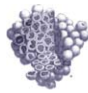
STEM CELLS TRANSLATIONAL MEDICINE®

PLURIPOTENT STEM CELLS

Enhancement of β-Globin Gene Expression in Thalassemic IVS2-654 Induced Pluripotent Stem Cell-Derived Erythroid Cells by Modified U7 snRNA

PHETCHARAT PHANTHONG,[a,b] SUPARERK BORWORNPINYO,[c,d] NARISORN KITIYANANT,[b] NATEE JEARAWIRIYAPAISARN,[e] LALANA NUNTAKARN,[f] JIRAWAT SAETAN,[g] TIWAPORN NUALKAEW,[e] KHANIT SA-NGIAMSUNTORN,[h] USANARAT ANURATHAPAN,[i] ANDRAS DINNYES,[j,k] YINDEE KITIYANANT,[a,b,l] SURADEJ HONGENG[i]

Departments of [a]Anatomy and [c]Biotechnology, and [d]Excellent Center for Drug Discovery, Faculty of Science, Mahidol University, Bangkok, Thailand; [b]Stem Cell Research Group, [e]Thalassemia Research Center, [l]Reproductive Biology Research Group, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand; Departments of [f]Obstetrics and Gynecology and [i]Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand; [g]Anatomy Department, Faculty of Science, Prince of Songkla University, Songkhla, Thailand; [h]Department of Biochemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand; [j]Biotalentum Ltd., Godollo, Hungary; [k]Molecular Animal Biotechnology Laboratory, Szent Istvan University, Godollo, Hungary

Correspondence: Suparerk Borwornpinyo, Ph.D., Department of Biotechnology and Excellent Center for Drug Discovery, Faculty of Science, Mahidol University, Bangkok, Thailand. Telephone: +669 5859 3777; Fax: +66 2354 7160; e-mail: suparerk.bor@mahidol.ac.th; or Suradej Hongeng, M.D., Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Telephone: +668 1616 7812; Fax: +66 2201 1495; e-mail: suradej.hon@mahidol.ac.th

Received March 16, 2016; accepted for publication December 21, 2016; published Online First on 18 February 2017.

©AlphaMed Press 1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/sctm.16-0121

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Key Words. Induced pluripotent stem cell • β-Thalassemia • U7 snRNA • Induced pluripotent stem cell-derived erythroblast

ABSTRACT

The therapeutic use of patient-specific induced pluripotent stem cells (iPSCs) is emerging as a potential treatment of β-thalassemia. Ideally, patient-specific iPSCs would be genetically corrected by various approaches to treat β-thalassemia including lentiviral gene transfer, lentivirus-delivered shRNA, and gene editing. These corrected iPSCs would be subsequently differentiated into hematopoietic stem cells and transplanted back into the same patient. In this article, we present a proof of principle study for disease modeling and screening using iPSCs to test the potential use of the modified U7 small nuclear (sn) RNA to correct a splice defect in IVS2-654 β-thalassemia. In this case, the aberration results from a mutation in the human β-globin intron 2 causing an aberrant splicing of β-globin pre-mRNA and preventing synthesis of functional β-globin protein. The iPSCs (derived from mesenchymal stromal cells from a patient with IVS2-654 β-thalassemia/hemoglobin (Hb) E) were transduced with a lentivirus carrying a modified U7 snRNA targeting an IVS2-654 β-globin pre-mRNA in order to restore the correct splicing. Erythroblasts differentiated from the transduced iPSCs expressed high level of correctly spliced β-globin mRNA suggesting that the modified U7 snRNA was expressed and mediated splicing correction of IVS2-654 β-globin pre-mRNA in these cells. Moreover, a less active apoptosis cascade process was observed in the corrected cells at transcription level. This study demonstrated the potential use of a genetically modified U7 snRNA with patient-specific iPSCs for the partial restoration of the aberrant splicing process of β-thalassemia. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1059–1069*

SIGNIFICANCE STATEMENT

This is the first study to demonstrate a success of combining antisense RNA and induced pluripotent stem cells (iPSC) technologies to correct β-thalassemia disease. Our results provide a proof of principle of using iPSCs as a modeling and a screening tool for an alternative approach in repairing RNA splicing defect of β-thalassemia disease mutations by the modified antisense RNA.

INTRODUCTION

β-thalassemia is an autosomal recessive inherited blood disorder caused by mutations in the beta hemoglobin gene (*HBB*) leading to the decrease or absence in production of functional β-globin [1, 2] and is commonly found in Southeast Asia [3]. The two most common β-thalassemic mutations affecting pre-mRNA splicing process are HbE (G to A at codon 26 of exon 1) and IVS2-654 (C to T in position 654 of the IVS2 intron) which are prevalent in Thailand and China [1, 4, 5] and compound heterozygote β-thalassemia/HbE affected over million people worldwide [6]. The HbE mutation results in activation of a cryptic 5′ splice site causing the aberrant splicing of β^E-globin pre-mRNA, leading to a deletion of the 3′ end in exon 1 (nucleotides 77–92) and producing a new premature stop codon [7]. However, correctly spliced β^E globin mRNA is still produced from HbE mutation and the homozygous HbE mutation causes only mild clinical symptoms [8]. In IVS2-654 mutation, it generates an aberrant 5′ donor splice site at position 652 and also activates a cryptic 3′ acceptor splice site at position 579. This leads to inclusion of an intronic sequence (nucleotides

 © 2017 The Authors
STEM CELLS TRANSLATIONAL MEDICINE published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

580–652) in the aberrantly spliced β-globin mRNA, which contains a premature termination codon preventing production of functional β-globin protein [9, 10]. The clinical phenotype of homozygous IVS2-654 mutation is classified as transfusion-dependent thalassemia major [4].

The aberrantly spliced β-globin mRNA has been successfully repaired by synthetic splice-switching oligonucleotides (SSOs) designed to block aberrant and cryptic splice sites in order to restore the correct splicing pathway and correctly spliced β-globin mRNA. Therapeutic potential of these SSOs has been demonstrated in several systems including Hela cells expressing human IVS2-654 or β^E-globin gene [11, 12], erythroid progenitor cells from IVS2-654 β-thalassemic/HbE patients and IVS2-654 β-thalassemic mice [13–16]. A vector such as U7 small nuclear (sn) RNA has been used to carry splice-switching sequences to promote long-term correction of aberrant splicing. A natural anti-histone sequence of the U7 snRNA has been replaced with the splicing-switching sequence generating the modified U7 snRNA that could restore the correct splicing in a manner similar to synthetic oligonucleotides. [17]. The modified U7 snRNA were lentivirally delivered into human primary erythroid precursor cells isolated from IVS2-745/IVS2-1 β-thalassemic patients to repair the aberrant splicing of the IVS2-745 β-thalassemic pre-mRNA. This resulted in a production of correctly spliced β-globin mRNA and HbA [18].

Recently, patient specific thalassemic induced pluripotent stem cells (iPSCs) with various β-globin mutations including the compound heterozygous codon 41/42 and codon 17 β-thalassemia [19, 20], homozygous codon 41/42 β-thalassemia [21], compound heterozygous nucleotide-28 (A–G)/codon 41/42 β-thalassemia [22], homozygous codon 39 (C–T), compound heterozygous IVS1-110 (G–A), IVS1-1 (G–A), IVS1-15 (G–C)/ codon 39 [23], homozygous IVS2-654 [24] have been generated as an excellent tool for screening the various designs of approaches for putative curing of β-thalassemia. Although various β-thalassemic patient specific iPSCs have been established, the iPSCs derived from the compound heterozygous IVS2-654 β-thalassemia/HbE patient have not been reported. In addition, β-thalassemia genotype diversities may or may not be accounted for clinical severity because the patients with the same mutation can give rise to different degrees of symptoms and other modifying factors such as nonsense-mediated mRNA decay (NMD) or the excess of α-globin chain are also reflected in the clinical spectrum of β-thalassemia/HbE [25]. Therefore, the patient-specific genotype in iPSCs is very valuable in order to elucidate the β-thalassemia disease phenotype-genotype correlations, disease onset development, the interaction of HbE and β-thalassemia, as well as to use such iPSCs as screening tools for the therapeutic potential of the new strategies.

In addition, modified U7 snRNA has the potential in the restoration of RNA mis-splicing β-thalassemia and can be combined with iPSCs technology. Therefore, in this report we present our results aimed to generate iPSCs derived from a compound heterozygous IVS2-654 β-thalassemia/HbE patient, differentiate them into erythroblasts and test the modified U7 snRNA lentiviral vector capability to correct aberrant splicing of IVS2-654 β-thalassemic pre-mRNAs.

Materials and Methods

Ethics Statement

Human and animal ethics protocols were reviewed and approved by Mahidol University Institutional Review Board (Protocol ID 80-54-31) and Institutional Animal Care and Use Committee, Institute of Molecular Biosciences, Mahidol University (MB-ACUC2012/005), respectively. Written informed consents were obtained from all participants.

