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RAPID COMMUNICATION

Therapeutic genome editing of an aberrant splice site in β-thalassemia by CRISPR/Cas9 with multiple sgRNAs

Genetic mutations cause aberrant splicing, one of the important molecular mechanisms in human diseases. IVS2-654, a mutation causing aberrant splicing of β-globin pre-mRNA and contributing to β-globin deficiency, is one of the most common diseases in Southeast Asia and China.[1] In our previous work, we found a TTTV protospacer adjacent motif (PAM) by the Cas12a system and the editing efficiency of IVS2-654 $C > T$ achieving 76.7%.[2] Here, we present that the efficiency and persistence of IVS2-654 $C > T$ can be rescued through electroporation of Cas9 ribonucleoprotein (RNPs) with multiple single guide RNAs (sgRNAs) into hematopoietic stem and progenitor cells (HSPCs), introducing high-efficiency indels of pre-mRNA with the aberrant splice sites disrupted and restoring normal β-globin expression. Notably, we found those multiple adjacent sgRNAs could induce large fragment deletion rather than regular small indels, leading to normal coding sequence (CDS) region and directly restoring the β-globin gene function of IVS2-654 $C > T$ without off-target effects. Our strategies could directly rescue the β-globin gene function of IVS2-654 $C > T$ with a higher restoration by multiple sgRNAs co-delivered.

CRISPR/Cas9 system has a more mature operation and higher editing efficiency, while there is no suitable NGG PAM at IVS2-654 $C > T$ aberrant splice site to target the directly for SpCas9. Therefore, we try to explore an appropriate neighboring NGG PAM to induce frequencies of small insertion/deletions (indels) and disrupt the conserved sequence of the aberrant splicing donor site ('AAGGTAATA'; Fig. 1A). We found a sgRNA (sg654-1) targeting an AGG PAM adjacent to the mutation site co-delivery with Cas9 RNP to the engineered HUDEP-2 cell lines (homozygous *T* at IVS2-654 locus) (Fig. S1A and Table S1). Targeted deep sequencing demonstrated high indel efficiency (96.39%) in sg654-1 co-delivery with Cas9 RNP (Fig. S1B, C). Sanger sequencing of amplified cDNA was performed to confirm the restoration of the normal CDS region (Fig. S1D). We further quantified the frequencies of indel rates by next-generation sequencing (NGS) and selected edited clones for detailed analysis. All clones were amplification by PCR analysis and genotyped by Sanger Sequence. As expected, each clone disrupted the aberrant splicing donor site and restored the correct splice product expression. Among them, the genotype of 6 bp deletion clone with the largest proportion (11.17%) showed significant restoration of the correct splice product with almost no aberrant splice product, while deletion '-GG' had a high proportion (10.12%) (−2 bp; Fig. S1C) while aberrant splicing product remained (Fig. S1E). Furthermore, we co-delivery sg654-1 with Cas9 RNP to patient-derived $CD34^+$ HSPCs with compound heterozygous for IVS2-654 $C > T$ and an *HBB* null mutation (Table S2). RT-PCR of β-globin spanning cDNA of the exon 2-3 junction and following gel electrophoresis represented the expression of correct and aberrant splice products in the differentiated erythroid cells from different donors. We observed a significant reduction of the aberrant splice product and restoration of the correct splice product (Fig. S1F, lower lane). RT-qPCR specific to the correct expression of β-globin mRNA (relative to α-globin) increased significantly compare to unedited samples (Fig. S1F, upper lane). To investigate the therapeutic potential of our approach, HSPCs from healthy donors after RNP electroporation with Cas9 of sg654-1 were transplanted into immunodeficient NOD B6. SCID $Il2r\gamma^{-/-}Kit^{W41/W41}$ (NBSGW)[3] mice. We assessed the human hematopoietic lineages in recipient bone marrow (BM) after transplantation. Subsequently, we extracted BM for analysis around 16 weeks after transplantation and found similar human marrow engraftment with edited and unedited $CD34^+$ HSPCs (Fig. S1G). Flow cytometry using an anti-human CD45 antibody showed that human cells made up about 82.33% of BM in all mice (similar to 86% in unedited $CD34^+$ cells) (Fig. S1H). Edited cells also

