รายงานฉบับสมบูรณ์

โครงการ การสร้าง induced pluripotent stem cells (iPSCs) **จากเซลล์ของผู้ป่วยเบต้าธาลัสซีเมียฮีโมโกลบินอี**

Generation of induced pluripotent stem cells (iPSCs) from beta-thalassmia hemoglobin E patients

โดย

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พฤษภาคม 2556

สัญญาเลขที่ DBG5380036

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ชื่อโครงการ **การสร้าง induced pluripotent stem cells (iPSCs) จากเซลล์ของผู้ป่วยเบต้าธาลัสซีเมียฮีโมโกลบินอี**

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ระยะเวลาโครงการ 3 ปี

เซลล์ที่ถูกชักนำให้เป็นเซลล์ต้นกำเนิด หรือ iPS cells (induced pluripotent stem cells) กำลังเป็นที่คาดหวังอย่างสูงในการวิจัยด้านการแพทย์ โดยเฉพาะอย่างยิ่งการใช้เพื่อเป็นแบบจำลองของโรคในมนุษย์,การตรวจคัดกรองยา,และการปลูกถ่ายยีนและเซลล์เพื่อการรักษา โรคบีตาธาลัสซีเมียซึ่งเป็นหนึ่งในโรคติดต่อทางพันธุกรรมซึ่งพบได้ทั่วไปในประชากรที่อาศัยอยู่ในภูมิภาคเอเชียอาคเนย์ สาเหตุเกิดจากการกลายพันธุ์ของยีนและส่งผลกระทบต่อกระบวนการตัดต่อลำดับเบสบนสายเอ็มอาร์เอ็นเอ ส่งผลให้ไม่มีการผลิตสายโกลบินชนิดเบตาหรือผลิตได้น้อยลงทำให้ผู้ป่วยมีฮีโมโกลบินอยู่ในระดับต่ำ เนื่องจากปัจจุบันยังไม่พบรายงานเกี่ยวกับการสร้าง iPS cells จากคนไข้hemoglobin (Hb) E-β(IVS2-654)-thalassemia. ดังนั้นจุดประสงค์หลักของการศึกษาครั้งนี้คือการสร้างiPS cells จากผู้ป่วยเบตาธาลัสซีเมียชนิดนี้ ซึ่งสามารถพบได้ในประชากรไทย ธาลัสซีเมียที่ใช้ในการศึกษาครั้งนี้ มีการกลายพันธุ์ของลำดับเบสเฉพาะที่(point mutation) 2 จุด คือ ที่ตำแหน่ง IVS2-654 ในยีนของสายโกลบินชนิด เบตาซึ่งก่อให้เกิดความผิดปกติในจุดตัดของเบสทางด้าน 5´ และทำให้เกิดความคลุมเครือของจุดตัดของเบสด้าน 3´ทำให้ยังคงมีลำดับเบสของส่วนอินทรอนค้างอยู่ในเอ็มอาร์เอ็นเอของโกลบินสายเบตา อีกจุดคือมีการกลายพันธุ์จากเบสG เป็น A ในโคดอนลำดับที่ 26 ของยีนHBB (ยีนโกลบินชนิดเบตา) ส่งผลให้เกิดการความคลุมเครือของจุดตัดของเบส ในโคดอนลำดับที่ 25 ทำให้ฮีโมโกลบิน อี ที่ปกติลดลง ในการศึกษาครั้งนี้ประสบความสำเร็จในการสร้างiPS cells จากผู้ป่วยเบตาธาลัสซีเมียซึ่งมีการกลายพันธ์แบบ IVS-2 654/β^Eและได้ผ่านการทดสอบคุณลักษณะจำเพาะของ iPS cells โดยการย้อมเอนไซม์อัลคาไลน์ ฟอสฟาเตส, ย้อมโปรตีนบ่งชี้ ของความเป็นเซลล์ต้นกำเนิด ได้แก่ Nanog, Sox 2, SSEA4, Oct4, TRA-1-60, TRA-1-81, และทดสอบการแสดงออกของยีนบ่งชี้ ของความเป็นเซลล์ต้นกำเนิด ได้แก่ *OCT4, SOX2, KLF4, c-MYC, NANOG, REX1,GDF3, DNM3TB, UTF1* ผลวิเคราะห์ระดับการเ ติมหมู่ Methyl ที่ลำดับเบส CpG ที่โปรโมเตอร์ของยีน OCT4 ด้วยวิธี bisulfite sequencing พบว่าใน iPS cells มีระดับการเกิดเติม Methyl ที่ต่ำกว่าในเซลล์ที่เป็นต้นแบบก่อนการชักนำให้เป็นเซลล์ต้นกำเนิด ซึ่งแสดงว่าโปรโมเตอร์ของยีน OCT4 ถูกกระตุ้นภายหลังจากการ reprogramming นอกจากนี้ iPS cells ที่สร้างขึ้นสามารถพัฒนาไปเป็นเซลล์ประสาท, เซลล์ตับ, และเซลล์กล้ามเนื้อเรียบเมื่อทำการทำสอบในระดับจานเพาะเลี้ยง เมื่อนำไปฉีดให้หนู ที่มีภูมิคุ้มกันต่ำ(NUDE mice) พบว่าเซลล์สามารถก่อรูปเป็นก้อนเนื้อที่ประกอบด้วยเซลล์ของเนื้อเยื่อของตัวอ่อนทั้ง 3 ชนิด ดังนั้นจึงสามารถใช้iPS cells ที่สร้างขึ้นจากผู้ป่วยธาลัส

ซีเมียเพื่อการศึกษากลไก, พยาธิสภาพ, และการคัดกรองยาสำหรับรักษาผู้ป่วยโรค เบตาธาลัสซีเมียซึ่งเกิดจากความผิดปกติแบบ IVS-2 654/β^{E} ไต้

Abstract

Project Code: DBG5380036

Project Title: Generation of induced pluripotent stem cells (iPSCs) from beta-thalassmia hemoglobin E patients

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Project Period: 3 years

Induced pluripotent stem cells(iPSCs) are promising tools in medical research exclusively with human disease modeling, drug screening, gene and cell replacement therapy. One such genetic disorder is β thalassemia, one of the most common genetic diseases among the people living in Southeast Asia. A large number of mutations affecting splicing consequently cause the decrease or absence in production of β-globin chain, resulting in low level of hemoglobin. At the present, there are no report about generating iPS cells from hemoglobin (Hb) E-β (IVS2-654)-thalassemia. In this study, we focus on establishment of iPS cells from a HbE-β-thalassemia patient carrying two specific point mutations observed in Thai population. The first one is the point mutation at position IVS2-654 in β-globin gene which creates the aberrant 5′ splice site and in turn activates a cryptic 3′ splice site, generating the inclusion of intronic sequences in spliced β-globin mRNA. Another one is G to A mutation in codon 26 of *HBB* (β-globin gene), resulting in activation of a cryptic 5′ splice site in codon 25 and decreasing of correctly spliced β^E-globin. Here, we report successful generation of iPSCs derived from a β-thalassemic patient carrying IVS-2 654/β^E mutations. The patient specific iPS cells were fully characterized by Alkaline Phosphatase staining, immunofluorescent staining of pluripotent markers; Nanog, Sox 2, SSEA4, Oct4, TRA-1-60, TRA-1-81, gene expressions of pluripotent markers; OCT4, SOX2, KLF4, c-MYC, NANOG, REX1,GDF3, DNM3TB, UTF1. Methylation status of OCT4 promoter in the iPS cells was lower than parental cells analyzed by

bisulfite sequencing which indicated that re-activation of OCT4 promoter was occurred after reprogramming. Moreover, the established iPSCs were capable of in vitro differentiation into neuron, hepatocyte and smooth muscle cells. The iPSCs were formed teratoma comprising of three-germ layer cell type after injection into immunosuppressive NUDE mice. Therefore, we can use the patient specific thalassemic iPSCs to study mechanisms, pathologies, drug screening, gene and cell therapy for β-thalassemic patient carrying IVS-2 654/β^E mutations.

Keywords: beta-thalassemia, IVS-2 654, hemoglobin E, induced pluripotent stem cells, disease modeling

1. Introduction

In the present, human stem cells research is widely public in both embryonic stem (ES) stem cells and somatic stem cells. The ultimate goal for these researches is to use stem cells for transplantation treatment or cell based therapy in many diseases e.g. Parkinson disease, thalassemia, age-related macular degeneration. However, immune rejection from ES cells or xeno-transplantation is a major hurdle for this regimen. Therefore, transplantation cells should be obtained from patient in order to reducing immunological incompatibility problem. Previously, bone marrow or thymic transplantation were operated before stem cell transplantation for decreasing immune response as well as using immune-suppressive drug. In 2006, a new strategy for somatic cell reprogramming was discovered by Takahashi and Yamanaka who screened 24 transcription factors that played crucial roles in the embryo and ES cell maintenances and introduced them into adult mouse fibroblast by retrovirus transduction. They observed that only four transcription factors, Oct4, Klf4, Sox2, and c-Myc, (known as Yamanaka's factors) were required for conversion of adult cells into ES like-cell state named as induced pluripotent stem cells(iPSCs)(Takahashi and Yamanaka 2006). This significant breakthrough reprogramming method rises an interesting in regenerative medicine because autologous transplantation may be possible. The defined factors for reprograming can be successfully delivered into the cells by two main strategies categorized by genomic modification with various efficiencies. First, genomic integration method, the reprogramming factors are integrated into host genome for driving reprogramming processes. These take in retrovirus, lentivirus (constitutive, inducible, excisable transgene expression), transposon, linearized plasmid, and phage integrase plasmid (Ye et al. 2010; Karow et al. 2011; Muenthaisong et al. 2012; review in Gonzalez et al. 2011). Second, genomic integration-free method, the exogenous factors are transiently expressed in the cells without genomic alteration. These delivery techniques include adenovirus, sendai virus, non-replicating or replicating episomal vector, minicircle vector, human artificial chromosome vector, synthetic mRNA, and cell penetrating recombinant proteins (Ban et al. 2011; Hiratsuka et al. 2011; Zhang et al. 2012a; Okita et al. 2013; review in Gonzalez et al. 2011). However, prevalent and high efficiency methods in

iPSC generation are retrovirus, lentivirus, sendai virus, andepisome. This study, we chose lentiviral vector for reprogramming factor delivery because the first successfulin gene therapy clinical trialfor human β-thalassaemia treatment was performed by using lentiviral vector (Cavazzana-Calvo et al, 2010)

In Thailand, α-thalassemia is found at 20-30% while β-thalassemia is 3-9%. Fifty two percent of β-thalassemia are hemoglobin (Hb) E and at least four percent are Hb constant spring. Hb E is the point mutation at exon 1of codon 26 (GAG->AAG) of *HBB* (β-globin) gene, resulting in activation of a cryptic 5′ splice site in codon 25 and decreasing of correctly spliced β^{E}-globin. β^{654}-thalassemia hasa mutation at position IVS2-654 in β-globin gene which creates the aberrant 5′ splice site and in turn activates a cryptic 3′ splice site, generating the inclusion of intronic sequences in spliced β-globin mRNA. Lifelong blood transfusion and iron chelation have been supportive, prolonged and managed the survival of the affected β-thalassemic patients. However, mortality and morbidity are still significant associated with this disease. In β-thalassemia, more than 200 mutations causing wide range of aberrant β-globin gene expression result in decrease (β+) or absence (β0) of β-globin production, leading to the precipitation of excess unbound alpha globin chains in erythroid progenitor cells. Consequently, the alpha globin aggregates trigger the damage of red blood cell membrane, early cell death and ineffective erythropoiesis.