Derivation, Cultivation, and Characterizations of iPSCs

Human mesenchymal stromal cells (MSCs) were obtained and isolated from bone marrow of healthy volunteer or IVS2-654/HbE thalassemic patient and maintained as described previously [26]. For iPSC derivation, human MSCs (1×10^5 cells) were transduced with a polycistronic lentiviral vector encoding for human OCT4, KLF4, SOX2, MYC, and a fluorescent reporter, dTomato (pRRL.PPT.SF.hOKSM.idTomato.preFRT) [27] at multiplicity of infection of 0.5 in the presence of 4 μg/ml Polybrene (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Lentiviral supernatant were produced by cotransfection of pRRL.PPT.SF.hOKSM.idTomato.preFRT and third generation packaging plasmid system (Addgene plasmid # 12251 (pMDLg/pRRE), #12251 (pRSV-REV), #12259 (pMD2.G), Cambridge, MA, http://www.addgene.org) into human embryonic kidney (HEK) 293T cells (Clontech, Mountain View, CA, http://www.clontech.com) using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany, http://www.lifescience.roche.com) and being concentrated by Lenti-X concentrator solution (Clontech) according to the manufacturer's instructions. Concentrated lentivirus was titrated on HEK 293T cells and determined for dTomato positive cells using FACSCanto flow cytometer (BD biosciences, San Jose, CA, http://www.bdbiosciences.com). Sodium butyrate (Sigma-Aldrich) was added to increase reprogramming efficiency at the concentration of 0.5 mM during day 2–11 post-transduction. The transduced MSCs were passaged at day 4 post-transduction onto a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (MEFs; Reprocell, Tokyo, Japan, http://www.reprocell.com) and cultured in conventional human embryonic stem (ES) medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone, Logan, UT, http://www.gelifesciences.com/hyclone), 20% Knockout Serum Replacement, 0.1 mM 2-mercaptoethanol, 1% MEM non-essential amino acid, 1% Glutamax, 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, http://www.thermofisher.com) and 10 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, http://www.peprotech.com) in a humidified atmosphere containing 5% CO_2 at 37°C. Human iPSCs were first maintained in human ES medium with feeder dependent and further adapted to home-made E8 medium or mTeSR1 [28] with Matrigel (BD biosciences)-coated surface. Established human iPSC lines were characterized by staining of pluripotency markers as follows; alkaline phosphatase (AP) (AP detection kit, Merck Millipore, Darmstadt, Germany, http://www.merckmillipore.com), NANOG, SOX2, SSEA4, OCT4, TRA-1-60, and TRA-1-81 antibodies (Santa Cruz, Dallas, TX, http://www.scbt.com). Gene expression profiling of pluripotency markers was performed by reverse transcription-polymerase chain reaction (RT-PCR). Bisulfite sequencing of OCT4 promoter was analyzed by EZ-DNA methylation-lighting kit (Zymo Research, Irvine, CA, http://www.zymoresearch.com). To test the ability of iPSCs to differentiate into three germ layers, the cells were in vitro spontaneously differentiated via embryoid body-stage into hepatocytes (endoderm) and smooth muscle cells (mesoderm), and induced to differentiate into neurons (ectoderm). Chromosome stability was examined by karyotyping with standard G-band staining performed at Human Genetics Unit, Department of Pathology, Ramathibodi Hospital, Thailand. Teratoma assay was performed by injection the mixture of Matrigel and human iPSCs from a 10-cm

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

dish into the dorsal flank of nude mice (BALB/cMlac-nu; National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand, http://www.nlac.mahidol.ac.th). After 8–12 weeks, mice were sacrificed and the teratomas were dissected, and then standard hematoxylin and eosin staining were performed.

Production of Lentiviral Vector Harboring the Modified U7 snRNA

The modified U7 snRNA cassette [18] was cloned into a pLL3.7 lentiviral vector [29] at XbaI and XhoI restriction sites to replace the U6 promoter in the original plasmid. The resulting plasmid was used to produce lentivirus as described in the previous section.

Lentiviral Delivery of the Modified U7 snRNA Into Patient-Specific iPSCs

iPSCs were adapted to culture on Matrigel coated dish in mTeSR1 medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) for at least five passages. The patient-specific iPSCs (passage 9) were transduced with lentivirus carrying green fluorescent protein (GFP) reporter and the modified U7 snRNA designed to specifically targeting the IVS2-654 β-globin pre-mRNA. The transduction was performed in one well of a 12-well plate by adding 10 μl of concentrated lentivirus (1.5×10^8 TU/ml) on 50% confluency growing iPSCs in mTeSR1 medium for overnight at 37°C, 5% CO_2. The culture medium was daily changed and the cells were daily observed for GFP expression under an epifluorescent microscope (Olympus IX71; Olympus, Tokyo, Japan, http://www.olympus-lifescience.com). The GFP positive colonies were marked and picked onto a new dish. After a week, the positive colonies were detached by exposure to 0.5 mM ethylenediaminetetraacetic acid (EDTA) for 2–5 minutes and seeded onto new Matrigel-coated dishes in mTeSR1 medium. During expansion, the positive colonies were manually selected. The selected colonies were finally sorted by a BD FACSAria II cell sorter (BD biosciences). The cells were treated with Accutase (Life Technologies) for 10 minutes and resuspended in magnetic cell sorter (MACS) buffer (Dulbecco's phosphate-buffered saline (DPBS) containing 0.5% w/v BSA, 2 mM EDTA) prior to be applied in the cell sorter. The sorted cells were treated with 0.4 μM PD0325901, 2 μM SB431542, 1 μM CHIR99021 (Merck Millipore), and 10 μM Y27632 (Tocris, Bristol, U. K., http://www.tocris.com) in mTeSR1 for an overnight. The MU002.A-hiPS.snRNA were transferred to E8 medium for expansion and erythroid differentiation experiment.

Erythroblast Differentiation of Human iPSCs

Human iPSCs (passages 25–35) were differentiated into erythroblasts according to a previous study [30], with a minor modification. Briefly, iPSCs were maintained on feeder free condition in E8 medium. At 70% confluency, the cells were detached by incubating with 1 mg/ml Collagenase IV (Life Technologies) treatment for 15 minutes, scraped by cell scraper (Corning, Corning, NY, http://www.corning.com), washed with DMEM/F12 basal medium (Hyclone) for 2 times, and resuspended in erythroid differentiation medium (alpha-minimum essential medium (MEM) without nucleoside [Hyclone], 10% defined fetal bovine serum [Hyclone], 100 μM 1-thioglycerol [Sigma-Aldrich], 50 μg/ml ascorbic acid [Sigma-Aldrich], 2 mM Glutamax, 1% penicillin/streptomycin). The cell suspension was seeded onto 3–5 days post-confluent OP9 cells (ATCC, Manassas, VA, http://www.atcc.org), at $1–1.5 \times 10^6$ cells per 10-cm dish in 20 ml erythroid differentiation medium. On the next day, total medium was replaced and at day 4 and 6, half of medium was changed. After 8 days of coculture on OP9 cells, the cells were harvested for colony forming cells (CFC) assay.

CFC Assay

The differentiated cells from a coculture system were harvested by manual picking and treating with 2 mg/ml Collagenase IV incubation for 30 minutes, and then with 0.25% Trypsin-EDTA (Life Technologies) for 20 minutes. The single cells were counted and seeded at $1–2 \times 10^5$ cell/ml into Methocult H4434 medium (StemCell Technologies) specified for CFC assay according to the manufacturer's protocol. The cells were maintained at 37°C, 5% CO_2 and the pattern of colony forming units (CFUs) was examined at day 12–13. At day 16, burst-forming unit erythroid colonies were identified and picked for CD235a (Glycophorin A [GPA]) positive cell selection by the MACS (Miltenyi Biotech, San Diego, CA, http://www.miltenyibiotec.com) according to the manufacturer protocols. The purified CD235a positive cells were used for determining hemoglobin chain expression pattern. The cell morphology was evaluated by being spun onto a glass slide using cytocentrifugation (Shandon Cytospin 4; Thermo Scientific, Waltham, MA, http://www.thermoscientific.com) at 1,000 rpm for 5 minutes and stained with Wright-Giemsa staining solution (Sigma-Aldrich).

RNA Isolation

For gene expression analysis of pluripotent markers, total RNA was extracted from the cells using RNAeasy Plus mini kit (Qiagen, Hilden, Germany, http://www.qiagen.com) following supplier's instructions with DNase I on column treatment. For hemoglobin expression analysis, total RNA was isolated from the purified CD235a positive cells using ArrayPure Nano-scale RNA Purification Kit (Epicenter Biotechnologies, Madison, WI, http://www.epibio.com) following the manufacturer's instructions. Briefly, 10,000 cells were lysed, and proteins were removed by precipitation solution. The supernatant containing the total RNA was precipitated by isopropanol, pelleted, and treated with DNase I for genomic DNA removal. The treated RNA pellet was precipitated again and washed with 70% (v/v) ethanol before being diluted in 6.5 μl of nuclease-free water. Concentration of the purified total RNA was determined by Nanodrop 2000 (Thermo Scientific).

