Targeted B-domain deletion restores F8 function in human endothelial cells and mice

Signal Transduction and Targeted Therapy (2022) 7:189 ; https://doi.org/10.1038/s41392-022-01016-9

Dear Editor,

Hemophilia A (HA) is the most common genetic bleeding disorder and affects 1 in 5000 male births.1 HA patients suffer spontaneous soft-tissue and joint bleeding or life-threatening intracranial hemorrhage. The main clinical treatment for severe HA patients is life-long maintenance of protein replacement therapy, which increases the risks of infection and induce FVIII inhibitors. Gene therapy is expected to cure HA, however, the current AAV-based gene addition strategy is limited by its loading capacity, which exerts coagulation function does not contain the B domain. In recent decades, a version of FVIII lacking most of the B domain (F8-BD-FVIII) was developed, and exogenous BDD-FVIII or BDD-F8 subsequently appeared to be safe and effective in preclinical studies or clinical management of HA patients.3 FVIII harbors 19 N-linked glycosylation sites within B domain. Partial B-domain-deleted FVIII, which retains several N-linked glycosylation sites, secretes more efficiently due to increased endoplasmic reticulum-to-Golgi transport.4 However, it remains unclear whether endogenous FVIII with either partial or entire F8-B deletion retains coagulation function. Recently, we discovered that reframed F8 within 54-bp deletion in F8-B could express functional FVIII, and hence hypothesized that in situ genetic manipulations of F8 to create an entire F8-B deletion might represent a therapeutic strategy for all HA patients with mutations in F8-B. Here we evaluated this strategy in HA patient-derived induced pluripotent stem cells (HA-iPSCs) and normal human iPSCs (N-iPSCs) using clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease (CRISPR/Cas9) (Fig. 1a). HA-iPSCs were previously generated from urine cells of an HA patient (c.3167delCTGA).5 To rule out re-cutting of the fused sequences (Ser743 fused to Gln1638) by CRISPR/Cas9, we retained the coding sequences for Gln744 and Asn745 to ensure the fusion of Asn745 to Pro1640 (Supplementary Fig. S1a). The dual single-guide RNAs (sgRNAs) F8-BDU-sg1 and F8-BDD-sg4 were designed with verification of the cleavage activity (Supplementary Fig. S1b). Plasmids expressing the CRISPR/Cas9 complex and sgRNAs along with the corresponding ssODN template were nucleofected into the HA-iPSC and N-iPSC lines. We achieved high targeting efficiency of 4.17% without any screening (Fig. 1b and Supplementary Fig. S1c). The stable BDD-F8 clones maintained pluripotency and a normal karyotype (Supplementary Fig. S1d–f). No off-target indels were observed (Supplementary Fig. S2). The reframed F8 transcript of the BD-iPSCs was detected (Supplementary Fig. S3a); however, almost no FVIII was detected in supernatant from iPSC culture (Supplementary Fig. S3b). Western blot revealed almost undetectable levels of lectin mannosere-binding 1 (LMAN1), which reportedly mediates FVIII secretion (Supplementary Fig. S3c).

Given that ECs are the main cell type secreting FVIII, we differentiated BD-iPSCs into EPCs/ECs and evaluated FVIII expression in BD-iPSC-derived EPCs (BD-iEPCs) and ECs (BD-iECs). The differentiation efficiencies ranged from 16 to 26% (Fig. 1c). Pure iEPCs expressing CD31 and CD144 and mature ECs expressing von Willebrand factor (vWF) were obtained (Supplementary Fig. S3d). F8 transcription in BD-iECs was detected (Supplementary Fig. S3e). The secreted FVIII was 1.00 ng/10^6 cells and 0.52 ng/10^6 cells from BD21-iECs and BD25-iECs respectively, both of which were higher than that from HA-iECs (0.17 ng/10^6 cells) and close to that from N-iECs (0.60 ng/10^6 cells) (Fig. 1d). We detected LMAN1 in ECs, possibly explaining why FVIII secreted from ECs but not from iPSCs (Supplementary Fig. S3c). Importantly, immunostaining by both N-terminal and C-terminal FVIII antibody revealed restoration of FVIII expression in BD-iECs, whereas the truncated FVIII was detected in HA-iECs by a N-terminal FVIII antibody (Fig. 1e). These results demonstrated an expression and secretion of endogenous BDD-FVIII in BD-iECs.

