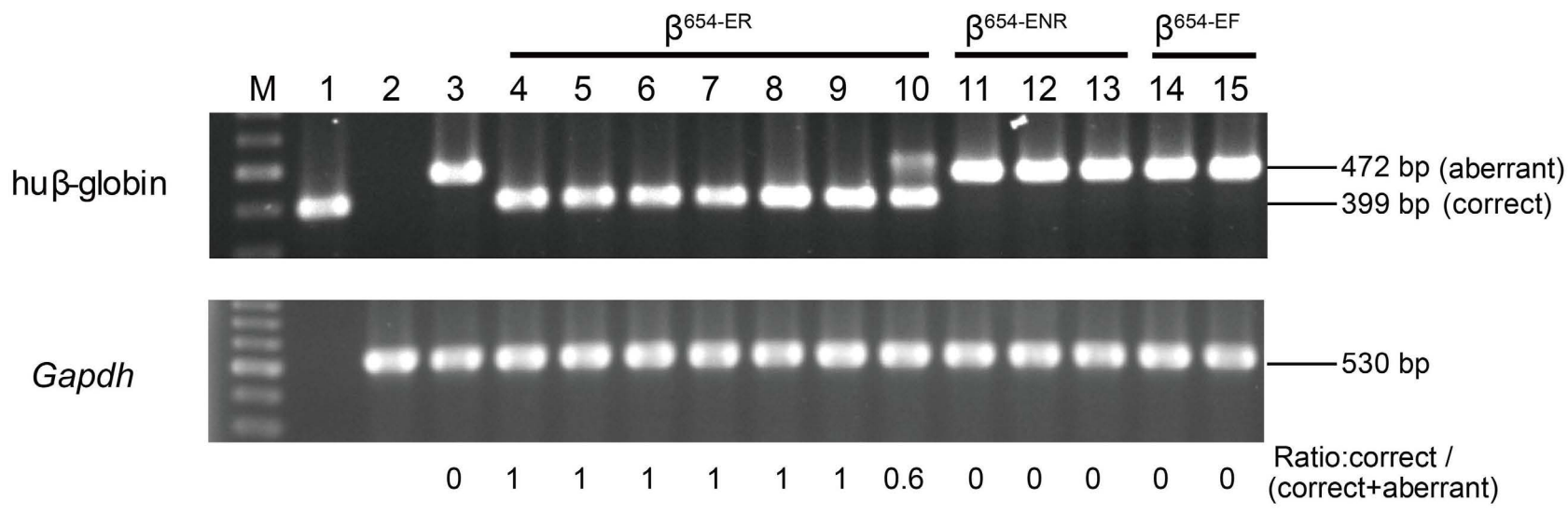
A



B

Mouse ID	Lane n. in Figure 2A,C and D	Edited RNA result	% of clones	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat gt aagaggt t t cat at t gct
β^{654}		β$^{654\text{-Ctrl}}$		t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat gt aagaggt t t cat at t gct
52	4	β$^{654\text{-ER}}$	100.0	t aa- // - - - - - - - - - - - - - - - - - // - t t t cat at t gct
53	5	β$^{654\text{-ER}}$	100.0	t aat act t t ccct aat ct ct t t c- - - - - - - - - - // - - - - - - - - - - - - - - - - - // - - - - - - - - - - - - - - - - - t aagaggt t t cat at t gct
64	6	β$^{654\text{-ER}}$	100.0	t aat act t t ccct aa- - - - - - - - - - - - - - - - - // - - - - - - - - - - - - - - - - - // - - - - - - - - - - - - - - - - - aagaggt t t cat at t gct
92	7	β$^{654\text{-ER}}$	100.0	t aat act t t ccct aat cagt a- - - - - - - - - - - - // - - - - - - - - - - - - - - - - - // - gct
87	8	β$^{654\text{-ER}}$	100.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct ggg- - - - - - - - // - - - - - - - - - - - - - - - - - aagaggt t t cat at t gct
90	9	β$^{654\text{-ER}}$	100.0	t aat act t t ccct aat ct ct t - - - - - - - - - - - - // - - - - - - - - - - - - aggt a// t aaat t gt aact - - - - - - - - - - gt t t cat at t gct
59	10	β$^{654\text{-ER}}$	55.0	t aat act t t ccct aa- - - - - - - - - - - - - - - - - // - - - - - - - - - - - - - - - - - // - - - - - - - - - - - - - - - - - t aagaggt t t cat at t gct
	10	β$^{654\text{-ENR}}$	45.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat - - - - gaggt t t cat at t gct
54	11	β$^{654\text{-ENR}}$	72.5	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat g- aagaggt t t cat at t gct
	11	β$^{654\text{-ENR}}$	27.5	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact - - - - - - - gaggt t t cat at t gct
74	12	β$^{654\text{-ENR}}$	47.5	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat - - - - - - ggt t t cat at t gct
	12	β$^{654\text{-ENR}}$	42.5	t aat act t t ccct aat t ct ct t t ct t t cagggc// at t t ct gggt t aaggt a// t aaat t gt aact gat aagat aagaggt t t cat at t
	12	β$^{654\text{-ENR}}$	10.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat gt t aagaggt t t cat at t gc
76	13	β$^{654\text{-ENR}}$	55.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat - - - - - - - gt t t cat at t gct
	13	β$^{654\text{-ENR}}$	45.0	t aat act t t ccct aat c- - t t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat - - - - - - - - - - - - - - - - - gt t t cat at t gct
80	14	β$^{654\text{-EF}}$	100.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat gt aagaggt t t cat at t gct
85	15	β$^{654\text{-EF}}$	100.0	t aat act t t ccct aat ct ct t t ct t t cagggca// at t t ct gggt t aaggt a// t aaat t gt aact gat gt aagaggt t t cat at t gct