Whole Transcriptome Amplification

Total RNA extracted from CD235a positive cells were amplified using CellAmp Whole Transcriptome Amplification Kit Ver.2 (Takara, Shiga, Japan, http://www.clontech.com) according to a manufacturer's instructions. Briefly, total RNA (∼15 ng) was converted into cDNA by a reverse transcriptase enzyme with oligo-dT primer. The synthesized cDNA was added with a dA tail at 3′ site by terminal deoxynucleotidyl transferase, resulting in cDNA containing 3′dA tail which was used as a template for cDNA amplification by PCR with condition suggested in manual using Ex Taq Hot Start DNA polymerase (Takara). Amplified cDNA was diluted at 1:40 and 2 μl of the diluted cDNA was used as template for detection of corrected β-globin mRNA and hemoglobin chain expression by RT-PCR and quantitative RT-PCR, respectively.

Human β-Globin Pre-mRNA Splicing Analysis

Amplified cDNA was used as template for detection of the aberrantly and correctly spliced β-globin mRNA. Conventional RT-PCR was carried out using β^A allele-specific primers (654S-F and 654S-R) to specifically amplify the β^A-globin mRNA but not the β^E-globin mRNA published previously [13] with KAPA2G Robust HotStart

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

ReadyMix PCR kit (Kapa Biosystems, Wilmington, MA, http://www.kapabiosystems.com/) following the manufacturer's manual on a thermal cycler (Takara). Temperature profile was used as follows: 1 cycle at 95°C for 3 minutes; 40 cycles at 95°C for 20 seconds, 65°C for 20 seconds, 72°C for 30 seconds; 1 cycle at 72°C for 3 minutes.

Gene Expression Analysis by Quantitative RT-PCR

The hemoglobin gene expression was compared among cDNAs from the purified CD235a positive cells derived from MU002.A-hiPS, MU002.A-hiPS.snRNA, and MU001.A-hiPS. The quantitative RT-PCR was performed in triplicate of 20-μl reactions using Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) as follows; 1 cycle at 50°C for 2 minutes; 1 cycle at 95°C for 10 minutes; 40 cycles at 95°C for 20 seconds and 55–62°C (depended on primers used) for 45 seconds (data acquisition). For correction analysis of correctly spliced β-globin mRNA, the 654C-F and 654C-R primers combined with the Ec blocker for blocking a detection of normal splicing derived from hemoglobin E mutation were used at final concentration of 900 μM in the reaction. Pluripotency marker expressions were analyzed by qPCR with StepOnePlus™ system (Thermo Scientific) using TaqMan probe assays (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) according to the manufacturer's protocols. The relative expression levels were calculated by ΔΔCt method and normalized with *GAPDH*. All primers used in this study were shown in Supporting Information Table 1. The 654S, Bi-OCT4, AIFM and GADH primers were reported elsewhere [14, 31–33].

Statistical Analysis

Value shown represented the mean ± SEM ($n = 3$–6). Statistical significance between groups was calculated with the unpaired Student's *t* test. Difference was considered significantly at $p < .05$.

Results

Generation and Characterization of iPSCs From IVS2–654/HbE β-Thalassemia Patient

To generate iPSCs, bone marrow derived MSCs from a healthy participant and a heterozygous IVS2-654 β-thalassemia/HbE patient were isolated and cultured. In this study, we focused on the IVS2-654 mutation because a patient with this mutation produces very small amount of correctly spliced β-globin mRNAs, and has symptoms like a severe form of β-thalassemia (β^0). The iPSCs were generated by transduction of a polycistronic lentivirus harboring cDNAs of OCT4, KLF4, SOX2, MYC, and dTomato into MSCs and the transduced MSC cells were seeded onto MEFs in conventional human ESC medium for 4 days after transduction (Supporting Information Fig. 1A). Sodium butyrate (0.5 mM) was included in the medium during day 2–11 post-transduction for increasing reprograming efficiency. The putative reprogrammed iPSC colonies were picked and identified by AP staining before expansion and adaptation onto feeder free condition. The potential fully reprogrammed iPSC colonies were selected based on the silencing of the exogenous gene, *dTomato* (Supporting Information Fig. 1B). This protocol resulted in many putative iPSC colonies (more than 30 colonies, data not shown) both in case of healthy control and patient cells. The IVS2-654 and HbE mutations on *HBB* gene were persisted in the established iPSCs as detected by genomic DNA sequencing (Fig. 1A). Expression patterns of endogenous pluripotency genes (*OCT4, SOX2, NANOG, TDGF1, DNMT3B, GABRB3*, and *GDF3*) in our iPSC colonies detected by quantitative reverse transcription (qRT)-PCR were similar as previously published for HEL11.4 human iPSCs [34] (Fig. 1B). Higher methylation degree on *OCT4* promoter region was observed in parental MSCs whereas lower methylation degree was observed in the iPSCs (Fig. 1C). iPSCs also expressed pluripotency protein markers, OCT4, SOX2, NANOG, TRA-160, TRA-1-81, and SSEA4 as shown by immunocytochemistry (Fig. 1D). Importantly, the iPSCs were able to differentiate into three germ layers in vitro (Fig. 1E) and formed typical teratomas upon injection into nude mice (Fig. 1F). Chromosomal integrity of the established iPSCs exhibited normal karyotype (Fig. 1G). We named the fully reprogrammed iPSCs from healthy participant and heterozygous IVS2-654 β-thalassemia/HbE patient as MU001.A-hiPS and MU002.A-hiPS, respectively.

Lentiviral-Mediated Delivery of the Modified U7 snRNA to MU002.A-hiPS

To study the ability of the modified U7 snRNA in restoration of correct splicing of IVS2-654 β-thalassemic pre-mRNA, MU002.A-hiPS were lentivirally transduced with the modified U7 snRNA designed to increase the expression of correctly spliced β-globin mRNA by specifically targeting the IVS2-654 β-thalassemic pre-mRNA and blocking the aberrant splicing pathway. Transgenic, GFP positive cells were observed within some colonies at day 5 post-transduction (Fig. 2A). Following expansion by a manual picking of GFP positive colonies, the positive cells were enriched. In order to purify and obtain a homogeneous cell population, the GFP-positive MU002.A-hiPS were propagated, dissociated into single cells, and sorted for GFP positive cells. We obtained and expanded homogenous transgenic cell colonies after sorting (Fig. 2B), henceforth called MU002.A-hiPS.snRNA. Sustainable expression of GFP MU002.A-hiPS.snRNA was observed after propagation, suggesting that the modified U7 snRNA cassette was stably integrated into a genome of the iPSC clones (Supporting Information Fig. 3A).

Erythroblast Differentiation of MU002.A-hiPS.snRNA

For modeling the β-thalassemia with IVS-2 654 mutation, MU001.A-hiPS, MU002.A-hiPS, and MU002.A-hiPS.snRNA were differentiated into erythroblasts by coculturing with OP9 cells. The iPSCs were detached and seeded onto over confluence OP9 cells for 8 days (Fig. 3A). The morphology of iPSCs was changed from flat human ES-like colony to differentiated colony with elevated central portion composed of compactly packed rounded cells, which is a typical characteristic of mesodermal colonies (Fig. 3B). During coculture, the GFP was not expressed in MU001.A-hiPS and MU002.A-hiPS while MU002.A-hiPS.snRNA exhibited sustained GFP expression (Fig. 3B). Following coculture, differentiated colonies were picked, dissociated into single cells, and then seeded into semisolid medium for 12–16 days. After CFC assay, several erythroid colonies were observed and GFP expression was not observed in MU002.A-hiPS-derived erythroblasts whereas it was still present in MU002.A-hiPS.snRNA-derived erythroblasts (Fig. 3C). The differentiated erythroblasts derived from MU001.A-hiPS, MU002.A-hiPS, or MU002.A-hiPS.snRNA showed the typical morphology of orthochromatic erythroblasts that are ovoid cells having a small eccentric dense nucleus and cytoplasm stained pink-orange or faint bluish-purple color, and low nucleus to cytoplasm ratio (Fig. 3C). This results indicated that all three established iPSC lines were able to differentiate into hemoglobinized erythroblasts, even though some variations were observed among cell lines. Besides erythroid differentiation, other blood lineage