At present, transplantation of allogeneic hematopoietic stem cells (HSCs) is the only definitive curative treatment for β-thalassemia patients. Following matched sibling donor, disease-free survival rates are found greater than 80% with a graft failure rate of about 10%. Unfortunately, most patients could not find a human leukocyte antigen (HLA) matched sibling. To overcome the limitations of the lack of available donors and immunologic side effects such graft-versus-host disease (GVHD) or graft rejection with allogeneic hematopoietic stem cell transplantation (HCT), autologous stem cell transplantation, or iPS cell-derived HSCs combined with gene transfer can potentially be the cure for β-thalassemia major. Beside potential in gene and cells based therapy from iPSCs for β-thalassemia, we can use the patient specific thalassemic iPS cells to study mechanisms, pathologies, and drug screening for some specific mutations in β-thalassemic patient. In this study, we report a successful generation of iPSCs derived from a β-thalassemic patient carrying IVS-2 654/β^{E} mutations. The patient specific iPSCs were fully characterized by Alkaline Phosphatase

staining, immunofluorescent staining of pluripotent markers; Nanog, Sox 2, SSEA4, Oct4, TRA-1-60, TRA-1-81, gene expressions of pluripotent markers; OCT4, SOX2, KLF4, c-MYC, NANOG, REX1,GDF3, DNM3TB, UTF1. Methylation status of OCT4 promoter in the iPSCs was lower than parental cells analyzed by bisulfite sequencing which indicated that re-activation of OCT4 promoter was occurred after reprogramming. Moreover, the established iPSCs were capable of in vitro differentiation into neuron, hepatocyte and smooth muscle cells. The iPSCs were formed teratoma comprising of three-germ layer cell type after injection into immunosuppressive NUDE mice. Importantly, the established thalassemic iPSCs and healthy iPSCs had ability to differentiate to erythroid cells which indicated that we probably use this patient specific iPSCs for further study in compound heterozygous Hb E/β^{654}-thalassemia.

2. Materials and Methods

2.1 The establishment of induced pluripotent stem cells (iPSCs)

2.1.1 Experimental specimens

Leftover of cultured bone marrow-mesenchymal stromal cells (MSCs) was obtained from routine pathology diagnosis of patients with ethical reviewed by Mahidol University Institutional Review Board (Protocol ID 80-54-31). MSCs were maintained in MSC growth medium containing alpha-MEM medium, 10% Fetal Bovine Serum (FBS, HyClone, Logan, UT, USA), 1% Glutamax, 1% penicillin-streptomycin, (Life Technologies, Carlsbad, CA, USA) at 37 °C, 5% CO_2. The medium was change every other day. When reaching 70-75% confluency, MSCs were detached by 0.05% trypsin-EDTA (Life technologies) treatment. The MSCs were used in passage 3-5 for iPSC generation.

2.1.2 Lentivirus productionand titration

All plasmids used in lentivirus production were produced by transformation of them into DH5alpha bacteria and extracted by NucleoBond® Xtra Maxi EF (Macherey-Nagel, Düren, Germany). Lentiviral particles were produced in human embryonic kidney (HEK) 293T cells (Clontech, Mountain View, CA, USA) by co-transfection of transfer and third generation packaging vectors using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the supplier's protocol at 1:3 ratio (DNA:reagent). The HEK 293T cells were grown in DMEM (Hyclone) supplemented with 10% FBS (Hyclone) and 1% Glutamax (Life technologies) at 37 °C, 5% CO_2. For 10-cm dish, $5x10^6$ HEK 293T cells were co-transfected with 10 ug of pRRL.PPT.SF.hOKSMco.idTomato.preFRT (lentiviral vector containing Oct4, Klf4, Sox2, c-Myc and fluorescent reporter gene, dTomato , kindly provided by Prof. Christopher Baum), 3.75 ug of pMDLg/pRRE (Gag, Pol), 3.75 ug of pRSV-REV (Rev), and 2.5 ug of pMD2.G (VSV-G, Addgene, Cambridge, MA, USA). After 48 to 72 h, the medium containing viral particles were harvested, filtered through a 0.45 μm PES membrane, and concentrated by Lenti-X concentrator solution (Clontech) according to manufacturer's instruction. Viral titration was

determined by 10 fold serial dilution of concentrated virus (10^0 -10^3). On the day of transduction, two wells of the cells were trypsinized and counted for titration calculation. Each dilution (5 µl) was transduced into HEK 293T cells pre-seeded one day before transduction at $5x10^4$ cells/well of 24 well-plate by spinoculation at 1000 x g, 32 °C for 30 min in growth medium (495 µl) containing 4 ug/ml Polybrene (Sigma, St. Louis, MO, USA). At 16-18 h post-transduction, fresh medium without Polybrene was changed. Three days after transduction, the cells were harvested and analyzed by flow cytometer (FAC atria II, BD). Less than 10% of transduction frequency was used for calculation. The titer was defined as follows: Transduction unit (TU)/ml = [cell number at transduction day x (average of deduced % positive cells/100)] / [volume of diluted virus added (µl) x dilution x 1 ml/1000 µl].

2.1.3 IPSC generation

MSCs were seeded at $1x10^5$ cells on 6 well-plate one day before transduction. The cells were transduced with concentrated lentivirus (multiplicity of infection (MOI) = 0.5) by centrifugation at 1000xg, 32 °C for 30 min in MSC growth medium containing 4 ug/ml Polybrene. The MOI was used to estimate the volume using in transduction as follows; volume of concentrated virus used (µl) = [cell number/ titer (TU/ml)] x MOI x 1000.

Following transduction for 16-18 h, MSC growth medium without Polybrene was changed and maintained in this medium for 5 days. Sodium butyrate (Sigma) was added at 0.5 mM into medium on day 2 to day 11 post-transduction. On day 4 post-transduction, transduced MSCs were split into two 10-cm dishes pre-seeded with Mytomycin C- inactivated mouse embryonic fibroblast (MEF) at $3.5x10^4$ cell/cm^2. Human iPSC medium containing DMEM/F12 (Hyclone), 20%Knockout Serum Replacement (KO-SR), 110 mM beta-mercaptoethanol, 1% non-essential amino acid (NEAA), 1% Glutamax, 1% penicillin-streptomycin, (Life Technologies), 10 ng/ml basic fibroblast growth factor (bFGF), was changed one day after splitting and daily changed. Human ESC like colony was observed and individually picked into new one well of 6-well plate on day 29-30 post-transduction. Human iPSC colony was preliminary identified by live staining two days before picking. Briefly, the cells were incubated with mouse anti human Tra 1-60 antibody (1:100 dilution, SantaCruz,) for

30 min in CO_2 incubator and gently washed two times with DPBS (Hyclone). Diluted Alexa 488 conjugated goat anti mouse IgG, IgM (1:1000 dilution, Life Technologies) was added and incubated for 30 min, then gently washed for two times with human iPSC medium. The positive colonies were observed under fluorescent microscope (Olympus) and marked for picking later. After passage 15, iPSCs were adapted to feeder free culture by picking the colonies to human ES-qualified Matrigel (BD) coated 6-well plate and cultured in mTeSR1 medium (Stem Cell Technologies) or home-made E8 medium (Chen et al, 2011) with daily medium change. On the feeder free condition, the iPSCs were routine passaged by 0.5 mM EDTA (Life Technologies) treatment for 2-5 min, pipette out the EDTA, and resuspended the cells in mTeSR1 or E8 medium before seeding onto new Matrigel coated dish. The selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK), Y-27632 (Torcis) were added into the medium at concentration 10 μM for overnight after passaging for increasing survival rate.

2.2 IPSC characterization

2.2.1 Alkaline Phosphatase (AP) staining

AP staining was done by AP detection kit (Millipore) following the supplier's instruction. Briefly, the iPSCs were fixed with 4% paraformaldehyde for 1-2 min, washed, and then incubated with Naphthol/Fast Red Violet Solution for 15 in dark place. Rinse the cells before observing the positive stained colonies.

2.2.2 Immunofluorescence staining

The iPSCs were fixed with 4% paraformaldehyde for 15 min, washed 3 times with PBS. Then, the cells were treated with permeabilizing/blocking solution containing 0.2% Triton-X, 3% bovine serum albumin, and 2% normal goat serum (Sigma) in PBS for 45 min. Primary antibody was incubated with cells overnight at 4 °C, followed by washing 3 times with 0.1% Tween 20 (Sigma) in PBS. Alexa 488 conjugated mouse IgG or IgM secondary antibodies (dilution 1:1,000, Molecular Probe) were applied for 1 h at room temperature (RT). After washing 3 times, cells were nuclear counterstained with Hoechst 33342 for 15 min at RT (dilution 1:1000, Molecular Probe). Following several times washing, fluorescent mounting medium

(DAKO) was mounted before capturing images with FV-LIV10 fluorescent confocal microscope (Olympus). Both primary and secondary antibodies were diluted in permeabilizing/blocking solution. Primary antibodies were used as follows; NANOG, SOX2, SSEA4, OCT4, TRA-1-60, TRA-1-81, alpha fetoprotein (AFP), alpha-smooth muscle actin (α-SMA), β-III tubulin (dilution, 1:100, all from SantaCruz).

2.2.3 Reverse-transcription (RT)-PCR of pluripotency genes

Total RNA was extracted from iPSCs using RNAeasy plus mini kit with additional on column DNAse I treatment (Qaigen) according to manufacturer's suggestion. First strand cDNA synthesis was performed from 200 ng of total RNA using oligo-dT primer with SuperScript III First-Strand Synthesis System (Invitrogen) following supplier's protocol. Two microliters of a diluted cDNA (1:10) were used for template in the reaction of PCR using Kappa2G robust hot-start ready mix PCR kit with 5 μM of each primer listed in table 1. PCR product amplification was carried out with TAKARA thermal cycler using temperature profile as follows: 1 cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 60-67°C for 30 s, 72 °C for 30 s; 1 cycle of 72 °C for 5 min. PCR product was resolved on 0.7-1% agarose gel.

2.2.4 Bisulfite sequencing of OCT 4 promoter

Genomic DNA was isolated by Genomic DNA mini kit (blood/cultured cell, Geneaid), and then measured a concentration by Nanodrop 2000 (Thermo scientific). Bisulfite conversion of DNA was done by starting with 200 ng of genomic DNA and then converted using the EZ DNA methylation-lighting kit (Zymo Research) as described by manufacturer's instruction. OCT4 promoter was amplified by PCR from converted DNA using published primers listed in table 1 with condition as follows: 1 cycle of 95 °C for 10 min; 35 cycles of 95 °C for 10 min, 58 °C for 1 min, 72 °C for 1 min; 1 cycle of 72 °C for 10 min. Amplified PCR product was checked on 1.5% agarose gel and purified by Hiyield Gel/PCR DNA extraction kit (RBC bioscience). Purified PCR product was ligated into PCR2.1 vector from TA cloning kit following manufacturer's suggestion (Invitrogen, Carlsbad, CA) and then transformed into competent bacterial cells DH5alpha. Single clone of bacteria was confirmed for insertion product by EcoRI digestion before sequencing with M13R-pUC primer

(5’CAGGAAACAGCTATGAC 3’) by Macrogen (Korea). OCT 4 promoter sequences from 10 clones of bacteria were analyzed for methylation status with QUMA (http://quma.cdb.riken.jp/). Each clone was described as row of circles which referred to one CpG position with methylation (black) or unmethylation (white) state.

