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Research Article

Robust, Efficient and Pure Induced Mesenchymal Stem Cells Generation from mRNA Induced Pluripotent Stem Cells in Suspension

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ABSTRACT

Mesenchymal Stem Cells (MSCs) are involved in many promising clinical trials tackling vastly complicated diseases. Many factors are determining the safety in these clinical trials such as the purity of tissue-derived MSCs cell population used in therapies. Also, the efficacy of the injected MSCs must be tested in-vitro, before application, through proliferation capacity and reproducibility over continuous passages. In addition to, the importance of choosing the right source of MSCs derivation for successful cellular therapy and transplantation. This study demonstrates robust generation of iMSCs from induced Pluripotent Stem Cells (iPSC) of healthy human donor (with full genetic test done prior) using non-integrative (mRNA) method. This conversion method comprises (i) differentiating a population of iPSCs in suspension without iMatrix, (ii) passaging the cells differentiated in step (i) in the presence of a conditioned MSC medium for a time and under conditions sufficient to produce iMSC in culture for long term with no sign of epigenetic memory.

Analysis of Pluripotent markers expression (Oct-4, SSEA-4, Sox-2, Tra-1-60) was confirmed by flow cytometry and Immunocytochemistry through Fluorescence microscope visual assessment. No teratoma was developed by in-vivo injection of the iMSC population in male hamsters, confirming the transformed purity of iMSCs and the immune-modulating property in culture without iPSC respectively. For cell cycle and senescence studies, pure in-vitro iMSCs were tested using flow cytometry using CD73, 90 and 105 expression analysis and compared with UC-MSC. Later, iMSCs demonstrated tri-differentiation of chondrocytes, osteocytes and adipocytes relative to UC-MSCs, which could make it possible to address the drawbacks of using adult MSCs and thus provide a valuable tool for future use in various clinical settings.

Keywords: Mesenchymal stem cells (MSCs); Induced pluripotent stem cells (iPSCs); Human iPSCs (hiPSCs); Induced MSC (iMSCs); Senescence; Cell cycle

INTRODUCTION

Induced Pluripotent Stem Cells (iPSCs) were first established in 2006 [1-4]. Later, human iPSCs were successfully derived by Thompson and many other groups [1]. In past years, integrative method was initially employed to deliver reprogramming factors for iPSC generation [1-3]. These methods had the potential to produce tumorigenic insertional mutations and residual or reactivation of transgene expression during iPSC differentiation [2-6]. To overcome these problems, various methods were explored to derive transgene-free iPSCs, such as plasmid vectors, minicircle DNA vectors, piggyBac, mRNA, adenovirus, Sendai virus, proteins, small molecules and episomal [7-20].

Expression of mRNA reprogramming factor provides another wa y of making transgene-free iPSCs. It has been shown that mRNAs transcribed *in vitro* ca

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n express reprogramming factors efficiently when transfected into human fibroblasts [6].

The mRNA is immediately converted into proteins by ribosomes after the delivery of synthetic mRNA into the cytosol and no entry into the nucleus is needed.

Induced MSCs (iMSCs) derivation and characterization from iPSCs are on the rise. When applied to a range of animal models, iMSCs have been shown to promote regeneration and healing; multiple sclerosis, limb ischemia, arthritis, liver damage, bone defects, wound healing, and brain hypoxia [10-35].

The primary human bone marrow Mesenchymal Stem Cells (MSCs) comprise a sub-population of multipotent stem cells that hold the capacity for osteogenic, chondrogenic and adipogenic differentiation [36-42]. Such multipotent MSCs are isolated from fetal femur in addition to adult sources [43]. MSCs offer significant benefits because of their highly proliferative, immune-modulatory properties and paracrine orchestration is therapeutic potential for an increasing aging demographics [32-48].

MSCs distinct from iPSCs (iMSCs) is a cell type derived from iPSCs that are of primary interest to bypass shortcomings associated with primary MSCs. It has already been shown the similarity of iMSCs to primary MSCs and their *in vivo* regenerative capacity [25-39]. In addition, donor age expression in iMSCs has been shown to be restored to a younger state and expressed in iMSCs from patients with early-onset aging syndromes [49-52]. iPSC-derived iMSCs are identified as a potential source of transplantable donor cells for regenerative therapies (Figure 1).

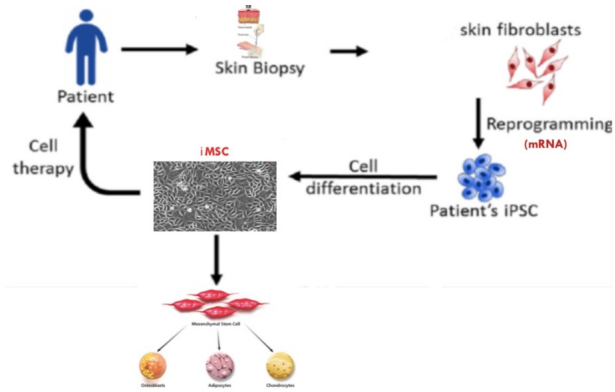

Figure 1: iMSC derived from mRNA iPSC can be a good source for cell therapy.