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

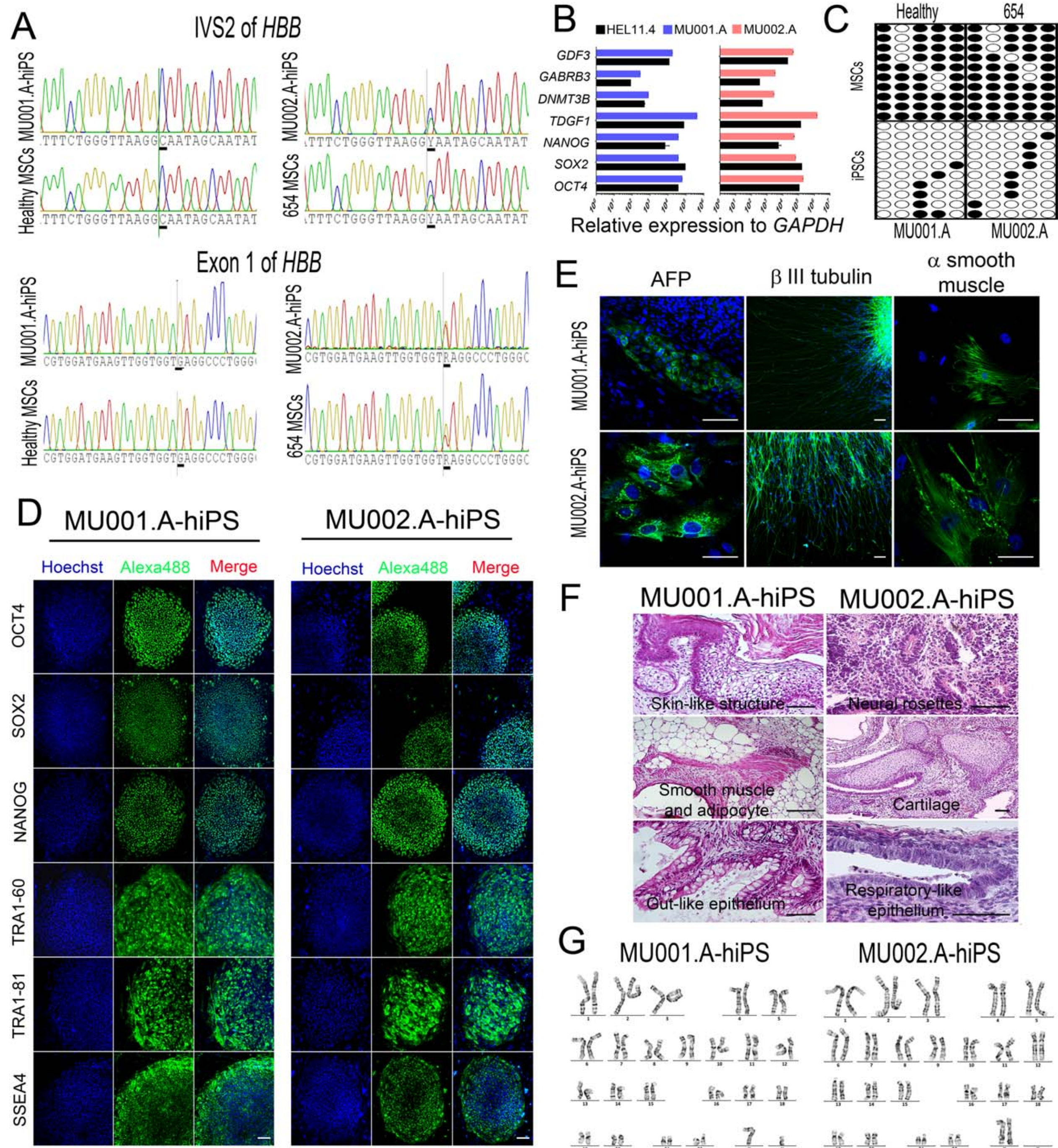

Figure 1. Generation and characterization of human iPSCs. (A): Sequencing results of the second intron (IVS2) at nucleotide position 654 (underline; upper panel) and exon1 at codon 26 (underline; upper panel) of *HBB* gene in healthy MSCs, MU001.A-hiPS, and patient-specific MSCs (C to T at IVS2-654 and G to A at codon 26), MU002.A-hiPS (C to T at IVS2-654 and G to A at codon 26). Y = C or T and R = G or A. (B): qRT-PCR analysis of pluripotent marker genes of the newly established iPSCs in comparison with the previously described HEL11.4 iPSC line [34]. Relative expression level was normalized to *GAPDH* and shown as the mean ± SD. (C): Methylation status analysis of *OCT4* promoter region by bisulfite genomic sequencing. White and black circles represented unmethylated and methylated cytosine guanine dinucleotides (CpGs) of each position (column) on amplified *OCT4* promoter region. Each row indicates bacterial clone used for sequencing. (D): Immunofluorescent staining for OCT4, SOX2, NANOG, TRA-160, TRA-1-81, and SSEA4 (green). Nuclei were localized by Hoechst 33342 (blue). Scale bars, 100 μM. (E): In vitro differentiation of iPSCs showed immunoreactivities (green) of AFP, βIII-tubulin and α-smooth muscle actin. Nuclei were localized by Hoechst 33342 (blue). Scale bars, 50 μM. (F): Hematoxylin/eosin staining of teratoma derived from iPSCs. Scale bars, 100 μM. (G): Normal karyotype of MU001.A-hiPS (46, XY) and MU002.A-hiPS (46, XX). Abbreviations: AFP, α-fetoprotein; HBB, beta hemoglobin; iPSCs, induced pluripotent stem cells; MSC, mesenchymal stromal cells.

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

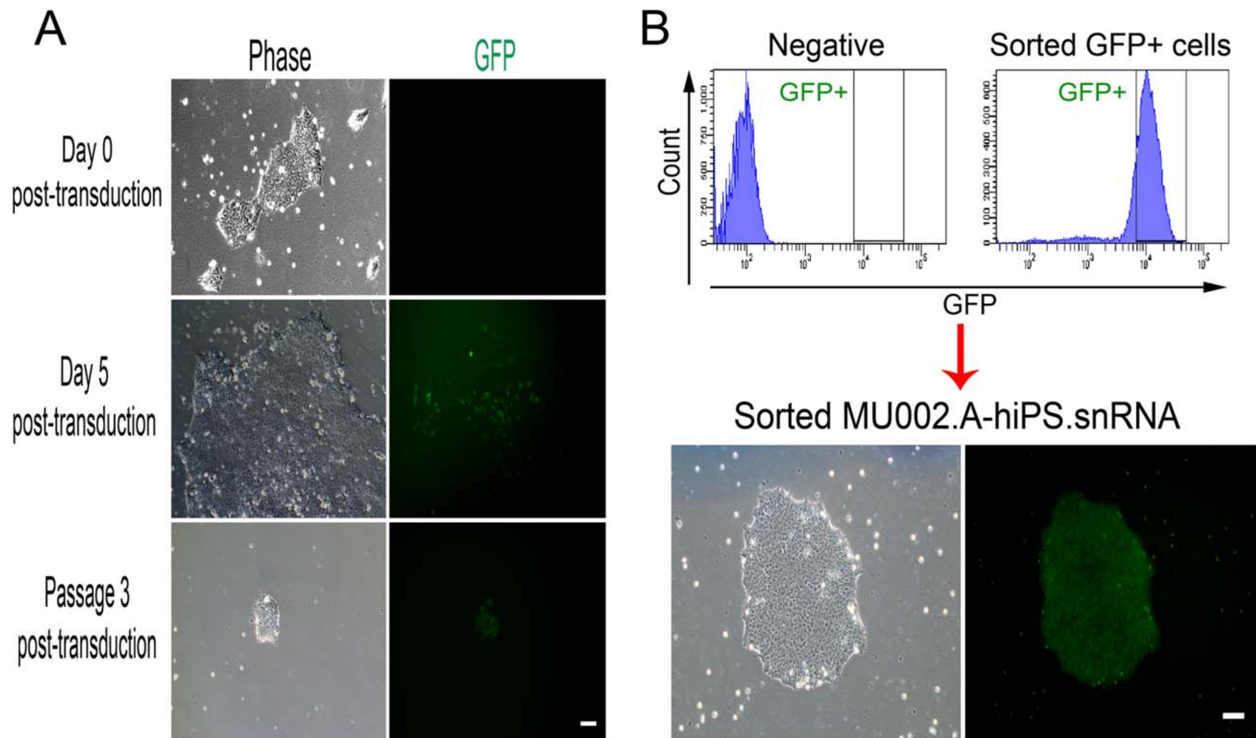

Figure 2. Transduction of MU002.A-hiPS by a lentiviral vector harboring the modified U7 snRNA and GFP reporter. **(A)**: GFP reporter expression in MU002.A-hiPS.snRNA. MU002.A-hiPS.snRNA were manually picked and observed for GFP expression at 5 day post-transduction and at the passage 3 post-transduction. Scale bars, 100 μm. **(B)**: Cell sorting for MU002.A-hiPS.snRNA expressing GFP. MU002.A-hiPS were used as a negative control. The sorted MU002.A-hiPS.snRNA were maintained under feeder free condition. Scale bars, 100 μm. Abbreviations: GFP, green fluorescent protein; snRNA, small nuclear RNA.

differentiations were also observed including CFU-granulocyte-macrophage, CFU-granulocyte, CFU-macrophage, CFU-mixed population of granulocytes, erythroid cells, macrophages, and megakaryocytes (Supporting Information Fig. 2). Furthermore, expression of erythroid cell-specific genes was detected in iPSC-derived erythroblasts. First, erythroid transcription regulator, *GATA1*, was highly expressed with no significant difference among the three lines. Second, the subtle expression of *KLF1* was detected in MU002.A-hiPS-derived erythroblasts and tended to be higher in MU001.A-hiPS- and MU002.A-hiPS.snRNA-derived erythroblasts. Finally, there were no significant difference ($p > .05$) of *HBA*, *HBG,* and *HBE* expression levels among the three lines (Fig. 3D) implying that the modified U7 snRNA did not disturbed the other globin chain expressions.