C



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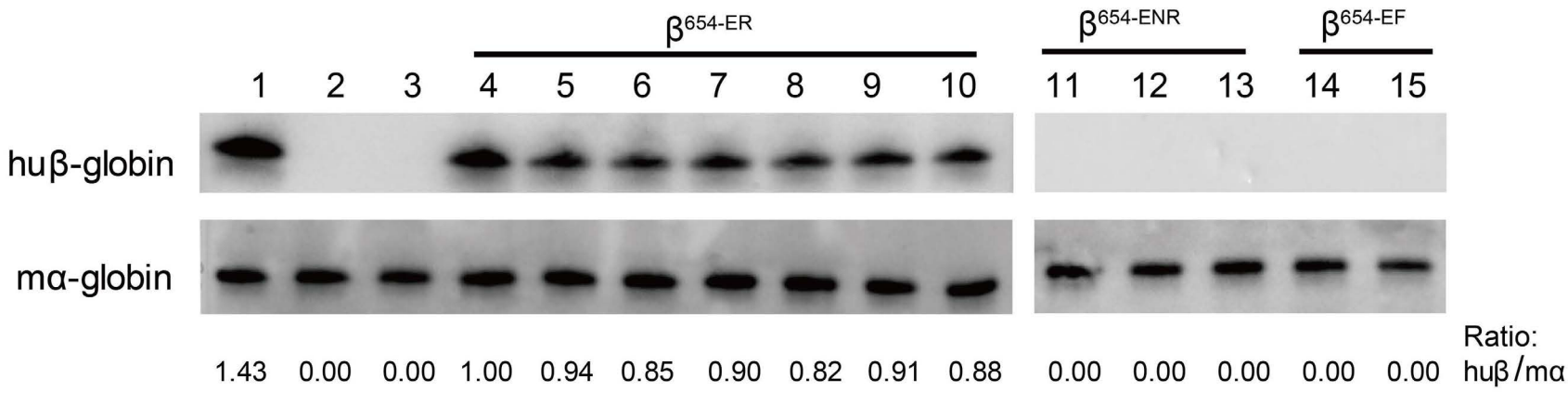

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Figure 2. Identification of human β-globin expression in β654-E mice. (A) Polymerase chain reaction (PCR) of genomic DNA identified β654-E mice. The unedited amplicon is 616 bp. Lane M: 100 bp DNA ladder; lane 1: no template control; lane 2: β654-Ctrl mouse; lane 3: wild-type mouse (WT); lanes 4-15: β654-E mice obtained following gene editing. The lane numbers corresponding to the Mouse ID are listed in panel B and are consistent across all panels of Figure2C, D. (B) Gene-editing sequence profiles. The underlined sequences are G1 and G2, and the yellow highlighted sequences are protospacer adjacent motifs. There is one founder embryo for each mouse ID. The red sequences represent inserted bases. (C) Reverse transcriptase PCR analysis of human β-globin RNA expression. The 399 bp band indicates the correctly spliced human β-globin (huβ-globin) transcript, and the 472 bp band indicates the aberrant β-globin transcript. The mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene with a 530 bp amplicon was used as an internal control. Lane M: 100 bp DNA ladder; lane 1: human; lane 2: WT mouse; lane 3: β654-Ctrl mouse; lanes 4-15 as described above for 2A. The ratios of correctly spliced amplicons *vs.* total amplicons (correct + aberrant) were measured by gel densitometry. (D) Western blot analysis of human β-globin protein expression. Lane 1: human; lane 2: β654-Ctrl mouse; lane 3: WT mouse; lanes 4-15: as described for 2A above. Mouse α-globin (mα-globin) served as an internal control. The intensity ratios of huβ-globin *vs.* mα-globin are shown below each lane.