The benefit of using iMSCs is that they can be generated with known HLA types from well-characterized and banked iPSCs. Another benefit of iMSCs is that they have been described as rejuvenated MSCs over their native counterparts. Although they are derived from pluripotent cells (which are tumorigenic by definition), iMSCs themselves are free from the possibility of tumor formation as a result that does not express oncogenic pluripotency-associated genes such as OCT4 [40-54].

In this research, we showed that iPSC was made from skin fibroblasts using the 3rd generation mRNA method in a private clinic and subsequently differentiated to pure iMSCs without iPSC in culture using a suspension process. Such iMSCs are contrasted with UC-MSC in cells based markers, cell cycle, senescence tests, and in-vitro differentiation.

MATERIAL AND METHODS

Ethical approval

Skin samples were obtained from a healthy volunteer donor (Full genetic test was conducted prior to the biopsy) from 38 yrs old male (human), with written permission from the Stem Cell 21 Ethics Committee, Bangkok (Thailand).

Fibroblasts culture

Fibroblasts have been isolated from 4 mm Healthy donor skin biopsy, which has been fully screened for any genetic mutations or abnormalities. The skin was chopped using sterile surgical instruments and plated in six-well dishes with a medium xeno-free fibroblast plate (FP) (Fibro-life Cat. No: LM-0001) and was grown for 14 days in a 5% CO_2 incubator at 37°C [4].

Reprogramming fibroblasts by mRNA

Skin fibroblasts of 60000 cells were reprogrammed using mRNA 3rd generation Reprogramming Kit (Stemgent Cat. No: 00-0076) according to manufacturer instructions. iPSC, were expanded under xeno-free conditions on iMatrix (Reprocell Cat. No: NP892-011) with Nutristem medium (Reprocell Cat. No: 01-0005) 5% CO_2 at 37°C as described previously [6].

Alkaline phosphatase staining

Putative iPSC colonies were tested for alkaline phosphatase (AP) using a diagnostic AP substrate kit according to the manufacturer's specification (Stemgent Cat. No: 00-0055).

Immunofluorescence staining

For immunocytochemistry, colonies were fixed and examined for OCT-4, SSEA-4, SOX-2 and TRA 1-60 using kit (Invitrogen Cat. No: A24881 and A25526).

A brief protocol using the above anti-bodies from the kit:

- Remove media from the cells
- Add fixative solution and incubate for 15 minutes at room temperature
- Remove fixative solution
- Add permeabilization solution and incubate 15 minutes at room temperature
- Remove permeabilization solution
- Add blocking solution and incubate 30 minutes at room temperature
- Add desired primary antibody (see Kit for co-staining options) directly to the Blocking Solution covering the cells to yield a 1X final dilution, mix gently, and incubate for 3 hours at 4C
- Remove the solution. Add wash buffer (diluted to 1X with water) and wait for 2-3 minutes. Repeat the wash procedure 2 more times so that the cells are washed a total of 3 times

- Remove the third Wash buffer and add the appropriate Secondary Antibody (diluted to 1X in Blocking Solution; as listed in Kit) and incubate for 1 hour at room temperature
- Remove the solution. Add Wash buffer (diluted to 1X with water) and wait for 2-3 minutes. Repeat the wash procedure 2 more times so that the cells are washed a total of 3 times
- Add 1-2 drops/mL of NucBlue™ Fixed Cell Stain (DAPI) into the last wash step and incubate for 5 minutes
- Image the cells immediately or store cells at 4C in the dark, wrapped with parafilm to prevent the samples from drying out, for up to 1 month

Furthermore, control cell lines with excluded primary antibodies were used as negative controls and the cells under a fluorescence microscope were examined (Nikon Eclipse TS2)

Embryoid bodies formation

The *in vitro* differentiation ability of the iPSCs was analyzed by spontaneous differentiation, according to our previous protocol. First, dissociated five hundred thousand iPSCs were cultured in ultralow adhesive plates (Costar 6 well plate Corning Lot. No 33818025) to each well with embryoid body induction medium (DMEM, 20% FBS, Penicillin/Streptomycin, 100XGlutamax, 2-β mercaptoethanol, and 100X Non-Essential Amino Acids) for 7 days to see spheroids with no medium change [4-8].

Flow cytometry

iPSCs were dissociated for 2-4 min at RT using Accutase (Innovative Cell Technologies), centrifuged for 5 min at 300 rcf and resuspended in PBS+2% FBS. At 1:800 dilutions, OCT-4, SSEA-4, PE-conjugated, antibody was added and incubated at RT for 20 min. SSEA-1 was used at the same final concentration ($3.12=10^{-5}$ mg/mL) as a negative command. A CytoFLEX flow cytometer (Beckman Coulter) and CytExpert 2.0 program analyzed the cells.

