Biochemical and Biophysical Research Communications 472 (2016) 144–149

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Targeting of the human *F8* at the multicopy rDNA locus in Hemophilia a patient-derived iPSCs using TALENickases

Jialun Pang, Yong Wu, Zhuo Li, Zhiqing Hu, Xiaolin Wang, Xuyun Hu, Xiaoyan Wang, Xionghao Liu, Miaojin Zhou, Bo Liu, Yanchi Wang, Mai Feng, Desheng Liang*

State Key Laboratory of Medical Genetics, School of Life Sciences, Central South University, Changsha, Hunan, China

ARTICLE INFO

Article history:
Received 13 February 2016
Accepted 19 February 2016
Available online 24 February 2016

Keywords:
Hemophilia A
iPSCs
Ribosomal DNA
Multicopy
TALENickases

ABSTRACT

Hemophilia A (HA) is a monogenic disease due to lack of the clotting factor VIII (FVIII). This deficiency may lead to spontaneous joint hemorrhages or life-threatening bleeding but there is no cure for HA until very recently. In this study, we derived induced pluripotent stem cells (iPSCs) from patients with severe HA and used transcription activator-like effector nickases (TALENickases) to target the factor VIII gene (*F8*) at the multicopy ribosomal DNA (rDNA) locus in HA-iPSCs, aiming to rescue the shortage of FVIII protein. The results revealed that more than one copy of the exogenous *F8* could be integrated into the rDNA locus. Importantly, we detected exogenous *F8* mRNA and FVIII protein in targeted HA-iPSCs. After they were differentiated into endothelial cells (ECs), the exogenous FVIII protein was still detectable. Thus, it is showed that the multicopy rDNA locus could be utilized as an effective target site in patient-derived iPSCs for gene therapy. This strategy provides a novel iPSCs-based therapeutic option for HA and other monogenic diseases.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Hemophilia A (HA) is a common X-linked recessive genetic disease due to lack of the clotting factor VIII (FVIII), encoded by the factor VIII gene (*F8*). The incidence of HA in male is approximately 1/5000. The main symptom of HA is spontaneous or traumatic bleeding, particularly in muscles and joints, which is often disabling and can even be life-threatening because of intracranial or joint hemorrhage [1]. The traditional treatment of HA is supplementation with FVIII derived from the plasma or recombinant protein. However, an ongoing concern is the development of inhibitory antibodies to plasma-derived FVIII or recombinant FVIII. And the short half-life of FVIII necessitate repeated infusions of the protein, making it a very costly and long-term treatment option. Because of these drawbacks, scientific interest is shifting to a gene-based approach for HA treatment [2].

Being a monogenic disease, HA is one of the most attractive models for gene therapy researchers, because FVIII does not involve complex pathways and even a small increase of FVIII can improve the patient's condition. The first generation of gene therapy involves the delivery of the normal genes into the cells of patients to replace or correct disease-causing genes using a viral vector such as lentivirus or retrovirus. This approach has been successful in various small and large animal models [3–5]. However, similar successes are not documented in clinical trials until very recently [6]. Moreover, this approach increases the risk of cancer for the reason of random insertional mutagenesis. The second generation of gene therapy involves the repair of virulence genes (in situ) or knock-in a normal gene at a specific site (ex situ) using gene editing technology, and individualized therapy based on patient-derived induced pluripotent stem cells (iPSCs) [7–9]. This strategy is markedly safer than the first generation of gene therapy.

Our group focuses on the identification of the ribosomal DNA (rDNA) locus as a safe and effective target site for gene therapy. We have reported successful targeted gene addition at the rDNA locus in human embryonic stem cells (hESCs) [10] and other cell types [11–13]. There are hundreds of copies of rDNA genes clustered on the short arms of the ten acrocentric chromosomes in diploid human cells. A group found that this area exhibits high recombinational activity during both meiosis and mitosis [14], indicating that the homologous recombination (HR) of this area might be effective. Another study proved that the loss or gain of some copies of the rDNA gene did not cause any abnormal phenotype and could be

* Corresponding author. State Key Laboratory of Medical Genetics, Central South University, 110 Xiangya Road, Changsha, Hunan 410078, China.
E-mail address: liangdesheng@sklmg.edu.cn (D. Liang).

http://dx.doi.org/10.1016/j.bbrc.2016.02.083
0006-291X/© 2016 Elsevier Inc. All rights reserved.

inherited stably. In addition, fragments with balanced translocation involving the short arms of acrocentric chromosomes showed a normal phenotype [15], suggesting that the gene translocated to this area could express normally. Therefore, the rDNA locus is considered a suitable harbor for transgenes.