Table 1. Hematologic analyses.

Group	N	RBC (10^6/μL)	HGB (g/L)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/L)	RET (%)
WT	28	9.2±1.1*	136.6±18.2*	44.6±5.7*	48.3±1.0*	14.8±0.4*	305.9±8.7*	3.3±0.6*
β654-ER	7	9.6±1.2*	145.0±17.5*	47.5±6.2*	49.5±1.6*	15.1±0.6*	304.4±6.5	3.8±1.2*
β654-ENR	3	6.0±0.6**	76.3±0.1**	22.8±2.9**	37.7±1.1**	12.8±0.9**	337.7±32.7**	15.9±3.1**
β654-Ctrl	21	6.5±0.7	82.3±7.6	25.7±2.3	39.4±2.2	12.6±0.8	320.8±14.3	19.2±3.6

Values represent mean ± standard deviation. Statistically significant differences are indicated for WT, β654-ER, or β654-ENR compared to β654-Ctrl. *P<0.01, **P>0.05. N: number of mice tested; RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RET: reticulocyte count.

β654-ER mice), and human peptides ranged from 50.1% to 52.5% of total β-globin (*Online Supplementary Figure S3*).

Improvement of hematologic indices in β654-ER mice

Hematologic parameters were measured once every 2 weeks for 3 months. Elevated levels of red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were noted in the β654-ER mice when compared to β654-Ctrl mice (P<0.01), while these parameters were not significantly different compared to those in WT mice (P>0.05) (Table 1). In addition, there was a dramatic reduction in reticulocyte numbers, from 19.17% to 3.78%. Altogether, this indicated a significant improvement of anemia symptoms in the seven β654-ER mice.

Peripheral blood smears indicated that anisocytosis and poikilocytosis were reduced, and almost no target cells were observed in the β654-ER mice (Figure 3A). The abnormal bone marrow proliferation and erythropoiesis with an increasing proportion of nucleated cells usually seen in β654-Ctrl samples were also decreased in β654-ER mice (Figure 3B).

Improvement of morphological changes in spleen and liver in β654-ER mice

To determine the extent of the improvement of extramedullary hematopoiesis in β654-ER mice, a morphological examination of spleens and livers from β654-ER 7-month-old mice and age-matched β654-Ctrl mice was conducted. Histopathology of the spleens showed that, compared to β654-Ctrl mice and β654-ENR mice, β654-ER mice showed significant reductions of red pulp and the marginal zone of white pulp was distinct (Figure 3C). The hemosiderin in the spleens of the β654-ER mice was dramatically reduced compared to that in the β654-Ctrl mice and β654-ENR mice (Figure 3D). Iron accumulation was rarely observed in the livers of the β654-ER mice (Figure 3E).

The extent of splenomegaly was investigated after dissecting and weighing spleens from the β654-E mice. Both the weight and sizes of spleens of β654-ER mice were remarkably reduced compared to values for the β654-Ctrl and β654-ENR mice. There was no significant difference between the values for β654-ER mice and WT mice (Figure 4).

Elevated survival rate and stable therapeutic effects in offspring of β654-ER mice

To investigate whether the therapeutic effect can be inherited and stable in the progenies of β654-ER mice, two founder mice (ID 52 and ID 53) were mated with WT mice to obtain F1 offspring. F2 animals were generated by full-sibling matings. The survival rates of β654-Ctrl F1 and F2 generations were only 29.0% and 43.4%, respectively, due to the severity of the thalassemia syndrome (Table 2).[17] Interestingly, elevated survival rates of 45.6% and 72.0% were observed in the F1 and F2 generations of β654-ER mice, respectively (Table 2), indicating a clinically significant improvement of survival rate of β654-ER mice follow-

ing gene editing. Furthermore, our gene-editing therapy resulted in the survival of homozygous $\beta^{654\text{-ER}}$ mice, while the unedited homozygous $\beta^{654\text{-ctrl}}$ mice are not usually born live.