To determine the expression efficiency of endogenous N8-FVIII, we constructed an in situ partial B-domain-deleted N8-FVIII variant harboring 271 amino acids at the N-terminus (Fig. 1f and Supplementary Fig. S4a). The iPSCs harboring the N8-FVIII variant were obtained with an efficiency of 14.58% (Supplementary Fig. S4b–d). N8-iPSCs and N8-46-iPSCs clones that expressed pluripotient genes, maintained a normal karyotype, and transcribed reframed N8-FVIII (Supplementary Fig. S4e–g) were selected for further experiments. Almost no FVIII was detected in culture supernatant of iPSCs (Supplementary Fig. S5a). Sanger sequencing revealed no indels at the putative off-target sites (Supplementary Fig. S5b). N8-iPSCs were differentiated to EPCs (N8-iEPCs) and ECs (N8-iECs) (Fig. 1c and Supplementary Fig. S6a) that were characterized by angiogenesis and Dil-acetylated-low-density-lipoprotein endocytosis (Supplementary Fig. S6b, c). The reframed N8-FVIII transcripts were detected in N8-iEPCs (Supplementary Fig. S6d), which were eightfold higher than those in HA-iEPCs and threefold higher than those in BD-iEPCs (Fig. 1g), although well lower than that in primary liver sinusoidal endothelial cells (LSECs) with highest FVIII production capacity described previously. FVIII levels in supernatant of N8-9-iEPCs and N8-46-iEPCs were significantly higher than that in BD-iEPCs (Fig. 1d). Meanwhile, FVIII expression in N8-iECs was confirmed by immunostaining (Fig. 1e), and LMAN1 was detected in ECs (Supplementary Fig. S6e).

To validate the therapeutic effects of endogenous BD-FVIII and N8-FVIII, we transplanted HA-iEPCs, reframed iEPCs, and N-iEPCs at the closure of injury to mice.
into HA mice via retro-orbital vein injection. By 2 weeks post-infusion, HA mice were subjected to a tail-clip challenge and plasma FVIII activity assays. The average survival time of HA mice transplanted with BD-iEPCs and N8-iEPCs (24.62 h and 29.25 h, respectively) were significantly longer than those of untreated mice (5.73 h) (Fig. 1h, i). Notably, three of the 18 HA mice transplanted with BD-iEPCs and 3 of the 18 HA mice with N8-iEPCs were alive 48 h after tail-clip challenge (the experiment endpoint). Furthermore, we observed higher plasma FVIII activities in HA mice transplanted with BD-iEPCs and N8-iEPCs (12.79% and
Functional restoration of FVIII in human ECs and mice via targeted entire or partial B-domain deletion of the endogenous F8 gene. **A**. Schematic representation of generation of B-domain targeted deletion and 8 N-linked glycosylation sites retained in B domain of the endogenous F8 gene. **B**. PCR screening of BD-iPSCs using the primers BUF/BDR. Sizes of the PCR products: B-domain deletion, 341 bp; N-iPSCs, 3023 bp; HA-iPSCs, 3019 bp. **C**. qRTPCR analysis of F8 expression in iPSCs using primers targeting exons 23 and 26 (E23-26), with GAPDH used as a loading control. **D**. Proportions of surviving mice after tail-clip challenge. HA mice, hemophilia A mice (n = 9); HA mice transplanted with HA-iPSCs (n = 9), BD21-iEPCs (n = 9), BD25-iEPCs (n = 9), N8-9-iEPCs (n = 9), N8-46-iEPCs (n = 9), N-iEPCs (n = 9), N-BD-iEPCs (n = 9), and N-N8-iEPCs (n = 9), respectively. n.s., not significant compared with HA mice. **p** < 0.05, ***p** < 0.001, vs. the BD25-iEPCs group. **E**. Immunofluorescence staining of FVIII (red) and vWF (green) in iECs, DAPI was used for nuclear staining. FVIII-N, F8 protein N-terminus, FVIII-C, F8 protein C-terminus. **F**. PCR screening of N8-iPSCs. The sizes of the PCR products using primers BUF/BDR for the N8-iPSCs was 1160 bp, N-iPSCs was 3023 bp, and HA-iPSCs was 3019 bp. **G**. qRT-PCR analysis of F8 expression in iECs using primers targeting exons 23 and 26 (E23-26), with GAPDH used as a loading control. **H**. Proportions of surviving mice after tail-clip challenge. HA mice, hemophilia A mice (n = 9); HA mice transplanted with HA-iPSCs (n = 9), BD21-iEPCs (n = 9), BD25-iEPCs (n = 9), N8-9-iEPCs (n = 9), N8-46-iEPCs (n = 9), N-iEPCs (n = 9), N-BD-iEPCs (n = 9), and N-N8-iEPCs (n = 9), respectively. Data represent the mean ± SEM, n.s., not significant compared with HA mice. **p** < 0.01, **p** < 0.001, vs. the HA-iEPC group. **I**. Relative FVIII activity detected at 2-week post-transplantation in HA mice, HA mice without transplantation (n = 6); HA mice transplanted with DPBS (n = 6), HA-iEPCs (n = 6), BD21-iEPCs (n = 6), BD25-iEPCs (n = 6), N8-9-iEPCs (n = 6), N8-46-iEPCs (n = 6), N-iEPCs (n = 6), N-BD-iEPCs (n = 6), and N-N8-iEPCs (n = 6), respectively. Data represent the mean ± SEM. **p** < 0.001, vs. the HA-iEPC group. **J**. Liver tissue sections of HA mice transplanted with BD-iEPCs were analyzed using immunofluorescence with anti-human vWF (green) and CD31 (red) antibodies. No signal was found in the DPBS group. DAPI was used for nuclear staining. Tissue sections of other organs from mice transplanted with BD-iEPCs were analyzed by immunofluorescence. Cells positive for anti-human vWF staining were observed in the spleen and lung. CD31 (red), vWF (green), with DAPI used for nuclear staining. **m**. No signal was found in the heart or kidney. CD31 (red), vWF (green), with DAPI used for nuclear staining.