Historically, targeted gene inactivation, replacement, or addition had been achieved by HR. However, the low efficiency of this approach in human cells markedly limited its utility. In recent years, clustered regularly interspaced short palindromic repeats (CRISPRs) and transcription activator-like effector nucleases (TALENs) had became powerful tools for genome editing, because they could increase the frequency of homology-directed repair (HDR) by several orders of magnitude [16]. By co-delivery of the site-specific nuclease and the donor plasmid bearing homology arms, transgenes could be efficiently integrated into specific locus. Hockemeyer showed that the transgene could be effectively targeted into five distinct genomic locus in both hESCs and iPSCs using TALENs [17]. In this study, the specificity of TALENs-mediated genome targeting had been further refined by the development of TALEnickases (TALENickases), which take advantage of the finding that the induction of nicked DNA stimulates HDR without activating the error-prone nonhomologous end joining (NHEJ) repair pathway [18]. Consequently, TALENickases led to less off-target mutagenesis and cytotoxicity compared with conventional double strand break (DSB) induced methods for genome editing, which was particularly important to the multicopy rDNA locus [19].

Here we reported for the first time that the targeting of *F8* at the multicopy rDNA locus in HA patient-derived iPSCs using TALENickases. And it was confirmed that more than one copy of the exogenous *F8* could be integrated into the rDNA locus. Importantly, we detected exogenous *F8* mRNA and FVIII protein in targeted HA-iPSCs. After they were differentiated into endothelial cells (ECs), the exogenous FVIII protein was still detectable.

2. Materials and methods

2.1. Generation of HA-iPSCs from urine cells

Renal tubular epithelial cells (urine cells) collected from urine of severe HA patients were infected with viral supernatants generated by transfection of HEK293T cells using lipofectamine 2000 (Invitrogen) with retroviral pMXs vectors (Addgene) producing human Oct4, Sox2, Klf4, and c-Myc transcription factors. Two rounds of infection were performed successively. Polybrene (Sigma) was added to increase infection efficiency. On day three or four, cells were trypsinized and 50,000 cells were seeded on mouse embryonic fibroblasts (MEF) feeders in the 10 cm culture dishes. VPA (Sigma) was added from day five to twelve. After infection the medium was renewed daily in all stages. From day 20, the colonies were big enough to be picked mechanically and expanded in hESCs medium on MEF feeders.

2.2. Plasmids and gene targeting

Based on the homologous recombination method, the targeting scheme was summarized in Fig. 2A. The plasmids pHrnF8 [11] and TALENickases [19] were applied in this research as described previously. For gene targeting, the iPSCs were incubated at 37 °C with trypLE™ Select (Invitrogen) for 3 min. Then the cells were immediately collected and counted. The centrifugated cells were resuspended with 100 uL Human Stem Cell Nucleofector Kit 2 (Lonza) with 5 μg linearized pHrnF8 and 5 μg both sides of TALENickases. Nucleofection was completed by Nucleofector II (Lonza) with program B016. And 50 μg/ml G418 (Sigma) selection was initiated on seven days after transfection. About ten days after selection, all resistant clones were picked mechanically and expanded on Matrigel (BD Bioscience) in mTeSR1 (Stemcell).

2.3. Southern blotting

After digested with pvuII restriction enzyme (NEB) overnight, 5 μg genomic DNA were electrophoresed on a 0.8% agarose gel in low voltage, then transferred to positively charged nylon membranes (Roche). λDNA Hind III (Takara) was used as molecular weight marker. The blots were hybridized with DIG-dUTP labeled probes overnight at 42 °C. After incubation with AP-conjugated DIG-Antibody (Roche) and appropriate washing, the signals were detected using CDP-Star (Roche) as a substrate for chemiluminescence.

2.4. Quantitative real-time PCR

2.4.1. For copy number of exogenous gene

Genomic DNA was isolated from the iPSCs using phenol/chloroform extraction. Quantitative PCR (qPCR) was performed using BIO-RAD CFX96 touch q-PCR system (Bio-Rad) with SYBR Premix Ex TaqTM (Takara) according to the manufacturer's instructions. The sex-determining region of Y-chromosome (*SRY*) gene was amplified as an endogenous control. Ct value was calculated using the Bio-Rad CFX Manager software (Bio-Rad). Copy numbers of targeted clones were calculated using the formula. To minimize the error associated with different quantities of applied template, the experiments were repeated three times and the values were reported as means ± standard error.

2.4.2. For transcription activity of exogenous gene

Total RNA was extracted using Trizol reagent (Sigma), RNA was treated with DNase (Thermo) to eliminate any DNA contamination. Quantitative reverse transcription PCR (qRT-PCR) analysis was performed using Bio-Rad CFX96 touch q-PCR system with HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme) according to the manufacturer's instructions. The hypoxanthine phosphoribosyltransferase (*HPRT*) gene was amplified as an endogenous control. Data processing method is consistent with the previously described.