Restoration of Correctly Spliced β-Globin mRNA by the Modified U7 snRNA

To detect the level of correctly and aberrantly spliced β-globin mRNA in iPSC-derived erythroblasts, total RNA extracted from isolated GPA-positive cells was analyzed by conventional RT-PCR (Fig. 4A). The result showed that the aberrantly spliced β-globin mRNA was highly expressed in MU002.A-hiPS-derived erythroblasts and this level was significantly reduced in MU002.A-hiPS.snRNA-derived erythroblasts with a concomitant increase in the level of correctly spliced β-globin mRNA. As expected, only the correctly spliced β-globin mRNA was detected in MU001.A-hiPS-derived erythroblasts. Moreover, this result was confirmed by a quantitative reverse transcription PCR using allele-specific primers (654C-F and 654C-R) to detect only correctly spliced β globin transcript, which also showed highly upregulated expression of the correctly spliced β-globin mRNA in the MU002.A-hiPS.snRNA-derived erythroblasts (Fig. 4B). These results suggested that the modified U7 snRNA stably integrated into the genome and expressed in MU002.A-hiPS.snRNA, leading to efficient splicing correction of IVS2-654 β-globin pre-mRNA. Stable production of correct splicing β-globin transcripts could be detected as late as passage 33 indicating that the correction could be maintained for many passages (Supporting Information Fig. 3B). The efficiency of restoration was estimated by comparing the correctly spliced β-globin mRNA level or band intensity from the MU002.A-hiPS.snRNA-derived erythroblasts (expression from only one allele) to the half of the expression level or band intensity of MU001.A-hiPS-derived erythroblasts (expression from two alleles). The restoration efficiency was approximately 80% (77% according to measurement of RT-PCR band intensity or 89% by qRT-PCR). This study shows that abnormal splicing of β-globin pre-mRNA could be repaired potentially by the modified U7 snRNA antisense-based technology.

Downregulation of Apoptosis-Related Genes, *CASP3* and *AIFM1*

In order to study beneficial effect of U7 snRNA-mediated correction of IVS2-654 mutation in iPSCs, we determined the expression levels of apoptosis-related genes, *CASP3* and *AIFM1*, in erythroblasts derived from iPSCs. The results showed that the expressions of *CASP3* and *AIFM1* in the healthy MU001.A-hiPS and the corrected MU002.A-hiPS.snRNA-derived erythroblasts were significantly lower than in the patient MU002.A-hiPS-derived erythroblasts (Fig. 4C). These results indicate that a less active

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

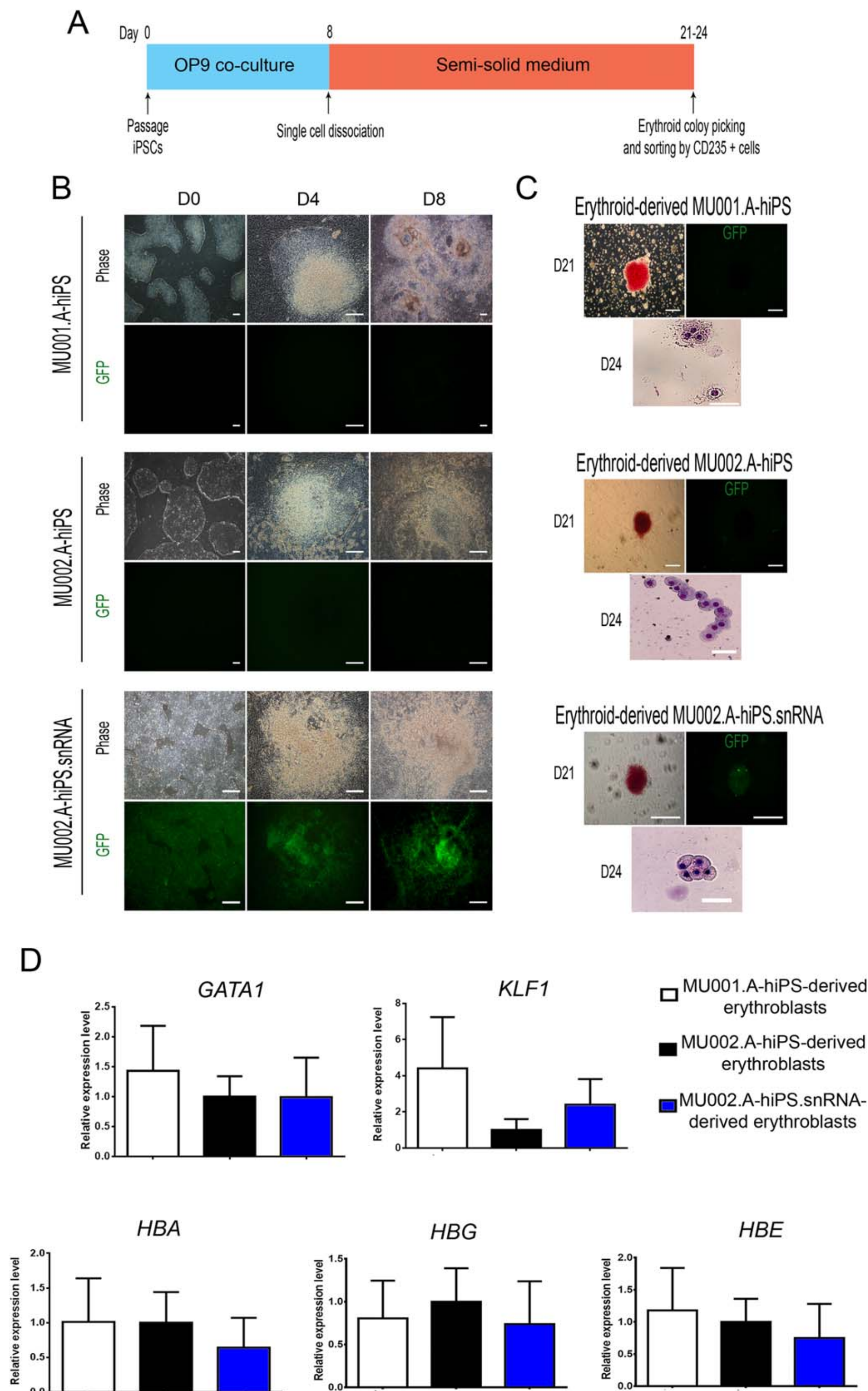

Figure 3. Erythroid cell differentiation of iPSCs. **(A)**: Schematic representation of erythroid cell differentiation protocol on OP9 cell coculture system. **(B)**: Phase contrast images of differentiated iPSCs on OP9 cells at day (D) 0, 4, and 8 (upper panel). GFP detections show under phase contrast images. Scale bars, 200 μm. **(C)**: Erythroid colony forming in semisolid medium (upper left panel) and GFP expression (upper right panel). Scale bars, 200 μm. Wright-Giemsa staining of erythroblasts (lower panel) derived from human iPSCs picked at D24 following differentiation. Scale bars, 50 μm. **(D)**: Quantitative reverse transcription (qRT)-PCR analyses of erythroid specific genes, *GATA1, KLF1, HBA, HBG,* and *HBE* were normalized to *GAPDH* and shown as the mean ± SEM ($n = 3$–6). Abbreviations: CD, cluster of differentiation; GFP, green fluorescent protein; iPSCs, induced pluripotent stem cells.