Freezing and thawing of iPSCs

Once cells reached a 70-80 percent confluence, the spent medium was removed and trypsin was used to raise the cells. The cells were then incubated for 4 min at 37°C and moved gently into conical tubes, leaving the cells in clumps. The cell suspension was centrifuged and a commercial (Cryofreeze medium) freezing solution resuspended the pellet. The suspension of cells was aliquoted into cryovials and positioned at -80°C in a cryofreezing tank. The cells are moved to a liquid nitrogen tank for long-term storage following overnight storage at-80°C. The cryovial was submerged in a 37°C water bath for 1-2 min for thawing and regeneration of cells. The cells were then gently moved to a conical tube of 15 ml containing Nutristem pre-warmed medium. The cells are centrifuged and supplemented with the pellet resuspended in 2 ml of Nutristem water. The cells are grown on 6-well plates covered by iMatrix. The next day, the cells were refreshed with fresh Nutristem medium. The medium was replaced daily thereafter until the cells are approximately 80% confluent. To determine the efficiency of the cell recovery following cell thawing, images were captured by phase-contrast microscopy at regular intervals [2-6].

iPSC differentiation to iMSC

iPSC differentiation to iMSC was done by using Minimum Essential Medium, Alpha 1X (Cat.No: 15-012-CV, Corning, USA) 500ml, 1% 100X Glutamax (Cat.No: 35050-061, Gibco, Japan), 1% 100X MEM Nonessential amino acids (Cat.No: 25-025-CL, Corning, USA).7% Embryo-max Fetal Bovine Serum, ES qualified (Cat.No: ES-009-B, Merck, Germany) and 2% 100X Penicillin/Streptomycin (Cat.No: 30-002-CL, Corning, USA),in suspension with no iMatrix plated and the medium was changed every day for 5 days till passage 1.

Umbilical cord MSC

Three Umbilical cord MSCs from 3 healthy individuals (UC382757, UC75259, UC5414502) were isolated and were stored for research purposes by the consent of the patients at Stem Cell 21 clinic, Bangkok. Thailand.

Cell cycle

iMSC cells and UC-MSCs were analysed for cell cycle stages by using EZcell Kit (Bio Vision, Cat.No: K290-100 and M1-86096) at P2, P3, and P4.

Brief cell cycle Protocol

Sample preparation: Grow cells of interest (2-5 × 105 cells/well) in desired medium and culture conditions preferably in 6-well plates for 24 hr prior to the experiment. Synchronize cells with culture medium containing 0.1% FBS for 24 hr. Treat cells with test compounds in culture medium containing 10% FBS for 4-24 hr. As controls, incubate cells of interest in culture medium with 10% FBS without any test compound. Harvest cells and centrifuge at 400 xg for 5 min. Remove the supernatant and wash cells in 2 ml ice-cold 1X Cell Cycle Assay Buffer, centrifuge cells at 400 xg for 5 min., remove the supernatant and save the cell pellet.

Nucleic acid labelling: Fix the cells by adding 2 ml ice-cold 70% ethanol (add drop by drop while vortexing) to the cell pellet, put on ice for at least 30 min. Centrifuge cells at 400 xg for 5 min. and carefully remove the supernatant. Wash cells in 2 ml of 1X Cell Cycle Assay Buffer, centrifuge cells at 400 xg for 5 min. and carefully remove the supernatant. Re-suspend cells completely with 500 µl of Staining Solution, protect from light exposure. Incubate at RT for 30 min.

Senescence

iMSC and UC-MSCs were analysed for senescence by using Millipore kit (Cat. No: KAA002RF) at P2, P3, P4, P5

A brief summary of the protocol:

Cells preparation: Collect 1 × 106 cells for seeding into 3 wells of 6 well-plate with seeding density of 3.33 × 105 cells per well.

- SA-β-Gal staining procedure:
- Aspirate the medium from the cells
- Wash cells once with 2 mL D-PBS and aspirate the wash
- Add 1 mL 1X Fixing Solution per well and incubate at room temperature for 10-15 minutes
- Remove 1X Fixing Solution and wash it twice with 2 mL D-PBS
- Add 2 mL of SA-β-Gal Detection Solution per well and add 2 mL D-PBS for -VE well

- Incubate at 37C without CO by wrapping the plate with parafilm, cover with foil and put it in incubator at least 4 hours
- Remove SA- β -Gal Detection Solution and wash it twice with 2 mL D-PBS
- Count the blue-stained cells under light microscopy
- (Optional) For long term storage, overlay the stained cells with 70% glycerol-diluted in 1X D-PBS and store at 4-8C

In-vivo injection of iMSC to Hamster

Protocols for animal studies have been accepted by the Institutional Animal Care and Use Committee of Stem Cell 21, Bangkok (Thailand). Male hamsters (n=3) were injected with 1 million iMSCs using intramuscular (quadriceps muscle) process, i.e. (1 million iMSCs dissolved in PBS to 200 µ L final volume) and control group (n=1) (only PBS to 200 µ L volume). This study was conducted to test any teratoma formation due to iMSCs.

