Construction of thalassemic mouse induced pluripotent stem cells for disease modeling

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have a great potential for disease modeling and therapeutic applications. One of the most common genetic diseases found in South East Asia is thalassemia. β-Thalassemia is an inherited single gene disorder caused by reduction or absence of β-globin chain production. The purpose of this study is to generate mouse iPSCs with β-thalassemia background. Mouse embryonic fibroblast (MEF) cells were obtained from transgenic β-thalassemic mouse, transduced with lentiviral vector carrying 4 transcription factors, Oct4, Klf4, Sox2 and c-Myc (OKSM), and cultured on a feeder layer. The embryonic stem (ES)-like colonies were manually picked up and expanded. The generated mouse iPSCs were similar to mouse embryonic stem cells (mESCs) in morphology, alkaline phosphatase activity and pattern of pluripotent gene expression and able to form teratoma containing tissues derived from all three germ layers. Therefore, we reported here an establishment of β-thalassemic mouse iPSCs which might be used for further disease modeling and therapeutic applications.

Keywords: induced pluripotent stem cells; β-thalassemia; lentiviral vector; disease modeling

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are reprogrammed somatic cells which undergo embryonic stem cell-like state by being forced expression of factors important for maintaining pluripotency properties in embryonic stem cells (ESCs). The first reprogramming of adult mouse fibroblasts into pluripotent stem cells was done by overexpression of four transcription factors *Oct4, Klf4, Sox2 and c-Myc* (Takahashi and Yamanaka, 2006). Human iPSCs generation were reported later by two groups that used two different sets of transcription factors, first are *Oct4, Sox2, Klf4,* and *c-Myc*, and another set contains *Oct4, Sox2, Nanog and Lin28* (Takahashi *et al.*, 2007, Yu *et al.*, 2007). Like ESCs, iPSCs are pluripotent, can grow indefinitely and differentiate into any cell types. Therefore iPSCs enable disease-specific stem cells to provide an alternative cell source for disease modeling, drug screening, gene therapy and personalized medicine. iPSCs have been made from the somatic cells of patients with a number of diseases including

adenosine deaminase deficiency, Gaucher disease, muscular dystrophies, Parkinson disease, Huntington disease, type 1 diabetes mellitus, amyotrophic lateral sclerosis, Down and Lesch-Nyhan syndromes and β-thalassemia (Dimos *et al.*, 2008, Park *et al.*, 2008, Soldner *et al.*, 2009, Ye *et al.*, 2009a, Ye *et al.*, 2009b)

β-thalassemia is one of the most common inherited single gene disorders caused by reduced or absent output of β-globin chains (Rund and Rachmilewitz, 2005). The reduction of β-globin production results in an imbalanced α- and β-globin chain synthesis, which leads to precipitation of excess α-globin chains on the erythroid membrane. The resulting oxidative membrane damage leads to early erythroid precursor cell destruction (ineffective erythropoiesis) and shortened red blood cell survival. Patients consequently have anemia, jaundice, hepatosplenomegaly, iron overload, and many other pathological changes of various organs such as cirrhosis, cardiac dysfunction and heart failure (Cao and Galanello, 2010).

The aim of this study is to establish mouse iPSCs from transgenic β-thalassemic mouse embryonic fibroblasts (MEFs) to be used for disease modeling, drug screening, and model for gene and cell therapies in the future.

MATERIALS AND METHODS

Thalassemic mice

The heterozygous $\beta^{IVSII-654}$ thalassemic mice ($\beta^{m/IVSII-654}$, 654) were kindly provided by Prof. Ryszard Kole, the Lineberger Comprehensive Cancer Center and Department of Pharmacology, University of North Carolina, Chapel Hill, NC, USA. The mice were created by replacing both mouse β-globin genes; β^{major} and β^{minor} on chromosome 7 with a copy of the human $\beta^{IVSII-654}$-thalassemic gene (Lewis *et al.*, 1998). Heterozygous mice are intermediate anemia with reduced hemoglobin levels, abnormal RBCs morphology, splenomegaly, and markedly increased reticulocyte counts. MEFs were obtained from E12.5 heterozygous 654 mice for iPS cell generation.