2.5. Differentiation of iPSCs and isolation of ECs

Targeted iPSCs were detached by 1 mg/ml dispase and replated on eight days old Mouse bone marrow stromal cell line (OP9) in OP9 differentiation medium. On day one after iPSCs planting, aspirated the medium and filled the dishes with 20 ml fresh. On coculture days four and six, changed half of the medium. On day eight of iPSCs/OP9 coculture, ECs were ready for isolation. The isolation was performed using human CD31 MicroBead Kit (Miltenyi) and LS Columns (Miltenyi) according to the manufacturer's instructions.

2.6. Enzyme linked immunosorbent assay (ELISA)

After culturing in suitable medium for three days, supernatants and lysate were collected from 12-well dishes. Total cells were trypsinized and counted. All samples were collected in triplicate. ELISA was performed using Paired Antibodies for ELISA - Factor VIII:C (Cedarlane) according to the manufacturer's instructions. Reference curves were constructed using serial dilutions of normal pooled plasma, with correlation coefficient (R2) of at least 0.990 using a 4-parameter logistic curve fit algorithm.

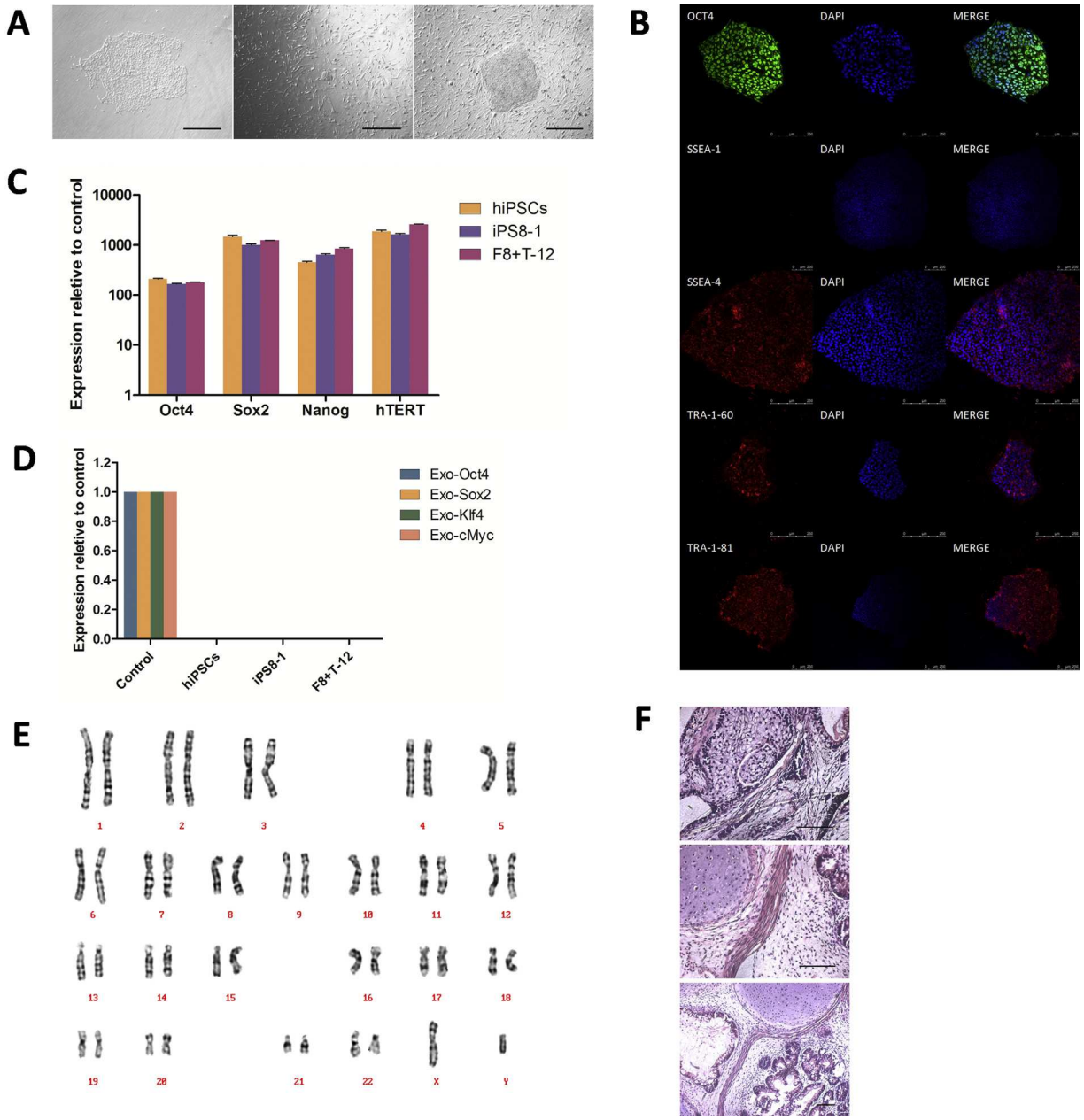

Fig. 1. Generation and characterization of HA iPSCs from urine cells. (A) Morphology of the urine cells from HA patients (left). Morphology of the small cell clump at the early stage of inducing (middle). Morphology of the expanded iPSCs from HA patients (right). (Scale bar, 500 μm) (B) Confocal immunofluorescence microscopy for the indicated human embryonic stem cell (ESC) markers of a representative iPSCs clone. (C) Quantitative reverse transcription PCR (qRT-PCR) for endogenous ESC transcription factors in the indicated iPSCs. hTERT indicates human telomerase