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

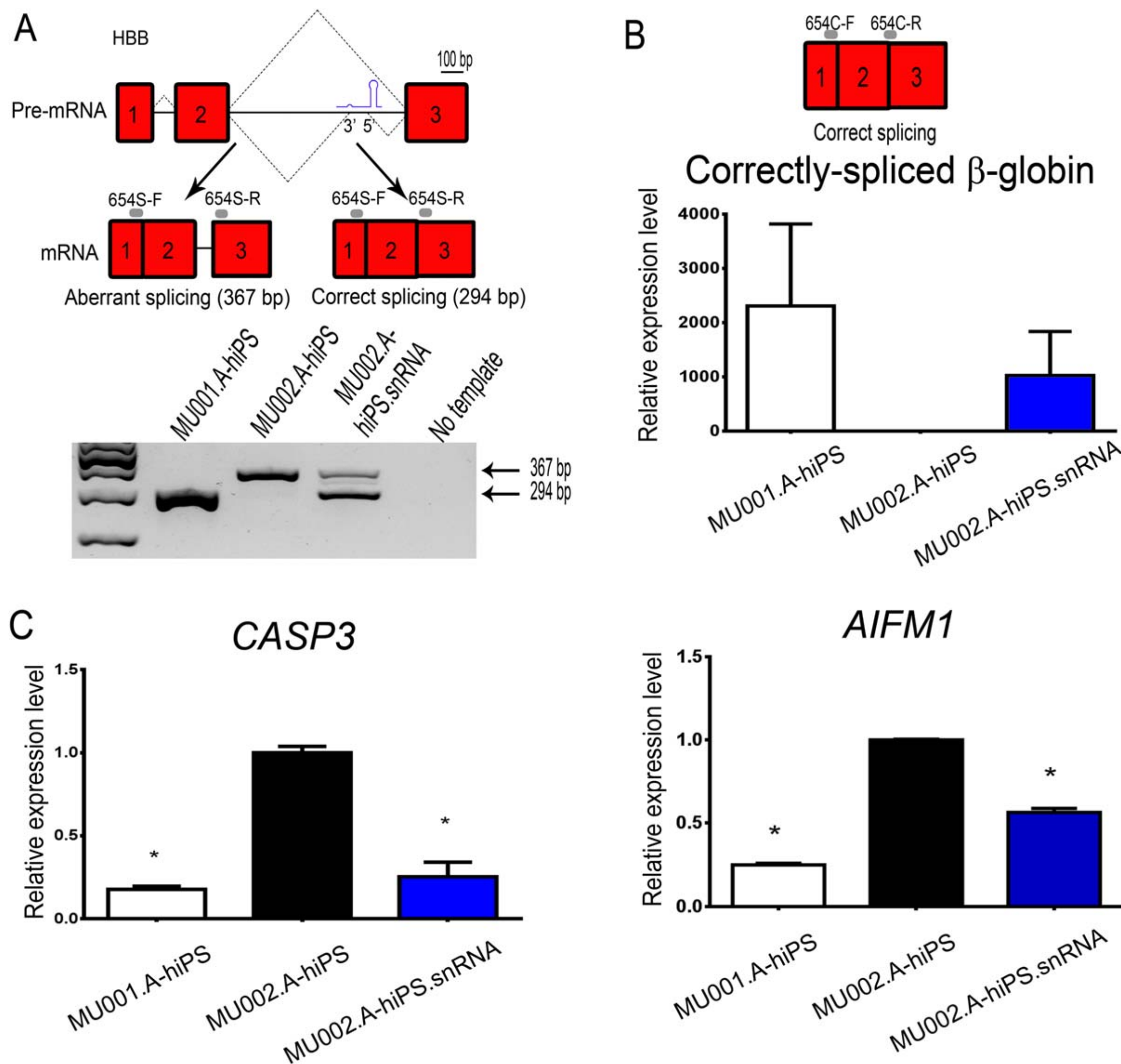

Figure 4. Restoration of correctly spliced β-globin transcripts in MU002.A-hiPS.snRNA. **(A)**: Upper panel, schematic representation of splicing correction of IVS2-654 β-thalassemic pre-mRNA by modified U7 snRNA, squares designate exons, lines designate introns, and primers (654S-F and 654S-R) for specific amplification β^A-globin mRNA are shown as short bars. The correct and aberrant splicing pathways are indicated by dashed lines above and below introns, respectively. The modified U7 snRNA specifically targeted IVS2-654 β-thalassemic pre-mRNAs is illustrated above the pre-mRNA. The PCR products from aberrantly and correctly spliced mRNA are 367 and 294 bps, respectively. Lower panel, conventional RT-PCR analysis of β-globin mRNA from MU001.A-hiPS-, MU002.A-hiPS-, and MU002.A-hiPS.snRNA-derived erythroblasts. No amplification products in no template control (water). **(B)**: Upper panel, diagram represents positions of primers 654C-F and 654C-R used to specifically amplify the correctly spliced β^A-globin mRNA in qRT-PCR. Lower panel, detection of correct β-globin mRNA by qRT-PCR. **(C)**: A representative experiment of relative expression level of *CASP3* and *AIFM1* transcripts from MU001.A-hiPS-, MU002.A-hiPS-, and MU002.A-hiPS.snRNA-derived erythroblasts. Relative expression level was normalized to *GAPDH* and shown as the mean ± SEM ($n = 3$–6), *, $p < .05$, compared to MU002.A-hiPS-derived erythroblasts. Abbreviation: HBB, beta hemoglobin; snRNA, small nuclear RNA.

apoptosis cascade process at transcription level might be the result of the correction by the modified U7 snRNA.

Discussion

In this study, we successfully demonstrated the first time that iPSCs could be fully reprogrammed from MSCs of β-thalassemic patient harboring compound heterozygous IVS2-654 β-thalassemia/HbE by using a polycistronic lentivirus harboring Yamanaka's factors [27]. Both IVS2-654 β-thalassemia and HbE are common mutations found in Thailand and China [1, 4, 35]. Therefore, the established MU002.A-hiPS from the patient who exhibits both mutations are an invaluable tool to serve as disease modeling for investigating the disease pathophysiology, phenotype-genotype correlation, disease onset development, the interaction of HbE and β-thalassemia, parasitology testing, screening drug toxicity or efficiency, and evaluating potential therapeutic strategies. In addition, β-thalassemia genotype diversities may or may not be accounted for clinical severity because patients with the same mutation can give rise to different degrees of symptoms [36] and other modifying factors such as the excess amount of α-globin chain are also reflected in the clinical spectrum of β-thalassemia/HbE [25]. Importantly, as the patient iPSCs can expand indefinitely and able to differentiate into various cell types the need for repeated collection of patient specimens for disease modeling is

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

diminished. In this study, the established iPSCs successfully differentiated into erythroid cells under a coculture system. However, the differentiation from human iPSCs into enucleated red cells expressing adult hemoglobin in vitro is still challenging since success on this topic is very limited except using a gene manipulation approach [37] or in vivo differentiation in mouse [38]. Only one report showed a high percentage of β-globin chain positive erythrocytes derived from human H1 ESCs with a coculture system of mouse fetal liver stromal cells [39]. The choice of cell source for iPSC generation might affect the efficiency of erythrocyte differentiation. Recently, it has been reported that genetically corrected human iPSCs derived from adult peripheral blood cells sickle cell disease patient, which epigenetically express adult hemoglobin, can differentiate into erythrocytes expressing adult hemoglobin in vitro [40]. Moreover, those iPSC-derived erythrocytes also highly expressed fetal hemoglobin suggesting that fully mature adult red blood cells could not be accomplished. Therefore, it is a challenge to generate the efficient red blood cell differentiation protocol from human iPSCs for in vitro β-hemoglobinopathy study. A recent publication on production of red blood cells from human pluripotent stem cells using a potent small molecule for hematopoietic stem cell differentiation and expansion is holding promises toward clinical applications [41]. Comparing to a success β-globin mRNA expression in human iPSC-derived erythroblasts by OP9 coculturing system from a previous study [24], we initially investigated an erythroblast differentiation potential in the established human iPSCs by this methodology. Interestingly, following OP9 coculture differentiation, our results showed that correct β-globin mRNA was detected in MU002.A-hiPS.snRNA-derived erythroblasts which is similar observation compared to previous studies involving genetically corrected iPSCs of β-thalassemia [24, 42]. Moreover, α-, ϵ-, and γ-globin were observed in iPSCs-derived erythroblasts indicating that fetal and embryonic hemoglobin were greatly produced in these cells and this observation was similar to previous reports [43, 44]. However, previously it was also demonstrated at mRNA level that the correctly spliced β^A-globin transcript was undetectable in patient specific iPSC-derived erythroid cells carrying IVS2-654/β^E mutations which is a characteristic of the IVS2-654 mutation [4]. Our results confirmed that patient-specific iPSC could be used for disease and phenotype interaction modeling between HbE and IVS2-654 thalassemia and needed to be further explored. We have chosen a lentiviral vector for genetic modifications because this allowed the modified U7 snRNA to be sustainably expressed in this system and lentiviral gene therapy for β-thalassemia in clinical trial showed the outstanding success in autologous transplantation of the corrected HSCs into the same patients [45–47]. Therefore, it is valuable to develop another strategy to permanently cure β-thalassemia using a lentiviral technology-based therapy. Treatment of hemoglobinopathies mediated iPSCs have been increasingly encouraging by using gene editing technology [21, 22, 24, 40, 42, 48–50] which imply the importance of the establishment of patient-specific iPSCs. Recently, the IVS2-654 mutation was corrected in β-thalassemia-derived human iPSCs by clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated 9 (Cas9) as well as transcription activator-like effector nucleases (TALENs) [42]. The CRISPR-Cas9 method provides a high target efficiency but unwanted off-target events also occurred, which is a risk factor of this technique. The authors decided to use TALEN-corrected iPSCs for differentiating into erythroid cells and showed the restoration of correctly spliced β-globin transcripts in concordance with previous TALEN-targeted IVS2-654 correction results from another team [24]. Moreover, other β-thalassemic mutations have been reported to be corrected by CRISPR-Cas9 in human iPSCs, restoring the β-globin mRNA in the corrected cells [22, 49]. The U7 snRNA correction used in this study resulted in comparable β-globin transcripts with the approaches mentioned above, providing another proof of principle that it is beneficial to use iPSCs for screening the potential of the gene editing methods in case of specific mutations.