2.2.5 Detection of beta hemoglobin (*HBB*) gene mutation

Genomic DNA from iPSCs and MSCs was isolated by Genomic DNA mini kit (blood/cultured cell, Geneaid). HBB gene fragment was amplified by PCR with high-fidelity hot-start DNA polymerase (Kapa Biosystems) and primers shown in table 1 at 1 cycle of 95 °C for 3 min; 35 cycles of 98 °C for 30 s, 58 °C for 30s, 72 °C for 30s; 1 cycle of 72 °C for 10 min. The amplicon was identified by agarose gel electrophoresis and purified with Hiyield Gel/PCR DNA extraction kit (RBC bioscience). The purified amplicon was sequenced by Macrogen (Korea) with M13-pUC primer (Macrogen). The mutations were recognized by alignment with reference *HBB* gene sequence (accession number NC_000011.10) acquired from www.ncbi.nlm.nih.gov using Geneious version 6 (Biomatters, New Zealand).

2.2.6 Karyotyping

The iPSCs were seeded on inactivated MEF in T25 flask until 80% confluency, and then incubated with 40 nM BrdU (Sigma) for overnight. The next day, the cells were treated with 0.1 ug/ml KaryoMAX colcemid solution (Life Technologies) for 3 h before harvesting by 0.25% (w/v) trypsin-EDTA (Life Technologies) treatment. Following washing with PBS, the cell pellet was incubated with hypotonic solution containing 0.4% (w/v) KCl, 20 mM HEPES, pH 7.4 for 30 min at 37 °C, followed by fixing with Carnoy’s fixative solution (3:1 (v/v) methanol/glacial acetic acid). The fixed cells were dropped onto glass slide, dried, and then treated with trypsin before staining with Giemsa (Sigma). The metaphase G-banded chromosomes were examined and analyzed by Human Genetics Unit, Department of Pathology, Ramathibodi Hospital.

2.2.5 In vitro differentiation

For spontaneous differentiation into smooth muscle cells and hepatocyte cells, iPSCs were formed embryoid body (EB) by incubating 1 mg/ml Collagenase IV (Life Technologies) for 30 min, and washed two times with DMEM/F12 basal medium. Cell clumps were cultured in human iPSC medium without bFGF for 8 days, followed by seeding onto Matrigel coated glass coverslip for another 8 days, and then fixed with 4% (w/v) paraformaldehyde for 15 min, washed 3 times with PBS. Fixed cells were stained with antibodies against AFP and alpha-smooth muscle actin according to topic 4.2.2. For direct differentiation into neuronal cells, iPSCs were adapted to feeder free condition in E8 medium, and then detached and cultured the cells for EB formation in the medium supplemented with 10 uM Y27632 (Torcis) and 0.5 mM Dorsomorphin (Merck). At day 4, EBs were cultured in the medium including 0.5 mM PD0325901, 0.5 mM Dorsomorphin, 15 mM SB431542 (Merck), and 10 uM Y27632 without bFGF for another 4 days. EBs were collected and seeded on Matrigel coated glass coverslip in medium containing Dorsomorphin, SB431542, and PD0325901 for next 4 day before fixing and staining with beta III tubulin antibody.

2.2.5 Teratoma formation assay

The iPSCs were detached from 10-cm dish using 1 mg/ml Collagenase IV treatment for 45 min, and then washed with human iPSC medium. The pellet was diluted in 100 ul mixture of 50% Matrigel and 50 % human iPSC medium supplemented with 10 uM Y27632 and without bFGF. The cell suspension (100 ul) was injected into dorsal flank of NUDE mice. After 8-12 weeks, mice were sacrificed and the teratomas were dissected, fixed in 10% (v/v) formalin, and dehydrated by a graded series of ethanol, and then embedded in paraffin. The embedded tissues were sectioned at 7 um thickness and stained with hematoxylin and eosin. Three germ layer structure were identified and captured by Nikon ECLIPESE E600 light microscope.

Table 1. Primers used in pluripotency expression, bisulfite sequencing Detection of beta hemoglobin (*HBB*) gene mutation.

Prmier name	Sequence	Ta (°C)
RT-PCR primers		
hKlLF4-F	ATTAATGAGGCAGCCACCTGG	66
hKLF4-R	CTCCCGCCAGCGGTTATTCG	
hOCT4-F	TGTACTCCTCGGTCCCTTTC	62
hOCT4-R	TCCAGGTTTTCTTTCCCTAGC	
hSOX2-F	GCTAGTCTCCAAGCGACGAA	62
hSOX2-R	GCAAGAAGCCTCTCCTTGAA	
hCMYC-F	CGGAACTCTTGTGCGTAAGG	62
hCMYC-R	CTCAGCCAAGGTTGTGAGGT	
hNANOG-F	CAGTCTGGACACTGGCTGAA	60
hNANOG-R	CTCGCTGATTAGGCTCCAAC	
hGDF3-F	TCCTGGAGATACTGGTCAAAGAA	62
hGDF3-R	GAGCATCTTAGTCTGGCACAG	
hREX1-F	TGCTCACAGTCCAGCAGGTGTTT	60
hREX1-R	TCTGGTGTCTTGTCTTTGCCCGTT	
hUTF-1-F	CCGTCGCTGAACACCGCCCTGCTG	68
hUTF-1-R	CGCGCTGCCCAGAATGAAGCCCAC	
hDNMT3B-F	AAGTCGAAGGTGCGTCGTGC	62
hDNMT3B-R	CCCCTCGGTCTTTGCCGTTGT	
hGAPDH-F	GAAGGCTGGGGCTCATTT	62
hGAPDH-R	CAGGAGGCATTGCTGATGAT	
Bisulfite primers		
Bi-OCT4-F	GAAGGGGAAGTAGGGATTAATTT	58
Bi-OCT4-R	CAACAACCATAAACACAATAACCA	
HBB amplification primers		
E-For	CTGACTCCTGAGGAGAAGTC	65
E-Rev	TCCCATAGACTCACCCTGAA	
654-For	ATACAATGTATCATGCCTCT	65
654-Rev	GCTTGGACTCAGAATAATCC	

3. Results and Discussion

3.1 The establishment of induced pluripotent stem cells (iPSCs)

3.1.1 Lentivirus production and titration

Lentivirus were produced from HEK 293T cells by co-transfection of packaging and reprogramming plasmids. The expression of dTomato in HEK 293T cells was observed after 48 h post-transfection (Figure 3.1) indicating co-expression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* because of tandem cloning of these genes in a single vector. Viral supernatants were collected after 48 h post-transfection and filtered through 0.45 µm cellulose acetate membrane in order to get rid of cell debris. Filtrated viral supernatants were concentrated with concentrator solution by centrifugation at 1,500 x g for 45 min at 4 °C and then titrated by transduction with HEK 293T cells at various volumes. Frequency of dTomato positive cells transduced with various dilutions of viral supernatants was examined by flow cytometer (Figure 3.2). At 10^{-1} dilution, the frequency was 2.5% in both sample set 1 and set 2 and used to calculate the titer as follows;

Transduction unit (TU)/ml = [cell number at transduction day x (average of deduced % positive cells/100)] / [volume of diluted virus added (µl) x dilution x 1 ml/1000 µl]

= 10^5 x [[((2.5%-0.2%)/100) + ((2.5%-0.2%)/100)]/2] / [5 µl x 10^{-1} x 1 ml/1000 µl]

= 4.6 x10^6 TU/ml

Henceforth, the titer, 4.6 x10^6 TU/ml, was used to estimate multiplicity of infection (MOI) or the proportion between amount of virus and transduction target that is the cells using for reprogramming experiment.

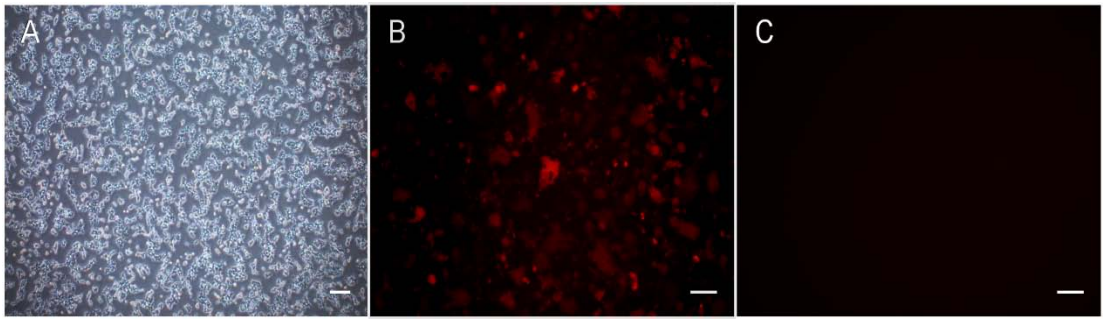
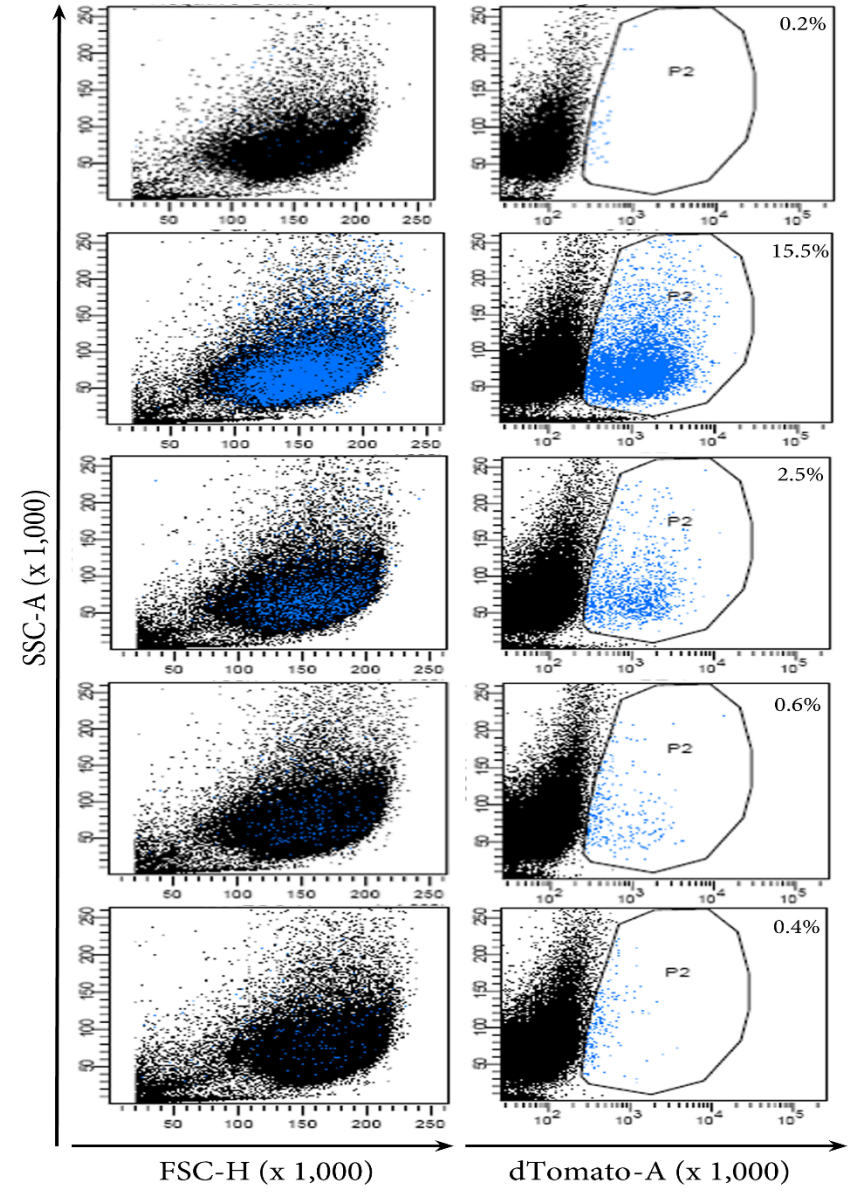


Figure 3.1 Lentiviral production in HEK 293T cells. **A**, the HEK 293T cells before co-transfection with packaging and reprogramming plasmids. **B**, Expression of dTomato in the HEK 293T after 48 h post-transfection. **C**, Non-transduced cells were used as negative control. Scale bars, 200 µm (**A**) and 100 µm (**B**, **C**).