Lentiviral production and titration

The polycistronic reprogramming lentiviral plasmid pRRL.PPT.SF.hOKSMco.idTom.pr.eFRT used in this study was kindly provided by Prof. Christopher Baum, Hannover Medical School, Germany (Warlich *et al.*, 2011). This plasmid contains human Yamanaka transcription factors, *Oct4, Klf4, Sox2* and *c-Myc* (*OKSM*) separated by three different 2A cleavage peptides (P2A, T2A and E2A respectively) and dimer Tomato (dTomato) under a SFFV promoter.

For lentiviral production, HEK 293T cells were co-transfected with the reprograming plasmid and packaging plasmids (pMDLg/pRRE, pRSV/Rev encoding packaging and pMD2.G encoding envelope glycoprotein) (Dull *et al.*, 1998) using X-tremeGENE HP DNA transfection reagent (Roche, Germany). The viral supernatant was harvested 48 hours after transfection and concentrated by lenti-Xconcentrator (Clontech, Japan) according to the manufacturer's protocols.

Induced pluripotent stem cells generation and culture

To generate iPSCs, $1x10^4$ MEF cells were transduced with lentivirus carrying *OKSM* at MOI of 2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin and 2 mM L-glutamine supplemented with 4 μg/ml polybrene. One day after transduction, the medium was replaced with mESC medium containing knockout DMEM supplemented with 5% FBS, 15% Knockout™ Serum Replacement (KOSR), 0.1 mM non-essential amino acid solution, 2 mM L-glutamine, 1% penicillin-streptomycin, 1 U/μl leukemia inhibitory factor (LIF) and 0.05 μM sodium butyrate.

ESC-like colonies were observed 10–13 days after transduction. The colonies were individually picked and expanded on feeder layer of MEFs for further characterization. iPSCs were routinely passaged by dissociation with 0.05% trypsin-EDTA (Thermo Scientifics, USA) and maintained on feeder layer in mESC medium.

Alkaline phosphatase staining

To check whether generated iPSCs were in pluripotency state, alkaline phosphatase staining was performed. Briefly, the iPS colonies cultured on feeder layer were stained with fast red violet and naphthol AS-BL phosphate and water mixed solutions in a ratio of 2:1:1. The iPS colonies were visualized under inverted microscope. Alkaline phosphatase-positive colony appears pink to purple while surrounding feeder cell and negative colony appear colorless. mESC and MEFs cells were used as positive and negative control, respectively.

Analysis of pluripotency gene expression

To check expression of pluripotency genes *Nanog*, *Sox2* and *Rex1*, RT-PCR analysis was performed. Briefly, total RNA were extracted from iPSCs using Trizol® solution (Thermo Scientifics, USA) and converted to cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientifics, USA) according to the manufacturer's protocols. PCR analysis was performed using primers specific to *Nanog*, *Sox2, Rex1* and GAPDH (Table 1) and *Taq* polymerase (Thermo Scientifics, USA) according to manufacturer's instruction. In brief, the reaction was carried out in a total volume of 50 μl containing 0.2 μM specific primer pairs for each gene, and PCR master mix (Thermo Scientifics, USA). The amplification was performed under the following condition: initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and the final extension step of 72°C for 10 minutes. The PCR products were analyzed in a 2% agarose gel and visualized under UV in gel documentation system (2000 Chemi Doc Gel Documentation System, Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.).

Table 1 List of the primers used in this study.

PCR primers	Sequence (5′⟶3′)	PCR product
UG1A	ACAAGACAGGTTTAAGGAGACCA	447 bp
LUG2A	GTCTGTTTCCCATTCTAAACTGTA	
HPRT-F	GATGGGAGGCCATCACATTGTAG	315 bp
HPRT-R	GCGACCTTGACCATCTTTGGATTA	
Mouse beta-F	TGAGAAGGCTGCTGTCTCTTG	260 bp
Mouse beta-R	CAGAGGATAGGTCTCCAAAGCTA	
Sox2 T-F	ACAGCTACGCGCACATGA	69 bp
Sox2 T-RNANOG-F	GGTAGCCCAGCTGCTCCT	
NANOG-R	GTGCATATACTCTCTCCTTCCC	209 bp
Rex1-F	AGCTACCCTCAAACTCCTGGT	
Rex1-R	GGAAGAAATGCTGAAGGTGGAGAC	262 bp
GAPDH-F	AGTCCCCATCCCCTTCAATAGC	
GAPDH-R	GTGTTCCTACCCCCAATGTG	215 bp
	GTCATTGAGAGCAATGCCAG	