reverse transcriptase. Values are referred to donor urine cells; hiPSCs were used as positive control. (D) qRT-PCR showing silencing of the exogenous transgenes in the indicated iPSCs; values are referred to transduced cells extracted at day 4. (E) Normal karyotype of representative iPSCs of male HA patients. (F) Teratoma formation in immunodeficiency mice by representative iPSCs. All three germ layers were observed in the ectoderm (top); in the mesoderm (middle); in the endoderm (bottom). (Scale bar, 200 μm).

3. Results

3.1. Generation and characterization of HA-iPSCs from urine cells

The cells from the urine of two unrelated patients with severe HA were isolated (Fig. 1A). We detected genotype of patients by inverse shifting PCR and sequencing (data not shown), and it turned out that one patient with intron 1 inversion and the other with intron 22 inversion. The isolated urine cells were infected with retroviral pMXs vectors that encode the four Yamanaka factors, and they appeared agglutinated and rounder on day eight after infection (Fig. 1A). Typical iPSCs colonies were observed 20 days after infection (Fig. 1A), which were called HA-iPSCs in this study. Pluripotent markers such as OCT4, SSEA1, SSEA4, TRA-1-60, and TRA-1-81 were detected in the HA-iPSCs using immunofluorescence staining (Fig. 1B). Moreover, the HA-iPSCs showed silencing of the exogenous four Yamanaka factors transgenes and expression of endogenous ESCs transcription factors, as determined by qRT-PCR (Fig. 1C and D). The HA-iPSCs were also karyotyped, and they possessed the normal 22 pairs of autosomes and a pair of sex chromosomes (Fig. 1E). Weeks after injecting into SCID mice, the teratomas produced from the HA-iPSCs contained multiple