3.1.2 The iPSC emerging and transgene silencing

The healthy or patient MSCs were transduced with concentrated lentivirus (MOI = 0.5) by centrifugation to increase transduction efficiency. Expression of dTomato was examined 48 h post-transduction (Figure 3.3 C, D and 3.4 C, D). The transduced cells were split on MEF and changed medium to hES medium to promote reprogramming process and colony formation. The morphology of dTomato positive cells were changed from fibroblast- like to epithelium-like cells around day 5 post-transduction (Figure 3.3 E, F and 3.4 E, F) and these cells divided and formed a small colony expressing dTomato on later day (Figure 3.3 G, H and 3.4 G, H). The colonies were larger and formed a tightly packed colony (Figure 3.3 I and 3.4 I). The expression of dTomato was undetectable in some colonies starting around day 13 and later on under fluorescence microscope which indicated the transgene silencing (Figure 3.3 J and 3.4 J). In addition morphological observation on patient iPSC colonies, live staining of TRA-1-60 antibody, a pluripotency surface marker, was performed to preliminary identify a potential fully reprogramming colony which the dTomato could not be detected (Figure 3.5). The TRA-1-60 positive colonies were marked and picked into new feeder dishes in order to expansion and characterizations later on.

A


DPBS
undiluted
10-1 diluted
10-2 diluted
10-3 diluted
SSC-A (x 1,000)
FSC-H (x 1,000)
dTomato-A (x 1,000)
P2
0.2%
15.5%
2.5%
0.6%
0.4%

B

viral supernatants (5 µl added)	% dTomato postive cells (set.1)	% dTomato postive cells (set.2)
DPBS	0.2	ND
undiluted	15.5	14.7
10^{-1} diluted	2.5	2.5
10^{-2} diluted	0.6	0.7
10^{-3} diluted	0.4	0.4

Figure 3.2 Titration of lentiviral supernatant analyzed by flow cytometer. **A**, Flow cytometer data set was expressed as scatter plots of the transduced HEK 293T cells (sample set.1) at various dilutions of concentrated viral supernatants (5 µl added of undiluted, 10^{-1} diluted, 10^{-2} diluted, and 10^{-3} diluted supernatants) or DPBS (negative control). The dTomato positive cells were gated in P2 and shown as percentage. **B**, Summary of dTomato positive cells analyzed by flow cytometer from sample set 1 and 2. ND, not determine. SSC-A, side-scattered lite (area). FSC-A, forward-scattered light (area).

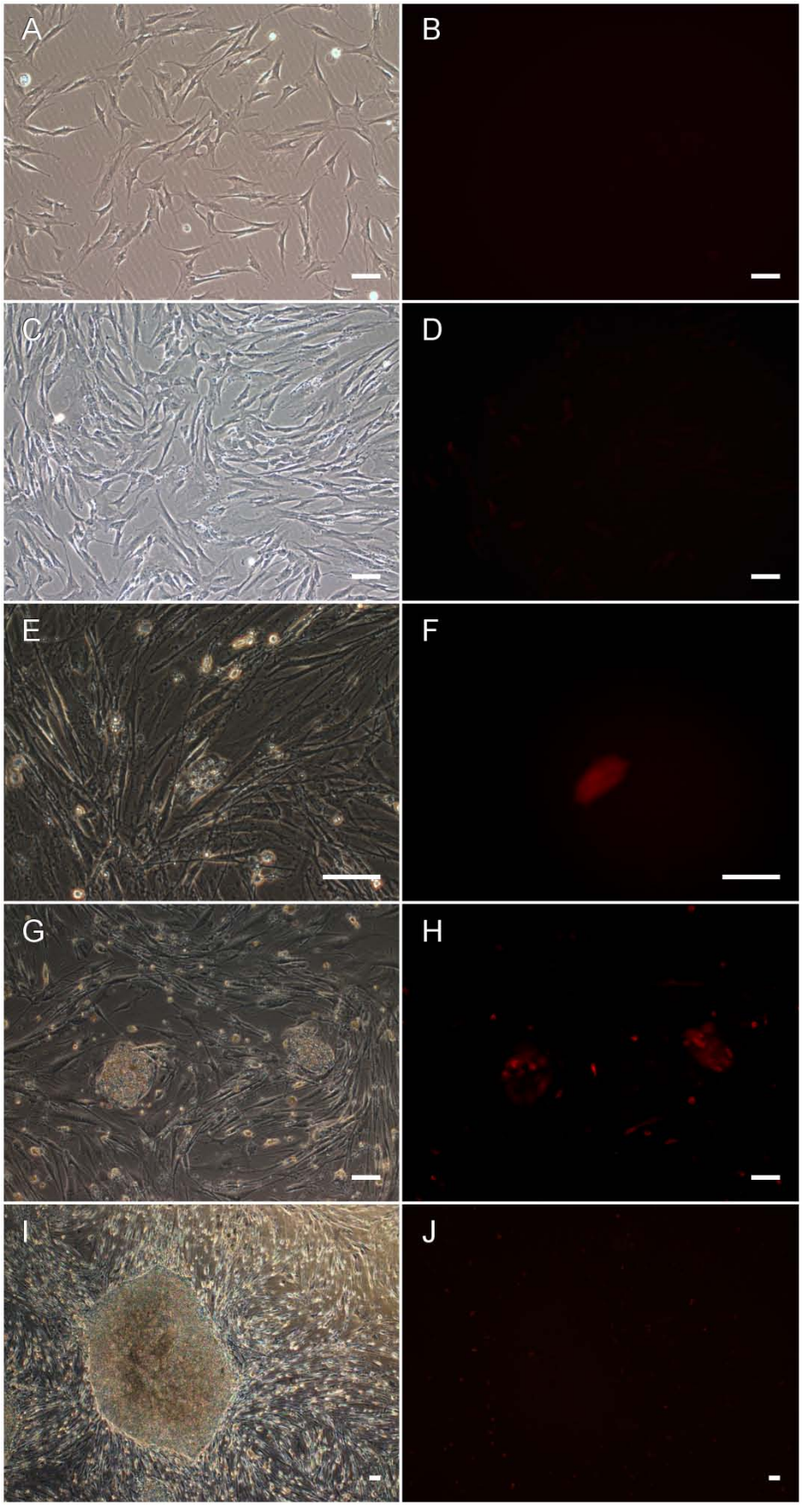
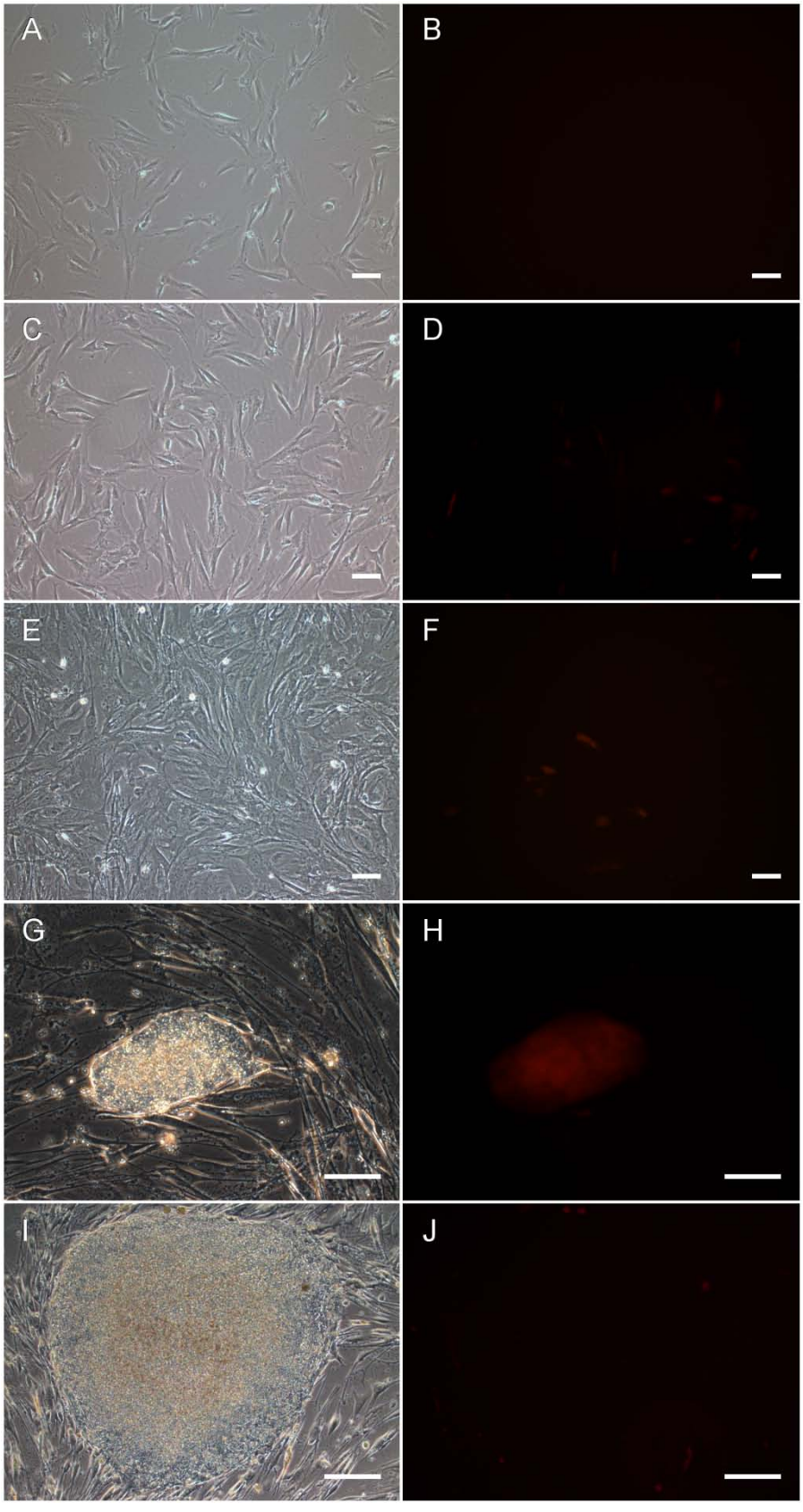

A
B
C
D
E
F
G
H
I
J

Figure 3.3 The healthy iPSC colony formation. The healthy MSCs were cultured in growth medium before transduction (**A**, **B**). Proliferation of MSCs and expression of dTomato could be observed 2 days after transduction (**C,D)**. Epithelium-like cells formed a small colony strongly expressing dTomato at day 5 (**E**, **F**). A few colonies could be obviously detected and were still expressed dTomato at day 11 (**G**, **H**). A large iPSC colony was observed after 24 days after transduction and could not detected the expression of dTomato (**I**, **J**). First column is the pictures taken under phase contrast and second column is under fluorescence microscope for dTomato detection. Scale bars, 100 μm.