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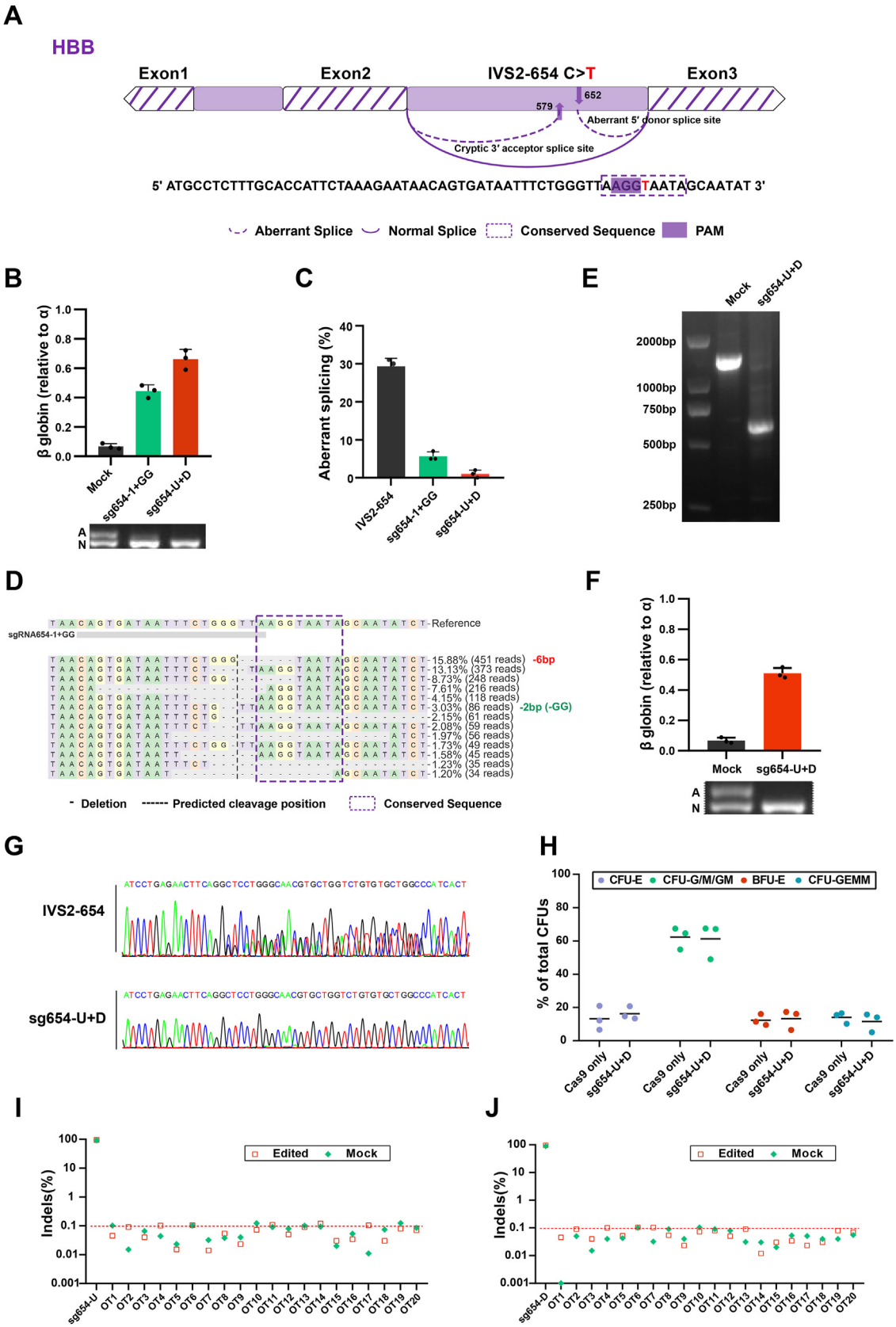

Figure 1 Efficient editing of CRISPR/Cas9 with multiple sgRNAs for restoring IVS2-654-globin pre-mRNA splicing. **(A)** Schema of strategy for correcting the IVS2-654 *C*> *T* mutation using CRISPR/Cas9 to induce double-strand break (DSBs) before the point mutation and conserved sequence of the aberrant splice donor site. **(B)** Successful restoration from different strategies to disrupt the aberrant splice sites in the engineered HUDEP-2 cell lines. The upper lane shows RT-qPCR analysis and the lower lane shows

showed a similar capacity for lymphoid, myeloid, and erythroid engraftment (Fig. S1I, J). The most general concern for genome editing in clinical applications is off-target genotoxicity. A total of 20 potential genomic sites with fewer mismatches relative to the on-target site were selected by a common off-target prediction platform Cas-OFFinder[4] (Table S3). All potential off-target sites show no Cas9-induced indel formation within the limit of detection (~0.1%) in edited cells compared to control cells (Fig. S1K). Thus, those edited HSPCs were preserved and the lineages survived, indicating that the CRISPR/Cas9 system could efficiently generate therapeutic levels in long-term engrafting HSCs without off-targeting effects.

It is remarkable that the genotype of 2 bp deletion '-GG' had a high proportion (10.12%) (Fig. S1C) while the aberrant splicing product remained (Fig. S1E). Then we wonder whether producing a re-disruption DSBs on the aberrant splice site can improve the editing efficiency on the aberrant splice site. Thus, we designed an sg654-GG co-delivery with sg654-1 to induce multiple DNA breaks on the aberrant splice site (Fig. 1A; Fig. S1A and Table S1). As expected, the results of gel electrophoresis, RT-qPCR, and the CDS region all showed high efficiency (Fig. 1B, C). Deep sequencing suggested higher indel frequency targeted on the abnormal splice site, and the corrected genotype of 6-bp increased from 11.17% to 15.88%. Meanwhile, the aberrant '-GG' went from 10.12% to 3.03% (Fig. 1D; Fig. S1C). These results indicate that multiple sgRNA targeting can improve the restoration of β-globin.