10.49%, respectively) than that in HA mice (3.88%) (Fig. 1f). These results indicated that endogenous FVIII with partial or entire deletion of F8-B could exert coagulation function and systematic infusion of the reframed iEPCs could rescue FVIII deficiency in HA mice, firstly demonstrating in vitro and in vivo functionality of FVIII which is encoded by endogenous F8 deletion with the deletion of the B domain. Using immunostaining of anti-human vWF antibody, we identified positive cells in the livers of HA mice transplanted with iEPCs but not in mice injected with Dulbecco's phosphate-buffered saline (DPBS) (Fig. 1k), and positive signals were observed in the lungs and spleens of iEPC-transplanted mice but not in the heart or kidney (Fig. 1l, m). Additionally, no obvious damage to the liver or kidney was observed within the 2-week observation period (Supplementary Fig. S6f–h).

In summary, we achieved an efficient targeted F8-B deletion in HA-iPSCs and the derived iEPCs could express functional FVIII. Transplantation with the F8-B-deleted iEPCs could restore FVIII function and rescue the bleeding phenotype in HA mice. These findings provide a proof of concept for the production of the functional FVIII from endogenous F8 with targeted B-domain deletion and preclinical validation of an autologous stem cell gene therapy for HA.

**DATA AVAILABILITY**

All data relevant to this work are included in this paper and Supplementary Information.

**ACKNOWLEDGEMENTS**

We thank the patients for participation in this study. This work was supported by grants from the National Natural Science Foundation of China (82101957, 81770200), the China Postdoctoral Science Foundation (2020TQ0362), the Natural Science Foundation of Hunan Province (grant number 2021J40806) and the National Key Research and Development Program of China (2016YFC0905102).

**AUTHOR CONTRIBUTIONS**

D.L. and M.Z. designed and supervised the study and edited the manuscript; Z.H., M.Z., Z.L., Y.W., and J.Z. performed the experiments; Z.H. drafted the manuscript; M.Z. prepared the figures; Z.L., Y.W., J.Z., and L.W. assisted in manuscript preparation; and all authors read and approved the final version of the manuscript.

**ADDITIONAL INFORMATION**

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-022-01016-9.

Competing interests: The authors declare no competing interests.

Ethics declarations: The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of the School of Life Sciences, Central South University (No. 2020-2-5 and No. 2020-1-6).

Zhiqing Hu1, Zhou Li1, Yong Wu1, Junya Zhao1, Lingqian Wu1, Miaojin Zhou1✉, and Desheng Liang2✉

1Center for Medical Genetics, School of Life Sciences, Central South University, Changsha, Hunan 410078, China

Correspondence: Miaojin Zhou (zhoumiaojin@sklmg.edu.cn) or Desheng Liang (liangdesheng@sklmg.edu.cn)

**REFERENCES**

1. Ragni, M. V. Hemophilia as a blueprint for gene therapy. Science 374, 40–41 (2021).
2. Bulcha, J. T., Wang, Y., Ma, H., Tai, P. W. L. & Gao, G. Viral vector platforms within the gene therapy landscape. Signal Transduct. Target Ther. 6, 53–77 (2021).
3. Pasi, K. J. et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. N. Engl. J. Med. 382, 29–40 (2020).
4. Miao, H. Z. et al. Bioengineering of coagulation factor VIII for improved secretion. Blood 103, 3412–3419 (2004).
5. Hu, Z. et al. ssODN-mediated in-frame deletion with CRISPR/Cas9 restores FVIII function in hemophilia A-patient-derived iPSCs and ECs. Mol. Ther. Nucleic Acids 17, 198–209 (2019).