Hematologic parameters were also measured once every 2 weeks for 3 months to monitor anemia in offspring. The red cell indices of heterozygous $\beta^{654\text{-ER}}$ mice showed significant improvement compared with the levels found in the $\beta^{654\text{-Ctrl}}$ mice, and there were no significant differences in the levels of red blood cells, hemoglobin and reticulocytes between heterozygous $\beta^{654\text{-ER}}$ mice and WT mice (*Online Supplementary Table S4*).

Off-target analysis of $\beta^{654\text{-ER}}$ mice

To identify off-target events after gene editing, whole-genome sequencing was performed on the seven $\beta^{654\text{-ER}}$ mice

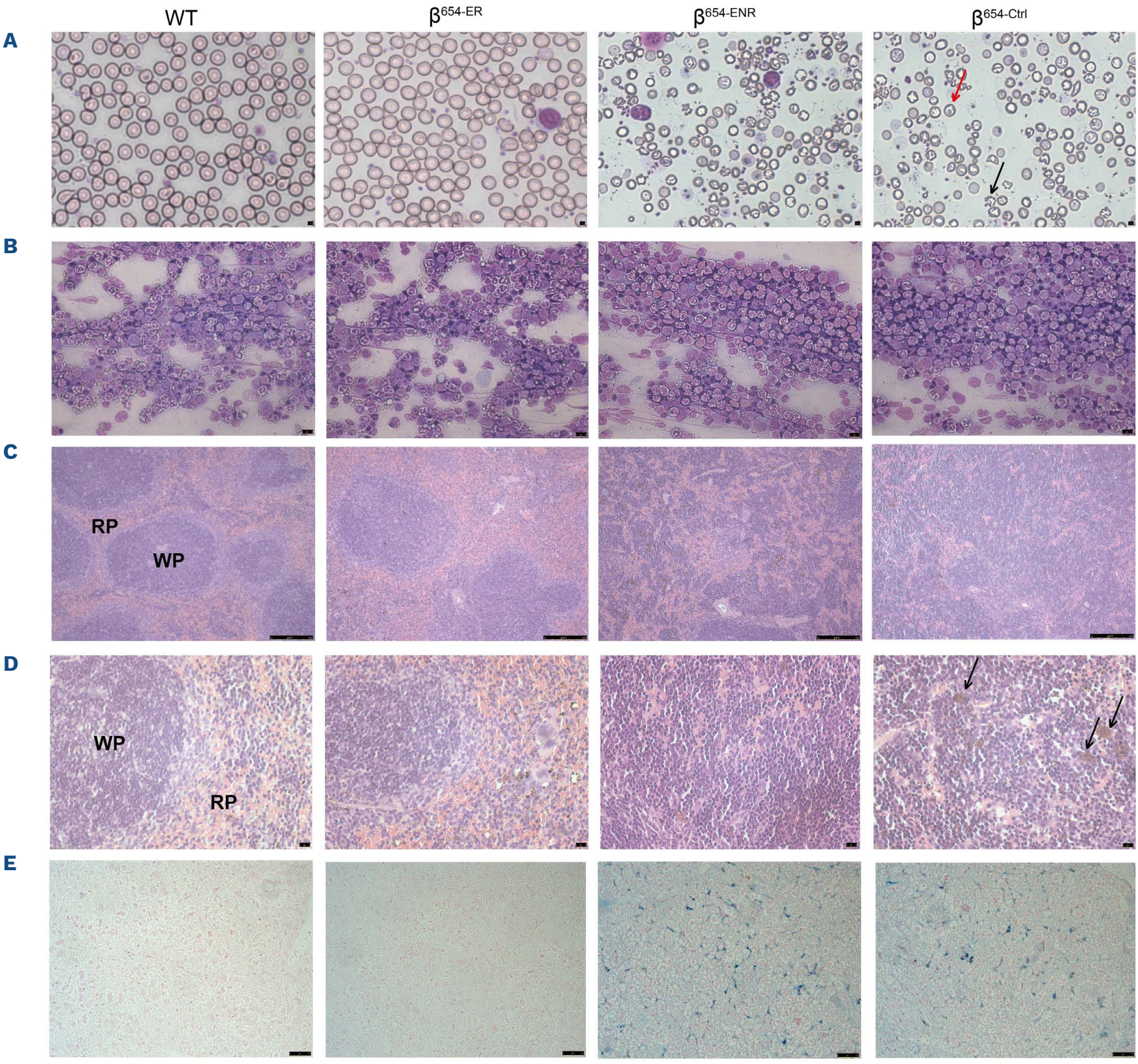


Figure 3. Red blood cell morphology and tissue pathology in representative $\beta^{654\text{-E}}$ mice. (A) Peripheral blood smears stained with Wright-Giemsa. WT, wild-type mouse; $\beta^{654\text{-ER}}$ mouse ID 64 (142 bp deletion); $\beta^{654\text{-ENR}}$ mouse ID 54 (2 bp or 4 bp deletion); $\beta^{654\text{-Ctrl}}$ mouse. The black arrow points to poikilocytosis, and the red arrow points to