Tri-lineage differentiation of UC-MSC and iMSC

Adipogenesis:

Seed 1 × 104 cells/cm2 into a 24 well plate and add 0.5 ml of MEM+

For 24 well plate, the seeding density would be 19000 cells per well

Since there will be 2 experimental well and 1 negative control well, the total cells needed is 57000 (5.70E+04) cells per specimen

Incubate the cells for one day

Replace MEM+ with complete adipogenesis differentiation medium

Exchange media in the cultures every 3-4 days

After 7 days (7-14 days) of culture, perform Oil Red O staining assay as follows:

- Remove media from the 24 well plate
- Rinse the plate with DPBS once
- Fix the cells with 4% formaldehyde solution for 30 minutes
- Rinse with distilled water
- Treat the cells with 60% isopropanol for 5 minutes
- Then, incubate with the Oil Red O staining solution for 5 minutes
- Rinse with distilled water
- Cells can be observed under a light microscope

Osteogenesis:

- Seed 5 × 103 cells/cm2 into a 24 well plate and 0.5 mL add MEM+
- For 24 well plate, the seeding density would be 9500 cells per well
- Since there will be 4 experimental wells and 2 negative control well, the total cells needed is 57000 (5.70E+04) per specimen

For alizarin red S staining:

Incubate the cells for one day

Replace MEM+ with complete osteogenesis differentiation medium

Exchange media in cultures every 3-4 days

After 7-14 days of culture, perform Alkaline phosphatase staining on 2 experimental wells as follows:

- Remove media from 24 well plate
- Rinse the plate with 0.5 mL PBST (prepared by adding 10ml of PBS with 5 µl of Tween) once
- Fix the cells with 0.5 mL fixing solution
- Incubate the cells in the dark for 5 mins
- Prepare the staining solution by adding equal amounts of solution A, B and C, which is 0.3 ml
- Once the cells are fixed, remove the fixing solution and wash with 0.5 mL PBST
- Remove the PBST and add 0.6 ml of staining solution
- Incubate in dark for 5-15 mins
- Once, the cells have stained red, stop the reaction by adding 1 mL of PBST.
- Observe the cells under the microscope

After 21 days of culture, perform Alizarin Red S staining assay as follows:

- Remove media from the 12 well plate
- Rinse the plate with DPBS once
- Fix the cells with 4% formaldehyde solution for 30 minutes
- Rinse twice with distilled water
- Stain the cells with Alizarin Red S staining solution for 2-3 minutes
- Rinse thrice with distilled water
- Cells can be observed under a light microscope

Chondrogenesis:

Prepare cell solution of 1.6 × 107 viable cells/mL

Seed 5 µ L (80,000 cells) droplets of cells solution into centre 24 well plate

Since there will be 2 experimental wells and 1 negative control well, the total cells needed is 960,000 (9.60E+05) per specimen

Incubate the cells for 2 hours

Add 0.5 mL of pre-warmed complete chondrogenesis media to the wells

Exchange media in cultures every 2-3 days

After 14 days of culture, perform Alcian Blue staining assay as follows:

- Remove media from the 12 well plates
- Rinse the plate with DPBS once
- Fix the cells with 4% formaldehyde solution for 30 minutes
- Rinse with DPBS
- Stain the cells with 1% Alcian Blue solution prepared in 0.1N HCL for 30 minutes
- Rinse wells 3 times with 0.1 N HCL
- Add distilled water to neutralize the acidity
- Cells can be observed under a light microscope

RESULTS

Human foreskin fibroblasts were expanded at low passages and reprogramed using mRNA Reprogramming Kit (Reprocell). Individual colonies were picked and subcultured into individual cell lines after 10-15 days and analyzed at cellular and genetic level to confirm successful reprogramming. After 25 days generated colonies displayed a typical human Embryonic Stem Cell (hESC) colony-like morphology with refractive edges as seen by bright field (BF) and phase-contrast (PC) microscopy and the cells had high nuclear/cytoplasmic ratio.

Once infected four times fibroblasts with mRNA, the spindle-like morphology changed to small compact cells similar to pluripotent cells Figure 2. A red stain assay called alkaline phosphatase has confirmed the pluripotent reprogramming of fibroblasts using the mRNA method.

