Induction of Pluripotency in Human Umbilical Cord Mesenchymal Stem Cells in Feeder Layer-Free Condition

ARTICLE in TISSUE AND CELL · APRIL 2015
Impact Factor: 1.25 · DOI: 10.1016/j.tice.2015.04.005

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PII: S0040-8166(15)00036-1
DOI: http://dx.doi.org/doi:10.1016/j.tice.2015.04.005
Reference: YTICE 941

To appear in: Tissue and Cell

Received date: 10-2-2015
Revised date: 9-4-2015
Accepted date: 13-4-2015

Please cite this article as: Daneshvar, N., Abdullah, R., Shamsabadi, F.T., Moeini, H., Mehrboud, P., Rahman, H.S., Borojerdi, M., Vellasamy, S., Induction of Pluripotency in Human Umbilical Cord Mesenchymal Stem Cells in Feeder Layer-Free Condition, Tissue and Cell (2015), http://dx.doi.org/10.1016/j.tice.2015.04.005

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Highlights:

- We optimize the best ratio of plasmid to Lipofectamine LTX to transfect UC-MSCs.
- We utilized a non-viral Minicircle vector to generate UC-MSC-derived iPSCs.
- We obtain iPSCs from UC-MSCs through a feeder-free method.
- We successfully examined the pluripotency of the iPSC through EBs formation assay.
Induction of Pluripotency in Human Umbilical Cord Mesenchymal Stem Cells in Feeder Layer-Free Condition

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Induced Pluripotent Stem Cells (iPSCs) has been produced by the reprogramming of several types of somatic cells through the expression of different sets of transcription factors. This study consists of a technique to obtain iPSCs from human umbilical cord mesenchymal stem cells (UCMSCs) in a feeder layer-free process using a mini-circle vector containing defined reprogramming genes, Lin28, Nanog, Oct4 and Sox2. The human MSCs transfected with the minicircle vector were cultured in iPSCs medium. Human embryonic stem cell (ESC)- like colonies with tightly packed domelike structures appeared 7 to 10 days after the second transfection. In the earliest stages, the colonies were green fluorescence protein (GFP)-positive, while upon continuous culture and passage, genuine hiPSC clones expressing GFP were observed. The induced cells, based on the ectopic expression of the pluripotent markers, exhibited characteristics similar to the embryonic stem cells. These iPSCs demonstrated in vitro capabilities for differentiation into the three main embryonic germ layers by embryoid bodies formation. There was no evidence of transgenes integration into the genome of the iPSCs in this study. In conclusion, this method offers a means of producing iPSCs without viral delivery that could possibly overcome ethical concerns and immune rejection in the use of stem cells in medical applications.
**KEYWORDS:** Induced Pluripotent Stem Cells (iPSCs); Umbilical cord mesenchymal stem cells; Embryoid bodies

1. **Introduction**

Induction of pluripotency in differentiated human cells to generate induced Pluripotent Stem Cells (iPSCs) is a relative new development in stem cell technology. The iPSCs provide a mean of deriving patient-special stem cells that would overcome ethical issues and immune rejection that plagues embryonic stem cell (ESC) applications [1, 2]. The key challenges in iPSCs development are identification of the useful techniques to transfer transcription factors in the reprogramming procedure, accessibility of various types of cells to be used, and determination of the ideal culture environments to generate iPSCs [3]. Earlier methods in iPSCs development used retroviral constructs or constitutive lentiviral transduction techniques, which have been condemned for the possible transgenes integration into the host DNA [4-6]. For that reason, there is a need to develop integration-free techniques in cell reprogramming. Current reprogramming methods use integration- free adenoviruses and reprogramming through excisable transposons, and non- viral vectors. Although these techniques overcome most of the problems caused by manifold proviruses on the genomic DNA of generated iPSCs, insertional mutagenesis may still occur. Several types of cells including neural progenitor cells and stomach cells may have been effectively reprogrammed [7, 8]. However, there is still need for several technical factors to be resolved, including elimination of genetic abnormalities, ease of induction, and determination of an absolutely ideal cell type before the technique can used in clinical applications [9].
Mesenchymal stem cells (MSCs) are interesting, multipotent stromal cells with great capability of differentiation into a large number cell types, including cartilage and fat cells [10]. There is enormous potential for the use of MSCs in gene therapy, regenerative medicine and immunotherapy, because these cells have exceptional ability of self-renewal and immune modulation and high plasticity [11, 12]. The MSCs are found in various tissues, such as the umbilical cord tissue [13], placenta [14] umbilical cord blood [15]. Umbilical cord MSCs, in fact, have a higher concentration of adult stem cells than umbilical cord blood, and these cells can easily be harvested from clinical wastes after child delivery [16-19].

Non-viral plasmids are interesting gene delivery systems for genetic modification and tumor gene therapy. From the viewpoint of efficiency and safety, a non-viral minicircle vector technique may be the most useful for UC-MSC-derived iPSCs production. Unlike viral plasmids, non-viral plasmid is easy to manufacture, safe and stable and have greater capability for gene encapsulation. In this study, a minicircle vector was used to produce human iPSCs from adipose-derived stem and neonatal fibroblast cells.