A
B
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I
J

Figure 3.4 The patient iPSC colony formation. The patient MSCs were cultured in growth medium before transduction (**A**, **B**). Proliferation of MSCs and expression of dTomato could be observed 2 days after transduction (**C,D)**. Change of fibroblast-like cells to epithelium-like cells expressing dTomato was found at day 5 (**E**, **F**). A few colonies could be obviously detected and were still expressed dTomato at day 11 (**G**, **H**). A large iPSC colony was observed at day 24 and could not detected the expression of dTomato (**I**, **J**). First column is the pictures taken under phase contrast and second column is under fluorescence microscope for dTomato detection. Scale bars, 100 µm.

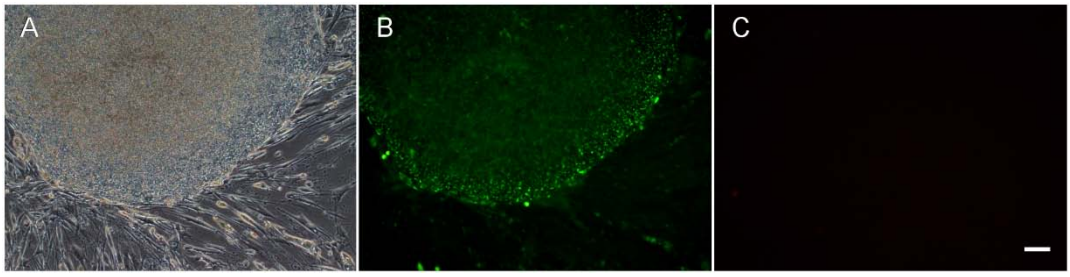

Figure 3.5 Live staining of patient iPSC colony against TRA-1-60 antibody, a pluripotency surface marker. The patient iPSC colony was grown on MEF for 25 days after transduction (**A**) and live stained with TRA-1-60 primary antibody followed by Alexa 488 conjugated goat anti mouse IgG, IgM. The TRA-1-60 positive colony was examined under fluorescence microscope shown in green (**B**). The dTomato expression could not be detected in this state (**C**). Scale bar, 100 µm.

3.2 The iPSC characterizations

3.2.1 Morphology of iPSCs

The established healthy and patient iPSCs on MEF were morphologically similar to typical human ESC colony in term of flat, dense colony, sharp colony border, high nucleus to cytoplasm ratio and prominent nucleoli (Figure 3.6). However, the morphologies of iPSCs on feeder and on feeder-free were not exactly the same as shown in Figure 3.6. We used Matrigel, undefined basement membrane matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcom, for feeder-free condition culture in mTESR or E8 medium. In this condition, the colonies were looser than on MEF (Figure 3.6) and easy to maintain and propagation.

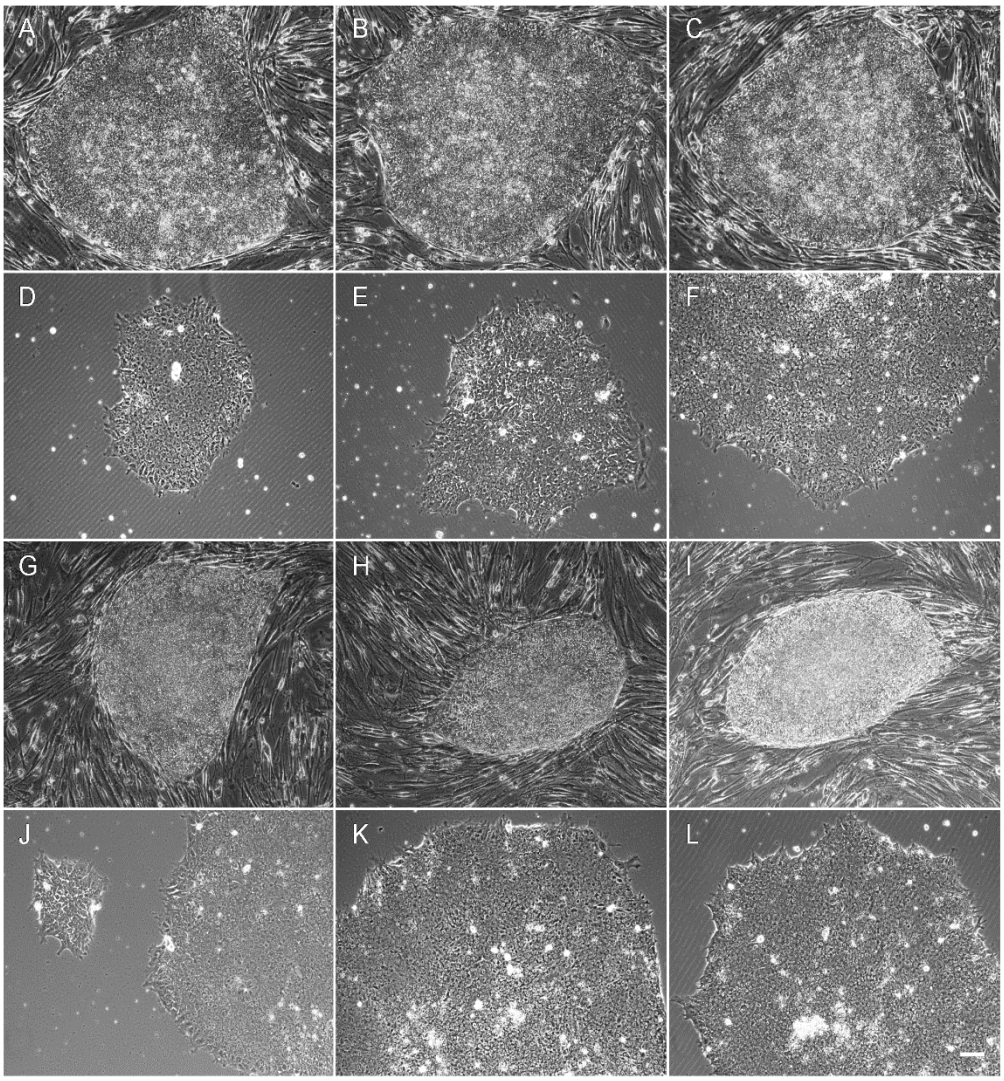

Figure 3.6 Morphology of healthy (**A-F**) and patient (**G-H**) iPSCs at passage 3. Individual clone of iPSC was picked and expanded on MEF (**A-C**, **G-I**) or Matrigel (**D-F**, **J-L).** On MEF, the colonies exhibited clear border and dense cells whereas the colonies on Matrigel were loose and the cells were easily identified but still showed high nuclease to cytoplasm ratio and prominent nucleoli. Healthy iPSC clone (C) #7 (**A, D**), C#8 (**B, E**), C#9 (**C, F**). Patient iPSC C#1 (**G, J**), C#2 (**H, K**), C#3 (**I, L**).Scale bar, 100 µm.

3.2.2 Pluripotent marker expressions

Otherwise morphologically observation, the established human iPSCs were confirmed the pluripoentcy by staining with various markers. The common marker for stem cells is alkaline phosphatase (AP) expression. We firstly detected AP activity and showed that all clones from both healthy and patient iPSCs were detected for alkaline phosphatase (AP) activity by change the substrate color to pink (Figure 3.7).

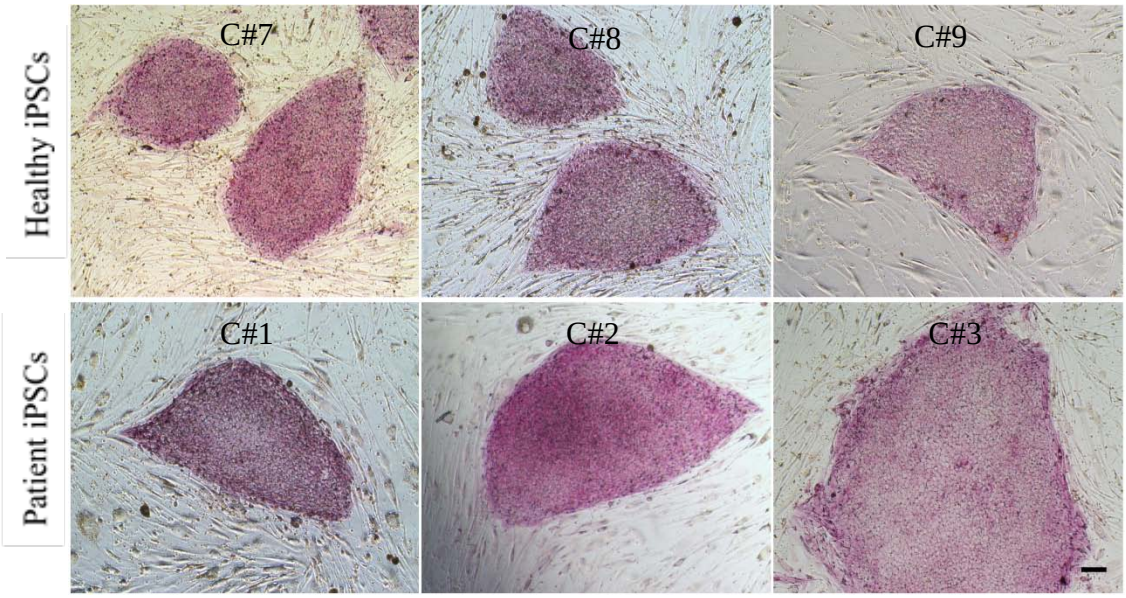

Figure 3.7 Alkaline phosphatase (AP) staining of healthy and patient iPSCs. Healthy iPSC clone 7 (C#7), C#8, C#9 and patient iPSC C#1, C#2, C#3 were positively stained pink of AP. Scale bar, 100 µm.