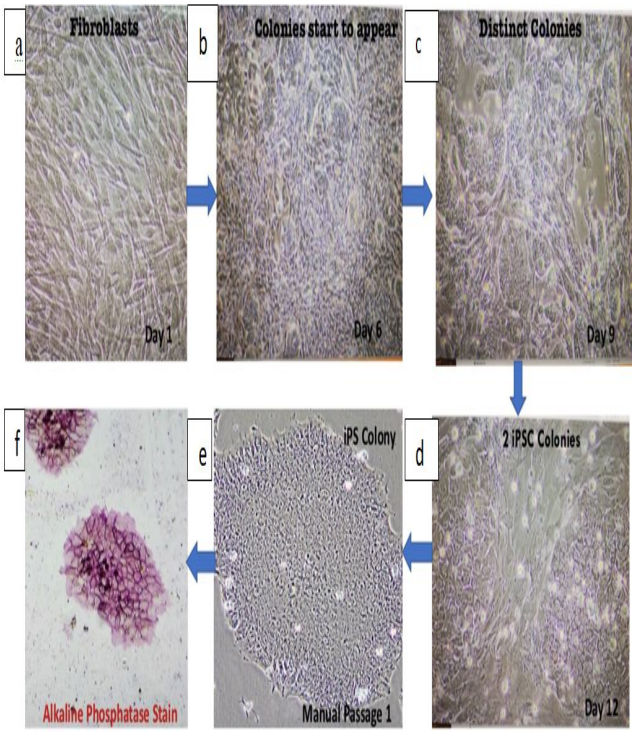

Figure 2: (a): Morphology of Fibroblasts is like spindle shape; (b-d): whereas iPSC lines generated from fibroblasts have well-defined edges; (e): densely packed and lacked differentiated cells; (f): iPSC colonies stained red with Alkaline phosphatase assay. Pluripotency was additionally assessed by immunocytochemistry.

Figure 3 displays bright field picture of iPS cells that were then used as surface markers for fluorescence staining of (i) OCT-4, SSEA-4 as green/red fluorescence and (ii) SOX-2, TRA 1-60 as green/red Fluorescence. To illustrate the viability of iPS cells, DAPI stained the nuclei. The combined merge image showed the distinct nuclei and cytoplasmic ratio. No color stain was detected in negative control with no primary antibody added.

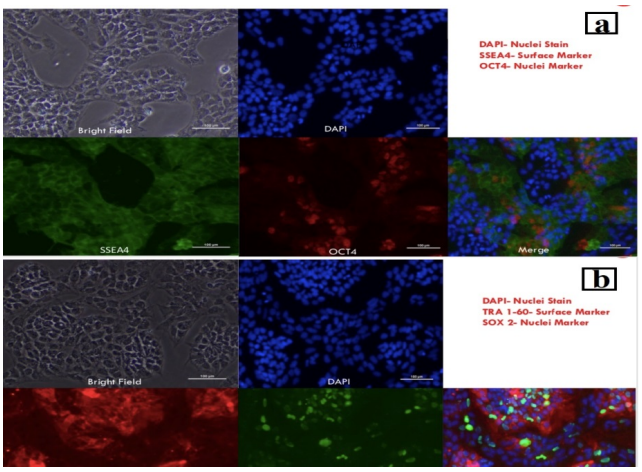

Figure 3: Expression of pluripotent stem cell-specific markers. The expression of pluripotent stem cell-specific markers was analysed using specific antibodies and fluorescence microscopy. iPSCs obtained by mRNA-based reprogramming showed a strong expression of (a) Oct4, SSEA-4, (b) Tra 1-60 and Sox2, having DAPI as a nuclei marker and Bright Field as colony appearance. Nuclei and cytoplasmic stain were merged along with DAPI in (a) and (b) simultaneously.

Figure 4 indicates that the flow cytometry test for OCT-4 is 90% positive as a nuclei marker and SSEA-4 is 99% as a surface marker, with SSEA-1 being negative at P1. It shows the high expression of fibroblasts reprogrammed with characteristics of pluripotency.

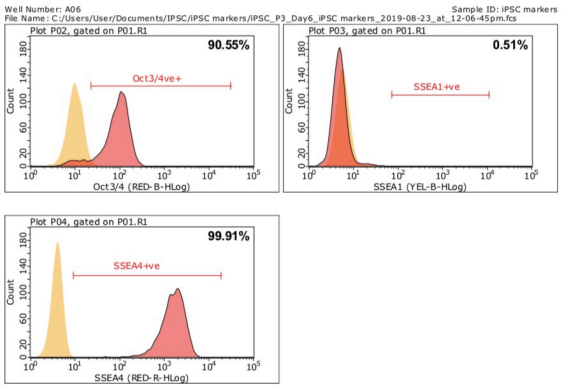

Figure 4: Additionally, Oct-4 (90% cells positive) and SSEA-4(99% cells positive) expression in iPSCs were also analysed by flow cytometry as a right shift (in Red) on the plot graph and having SSEA-1 as a left shift in orange (negative control). Both human stem cell markers were highly expressed in iPSC line generated by mRNA-based reprogramming at Passage 2.