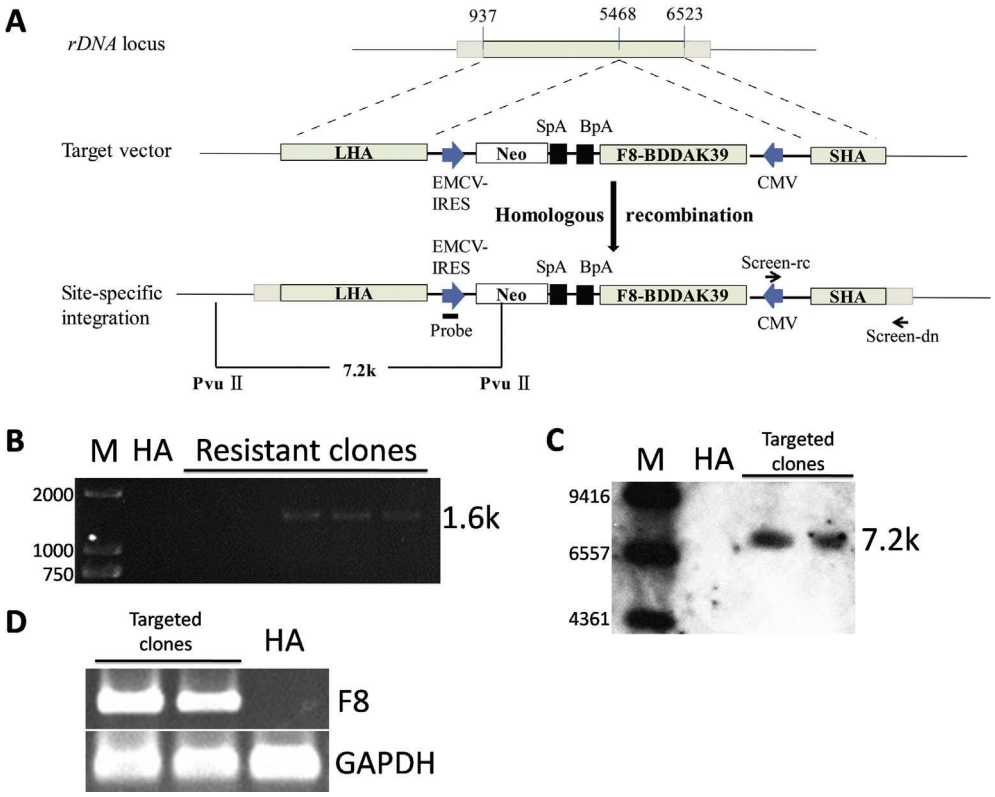

Fig. 2. Characterization of gene targeting at rDNA locus in HA patient-derived iPSCs. (A) Schematic representation of the rDNA locus, targeting vector, and site-specific integration after homologous recombination. The Neo cassette consisted of an IRES element from the encephalomyocarditis virus (EMCV). Neo is promoterless and activated by the promoter of the rRNA gene after homologous recombination. The BDD-FVIII gene is reversed and driven by a CMV promoter. (B) PCR analysis of genomic DNA from resistance clones. The sizes of the PCR products for homologous recombinants were 1.6 kb (C) The PCR-positive clones were confirmed by Southern bloting analysis of the PvuII digested genomic DNA. (D) RT-PCR was used to detect expression of exogenous F8. GAPDH was used as a loading control.

derivatives of the 3 germ layers (Fig. 1F), including squamous epithelium (ectoderm), cartilage (mesoderm), smooth muscle (mesoderm), and gastrointestinal epithelium (endoderm). Thus, it was showed the successful generation of human iPSCs from urine cells of patients with HA avoiding an invasive biopsy.

3.2. Targeting of F8 at the rDNA locus in HA-iPSCs by TALENickases

In this study, the rDNA target plasmid pHrnF8 and TALENickases were applied which were constructed in previous research [11,19]. The pHrnF8 introduced a promoterless neomycin (Neo) resistance cassette and a reversed CMV promoter-driven human B-domain-deleted factor VIII (BDD-FVIII) open reading frame (ORF) into the 45S pre-RNA gene. The two cassettes were flanked by a 5′ long homologous arm (4.5 kb) and a 3′ short homologous arm (1.1 kb). The first cassette contained an encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), which enabled resistant gene expression under the control of endogenous RNA polymerase I (Pol I) promoter upstream after HR (Fig. 2A). In this study, we applied the HA-iPSCs with intron 22 inversion, which most commonly existed in severe HA patient.

Three nucleofections were performed and a total of 20 Neo-resistant clones were picked up. Among these 20 resistant clones, some were not site specifically integrated with HR, because random integration into the downstream of any promoter could enable resistant gene expression as well. To identify the clones with site-specific integration, these clones were screened for HR product by PCR amplification using Screen-rc and Screen-dn primers (Fig. 2B). Screen-rc and Screen-dn bonded to the reversed CMV promoter and the 5.8S RNA coding sequence beyond the homologous sequence respectively, and the PCR product is 1.6 kb in length. Based on the above method, we finally identified 9 HR-positive clones. The HR led to knock-in of the Neo ORF to the rDNA locus causing an addition of PvuII site, resulting in a 7.2 kb fragment after digestion in Southern blotting. All the 9 HR-positive clones could be detected with this fragment by Southern blotting (Fig. 2C), which were called targeted clones in this study. In addition, the rDNA target plasmid pHrnF8 is applied in the first nucleofection without TALENickases, and none of clones with site specific integration was detected. Then, we employed both rDNA target plasmid pHrnF8 and TALENickases in latter two nucleofections. And we succeeded in obtaining 3 and 6 targeted clones at the targeting efficiency of 75% and 43%, respectively (Table S2).

Moreover, we designed specific primers based on the sequence crossing the B-domain of *F8*, to detect the transcription of *F8* with the method of reverse transcription PCR (RT-PCR). The results showed the transcripts crossing the B-domain of *F8* could be detected in targeted iPSCs but not in HA-iPSCs (Fig. 2D), while exogenous *F8* of targeted iPSCs was B-domain-deleted. To exclude the off-target cleavage caused by TALENickases, we tested three most likely potential off-target sites in targeted clones by sequencing, and none occurred (Table S3, Fig. S1).