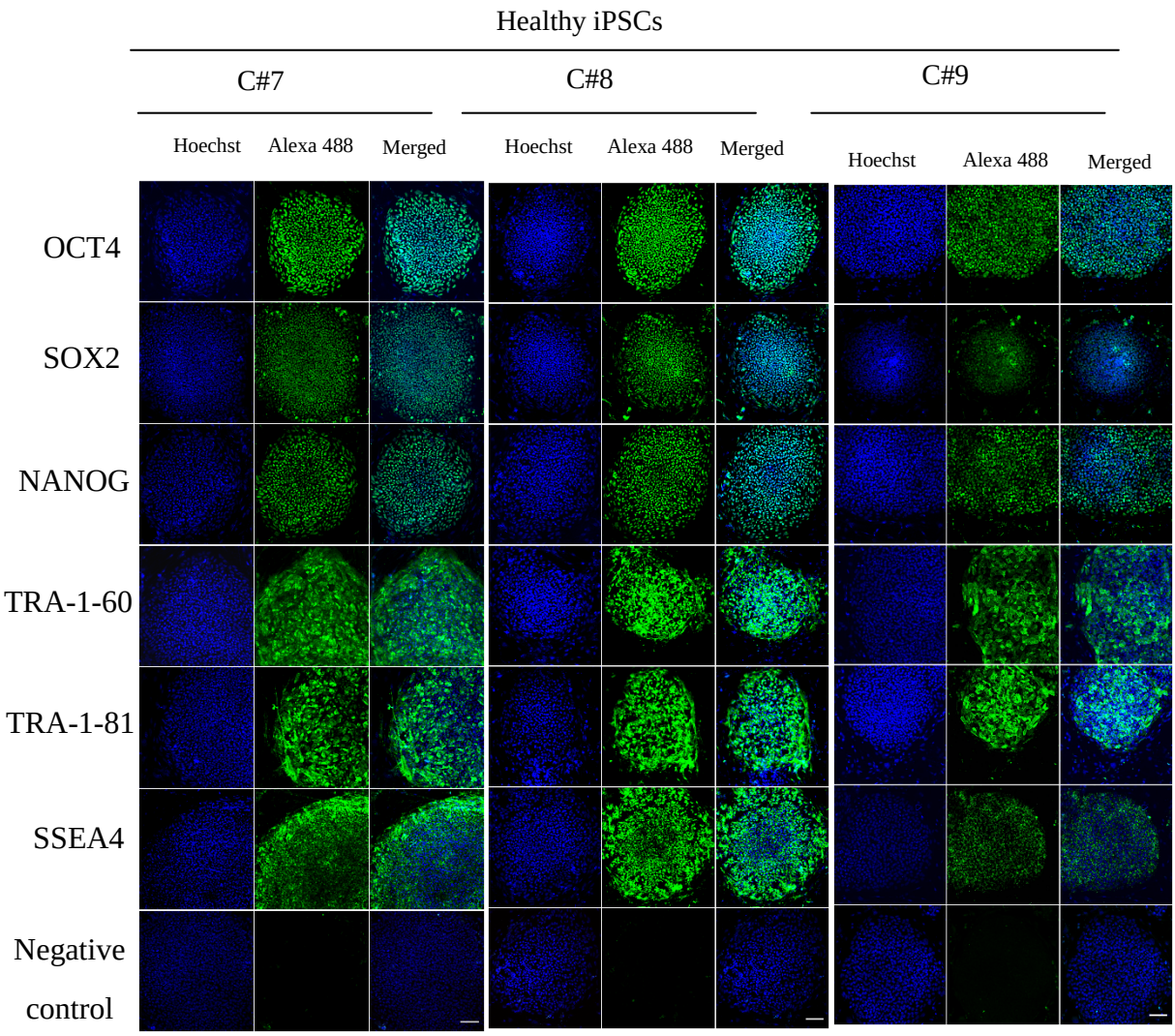

Figure 3.8 Immunofluorescence staining for OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81, and SSEA4 of healthy iPSCs C#7, C#8, and C#9. Staining without primary antibody was used as negative control. Alexa-488 (green) conjugated secondary was used. Nuclei were localized by Hoechst 33342 (blue). Scale bars, 100 µm.

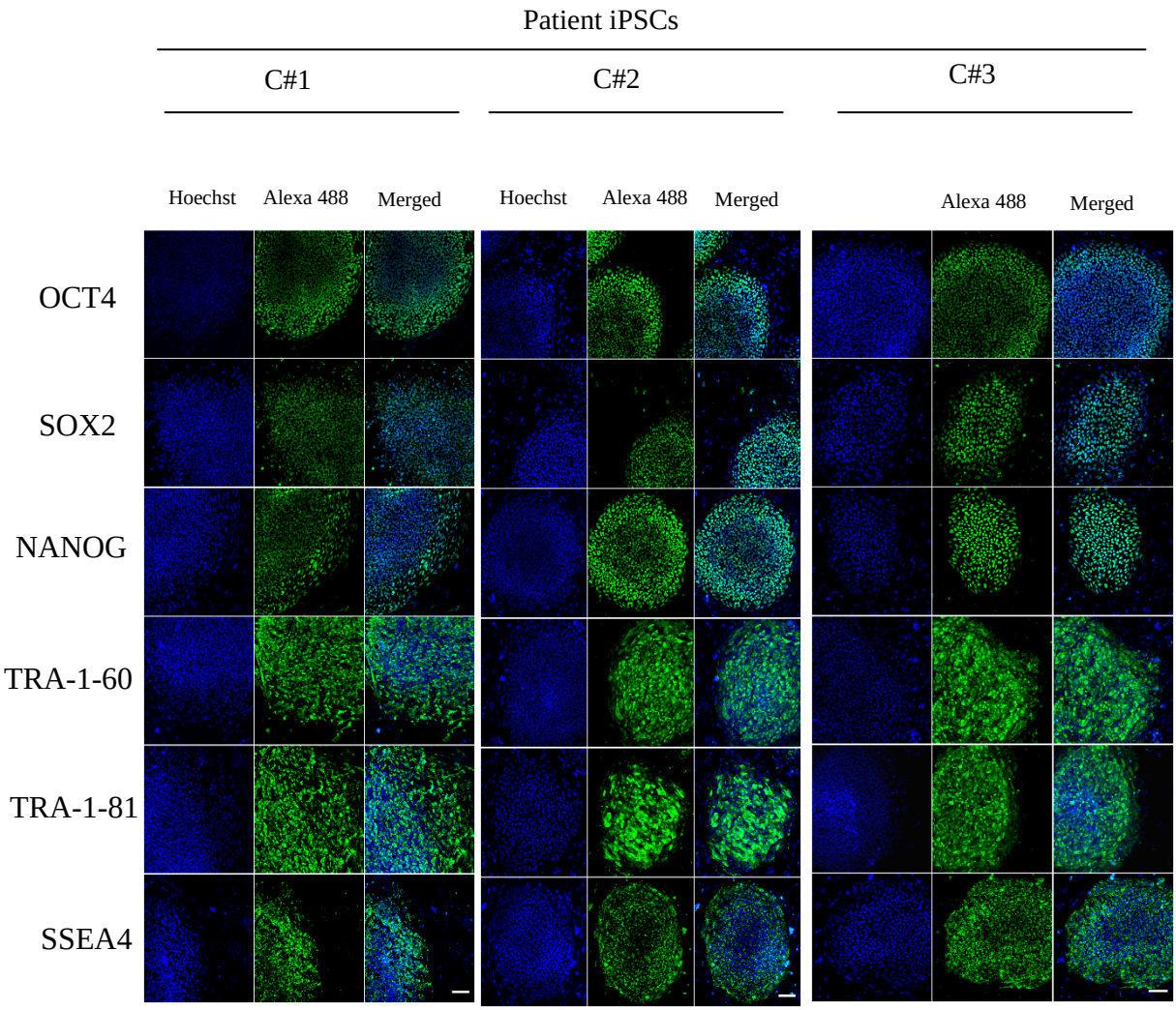

Figure 3.9 Immunofluorescence staining for OCT4, SOX2, NANOG, TRA-160, TRA-1-81, and SSEA4 of patient iPSCs C#1, C#2, and C#3. Nuclei were localized by Hoechst 33342 (blue). Alexa 488 conjugated secondary was used (green). Scale bars, 100 µM.

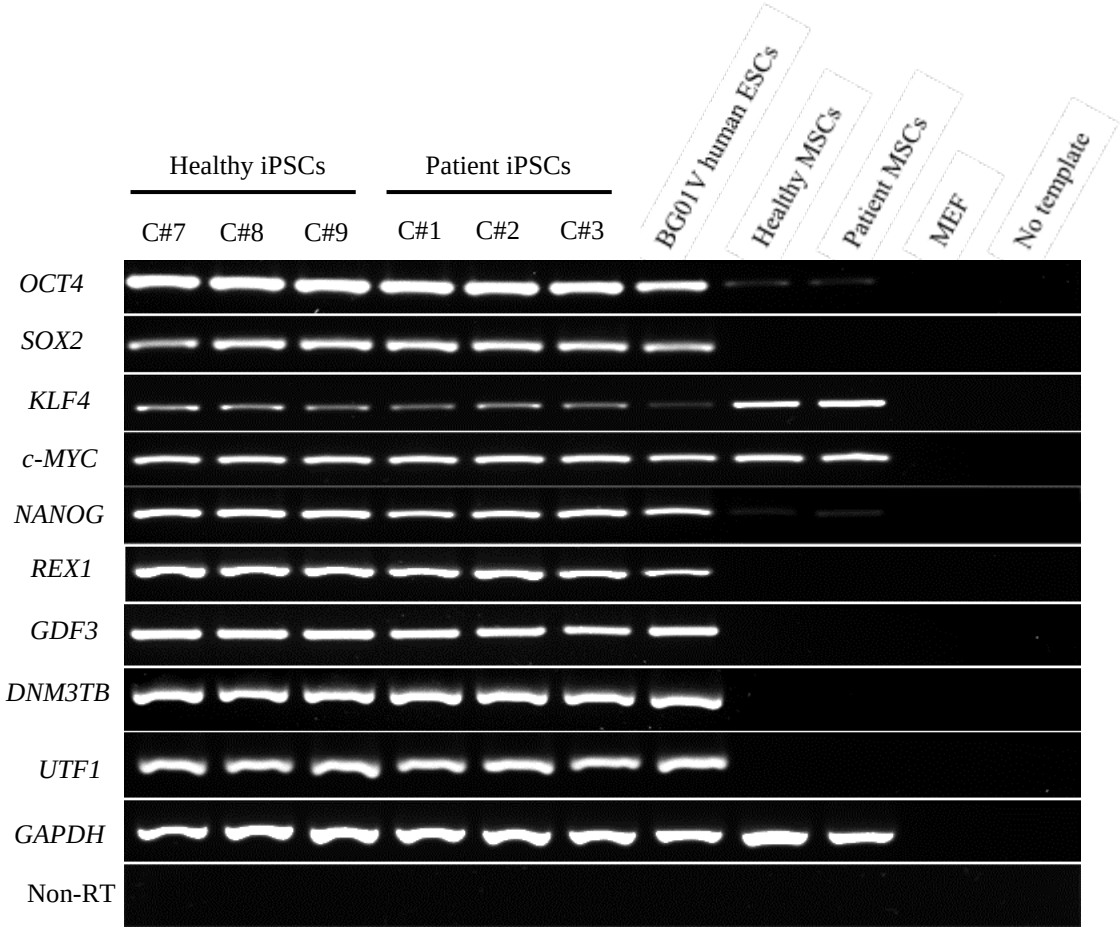

Figure 3.10 Gene expressions of pluripotency markers determined by RT-PCR. Both healthy and patient iPSCs were similarly exhibited expression profile of pluripotent markers as well as BG01V human ESCs (positive control). Some endogenous reprogramming factors and *NANOG* could be detected in parental MSCs. No detectable band was amplified from MEF, no template (H_2O), and non-RT (no reverse transcriptase of *GAPDH*).

3.2.3 Epigenetic reprograming

In order to study epigenetic reprograming in iPSC, methylation status of OCT4 promoter was elucidated. The unmethylation at CpGdinucletide in the iPS cells was shown in higher rate than parental MSCs (Figure 3.11) analyzed by bisulfite sequencing which indicated that re-activation of OCT4 promoter was occurred after reprogramming.