target cells in $\beta^{654\text{-Ctrl}}$ mice. Scale bars are 50 μm in all images. (B) Bone marrow smears stained with Wright-Giemsa. (C) Spleen histological sections stained with hematoxylin-eosin. RP: red pulp; WP: white pulp. (D) Spleen histological sections stained with hematoxylin-eosin. Black arrows point to hemosiderin granules found in $\beta^{654\text{-Ctrl}}$ mice. (E) Ferrocyanide iron staining of liver samples. Blue staining in the right two panels indicates iron accumulation.

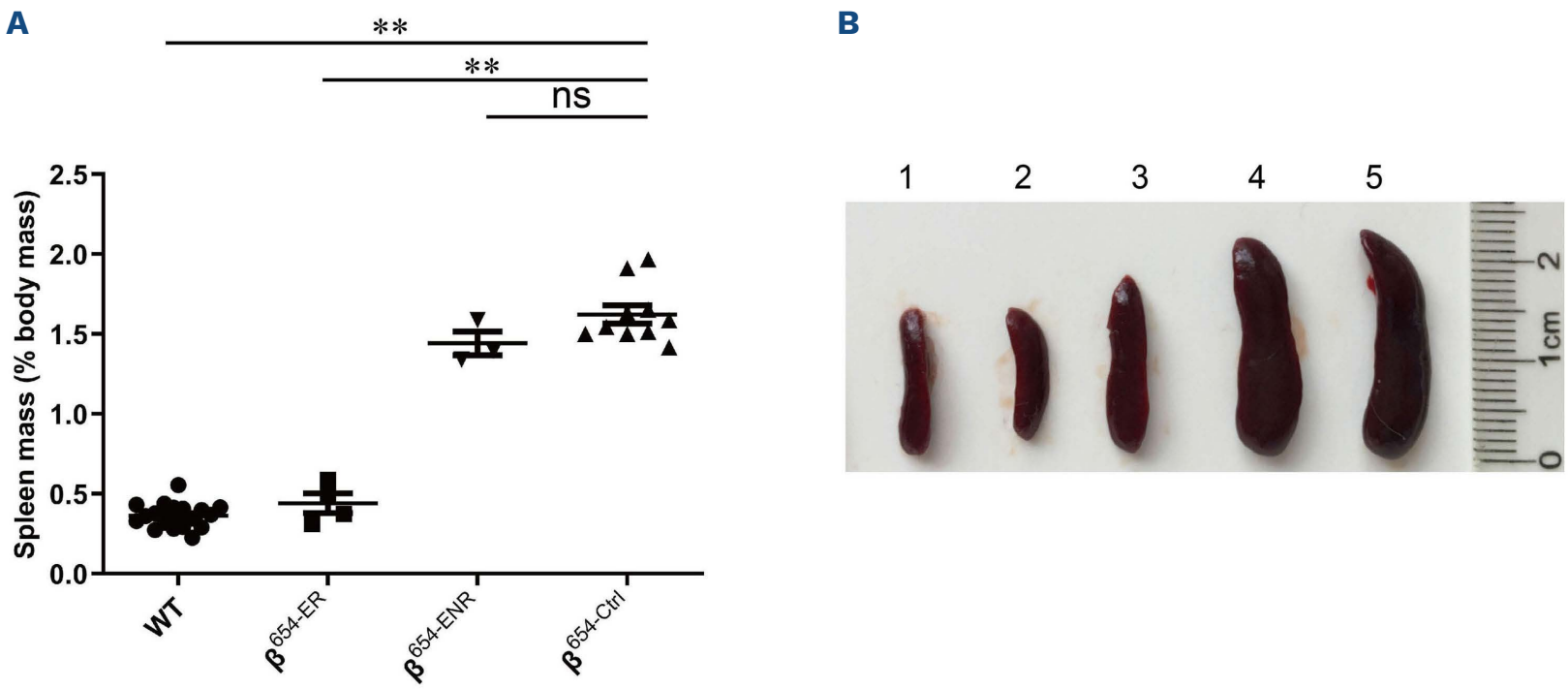


Figure 4. Improvement of splenomegaly in $\beta^{654\text{-ER}}$ mice. (A) Spleen mass in $\beta^{654\text{-ER}}$ mice. The spleen relative mass is represented as % body mass. **Statistically significant difference *P*<0.01; ns: not significant. Wild-type (WT) n=18; $\beta^{654\text{-ER}}$ n=4 (mouse ID 52, 53, 64 and 59); $\beta^{654\text{-ENR}}$ n=3 (mouse ID 54, 74, and 76); β^{654} n=10. (B) Representative spleen images. 1: spleen from a WT mouse; 2-3: spleens from $\beta^{654\text{-ER}}$ mice 52 and 53; 4-5: spleens from $\beta^{654\text{-Ctrl}}$ mice.