3.3. Integration of multicopy exogenous gene and expression of exogenous F8

There are hundreds of copies of rDNA in the genome, theoretically, more than one copy of the exogenous gene could be

integrated into the genome. After confirming the integration, the copy number of site-specific integrated transgene was measured in each clone derived from single cell. We designed specific primers for detecting the exon 7 of *F8* (Table S1), and the method of q-PCR could measure the total copy number of *F8* with the above primers. There exits only one copy of *F8* in HA-iPSCs, in this case, the copy number of integrated exogenous *F8* could be calculated. The results turned out that about half of the clones could be detected with one copy of the transgene, whereas the others with two copies (Table S4).

The transcription of endogenous *F8* in HA patients would cease before the sequence of exon 23. In this case, we designed specific primers according to the sequence around the breakpoint of intron 22 inversion (Table S1). By this way, the transcription of endogenous *F8* in HA patients could not be detected. The method of qRT-PCR was applied to detect the expression of *F8*. It was revealed that the integrated exogenous *F8* could express efficiently in the targeted iPSCs (Fig. 3A).

Futhermore, we collected supernatant and lysate to determine the exogenous FVIII protein by ELISA, respectively from HA-iPSCs and targeted iPSCs. It turned out that the expression of FVIII protein increased markedly in the lysate of targeted iPSCs compared to HA-iPSCs (Fig. 3B), however, the expression could not be detected in the supernatant of both HA-iPSCs and targeted iPSCs.

Human iPSCs hold the ability to differentiate into all somatic cells. And the ECs derived from the mesoderm are a major source of FVIII expression [20]. Therefore, we differentiated both HA-iPSCs and targeted iPSCs into ECs respectively, and checked the expression of markers of ECs and the FVIII protein in both ECs by immunocytochemistry. As expected, all the differentiated ECs expressed marker protein for mature ECs, but only ECs derived from the targeted iPSCs expressed the FVIII protein (Fig. 3C).

4. Discussion

A typical genome editing approach for gene therapy is to target the disease locus itself. However, the proportion of alleles successfully edited may not express sufficient levels of protein to alleviate the disease phenotype. Alternatively, integration into a locus with high transcriptional activity ("safe harbor") would address this limitation and provide a versatile platform to express various proteins, substituting the donor for each respective therapeutic transgene. To date, there are three common sites in the human genome have been used for targeted transgene addition: the adeno-associated virus site 1 (AAVS1) [21], the chemokine (CC motif) receptor 5 (CCR5) gene locus [22] and the human ortholog of the mouse ROSA26 locus [23]. All three, however, are in fairly gene-rich regions and are near genes that have been implicated in cancer. Besides, each of these locus permits only one transgene to integrate into the genome at the same time [24]. In contrast, there are hundreds of copies of rDNA per cell serving as potential targets for transgenes. Variations in rDNA copy number are common among healthy individuals and balanced chromosomal translocation involving the rDNA occurs without any apparent phenotypic effect. And the rDNA locus permits two, even three or four transgenes to integrate into the genome at the same time which could regulate the expression of multiple genes. It appears great promise in gene and cell therapy for the monogenic diseases, as well as for other genetic diseases involving multiple genes.

Human iPSCs can be expanded unlimitedly in culture and hold the ability to differentiate into all somatic cells. Patient-derived iPSCs are the perfect model for testing genome editing approach described above. Genome editing has long been a difficult problem in gene therapy. However, the development of DNA cleaving enzymes such as TALENs and CRISPRs has made it possible to achieve far greater genome editing efficiencies. With the help of nucleases, genome editing has been successfully applied in various preclinical models [7,25,26]. Because of the multicopic property of the rDNA locus, the TALENs would fragment the rDNA units by multiple double-stranded DNA cleavage that is very likely to be fatal to the cells. So the TALENickases as the improved version were used in this study which lead to less off-target mutagenesis and cell toxicity compared with TALENs. And we did not detect any off-target cleavage occurred at three most likely potential off-target sites in targeted clones.