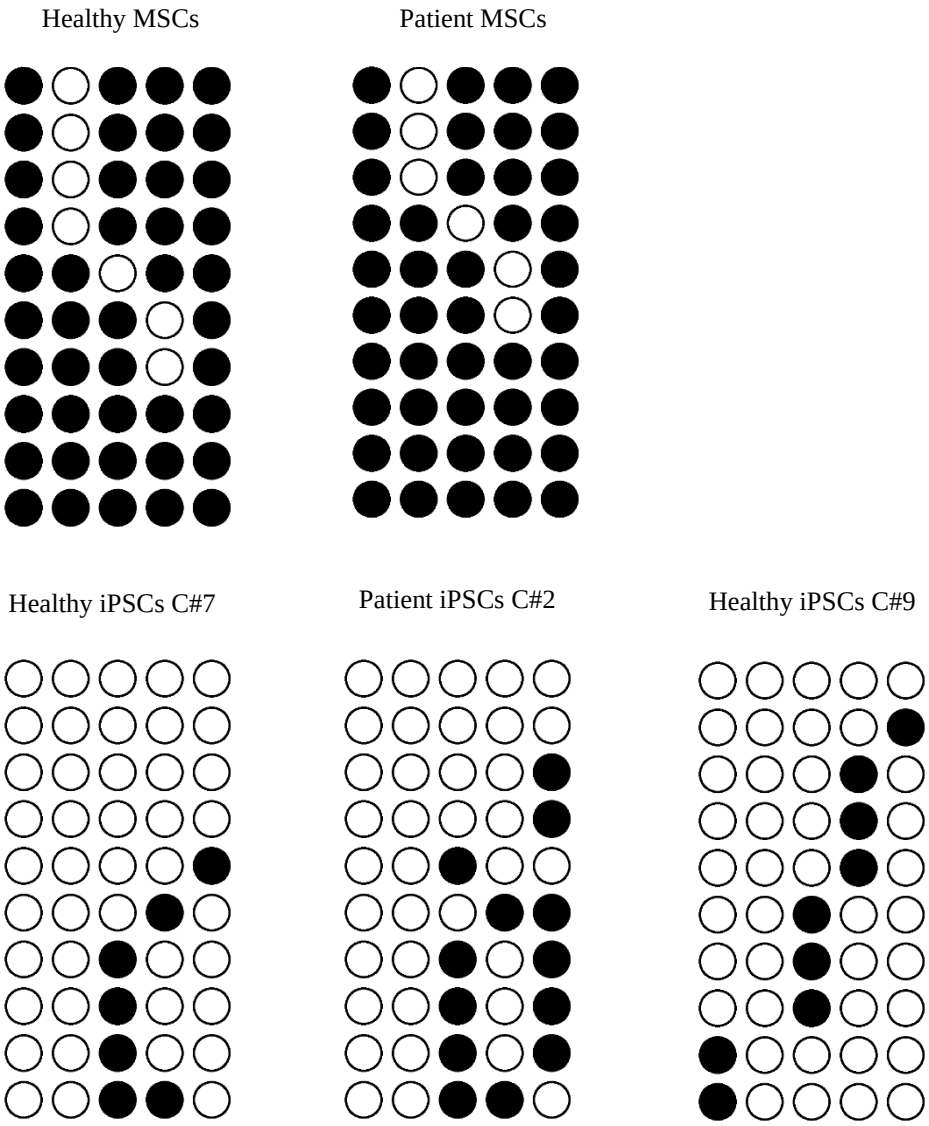

Figure 3.11 Methylation status analysis of *OCT4* promoter region by bisulfite genomic sequencing. White and black circles represent unmethylated and methylated cytosine guanine dinucleotides (CpGs) of each position (column) on amplified OCT4 promoter region. Each row indicates bacterial clone used for sequencing.

3.2.4 Detection of beta hemoglobin (HBB) gene mutation

MSCs and iPSCs from healthy volunteer were not exhibited point mutation at codon 26 and IVS2-654 whereas MSCs and iPSCs fromHbE-β^{654}-thalassemia patient showed the mutation from G to A at condon 26 (Hb E) and C to T at IVS2-654 (β^{654}) as illustrated in Figure 3.12 and 3.13. These results indicate that established iPSCs were not change genotype on *HBB* gene after performing reprogramming. Therefore, we possibly use these cell lines to study HbE-β^{654}-thalassemia.

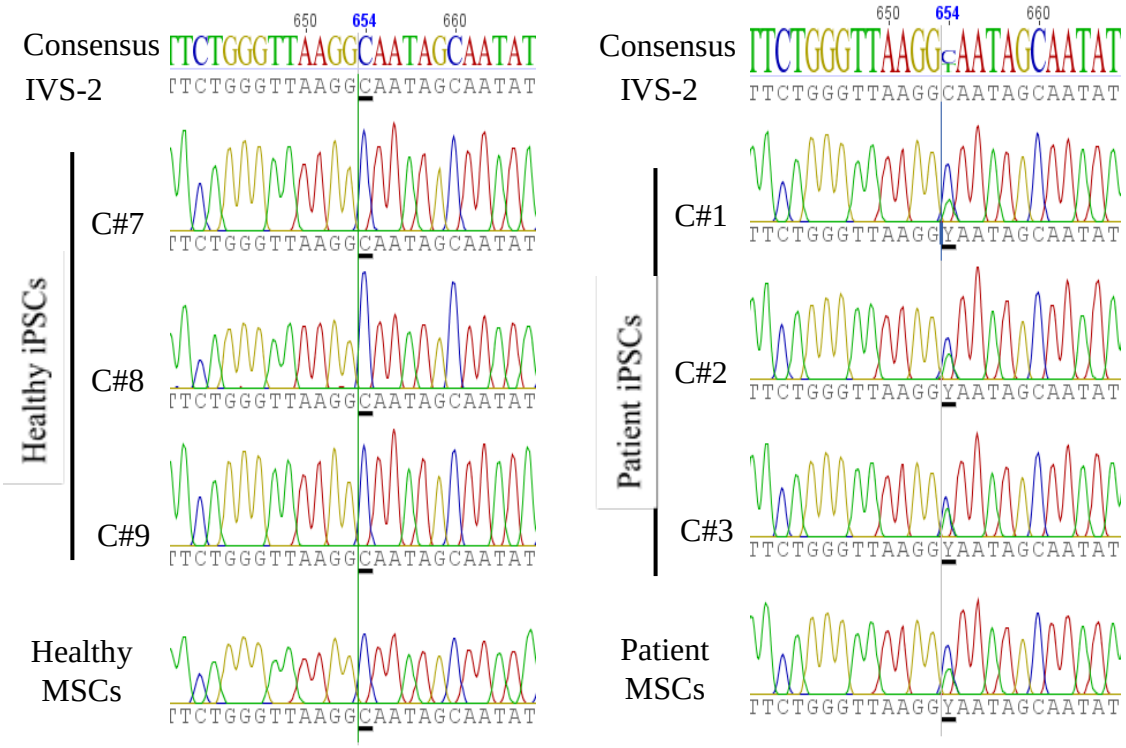

Figure 3.12 Sequencing analyses of intervening sequence-2 (IVS-2) of β-hemoglobin (*HBB*) gene. Healthy MSCs and healthy iPSCs C#7, 8, and 9 exhibited normal cytosine (C) nucleotide at position 654 (underline) of IVS-2 whereas patient MSC and patient iPSCs C#1, 2, and 3 exhibited heterozygous point-mutated allele from cytosine (C) to thymine (T, underline) or IVS-2 654 (C>T) point mutation. Y = C or T.

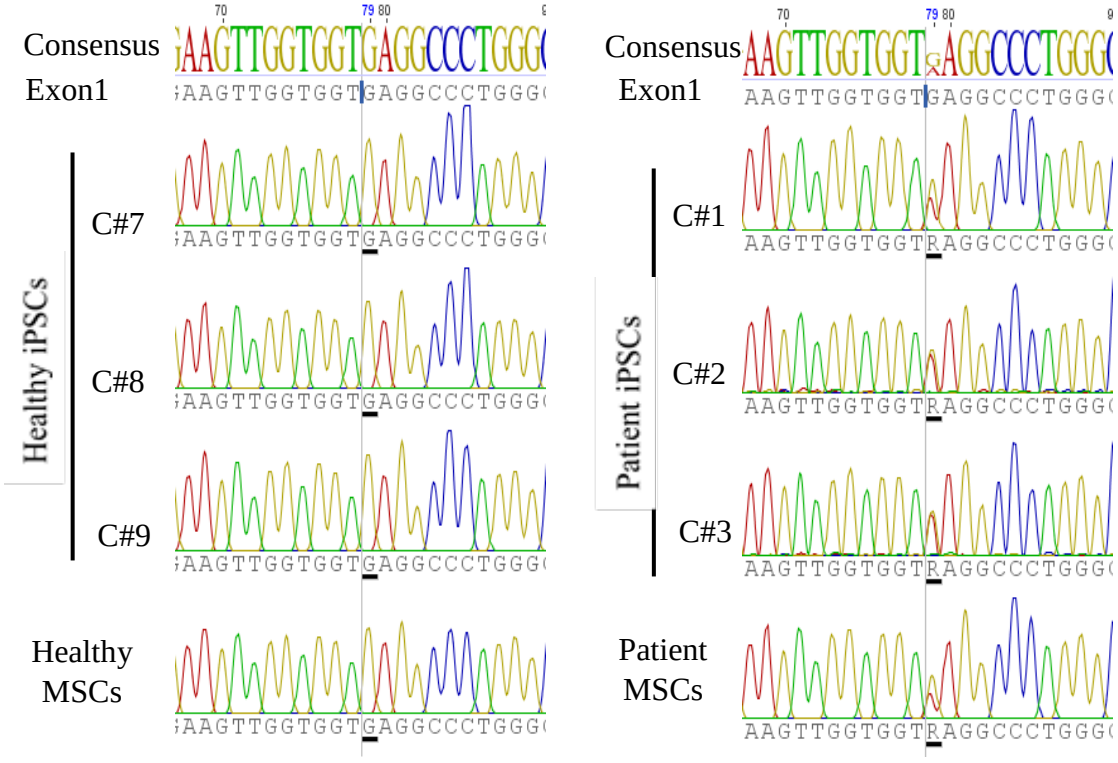

Figure 3.13 Sequencing analyses of exon1 of β-hemoglobin (*HBB*) gene. Healthy MSCs and healthy iPSCs C#7, 8, and 9 exhibited normal guanine (G) at codon 26 (nucleotide position 79, underline) of exon1 whereas patient MSC and patient iPSCs C#1, 2, and 3 exhibited heterozygous point-mutated allele from guanine (G) to adenine (A, underline). R = G or A.

3.2.5 Karyotype

After reprogramming, both healthy and patient iPSCs were demonstrated normal karyotype as shown in Figure 3.14. Therefore, reprogramming processes by lentiviral transduction of OCT4, SOX2, KLF4, c-MYC were not changed chromosomal integrity.