and three $\beta^{654\text{-Ctrl}}$ mice. Structural variants (including large deletions and insertions, duplications, translocations, and inversions), short insertions or deletions (indels), as well as single nucleotide variants were determined after alignment and comparison to the mm10 mouse reference genome. There were no significant differences in the average number of structural variants and indels between $\beta^{654\text{-ER}}$ mice and $\beta^{654\text{-Ctrl}}$ (Figure 5A, B and *Online Supplementary Table S5*). Total single nucleotide variant counts were significantly different (Figure 5C) with fewer variants in $\beta^{654\text{-ER}}$ mice. Thirty-five off-target sites predicted by the CRISPOR program (19 for G1 and 16 for G2) (*Online Supplementary Table S6*) were examined in detail in the whole genome sequencing data. No sequence variations were observed at the predicted off-target sites in any of the seven $\beta^{654\text{-ER}}$ mice.

Discussion

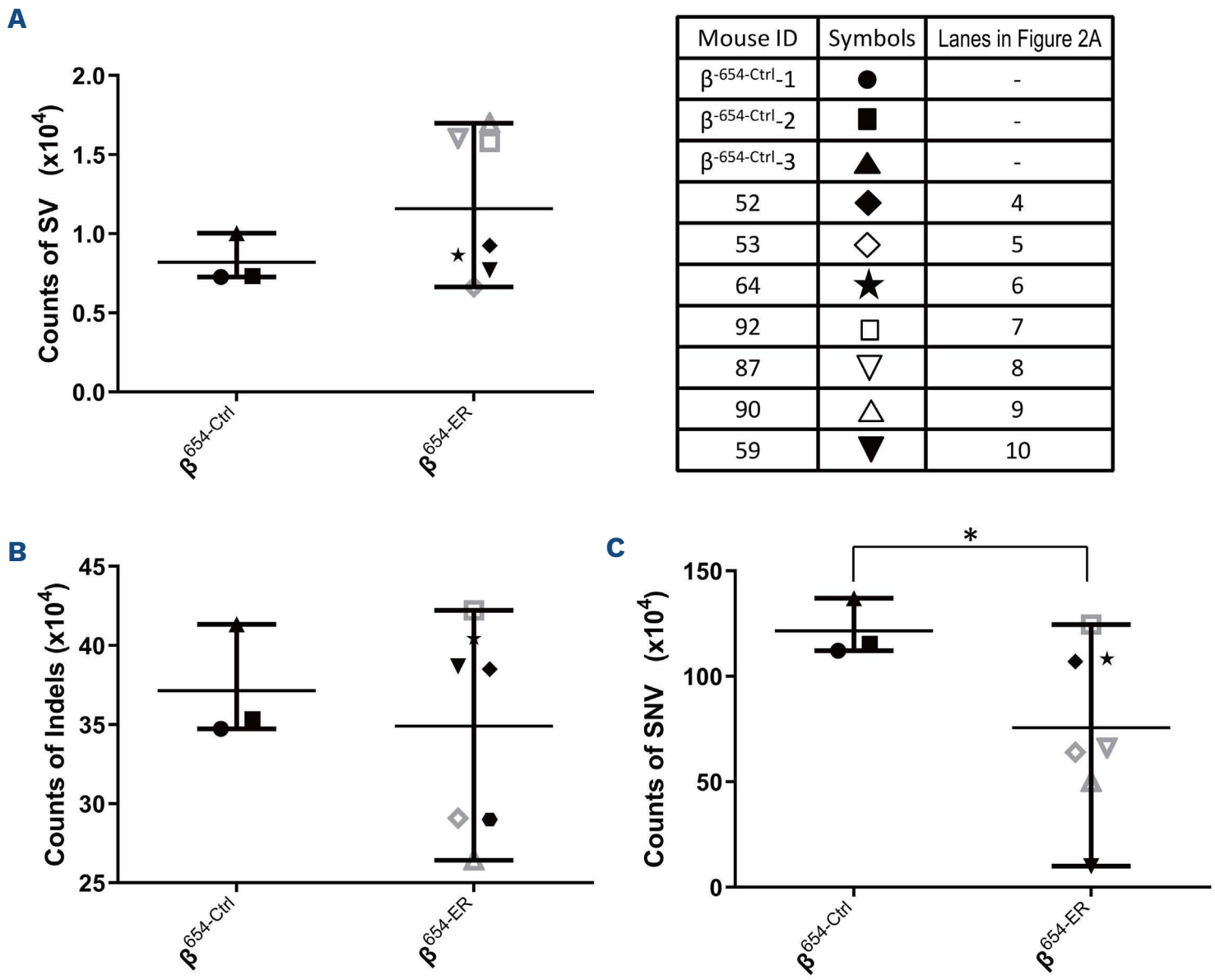
β^{654}-thalassemia is one of the most common β-thalassemias found in Chinese populations and is caused by abnormal RNA processing. According to the Human Gene Mutation Database (*http://www.hgmd.cf.ac.uk/ac/index.php*), 10%-15% of point mutations in genetic diseases can cause abnormal mRNA splicing,[20] and similar RNA processing defects caused by a point mutation were also found in other β-thalassemia diseases (e.g., position at IVS-2-705 and IVS-2-745).[21,22] Therefore, the strategy employed to treat β^{654}-thalassemia could also be expanded to other genetic diseases caused by abnormal RNA splicing for a range of clinical disorders. Here, we report the restoration of normal splicing in β^{654} mice using CRISPR/Cas9-mediated NHEJ with two sgRNA and described therapeutic efficacy in treating β^{654}-thalassemia.