In this research, FVIII protein was slightly detected in the lysate

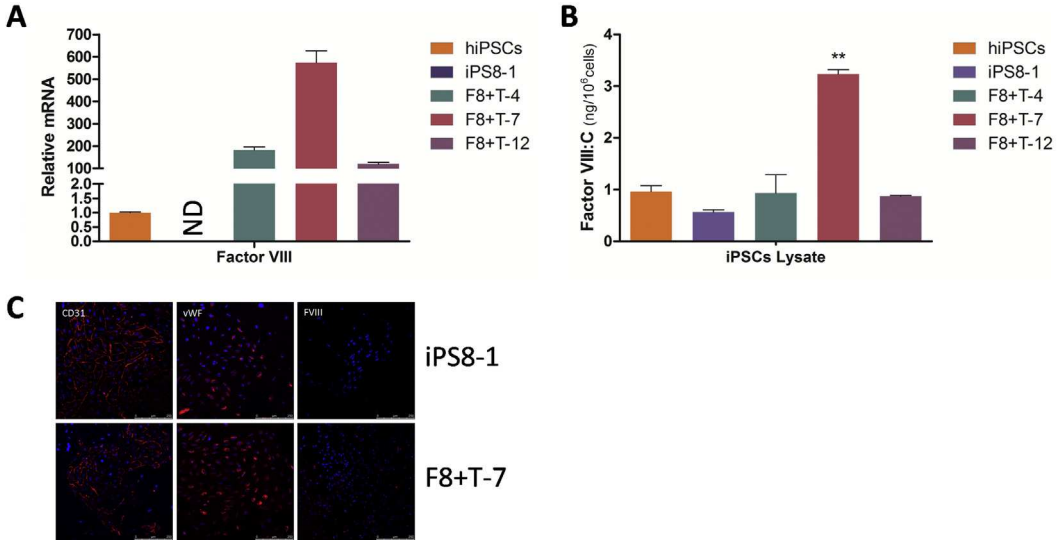

Fig. 3. Expression of the exogenous transgene in targeted iPSCs and ECs. (A) qRT-PCR was used to detect expression of F8 in normal (hiPSCs), patient (iPS8-1) and targeted iPSC lines (F8 + T-4; F8 + T-7; F8 + T-12). ND, not detected. (B) ELISA analysis of lysate of iPSCs from normal, patient and targeted iPSC lines. The different clones expressed FVIII protein at different levels. (**$p < 0.01$). (C) Expression of the FVIII protein in ECs differentiated from HA-iPSCs and targeted iPSCs was tested by immunocytochemistry. DAPI signals (blue) indicate the total cell presence in the image. FVIII, FVIII protein; CD31 and vWF (marker protein for mature ECs). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of HA-iPSCs with intron 22 inversion which was consistent with the fact that individuals with intron 22 inversion synthesize FVIII protein as two polypeptides intracellularly [27]. While in the lysate of targeted iPSCs, the FVIII protein could be detected significantly. However, there was no detectable FVIII protein in the supernatant of both HA-iPSCs and targeted iPSCs. It suggested that the secretion of FVIII protein might be an issue for our strategy. In 2008, Neyman found that FVIII could be stored in the α-granules of platelets [28]. When vascular injury occurred, activated blood platelets would adhere to a wound site and secrete biologically active FVIII without an inhibitor [4]. Besides, the broad-spectrum CMV promoter applied in this study can trigger the expression of exogenous genes in many cell types. Based on the above theories, the platelets differentiated from targeted iPSCs might produce and store exogenous FVIII intracellularly, and they might secrete biologically active FVIII when activated. This might be a good solution to the issue of secretion.

In summary, we used TALENickases to target exogenous *F8* into the rDNA locus of HA-iPSCs, and it is showed that both the targeted iPSCs and ECs differentiated from them expressed the FVIII protein in vitro. To the best of our knowledge, this is the first demonstration of the addition of genes into the rDNA locus using TALENickase in patient-derived iPSCs. More importantly, it was confirmed that more than one copy of the exogenous gene could be integrated into the rDNA locus. The rDNA targeting system needs to be optimized with further researches, which shows great promise in gene and cell therapy for the monogenic disease and for other genetic diseases involving multiple genes.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Targeting of the Human F8 at the Multicopy rDNA Locus in Hemophilia A Patient-Derived iPSCs Using TALENickases”.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81400101, 31571313, 81470299, 81272540 and 81271944), the National Basic Research Program of China (2010CB529903) and China Postdoctoral Science Foundation funded project (2015M580697).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.02.083