Healthy iPSCs C#7 Healthy iPSCs C#9 Patient iPSCs C#2

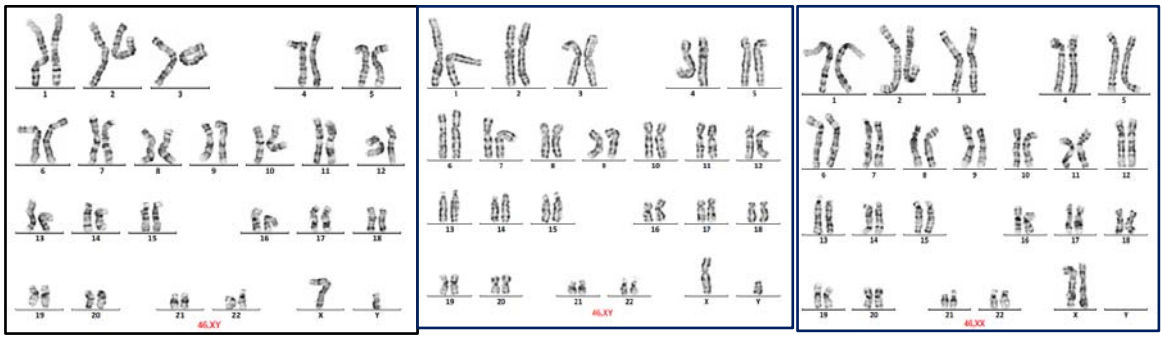

Figure 3.14 Karyotyping analyses for human iPSC lines. Metaphase spread of healthy iPSC C#7, #9 and patient iPSC C#2 exhibited normal karyotype after passage 20.

3.2.6 In vitro differentiation

Healthy and patient iPSCs were capable of differentiate into a represent cell type derived from embryonic three germ layer i.e. endoderm-derived liver cells (α-fetoprotein, Figure 3.15**A, D, G**) , mesoderm-derived smooth muscle cells (α-smooth muscle actin, Figure 3.15**B, E, H**), and ectoderm-derivedneuronal cells (βIII-tubulin, Figure 3.15**C**,**F**,**I**). This characteristic is important shown that the established iPSCs feasibly differentiate into other cell types for thalassemia modeling e.g. erythroid cells.

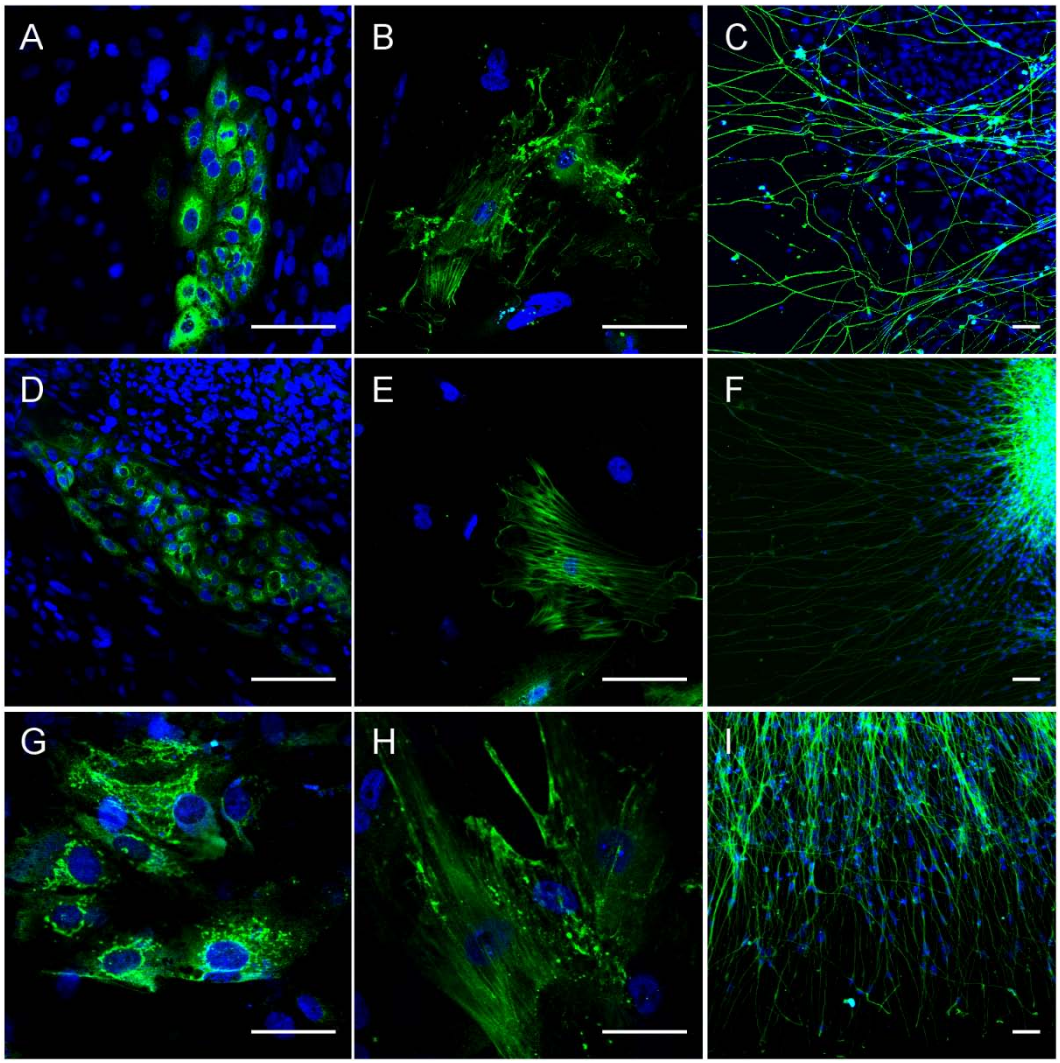

Figure 3.15 In vitro differentiation of human iPSCs. Embryoid body-mediated spontaneous differentiation of healthy iPSCs C#7 (**A-B**), C#9 (**D-E**) and patient iPSCs C#2 (**G-H**) showed immunoreactivities of α-fetoprotein (**A, D, G**) and α-smooth muscle actin (**B, E, H**). Neuronal direct differentiation of healthy iPSCs C#7 (**C**), C#9 (**F**) and patient iPSCs C#2 (**I**) showed reactivity of βIII-tubulin.Nuclei were localized by Hoechst 33342 (blue). Alexa 488 conjugated secondary was used (green). Scale bars, 50 µM.

3.2.7 Teratoma formation

A gold standard for characterization of iPSCs is teratoma formation which indicates fully reprogramming of somatic cells. The healthy and patient iPSCs were formed teratoma after at least 2 months post-transplantation into NUDE mice. All teratomas contain tissues derived from three germ layers. Ectoderm-derived keratinocyte (Figure 3.16 **A**), neural rosette (Figure 3.16 **D, G, J, M**), mesoderm-derived smooth muscle, adipose tissue (Figure 3.16 **B**), cartilage (Figure 3.16 **E, H, K, N**), and endoderm-derived gut like (Figure 3.16 **C, F, I, L**), respiratory like (Figure 3.16 **O**) epithelium could be detected after H&E staining.

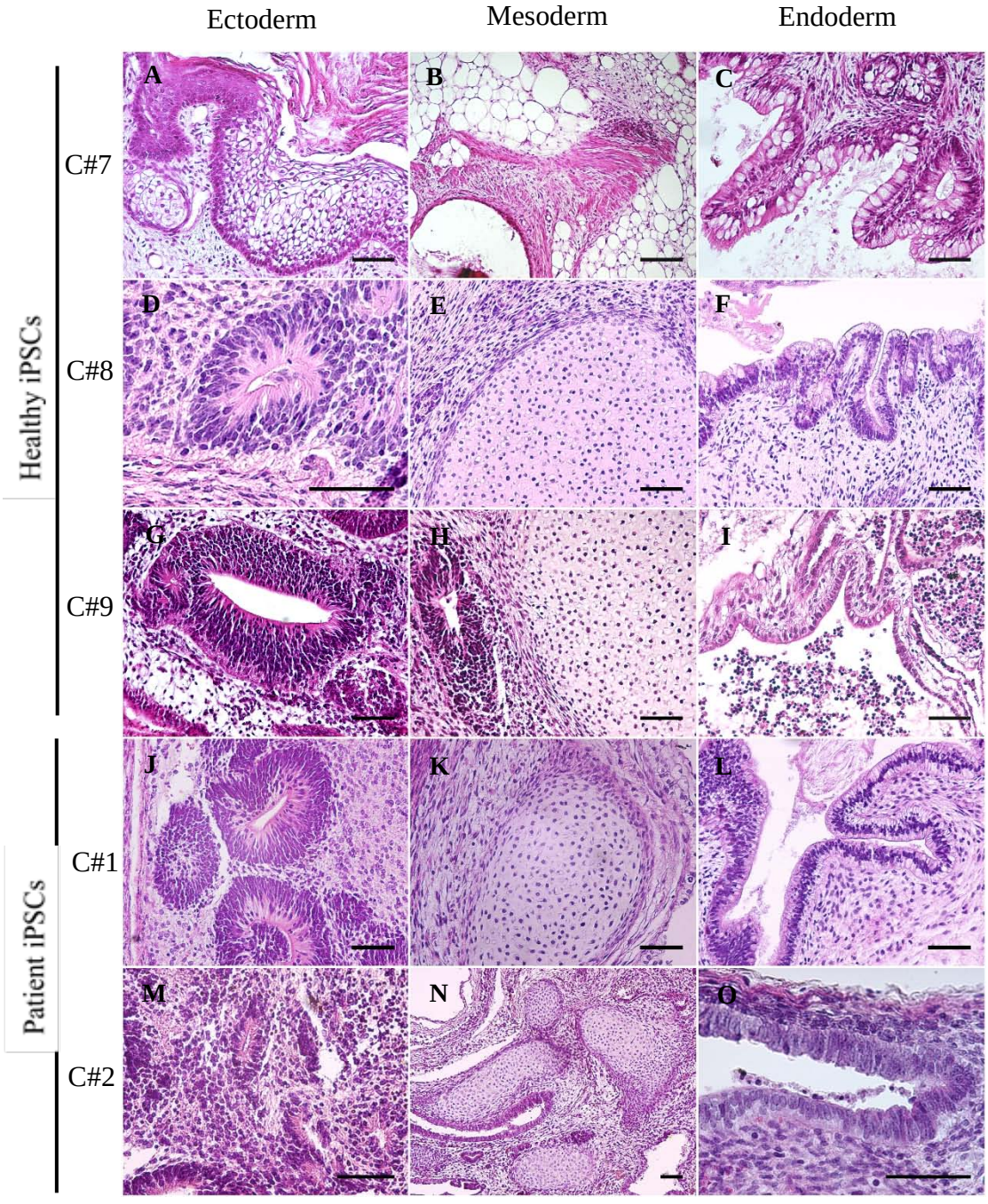

Figure 3.16 Teratoma derived from human iPSCs. Healthy iPSCs C#7, C#8, and C#9 and patient iPSCs C#1, C#2 could produce teratoma containing three germ layers. Ectoderm-derived keratinocyte (**A**), neural rosette (**D, G, J, M**), mesoderm-derived smooth muscle, adipose tissue (**B**), cartilage (**E, H, K, N**), and endoderm-derived gut like (**C, F, I, L**), respiratory like (**O**) epithelium could be detected. Scale bars, 100 μM.

4. References

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5. Outputs:

Suparerk Borwornpinyo, Phetcharat Phanthong, Narisorn Kitiyanant, Natee Jearawiriyapaisarn, Lalana Nuntakarn, Yindee Kitiyanant, Suradej Hongeng. Genetic Correction of Aberrant Splicing of β-Thalassemic Erythroid Cells with IVS-2 654/β^E Derived from Patient-Specific iPS Cells Expressing Antisense U7 snRNA. The ISSCR regional forum meeting, November 5-7, 2014, Singapore.