Furthermore, as the mutation site located in intron regions does not code for functional proteins, we wondered whether adjacent sgRNAs spaced a hundred bps spanning over the aberrant splice site could result in large fragment deletion and eliminate the abnormal splicing by deleting the aberrant splice donor site and accept site formed in the intron (73 nt). Therefore, we designed 5 pairs of sgRNAs upstream and downstream of the abnormally spliced donor and receptor site (Fig. S1A). We preselected sgRNAs in patient cells, and the editing efficiency was identified by Sanger sequencing and DNA gel electrophoresis. The sgRNA U+D showed the highest efficiency of large fragments deletion and causes the significant elimination of abnormal splicing (Fig. S2A, B). We also found much higher restoration efficiency than mock and sg654-1/-GG, leading to a spliced β-globin mRNA and a normal CDS region (Fig. 1B–E). Thus, we chose sgRNA U+D for the subsequent experiment. To confirm the feasibility of our strategy, we further edited the patient-derived $CD34^+$ HSPCs (Donor #3) under the same condition of co-delivery sg654-U+D with Cas9 RNP. As expected, the strategy led to a spliced β-globin mRNA and normal CDS region (Fig. 1F, G). Then, we performed the colony-forming unit (CFU) assay to evaluate the progenitor cell capacity of HSPCs. Edited HSPCs formed all types of colonies (erythroid, granulocyte/macrophage, and multilineage) to a comparable extent as mock-electroporated cells (Fig. 1H). Potential off-target sites also show no Cas9-induced indel formation within the limit of detection (~0.1%) in edited cells compared to control cells (Fig. 1I, J, sg654-U and sg654-D and Table S3). Moreover, those adjacent sgRNAs could target both alleles (IVS2-654 T and IVS2-654 C allele), and efficient editing did not affect healthy donor-differentiated erythroid cells (Fig. S3A, B). Overall, our strategies could directly restore the β-globin gene function of IVS2-654 $C > T$ with high efficiency, using CRISPR/Cas9 technology simplicity with a higher restoration of multiple sgRNAs designs.

Here, we demonstrated that NHEJ-based genetic disruption of the aberrant splice site was a simple and highly efficient approach for the elimination of a conserved sequence, achieving a desired therapeutic outcome without integration risk. This strategy takes advantage of the simplicity and with a higher restoration of sgRNA design, providing a new idea for mutation sites without suitable PAM, especially pathogenic mutations in non-coding regions. Further studies are needed to improve safety by disrupting the disease sites in the intron region.

Author contributions

Y.W., D.L., and F.Y. designed the experiments and wrote the manuscript; F.Y. and S.X. performed all experiments and analyzed the data; Z.S., Q.K., and C.H. helped produce Cas9 proteins; Y.H. advised on the editing of the manuscript; Y.Y. and Y.L. provided the HPSCs and analyzed the data; D.L. and Y.W. conceived and supervised the study; all authors discussed the results and commented on the manuscript, and contributed to the final manuscript.

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normal splicing after therapeutic editing. **(C)** The level of abnormal splicing in CDS region from different strategies in HUDEP-2 cell lines. **(D)** Deep sequencing results showed frequent indels after sg654-1/-GG editing. **(E)** Separation of the PCR products on agarose gel shows the deletion fragment of sg654-U+D after editing compared to mock cells. **(F)** The feasibility of co-delivery sg654-U+D with Cas9 RNP in patient-derived $CD34^+$ HSPCs. The upper lane shows RT-qPCR analysis and the lower lane shows normal splicing after therapeutic editing. **(G)** PCR and Sanger sequencing of CDS region in $CD34^+$ HSPCs (Donor 3). **(H)** HSPCs were sorted into a 6-cm dish containing methylcellulose. CFUs were counted 14 days after sorting the total number (percentage colony formation) ($n = 3$). Representative lineage-restricted progenitors (BFU-E, CFU-E, CFU-G/M/GM) and multipotent progenitors (CFU-GEMM). Morphology: CFU-E (CFU-erythroid), BFU-E (burst forming unit-erythroid), CFU-G/M/GM (CFU-granulocyte/macrophage), CFU-GEMM (CFU-granulocyte/erythrocyte/macrophage/megakaryocyte). **(I, J)** Potential off-target sites with fewer mismatches relative to the on-target site (sg654-U, sg654-D) were evaluated by amplicon deep sequencing. The dotted line at 0.1% denotes the sensitivity of deep sequencing to detect indels. In all graphs, data are plotted as mean ± standard deviation. Error bars indicate standard deviation ($n = 3$ independent experiments).

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Conflict of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.01.010.

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