A large number of EBs (Figure 5) on a bacteriological grade Petri-dish, ultra-low adherence plate or Petri-dish coated with a cell adherence inhibitor such as poly2-hydroxyethyl methacrylate (poly 2-HEMA) were developed by iPSC seven days in culture with the embryoid medium in static suspension culture, allowing spontaneous aggregation of cells into spheroids.

Although simple, this approach allows for little control over the size and shape of EBs. The result is frequent agglomeration of EBs into wide, irregular masses due to the likelihood that iPSC would encounter each other unintentionally.

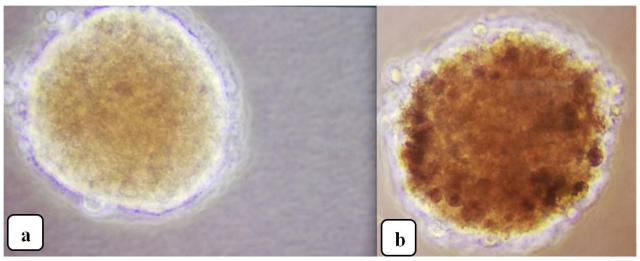

Figure 5: Formation of distinct circular Embryoid bodies (a) and (b) *in vitro* to show in future its differential potential to 3 germ layers (ectoderm, endoderm and Mesoderm).

To differentiate iPSCs from MSCs, we transferred iPS cells from iPS medium to medium consisting of Apha-MEM (Biological Industries), 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% FBS (Gibco), as previously described. At 24 hrs, 48 hrs, and 72 hrs Figure 6, the iPSCs started to lose the standard iPSC morphology and acquired a spindle-shaped morphology at the colonial border at Passage 1. The differentiated iPSCs were transferred after two weeks of differentiation, resulting in a complete morphological change of the cells to a fibroblastic form after three passages.

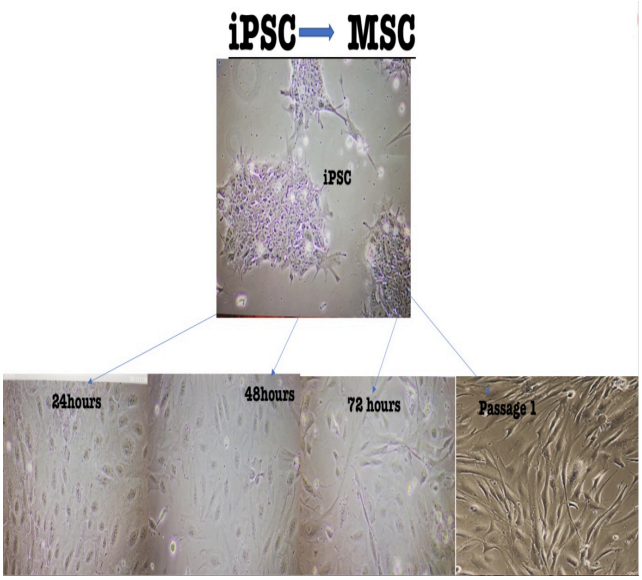

Figure 6: 1 million iPSCs were plated in suspension in T75 flask along with MSC medium and showed morphology of MSC after 24 hr, 48 hr, 72 hr and at Passage 1.

At Passage 1 UC-MSC and iMSCs were analyzed by flow cytometry for human mesenchymal markers (CD90, CD73, CD105) to investigate if the fibroblastic-like cells differentiated from iPSCs were iMSCs as shown in Figure 7. Furthermore, as shown in the flow cytometry graph, the percentage of positive MSC markers simultaneously increased in iMSCs from P1 to P5, showing robust potential of MSCs. Figure 8 shows that iMSC are 99 percent positive for CD 105, CD 90 and CD 73 compared to three UC-MSCs from P2-P5.

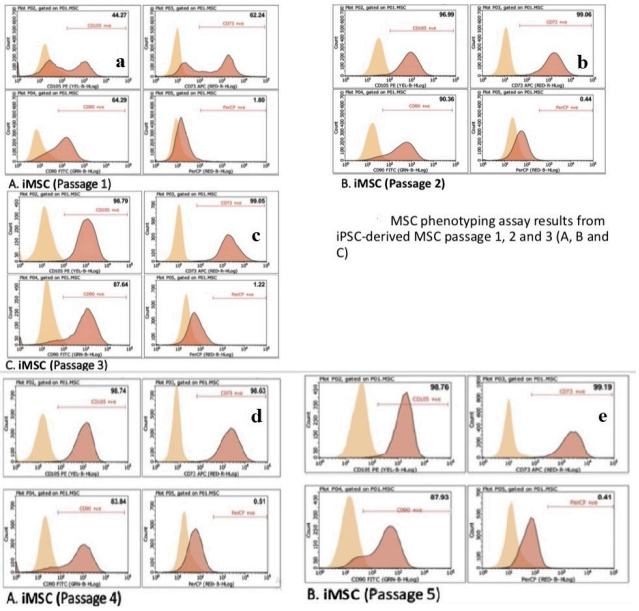

Figure 7: The flow cytometry was performed to test multi-potent markers for iMSCs at (a): Passage 1; (b): P2; (c): P3; (d): P4 and (e): P5. For positive CD 105, CD 90, and CD 73, all iMSCs show the right shift as red color in the plot graph and the left shift as negative in orange in all passages.