Previous gene editing approaches to target the IVS2-654 C→T mutation site have been reported.[10,11] However, the CRISPR/Cas9 tactic can be limited by the requirement for a protospacer adjacent motif sequence, so proper sgRNA directly targeting the mutation sites may not always be available. To find alternative target candidates, the splicing donor site (at IVS-2-579) and the splicing acceptor site (at IVS-2-654) were both considered. Normal human β-globin splicing in $\beta^{654\text{-ER}}$ mice was observed after the extra 73-nt exon sequence was eliminated by this approach. The clinical β-thalassemia symptoms were remarkedly relieved, and the survival rate of $\beta^{654\text{-ER}}$ mice improved significantly. Various sequence profiles in gene-edited human hematopoietic stem cells were observed when multiple sgRNA were used.[23,24] In this study, the percentage of dual cut deletions of both aberrant splicing sites in founder $\beta^{654\text{-ER}}$ mice and 293T cells was consistent (more than 40%).

Table 2. The elevated survival rate of β^{654} mice following gene editing.

Mouse	Heterozygous β^{654}	Homozygous β^{654}	Total	β^{654} Survival rate (%)
F1	26	0	57	45.61*
F1 controls	45	0	155	29.03
F2	28	8	50	72.00**
F1 controls	53	0	122	43.44

F1 mice were produced by crossing $\beta^{654\text{-ER}}$ with wild-type mice. Controls were produced by mating $\beta^{654\text{-Ctrl}}$ with wild-type mice. The F2 generation was generated by full-sibling matings of $\beta^{654\text{-ER}}$ mice. Controls were produced by full-sibling matings of $\beta^{654\text{-Ctrl}}$ mice. *P<0.05, **P<0.01.



Mouse ID	Symbols	Lanes in Figure 2A
β$^{654\text{-Ctrl}}$-1	●	-
β$^{654\text{-Ctrl}}$-2	■	-
β$^{654\text{-Ctrl}}$-3	▲	-
52	◆	4
53	◇	5
64	★	6
92	□	7
87	▽	8
90	△	9
59	▼	10

Figure 5. Whole-genome sequence analysis of CRISPR/Cas9 off-target alterations. The total counts of sequence variants relative to the mouse mm10 reference genome are plotted for three β$^{654\text{-Ctrl}}$ mice (Control) and seven experimental mice (β$^{654\text{-ER}}$). Variant categories include: (A) large structural variants; (B) short indels; and (C) single nucleotide variants. The horizontal line is the group average, the vertical line is ±1 standard deviation, *Statistically significant difference P<0.05, and data symbols are consistent for individual mice across the three plots (detailed in the table at the right of Figure 5A). SV: structural variants; indels: insertions or deletions; SNV: single nucleotide variants.