References

[1] P.M. Mannucci, M. Franchini, Haematology clinic: haemophilia A, Hematology 19 (2014) 181–182.
[2] M.E. Fomin, P.P. Togarrati, M.O. Muench, Progress and challenges in the development of a cell-based therapy for hemophilia A, J. Thromb. Haemost. 12 (2014) 1954–1965.
[3] J.M. Johnston, G. Denning, C.B. Doering, et al., Generation of an optimized lentiviral vector encoding a high-expression factor VIII transgene for gene therapy of hemophilia A, Gene Ther. 20 (2013) 607–615.
[4] L.M. Du, P. Nurden, A.T. Nurden, et al., Platelet-targeted gene therapy with human factor VIII establishes haemostasis in dogs with haemophilia A, Nat. Commun. 4 (2013) 2773.
[5] M. Moayeri, T.S. Hawley, R.G. Hawley, Correction of murine hemophilia A by hematopoietic stem cell gene therapy, Mol. Ther. 12 (2005) 1034–1042.
[6] G.L. Rogers, R.W. Herzog, Gene therapy for hemophilia, Front. Biosci. (Landmark Ed) 20 (2015) 556–603.
[7] C.Y. Park, D.H. Kim, J.S. Son, et al., Functional correction of large factor VIII Gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9, Cell Stem Cell 17 (2015) 213–220.
[8] R. Sharma, X.M. Anguela, Y. Doyon, et al., In vivo genome editing of the albumin locus as a platform for protein replacement therapy, Blood 126 (2015) 1777–1784.
[9] C.Y. Park, J. Kim, J. Kweon, et al., Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 9253–9258.
[10] X. Liu, Y. Wu, Z. Li, et al., Targeting of the human coagulation factor IX gene at rDNA locus of human embryonic stem cells, PLoS One 7 (2012) e37071.
[11] X. Liu, M. Liu, Z. Xue, et al., Non-viral ex vivo transduction of human hepatocyte cells to express factor VIII using a human ribosomal DNA-targeting vector, J. Thromb. Haemost. 5 (2007) 347–351.
[12] J. Yang, X. Liu, J. Yu, et al., A non-viral vector for potential DMD gene therapy study by targeting a minidystrophin-GFP fusion gene into the hrDNA locus, Acta Biochim. Biophys. Sin. (Shanghai) 41 (2009) 1053–1060.
[13] Y. Hu, X. Liu, P. Long, et al., Nonviral gene targeting at rDNA locus of human mesenchymal stem cells, Biomed. Res. Int. 2013 (2013) 135189.
[14] D.M. Stults, M.W. Killen, H.H. Pierce, et al., Genomic architecture and inheritance of human ribosomal RNA gene clusters, Genome Res. 18 (2008) 13–18.
[15] P. Steinbach, M. Djalali, I. Hansmann, et al., The genetic significance of accessory bisatellited marker chromosomes, Hum. Genet. 65 (1983) 155–164.
[16] A.A. Nemudryi, K.R. Valetdinova, S.P. Medvedev, et al., TALEN and CRISPR/Cas genome editing systems: tools of discovery, Acta Naturae 6 (2014) 19–40.
[17] D. Hockemeyer, H. Wang, S. Kiani, et al., Genetic engineering of human pluripotent cells using TALE nucleases, Nat. Biotechnol. 29 (2011) 731–734.
[18] A. McConnell Smith, R. Takeuchi, S. Pellenz, et al., Generation of a nicking enzyme that stimulates site-specific gene conversion from the I-AniI LAGLIDADG homing endonuclease, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 5099–5104.
[19] Y. Wu, T. Gao, X. Wang, et al., TALE nickase mediates high efficient targeted transgene integration at the human multi-copy ribosomal DNA locus, Biochem. Biophys. Res. Commun. 446 (2014) 261–266.
[20] T. Shahani, R. Lavend'homme, A. Luttun, et al., Activation of human endothelial cells from specific vascular beds induces the release of a FVIII storage pool, Blood 115 (2010) 4902–4909.
[21] C.J. Ramachandra, M. Shahbazi, T.W. Kwang, et al., Efficient recombinase-mediated cassette exchange at the AAVS1 locus in human embryonic stem cells using baculoviral vectors, Nucleic Acids Res. 39 (2011) e107.
[22] J.B. Rottman, K.P. Ganley, K. Williams, et al., Cellular localization of the chemokine receptor CCR5. Correlation to cellular targets of HIV-1 infection, Am. J. Pathol. 151 (1997) 1341–1351.
[23] S. Irion, H. Luche, P. Gadue, et al., Identification and targeting of the ROSA26 locus in human embryonic stem cells, Nat. Biotechnol. 25 (2007) 1477–1482.
[24] M. Sadelain, E.P. Papapetrou, F.D. Bushman, Safe harbours for the integration of new DNA in the human genome, Nat. Rev. Cancer 12 (2012) 51–58.
[25] B. Song, Y. Fan, W. He, et al., Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system, Stem Cells Dev. 24 (2015) 1053–1065.
[26] H.L. Li, N. Fujimoto, N. Sasakawa, et al., Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9, Stem Cell Rep. 4 (2015) 143–154.
[27] G.S. Pandey, C. Yanover, L.M. Miller-Jenkins, et al., Endogenous factor VIII synthesis from the intron 22-inverted F8 locus may modulate the immunogenicity of replacement therapy for hemophilia A, Nat. Med. 19 (2013) 1318–1324.
[28] M. Neyman, J. Gewirtz, M. Poncz, Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots, Blood 112 (2008) 1101–1108.