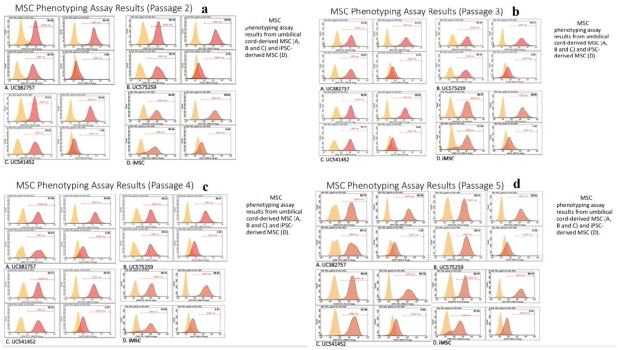

Figure 8: Flow cytometry was performed to test multipotent markers for UC-MSCs (3 different samples) and iMSCs at (a): Passage 2; (b): P3; (c): P4 and; (d): P5. Show the right shift as red color in the plot chart for CD 105, CD 90, and CD 73 positive, and the left shift in orange as negative.

Figure 9 shows a comparative percentage from P2-P5 of G0/G1 cell stage among the three UC-MSC vs iMSC samples and the graph with iMSC showing the highest percentage in all passages compared to UC-MSC. The G0 phase is a time in a quiescent state in the cycle of cells. The G0 phase is either considered as an extended G1 phase in which the cell does not divide or plan to divide, or as a separate quiescent phase outside the cell cycle. The comparative analysis graph shows iMSC's ability to convert to G0/G1 cells more robust than UC-MSC.

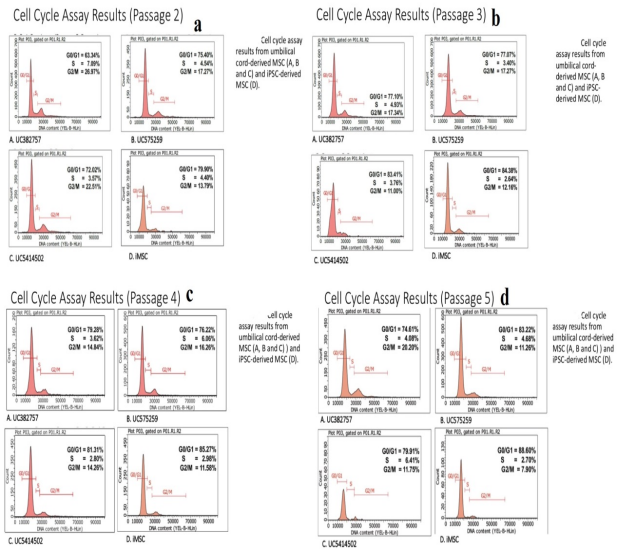

Figure 9: Overall comparison of cell cycle stages and percentages of G0/G1, S, G2/M between UC-MSC (3 different samples) and iMSC at (a): Passage 2; (b): P3; (c): P4 and (d): P5 in a flow cytometry analysis graph.

Figure 10 shows that the quality of iMSC is higher than that of UC-MSCs without blue stain cells, while all figures showed senescence in blue stain in UC-MSC as marked in circles. The classic characteristics of MSC's senescence phenotype include halting cell cycle growth in the G1 stage, prolonged or flattened morphology, increased senescence-associated β-galactosidase (SA-β-gal) and senescence-associated expression of α-L-fucosidase (SA-α-Fuc) and surface marker modification. Senescence marker, SA-β-gal, is commonly used for assaying cells. Figure 11 shows (i) Positive aggregation of lipid droplets as seen by Alizarin Red stain showed UC-MSC and iMSCs for adipogenic differentiation. (ii) UC-MSCs and iMSCs exposed to chondrogenic differentiation medium have been positive for glycosaminoglycan cartilage as detected by Alcian Blue staining. (iii) UC-MSC and iMSC are full of mineral accumulation as detected by Alizarin Red stain for osteogenic differentiation.