Deletions involving single cuts repaired by NHEJ at only the 5' or 3' sites were observed in some β$^{654\text{-E}}$ mice, and the single deletion sizes ranged from 4 bp to 79 bp. The therapeutic efficacy appeared to be related to the location of the deletion. Gene-edited mice with deletions including one or two aberrant splicing sites showed correction of the abnormal splicing pathway. It is interesting to note that the deletion of the IVS-2-579 splicing donor site alone can restore the normal expression of β-globin. Thus, the cryptic splicing donor site could be an alternative target for human genetic diseases caused by splice site mutations.

One mosaic β$^{654\text{-E}}$ mouse with 55% deletion of the extra exon DNA produced 60% correctly spliced mRNA, and a therapeutic effect was observed. This suggests that partially effective gene editing may be sufficient to relieve the disease symptoms. Similar dose effects were found in previous work in which 30% chimerism by induced pluripotent stem cells in β^{654}-thalassemia mice reversed the pathology of anemia.[25] Therefore, mosaicism provides a good model to explore the dosage effect of gene editing in β^{654}-thalassemia gene therapy. A study of human embryo gene editing reported mosaicism was prone to include off-target genetic modifications, which occurred after the first cell cycle.[21] More effort is needed to restrict the activity of Cas9 at the single-cell stage and reduce on-target and off-target mosaicism; such work may include developing anti-CRISPR proteins.[26]

Safety issues concerning CRISPR-mediated gene therapy must be considered. Off-target events may result in the production of unwanted mutations.[27,28] Several recent studies have shown that double-strand breaks induced by Cas9 can result in more extensive genetic changes, including loss of partial or whole chromosomes,[28] frequent loss of heterozygosity in human embryos,[29] chromothripsis in human hematopoietic stem cells,[30] and more complex genomic rearrangements in mouse embryos or embryonic stem cells.[31,32] Our study did not detect an obvious increase in sequence variants in the β$^{654\text{-ER}}$ mice, and these results are consistent with those of studies in other mouse em-

bryos[33] and hematopoietic stem cells.[11,34] On the other hand, off-target events may become apparent with expanded sample size, and a lack of overall increases in variation does not preclude the occurrence of individual pathogenic changes. We did not observe alterations at any of the *in silico* predicted off-site targets after editing cultured cells and embryos. Recent efforts have been made to increase the specificity of the CRISPR system by several orders of magnitude through adding an engineered hairpin secondary structure onto the spacer region of sgRNA.[35] Alternative tools that do not require double-strand breaks, such as base or primer editing,[36,37] may relieve the worries of adverse outcomes associated with double-strand breaks. Protospacer adjacent motif limitation and base changes at sites flanking the targeted base are also aspects amenable to continuing improvement of CRISPR/Cas9 technology for future clinical therapy.

In this study, the sgRNA and Cas9 mRNA were injected into single-cell embryos to examine the therapeutic efficacy for β^{654}-thalassemia. As human germline therapy is prohibited due to the technical challenges of editing efficiency and safety, as well as ethical and legal factors, somatic cell therapy, such as autologous hematopoietic stem cell transplantation with gene modification, is considered to be a practical clinical alternative. Autologous hematopoietic stem cells modified with a lentiviral vector carrying the human β-globin gene, or gene editing targeting the enhancer of *BCL11A*, have proven effective in clinical trials, and a large number of patients have received successful somatic gene therapy.[38-42] The consequences of somatic cell gene editing seem more predictable and acceptable since fewer chromosomal changes in hematopoietic stem cells via NHEJ were found in human embryo studies.[30,43] Thus, future careful examination employing the strategy presented in this study for somatic gene therapy in hematopoietic stem cells could lead us one step closer to clinical therapy for β^{654}-thalassemia.

In conclusion, our study showed that a CRISPR/Cas9-mediated editing approach to eliminate the extra 73-nt exon could correct the aberrant splicing pathway in β^{654} mice, significantly relieve the symptoms of the β^{654} phenotype, and elevate survival rates of heterozygote $\beta^{654\text{-ER}}$ mice. Notably, the live birth of homozygous $\beta^{654\text{-ER}}$ mice (no live homozygous $\beta^{654\text{-ctrl}}$ mice observed previously) also demonstrates the value of our strategy. The gene-editing study presented here appears to be both effective and efficient and could be applied to somatic cell engineering as a novel β^{654}-thalassemia therapeutic approach.

Disclosures

No conflicts of interest to disclose.

Contributions

DL designed and performed experiments, analyzed the data, and drafted the manuscript; XG, YF, XG, and YC conducted experiments; FY and GZ performed the genomic and targeted deep sequencing data analysis; QM and YT reviewed the design and edited the manuscript; FZ designed the project and drafted the manuscript.

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