Analysis of Oct4 and SSEA-1 by immunofluorescent staining

Intracellular pluripotency marker proteins, Oct4 and SSEA-1, was determined by immunofluorescent staining. Briefly, iPSCs were cultured for 2–3 days on 0.1% gelatin coated glass coverslips with a feeder layer until the colonies were 70-80% confluency. Then, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% triton X-100. The iPSCs were stained with primary antibodies, anti-mouse *Oct4* monoclonal antibody (1:100, Merck KGaA, Darmstadt, Germany) or SSEA-1 mouse monoclonal antibody (1:100, Merck KGaA, Darmstadt, Germany) and secondary antibody, Alexa Fluor®488 conjugated goat anti-mouse IgG, IgM (1:1000, Thermo Scientifics, USA). Visualization was performed under a fluorescence microscope (TI-SR, Nikon, Japan).

***In vivo* differentiation**

To test whether mouse thal-iPSCs could differentiate into cells derived from all three germ layers, *in vivo* differentiation by teratoma formation was performed as previously described (Tubsuwan *et al.*, 2013). Briefly, 1–2x10^6 cells of the mouse iPSCs were injected subcutaneously on the dorsal area of nude mice. After 4–8 weeks of injection, when the tumors reach approximately 0.5–1.0 cm of diameter, they were surgically removed from the skin and proceeded for histological analysis. Tumors were fixed in 10% formalin, sectioned at 3–5 µm and stained with hematoxilin and eosin. The morphology of differentiated cells in three germ lines was confirmed under a light microscope.

Confirmation of the genotype of the established iPSCs

Thalassemic genotyping of the established iPSCs was confirmed by multiplex PCR using three different primer pairs including mouse β-globin primers for detection of mouse β-globin gene, HPRT primers for detection of HPRT gene and LUG1A/LUG2A primers for detection of human thalassemic $\beta^{IVSII\text{-}654}$ gene which determined heterozygous 654 (Table 1). The multiplex PCR reaction was carried out in 25 µl volume containing 2.5 mM magnesium chloride, 200 µM dNTP, 0.2 µM mouse β-globin primers, 0.3 µM HPRT primers, 0.4 µM LUG1A/LUG2A primers, 0.5 units of *Taq* DNA polymerase and 100 ng DNA sample. The amplification was performed under the following condition: initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 minutes. And the final extension step of 72°C for 10 minutes. The PCR products were analyzed in a 2% agarose gel.

RESULTS

Induced pluripotent stem (iPS) cells production

In this study, 1x10^4 cells of heterozygous 654 mouse embryonic fibroblasts were reprogrammed into induced pluripotent stem cells using a lentiviral vector expressing *OKSM* at MOI of 2. The transduced cells were cultured in mESC medium containing sodium butyrate, which was changed every other day until iPS-like colonies were picked. Approximately 22 iPS-like colonies from 2 independent experiments emerged after 10–13 days post-transduction. All of the iPS-like colonies were individually and manually picked around day 15–17 after transduction under the cultured condition that had no sodium butyrate and the mESC medium was changed every day. These colonies showed sharp-edged, and compact similar to mESCs (Figure 1). All emerged iPSC-like colonies expressed dTomato, a red fluorescent protein under control of the same promoter as reprogramming factors in the viral

vector, in couple days after viral transduction, indicating expression of reprogramming factors, which was rapidly silenced after a few passages (data not shown). Based on number of iPSC-like colonies, the reprogramming efficiency was 0.11%. Only one of mouse thal-iPSC colonies, clone 11, was selected for further characterization.

Induced pluripotent stem cell characterization

Alkaline phosphatase expression indicates undifferentiated cells with the potential to self-renew. Similar to mESCs, the thalassemic mouse -iPSCs were positive for alkaline phosphatase as indicated by the pink color colonies while surrounding feeder appeared colorless (Figure 1). Immunofluorescent staining analysis of Oct4 and SSEA-1 was performed. The results showed that the iPSC expressed Oct4 and SSEA-1 which were comparable to mESCs (Figure 2), RT-PCR showed mouse thal-iPSCs but not parental MEFs expressed several pluripotent gene markers, such as *Nanog, Sox2* and *Rex1* similar levels to those in the mESCs (Figure 3)

To further evaluate *in vivo* differentiation ability of mouse thal-iPSCs, the cells were subcutaneously injected into the dorsal area of immunodeficiency mice (nude mice). After 4 weeks, teratomas were formed and hematoxilin-eosin-stained teratoma section showed the presence of cell derived from all three germ layers including keratinized epithelium (ectoderm), cartilage (mesoderm), and intestinal epithelium (endoderm) (Figure 4). These results indicated pluripotency properties of the generated thalassemic mouse iPSCs.

