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

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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 βE-globin premRNA, 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 βE-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...
S80–652) 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-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 41/2 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-thalassemic/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 B0-54-31) 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 × 10⁶ cells) were transduced with a polycistronic lentiviral vector encoding for human OCT4, KLF4, SOX2, MYC, and a fluorescent reporter, dTomato (pRL.PPT.SF.HOKSM.idTomato.preERT) [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 supernatants were produced by cotransfection of pRL.PPT.SF.HOKSM.idTomato.preERT and third generation packaging plasmid system (Addgene plasmid # 12251 (pMDIg/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.lifesience.roche.com) and being concentrated by Lent-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% CO₂ at 37°C. Human iPSCs were first maintained in human ES medium with feeder dependent and further adapted to home-made EB 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, 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
dish into the dorsal flank of nude mice (BALB/cN-Lac; 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 pL3.7 lentiviral vector [29] at XbaI and Xhol 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 \(\beta\)-globin pre-mRNA. The transduction was performed in one well of a 12-well plate by adding 10 \(\mu\)l of concentrated lentivirus (1.5 \(\times\) 10^8 TU/ml) on 50% confluency growing iPSCs in mTeSR1 medium for overnight. The MU002.A-hiPS.snRNA were transferred to E8 medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) for at least five passages. The patient-specific iPSCs were adapted to culture on Matrigel coated dish in mTeSR1 medium.

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 EB medium. At 70% confluence, the cells were detached by incubating with 1 mg/ml Collagenase IV (Life Technologies) treatment 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 \(\mu\)M P0325901, 2 \(\mu\)M SBA31542, 1 \(\mu\)M CHIR99021 (Merck Millipore), and 10 \(\mu\)M Y27632 (Tocris, Bristol, U. K., http://www.tocris.com) in mTeSR1 for an overnight. The MU002.A-hiPS.snRNA were transferred to EB medium for expansion and erythroid differentiation experiment.

Erythroblast Differentiation

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\(^\circ\)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.miltenyibiotech.com) according to the manufacturer protocols. The purified CD235a positive cells were used for determining hemoglobin chain expression pattern.

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\(^\circ\)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.miltenyibiotech.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 cytospin centrifugation (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 (Epigen Biotechnologies, Madison, WI, http://www.epibi.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 \(\mu\)l 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 \(\mu\)l of the diluted cDNA was used as template for detection of corrected \(\beta\)-globin mRNA and hemoglobin chain expression by RT-PCR and quantitative RT-PCR, respectively.

Human \(\beta\)-Globin Pre-mRNA Splicing Analysis

Amplified cDNA was used as template for detection of the aberrantly and correctly spliced \(\beta\)-globin mRNA. Conventional RT-PCR was carried out using \(\beta\)A \(\alpha\) allele-specific primers (6545-\(\alpha\) and 6545-\(\beta\)) to specifically amplify the \(\beta\)A-globin mRNA but not the \(\beta\)A-globin mRNA published previously [13] with KAPA2G Robust HotStart
qPCR with StepOnePlus™ system (Thermo Scientific) using TaqMan
colonies, data not shown) both in case of healthy control and
globin mRNA, the 654C-F and 654C-R primers combined with the Ec
exogenous gene,
ated by transduction of a polycistronic lentivirus harboring
adaptation onto feeder free condition. The potential fully reprog-
erated biosystems.com) following the manufacturer’s manual on a
significance between groups was calculated with the unpaired Stu-
protocol resulted in many putative iPSC colonies (more than 30
mRNA was used at final concentration of 900 nM in
reprograming efficiency. The putative reprogrammed iPSC colonies
were isolated and cultured. In this study, we focused on the IVS2-
toms like a severe form of
To generate iPSCs, bone marrow derived MSCs from a healthy par-
ticipant and a heterozygous IVS2-654
were used at final concentration of 900 nM in
methylation degree on
transduction of a polycistronic lentivirus harboring
declared 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
were propagated, dissociated into single cells, and
sorted for GFP positive cells. We obtained and expanded homoge-
ous 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).
Lentiviral-Mediated Delivery of the Modified U7 snRNA
to MU002.A-hiPS
To study the ability of the modified U7 snRNA in restoration of cor-
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
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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 confluent 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 sus-
tained 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 cyto-
plasm ratio (Fig. 3C). This results indicated that all three estab-
ished iPSC lines were able to differentiate into hemoglobinized
erthroblasts, even though some variations were observed among
cell lines. Besides erythroid differentiation, other blood lineage

GDF3) in our iPSC colonies detected by quantitative reverse tran-
scription (qRT)-PCR were similar as previously published for
HE11.4 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 immunocyto-
chemistry (Fig. 1D). Importantly, the iPSCs were able to differenti-
ate 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 partici-

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transduction (Fig. 2A). Following expansion by a manual picking of
GFP positive colonies, the positive cells were enriched. In order to
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ished iPSC lines were able to differentiate into hemoglobinized
erthroblasts, even though some variations were observed among
cell lines. Besides erythroid differentiation, other blood lineage
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): Immuno-fluorescent staining for OCT4, SOX2, NANOG, TRA-1-60, 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.
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 disturb 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 correctly 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...
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.
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 no longer required.

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diminished. In this study, the established iPSCs successfully differentiated into erythroid cells under a coculture system. However, the differentiation from human iPSCs into nucleated 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-hiPSC-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-globin transcript was undetectable in patient specific iPSC-derived erythroid cells carrying IVS2-654/β5 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-hiPSC and MU002.A-hiPSC.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 erythrocyte cells after treatment with antisense oligonucleotides suggesting that restoration of HbF 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
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.

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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.

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