Usually, the U7 snRNA is not a part of spliceosome and it participates in histone RNA processing [51]. The U7 snRNA contains a unique sequence at 5′ end that specifically RNA base-pairs with a purine rich sequence or histone downstream element (HDE) of histone mRNA and a Sm binding sequence which is different with consensus sequence of spliceosomal snRNA [52]. In order to use U7 snRNA, the HDE-specific sequence is replaced by a specific RNA antisense against target sequence and original Sm binding site is modified by consensus sequence of spliceosomal Sm binding sequence or SmOPT [53, 54]. For inhibiting a splicing defect at mRNA level, the modified U7 snRNA is an optimal choice because it is accumulated in the nucleus similarly to other snRNAs and it is small size and very stable [55]. Moreover, the modified U7 snRNA has been reported for treatment of some RNA mis-splicing diseases such as Duchenne muscular dystrophy [56], dysferlinopathies [57], spinal muscular atrophy [58, 59], as well as β-thalassemia [18].

Our results demonstrated that MU001.A-hiPS and MU002.A-hiPS.snRNA apparently expressed high amount of correctly spliced β-globin mRNA and this amount was estimated approximately 80% restoration in comparison to healthy cells. Although a complete restoration of correctly spliced β-globin mRNA was not achieved, it is estimated that only 10% level of correction would already lead to a clinically applicable result [60]. This incomplete correction may be the consequence of a lower production of functional U7 snRNA by the host cells due to their limited processing capacity, as reported previously [61, 62]. Although no significant downregulation of *HBA* and *HBE* genes was found in the corrected cells, they were prone to express these genes at a lower level than patient or healthy cells. Nonetheless, this tendency may not prove an obvious interference effect of U7 snRNA in the corrected cells and could be explained by a variation in erythroblast maturation efficacy during differentiation protocol using coculture with OP9 cells. A more controllable differentiation protocol might be needed to get a conclusive explanation. Likewise, previous studies [13, 14] reported that HbF was decreased in β-thalassemic erythroid cells after treatment with antisense oligonucleotides suggesting that restoration of HbA repressed HbF production. At this point, our results demonstrated that the modified U7 snRNA could be used to partially restore the aberrant splicing process. To support the therapeutic potential of this approach, the protein level of a correctly spliced β-globin chain is needed to be analyzed further and confirmed using an efficient protocol to differentiate fully mature red blood cells from iPSCs.

Apoptotic cell death is a common mechanism observed by a decrease of erythroblast cell number in human β-thalassemia patient [63, 64]. Moreover, the apoptotic effector protein, activated Caspase 3, was also significantly expressed in a synthetic erythropoiesis model of β-thalassemia derived by knocking down *β-globin* gene of normal $CD34^+$ cells [65]. The supporting evidence by phosphoproteomic analysis from hemoglobin E/β-thalassemia

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

CD34$^+$ cells showed the various apoptosis-related protein upregulations including apoptosis-inducing factor, mitochondria associated 1 (AIFM1) [66]. Therefore, in this study we demonstrated that the apoptosis related-genes, *CASP3* (caspase 3) and *AFFM1*, were highly expressed in patient erythroblasts and decreased in the corrected cells suggesting that the apoptosis cascade process might be less active as a consequential result of the partial restoration of correctly spliced β-globin mRNA. Similarly, lower expression of *CASP3* has been reported in β-thalassemia iPSCs gene-corrected with a CRISPR/Cas9 system [49]. However, the other apoptotic genes or proteins should be additional elucidated. Further studies for improving the safety and correction by the modified U7 snRNA are needed, for example, better monitoring of integration number and sites of the lentiviral vectors, addition of tissue specific enhancer sequences into the constructs, improving the U7 snRNA delivery strategies.

Conclusion

This is the first study to demonstrate a success of combining antisense U7 snRNA and iPSC technologies to reduce aberrant splicing of β-globin gene in IVS2-654 β-thalassemia disease. In conclusion, our results provide a proof of principle of using iPSCs as a modeling and a screening tool for an alternative approach in repairing RNA splicing defect of IVS2-654 β-thalassemia disease mutations by the modified antisense U7 snRNA. Besides IVS2-654 correction, HbE mutation may be further rescued by using this cellular model for searching a new strategy in ameliorating abnormal splicing process within *HBB*. Our strategy may not be confined to only β-thalassemia but might have implications to other RNA mis-splicing diseases, as well.

Acknowledgments

This work was supported by Research Chair Grant from the National Science and Technology Development Agency (NSTDA), Mahidol University Research Fund, the Office of the Higher Education Commission and Mahidol University under the National Research University Initiative, Thailand Research Fund, and the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7-2012-PEOPLE-IAPP under REA grant agreement 324451 (STEMMAD). P.P. was supported by the program Strategic Scholarships for Frontier Research Network, the Joint PhD Program Thai Doctoral Degree from the Office of the Higher Education Commission, Ministry of Education. We would like to thank Jim Vadolas for providing pLL3.7 lentiviral vector and Christopher Baum and Axel Schambach for providing reprogramming vector. We thank Pirut Thong-ngam and Yongyut Pewklaing for qPCR analysis.

Author Contributions

P.P. and N.K.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; N.J.: data analysis and interpretation, manuscript writing, final approval of manuscript; J.S., L.N., T.N., U.A., and K.S.: collection and/or assembly of data, final approval of manuscript; A.D. and Y.K.: manuscript writing, final approval of manuscript; S.B. and S.H.: conception/design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Cao A, Galanello R. Beta-thalassemia. Genet Med 2010;12:61–76.

2 Mettananda S, Gibbons RJ, Higgs DR. α-Globin as a molecular target in the treatment of beta-thalassemia. Blood 2015;125:3694–3701.

3 Fucharoen S, Winichagoon P. Haemoglobinopathies in Southeast Asia. Indian J Med Res 2011;134:498–506.

4 Cheng TC, Orkin SH, Antonarakis SE et al. Beta-thalassemia in Chinese: Use of in vivo RNA analysis and oligonucleotide hybridization in systematic characterization of molecular defects. Proc Natl Acad Sci USA 1984;81:2821–2825.

5 Lewis J, Yang B, Kim R et al. A common human beta globin splicing mutation modeled in mice. Blood 1998;91:2152–2156.

6 Vichinsky E. Hemoglobin E syndromes. Hematology Am Soc Hematol Educ Program 2007:79–83.

7 Orkin SH, Kazazian HH Jr., Antonarakis SE et al. Abnormal RNA processing due to the exon mutation of beta E-globin gene. Nature 1982;300:768–769.

8 Fucharoen S, Weatherall DJ. The hemoglobin E thalassemias. Cold Spring Harb Perspect Med 2012;2.

9 Zhang JZ, Cai SP, He X et al. Molecular basis of beta thalassemia in south China. Strategy for DNA analysis. Hum Genet 1988;78:37–40.

10 Huang SZ, Zeng FY, Ren ZR et al. RNA transcripts of the beta-thalassaemia allele IVS-2-654 C-->T: A small amount of normally processed beta-globin mRNA is still produced from the mutant gene. Br J Haematol 1994;88:541–546.

11 Shirohzu H, Yamaza H, Fukumaki Y. Repression of aberrant splicing in human beta-globin pre-mRNA with HbE mutation by antisense oligoribonucleotide or splicing factor SF2/ASF. Int J Hematol 2000;72:28–33.

12 Suwanmanee T, Sierakowska H, Fucharoen S et al. Repair of a splicing defect in erythroid cells from patients with beta-thalassemia/HbE disorder. Mol Ther 2002;6:718–726.

13 Suwanmanee T, Sierakowska H, Lacerra G et al. Restoration of human beta-globin gene expression in murine and human IVS2-654 thalassemic erythroid cells by free uptake of antisense oligonucleotides. Mol Pharmacol 2002;62:545–553.

14 Lacerra G, Sierakowska H, Carestia C et al. Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. Proc Natl Acad Sci USA 2000;97:9591–9596.

15 Svasti S, Suwanmanee T, Fucharoen S et al. RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice. Proc Natl Acad Sci USA 2009;106:1205–1210.

16 Bauman J, Jearawiriyapaisarn N, Kole R. Therapeutic potential of splice-switching oligonucleotides. Oligonucleotides 2009;19:1–13.