To confirm that the generated iPSC lines were derived from the β-thalassemic MEFs, multiplex PCR using specific primers of mouse β-globin and human β-globin was performed in both the iPSCs and parental MEFs. As shown in Figure 5, three bands of PCR products from mouse β-globin (260-bp), HPRT gene (315 bp), and human β-globin (447-bp) were observed in both the iPSCs and the parental MEFs.

DISCUSSION

Disease-specific iPSCs holds great promise as alternative cell source for disease modeling, drug screening, gene therapy and regeneration medicine. In this study, we have successfully generated mouse thalassemic iPSCs line from β-thalassemic MEFs by using a lentiviral vector expressing *OKSM.* The iPSCs exhibited characteristic similar to mESCs including alkaline phosphatase staining and expression of pluripotent markers, SSEA-1, *Nanog*, *Sox2* and *Oct4*. Alkaline phosphatase is a universal pluripotent marker for all pluripotent stem cell types including embryonic stem cells, induced pluripotent stem cells, and embryonic germ cells. Maintenance of pluripotency or initiation of differentiation depends

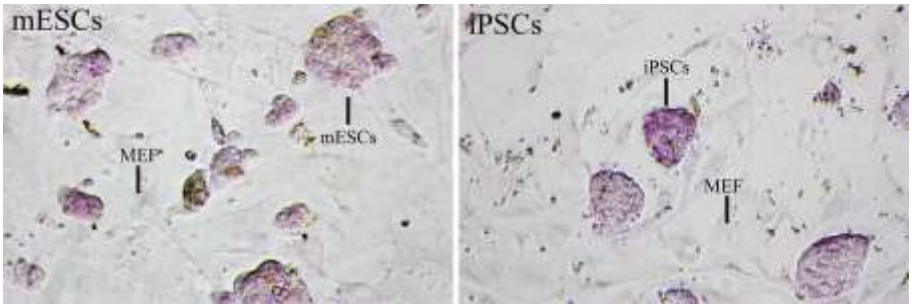

Figure 1 Alkaline phosphatase staining. Both thalassemic mouse iPSCs and mESC colonies were positive for alkaline phosphatase (pink color) while mouse embryonic fibroblasts were negative for the staining (colorless).