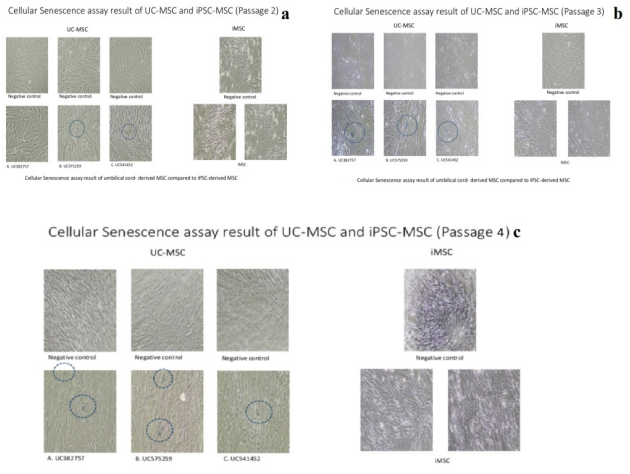

Figure 10: Cell Senescence comparative study between UC-MSC (3 different samples) iMSC at (a): Passage 2; (b): P3; (c): P4 using blue-colored SA-β-gal assay as shown in the circle.

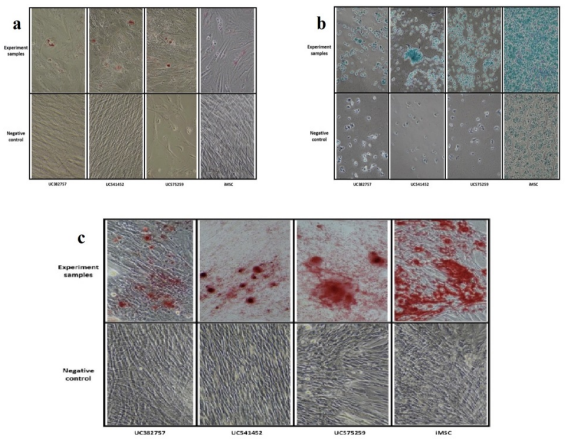

Figure 11: Tri-lineage differential potential of UC-MSC (3 different samples) and iMSC to (a): adipocytes in red dots using Alizarin Red staining; (b): chondrocytes in blue color using Alcian Blue staining; (c): Osteocytes in red aggregates using Alizarin Red staining at Passage 2 along with negative control at Passage 2.

DISCUSSION

Over the past few years, remarkable progress has been made with virus-and/or vector-free methods in generating clinically compliant and safer human iPSCs. The iPSCs we generated by mRNA reprogramming closely summarized the characteristics of human ESCs and are comparable to the iPSCs produced from other groups using the same process. This study further showed that these iPSCs are an attractive stem cell source for deriving robust mesenchymal stem cells towards clinical applications.

The healthy donor foreskin fibroblasts were expanded at low passages and reprogramed using mRNA Reprogramming Kit (Reprocell). Individual colonies were picked and sub-cultured into individual cell lines after 15-20 days and analysed common validation steps to confirm successful reprogramming. After Passage 1, the colonies showed a typical human Embryonic Stem Cell (hESC) colony-like morphology with refractive edges as seen by microscopy of the Bright Field (BF) with high nuclear/cytoplasmic ratio of the cells. The pluripotency markers OCT-4, SOX2, SSEA-4 and TRA-1-60 and the flow cytometry for the SSEA-4 pluripotency marker were further evaluated by immunocytochemistry. It is established that alkaline phosphatase (AP) is more involved in hiPSCs and the colorimetric assay depicting its operation indicated that the colonies selected for hiPSC are indeed pluripotent. Without positive results, the mycoplasma was checked regularly. Embryoid body formation *in vitro* was shown to test the hiPSC line potential to generate derivatives of three germ layers.

The human iMSCs we generated were approximate 99% positive to CD 105, CD 90, and CD73 recapitulating mesenchymal stem cell markers, making them capable of differentiating into specific lineages such as osteocytes, adipocytes, and chondrocytes.

iMSCs, when injected into the hamster hind leg, did not show any teratoma formation, and hence showed the same characteristics of been immune-modulant as UC-MSC. The

comparative analysis between UC-MSC also showed its superior characteristics in terms of numbers, cell cycle and senescence from early to late passages. Furthermore, this is the first report that shows robust, active and pure iMSC generation using cell suspension method (no iMatrix used) without iPSC contamination, tested by adding parallel iPSC medium to each iMSC culture passage. Consequently, we assume these features would strengthen them for future clinical applications.

CONCLUSION

Takahashi and Yamanaka's discovery of iPSCs is really a decade-long breakthrough in stem cell science. The last decade has seen tremendous improvement in our understanding of induced pluripotency molecular mechanisms, and in 2014 we moved from the "bench to bedside."

The latest long-term study involving the application of dopaminergic neurons in primate derived from human iPSC at the Center for iPS Cell Research and Development, Kyoto University, Japan, primate Parkinson's disease (PD) models shows that human iPSCs are medically relevant to the care of PD patients. The cell therapy based on the iPSC is still in its infant stage. The remaining obstacles that block the path to effective clinical therapy implementation of this technology must be resolved.

The footprint-free iPSCs obtained through mRNA-based reprogramming are promising cells for clinical application to generate desired cell types. Highly efficient footprint-free iPSC generation and effective differentiation into iMSC will increase this technology's potential in translational research, therapy, and disease modeling.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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