17 Gorman L, Mercatante DR, Kole R. Restoration of correct splicing of thalassemic beta-globin pre-mRNA by modified U1 snRNAs. J Biol Chem 2000;275:35914–35919.

18 Vacek MM, Ma H, Gemignani F et al. High-level expression of hemoglobin A in human thalassemic erythroid progenitor cells following lentiviral vector delivery of an antisense snRNA. Blood 2003;101:104–111.

19 Fan Y, Luo Y, Chen X et al. Generation of human beta-thalassemia induced pluripotent stem cells from amniotic fluid cells using a single excisable lentiviral stem cell cassette. J Reprod Dev 2012;58:404–409.

20 Wang Y, Jiang Y, Liu S et al. Generation of induced pluripotent stem cells from human beta-thalassemia fibroblast cells. Cell Res 2009;19:1120–1123.

21 Wang Y, Zheng CG, Jiang Y et al. Genetic correction of beta-thalassemia patient-specific iPS cells and its use in improving hemoglobin production in irradiated SCID mice. Cell Res 2012;22:637–648.

22 Xie F, Ye L, Chang JC et al. Seamless gene correction of beta-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. Genome Res 2014;24:1526–1533.

23 Papapetrou EP, Lee G, Malani N et al. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nat Biotechnol 2011;29:73–78.

24 Ma N, Liao B, Zhang H et al. Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

beta-thalassemia induced pluripotent stem cells. J Biol Chem 2013;288:34671–34679.
25 Thein SL. Genetic modifiers of the beta-haemoglobinopathies. Br J Haematol 2008; 141:357–366.
26 Sa-ngiamsuntorn K, Wongkajornsilp A, Kasetsinsombat K et al. Upregulation of CYP 450s expression of immortalized hepatocyte-like cells derived from mesenchymal stem cells by enzyme inducers. BMC Biotechnol 2011;11:89.
27 Warlich E, Kuehle J, Cantz T et al. Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming. Mol Ther 2011;19:782–789.
28 Chen G, Gulbranson DR, Hou Z et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8: 424–429.
29 Rubinson DA, Dillon CP, Kwiatkowski AV et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003;33:401–406.
30 Dias J, Gumenyuk M, Kang H et al. Generation of red blood cells from human induced pluripotent stem cells. Stem Cells Dev 2011; 20:1639–1647.
31 Freberg CT, Dahl JA, Timoskainen S et al. Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. Mol Biol Cell 2007;18:1543–1553.
32 Fitzhugh DJ, Naik S, Caughman SW et al. Cutting edge: C-C chemokine receptor 6 is essential for arrest of a subset of memory T cells on activated dermal microvascular endothelial cells under physiologic flow conditions in vitro. J Immunol 2000;165:6677–6681.
33 Ho YT, Lu CC, Yang JS et al. Berberine induced apoptosis via promoting the expression of caspase-8, -9 and -3, apoptosis-inducing factor and endonuclease G in SCC-4 human tongue squamous carcinoma cancer cells. Anticancer Res 2009;29:4063–4070.
34 Mikkola M, Toivonen S, Tamminen K et al. Lectin from *Erythrina cristagalli* supports undifferentiated growth and differentiation of human pluripotent stem cells. Stem Cells Dev 2013;22:707–716.
35 Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987;11:65–88.
36 Tubsuwan A, Munkongdee T, Jearawiriyapaisarn N et al. Molecular analysis of globin gene expression in different thalassaemia disorders: Individual variation of beta(E) pre-mRNA splicing determine disease severity. Br J Haematol 2011;154:635–643.
37 Kurita R, Suda N, Sudo K et al. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS One 2013;8:e59890.
38 Kobari L, Yates F, Oudrhiri N et al. Human induced pluripotent stem cells can reach complete terminal maturation: In vivo and in vitro evidence in the erythropoietic differentiation model. Haematologica 2012;97:1795–1803.
39 Ma F, Ebihara Y, Umeda K et al. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. Proc Natl Acad Sci USA 2008;105: 13087–13092.
40 Huang X, Wang Y, Yan W et al. Production of gene-corrected adult beta globin protein in human erythrocytes differentiated from patient iPSCs after genome editing of the sickle point mutation. Stem Cells 2015;33: 1470–1479.
41 Smith BW, Rozelle SS, Leung A et al. The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation. Blood 2013;122:376–385.
42 Xu P, Tong Y, Liu XZ et al. Both TALENs and CRISPR/Cas9 directly target the HBB IVS2-654 (C > T) mutation in beta-thalassemia-derived iPSCs. Sci Rep 2015;5:12065.
43 Chang KH, Bonig H, Papayannopoulou T. Generation and characterization of erythroid cells from human embryonic stem cells and induced pluripotent stem cells: An overview. Stem Cells Int 2011;2011:791604.
44 Chang KH, Nelson AM, Cao H et al. Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin. Blood 2006;108:1515–1523.
45 Cavazzana-Calvo M, Payen E, Negre O et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 2010;467:318–322.
46 Thompson AA, Rasko JE, Hongeng S et al. Initial results from the Northstar Study (HGB-204): A phase 1/2 study of gene therapy for β-thalassemia major via transplantation of autologous hematopoietic stem cells transduced ex vivo with a Lentiviral βA-T87Q-Globin Vector (LentiGlobin BB305 Drug Product). Blood 2014;124:549–549.
47 Cavazzana M, Ribeil J-A, Payen E et al. Study Hgb-205: Outcomes of gene therapy for hemoglobinopathies via transplantation of autologous hematopoietic stem cells transduced ex vivo with a Lentiviral βA-T87Q-Globin Vector (LentiGlobin® BB305 Drug Product). Blood 2014;124:4797–4797.
48 Chang CJ, Bouhassira EE. Zinc-finger nuclease-mediated correction of alpha-thalassemia in iPS cells. Blood 2012;120:3906–3914.
49 Song B, Fan Y, He W et al. Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. Stem Cells Dev 2015;24:1053–1065.
50 Zou J, Mali P, Huang X et al. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood 2011;118:4599–4608.
51 Marzluff WF, Wagner EJ, Duronio RJ. Metabolism and regulation of canonical histone mRNAs: Life without a poly(A) tail. Nat Rev Genet 2008;9:843–854.
52 Mowry KL, Steitz JA. Identification of the human U7 snRNP as one of several factors involved in the 3′ end maturation of histone premessenger RNA's. Science 1987;238:1682–1687.
53 Gorman L, Suter D, Emerick V et al. Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. Proc Natl Acad Sci USA 1998;95:4929–4934.
54 Grimm C, Stefanovic B, Schumperli D. The low abundance of U7 snRNA is partly determined by its Sm binding site. EMBO J 1993;12:1229–1238.
55 Spycher C, Streit A, Stefanovic B et al. 3′ end processing of mouse histone pre-mRNA: Evidence for additional base-pairing between U7 snRNA and pre-mRNA. Nucleic Acids Res 1994;22:4023–4030.
56 Goyenvalle A, Babbs A, van Ommen GJ et al. Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: Promising tool for DMD therapy. Mol Ther 2009;17:1234–1240.
57 Wein N, Avril A, Bartoli M et al. Efficient bypass of mutations in dysferlin deficient patient cells by antisense-induced exon skipping. Hum Mutat 2010;31:136–142.
58 Madocsai C, Lim SR, Geib T et al. Correction of SMN2 Pre-mRNA splicing by antisense U7 small nuclear RNAs. Mol Ther 2005; 12:1013–1022.
59 Geib T, Hertel KJ. Restoration of full-length SMN promoted by adenoviral vectors expressing RNA antisense oligonucleotides embedded in U7 snRNAs. PLoS One 2009;4: e8204.
60 Tisdale J, Sadelain M. Toward gene therapy for disorders of globin synthesis. Semin Hematol 2001;38:382–392.
61 Eckenfelder A, Tordo J, Babbs A et al. The cellular processing capacity limits the amounts of chimeric U7 snRNA available for antisense delivery. Mol Therapy Nucleic Acids 2012;1:e31.
62 De Angelis FG, Sthandier O, Berarducci B et al. Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. Proc Natl Acad Sci USA 2002;99:9456–9461.
63 Centis F, Tabellini L, Lucarelli G et al. The importance of erythroid expansion in determining the extent of apoptosis in erythroid precursors in patients with beta-thalassemia major. Blood 2000;96:3624–3629.
64 Mathias LA, Fisher TC, Zeng L et al. Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol 2000; 28:1343–1353.
65 Lee YT, Kim KS, Byrnes C et al. A synthetic model of human beta-thalassemia erythropoiesis using CD34+ cells from healthy adult donors. PLoS One 2013;8:e68307.
66 Ponnikorn S, Panichakul T, Sresanga K et al. Phosphoproteomic analysis of apoptotic hematopoietic stem cells from hemoglobin E/ beta-thalassemia. J Transl Med 2011;9:96.

See www.StemCellsTM.com for supporting information available online.

© 2017 The Authors Stem Cells Translational Medicine published by Wiley Periodicals, Inc. on behalf of AlphaMed Press