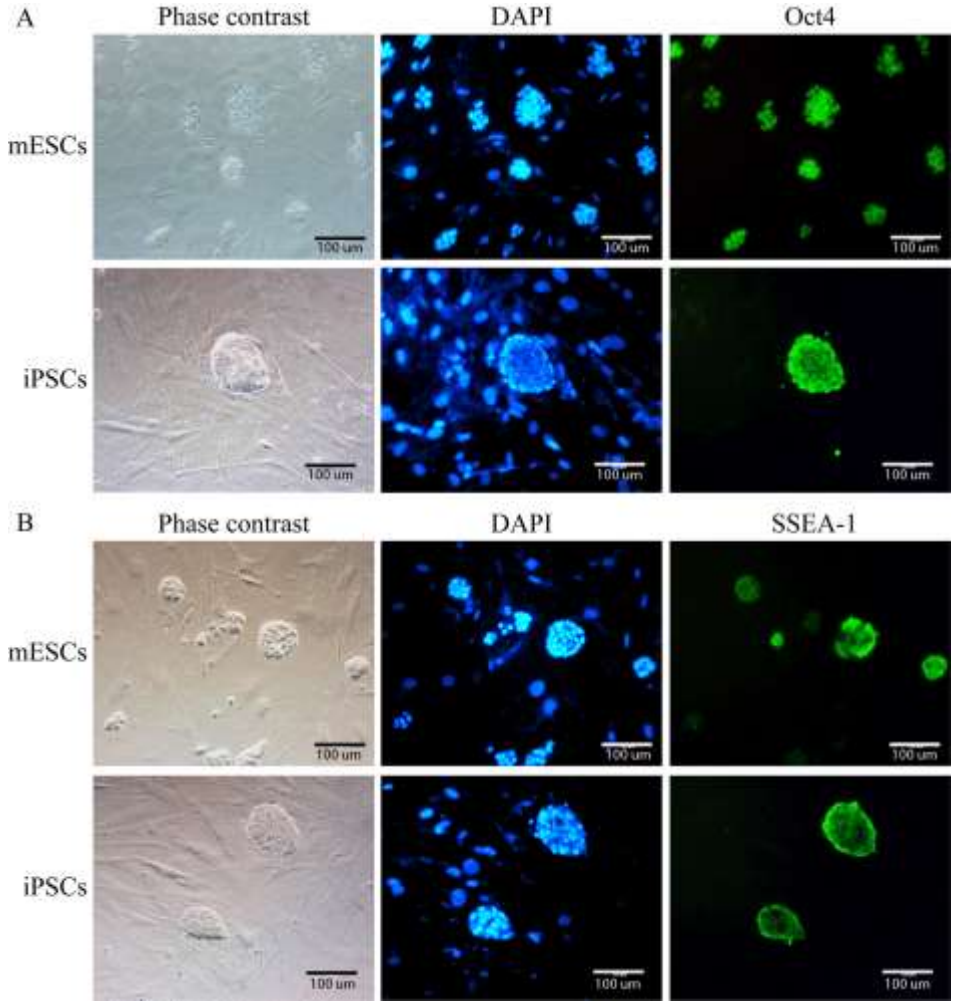

Figure 2 Morphology and immunocytochemical characterization of thalassemic mouse iPSCs. The mouse iPSCs and mESCs were immunofluorescently stained for stem cell markers, OCT4 (A) and SSEA-1 (B), and nuclear stained with DAPI. The thalassemic mouse iPSCs showed similar colony morphology compared to mESCs. Immunostaining of Oct4 and SSEA-1 was comparable to mESCs. Scale bar = 100 μm.

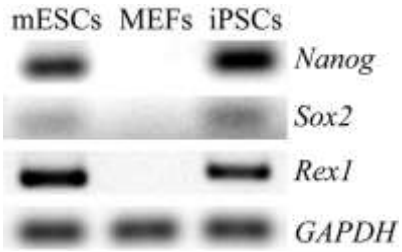

Figure 3 RT-PCR analysis of pluripotent genes. The expression of *Nanog, Sox2* and *Rex1* in mESCs, thalassemic mouse iPSCs (iPSCs) and parental MEF (MEFs) were observed in both iPSCs and mESCs but not in parental MEF cells. *GAPDH* was used as an internal control.

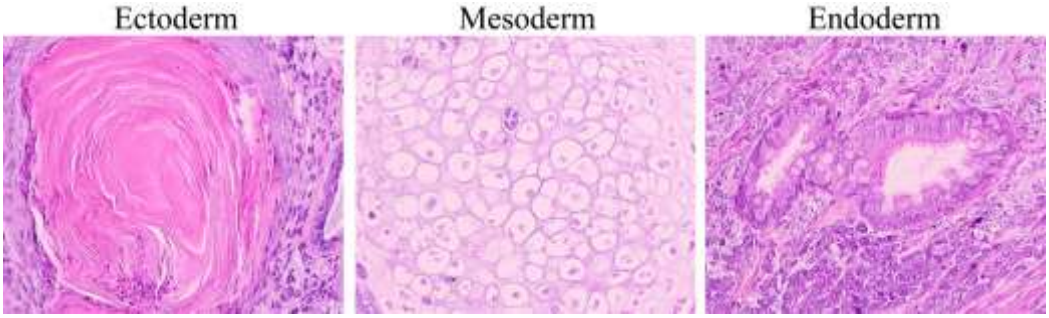

Figure 4 Hematoxylin and eosin-stained teratoma section. Various tissues of the three embryonic germ layers were detected. (A) Keratinized epithelium (ectoderm), (B) cartilage (mesoderm), and (C) intestinal epithelium (endoderm).

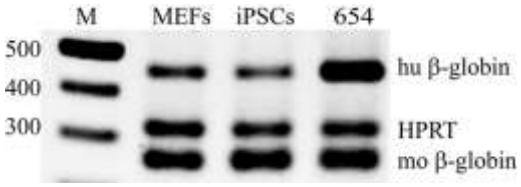

Figure 5 Confirmation of mouse genotyping by multiplex PCR assay. Three primer pairs specific to the human $\beta^{IVSII-654}$ gene (447 bp), HPRT gene (315 bp), and the mouse β-globin gene (260 bp) were used to identify β-thalassemia genotypes. M: 100 bp markers, MEF: parental mouse embryonic fibroblast, iPSCs: thalassemic mouse iPSCs, and 654: positive control from heterozygous 654 mouse.

on a highly regulated equilibrium between *Oct4*, *Nanog*, and *Sox2*. *Nanog*, a transcription factor, is nescessary for its interaction with others crucial factors in pluripotency network (Glauche *et al*., 2010). Our iPSCs were positive for all the pluripotent markers and able to differentiate into all three germ layers *in vivo*.

The only curative treatment for β-thalassemia is bone marrow transplantation, however it would be complicated by the limited availability of matched donor. Gene therapy is a promising therapeutic approach. Although successful treatment of a β-thalassemia patient by *ex vivo* transfer of a functional β-globin gene into the patient's own somatic hematopoietic stem cells has been reported (Cavazzana-Calvo *et al*., 2010), many concerns are still needed to be addressed such as potential oncogenic genotoxicity, improvement of transgene expression, and increased number of chimerism. Since iPSCs can be readily cultured before inducing their differentiation into an hematopoietic stem-like cell type, thus allowing for cell expansion and either gene correction or gene addition followed by selection of cell clones bearing vectors at sites of chromosomal integration with relatively safe area (Papapetrou *et al*., 2011, Tubsuwan *et al*., 2013).

Correction of the genetic defect by genome editing provided a hope to cure the hemoglobinopathies. Zinc finger nucleases (ZFNs) are engineered zinc finger DNA binding domain fused to the FokI endonuclease. In presence of homologous DNA template, the technology enable double stranded breaks and thus induce specific homologous recombination, It has been demonstrated that genetic defect of α-thalassemia (Bart's hydrop

fetalis) iPSCs has been corrected using ZFNs mediated insertion of α globin transgene in the AAVS1 site of human chromosome 19 (Chang and Bouhassira, 2012). Later gene correction of β-thalassemia iPSC has been done using a synthetic nucleases called TALENs providing an easier way to target specific DNA (Sun and Zhao, 2013). CRISPR-Cas9 systems are the most efficient genome editing technologies and can target more genes than those earlier techniques (Sander and Joung, 2014). β-thalassemia patient-specific iPSCs have been recently used to explore the potential of β-thalassemia gene correction using CRISPR-Cas9 (Xie *et al.*, 2014, Song *et al.*, 2015). Although generation of human β-thalassemia iPSC has been made and genetic correction of those β-thalassemia iPSCs has been evaluated *in vitro* (Varela *et al*., 2014, Song *et al*., 2015), *in vivo* therapeutic potential evaluation of the genetically corrected human iPSC-derived hematopoietic stem cells (HSC) is not feasible because of low reconstitution ability of human iPSC derived HSCs in the immune deficiency mouse (Tubsuwan *et al.*, 2013). Gene correction by homologous recombination in mouse iPSC derived from a humanized sickle cell disease mouse model, followed by successful transplantation of differentiated hematopoietic cells into isogenic mice has been applied to cure sickle cell disease phenotypes. Thus β-thalassemia mouse iPSC would be a good model for studying new therapeutic approach in animal model prior of clinical trial.

In conclusion, mouse thal-iPSCs were constructed and the traditional features of pluripotent cells were shown. These cells are expected to be used for further study on pathophysiology, drug screening and therapeutic applications for cells and molecular therapy such as gene editing, gene therapy, RNA based therapy including siRNA and antisense oligonucleotide, and production of hematopoietic cells *in vitro* as a source of autologous transplantation.

ACKNOWLEDGEMENTS

We would like to thank Mrs. Thanthiwa Chanphakhaphat for technical assistance. This work was supported by National Research Council of Thailand (NRCT), the Thailand Research Fund (IRG5780009), Office of the Higher Education Commission and Mahidol University under the National Research University Initiative and the Research Chair Grant, the National Science and Technology Development Agency (NSTDA), Thailand.

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