2. Materials and Methods

2.1. Reagents and chemicals. All cell culture medium and supplements, including Dulbecco’s Modified Eagle Medium-F12 (DMEM-F12), knockout DMEM, ES cell, 0.1 mM nonessential amino acids (NEAA), 2-mercaptoethanol, L-Glutamine, Fetal Bovine Serum (FBS), Penicillin/Streptomycin and Fungizone, were purchased from Gibco (Life Technologies, California, USA) and bFGF was prepared from Merck (Millipor, Germany). STEMcircles™-LGNSO plasmid (pDNA) was obtained from Stem Cell Technologies (Canada) and human umbilical cord MSC (UC-MSC) was obtained from Immunology Laboratory, Department of
Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Lipofectamine® LTX Reagents were purchased from (Carlsbad, CA, USA). Formaldehyde solution, Gelatin, Triton X-100 and all blocking solution reagents including BSA, Goat serum and tween 20% were obtained from Sigma (St. Louis, MO, USA). ALP Staining Kit was purchased from Stemgent (San Diego, CA, USA), Trizol and QuantiFast SYBR Green RT-PCR kits were provided by Life Technologies (California, USA) and Qiagen (Germany), respectively. Ultra-low attachment culture dishes were obtained from Corning Life Sciences (California, USA). PCR tubes were bought from Bio-Rad (Hercules, CA, USA) and collagenase type IV, DNA ladder and loading dye were obtained from Invitrogen (Carlsbad, CA, USA). All primary and secondary antibodies were purchased from Abcam, UK. GeneJET™ RNA Purification Kit and Tetro cDNA Synthesis Kit were provided by Fermentas (Canada) and Bioline (Germany), respectively.

2.2. Transfection of the DNA Plasmid. The MSCs were cultured in DMEM in a 24-well plate. In the wells of a different 24-well plate, 3 µg of the DNA plasmid in 300 µL serum free medium was mixed with 6 µL Lipofectamine LTX in 300 µL serum-free medium. After 20 minutes incubation at room temperature, the mixture was added to the wells containing confluent cells, and the plate rocked at 37°C in a CO2 incubator for 5 hours. Thereafter, 200 mL MSCs medium supplemented with 10% FBS (PAA, USA) and 150 µL was added to the wells. The cells were analysed for GFP expression under a fluorescence microscope (Nikon Eclipse TE 2000E) at 18, 48 and 72 hours post-transfection.
2.3. Flow cytometry Analysis. The expression of green fluorescent protein (GFP) reporter gene in the transfected MSCs was evaluated via flow cytometry assay. The cells were counted and $1 \times 10^6$ cells were placed into 12×15 mm test tubes and washed with 1×PBS and centrifuged at 300 × g at 4°C for 5 minutes. The supernatant was aspirated and the cell pellets resuspended in cold 1×PBS and fixed with 2% formaldehyde solution at 4°C for 30 minutes. After washing and resuspending the fixed cells in cold PBS, GFP expression was observed in a FACScalibur (BD Biosciences, USA). Non-transfected MSCs served as negative control.

2.4. Transfection and Cell Culture. Fully characterized human umbilical cord MSCs (UC-MSCs) were cultured in DMEM-F12 supplemented with 1% Penicillin/Streptomycin, 0.5% Fungizone and 10% FBS. On transfection day, the pDNA/lipofectamine LTX complexes were prepared as described above and added to the plate (150 μL/well) and incubated at 37°C under 5% CO₂. Four hours post-transfection, fresh MSCs medium was added to cells. A second transfection was carried out 2 days later using the same procedure described earlier and the cells were seeded in gelatin-coated dishes containing iPSCs media with knockout DMEM, 20% ES cell FBS, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-Glutamine, 0.5% penicillin-streptomycin and 10 ng/mL bFGF. Four to five days after the last transfection, some colonies began to appear in the gelatin-coated culture dishes. The morphology of the colonies was observed under a light microscope at 10× and 40× magnifications.

2.5. Immunocytochemistry (ICC) Assay. The induced cells were cultured in a 24-well plate until they reached more than 50% confluency. The cells were washed with cold PBS and fixed in 4% paraformaldehyde. After 3 washings, the cells were permeabilized with 1% Triton X-100 in PBS
and blocked with blocking solution (1% BSA, Goat serum, tween 20%) in PBS. The cells were then incubated with anti-Oct4 primary antibody (5 μg) overnight at 4°C. After washing, the cells were incubated with Donkey Polyclonal Secondary antibody IgG (5 μg) for 2 hours in the dark at room temperature. Finally, the cells were washed and counterstained with Propidium Iodide (PI) as DNA stain, stored in the dark at 4°C until viewed under a florescent microscope (Olympus BX51, Japan).

2.6. RNA Extraction and Quantitative Real-time PCR. Total RNA were extracted from the cells using Trizol reagent (Life Technologies, USA). Briefly, the cells were frozen-thawed 3 times and then centrifuged at high speed for 10 minutes in a sterile microtube, the supernatant was treated with Trizol for 5 minutes at room temperature. After adding chloroform, the samples were vigorously shaken and then incubated at room temperature for 15 minutes followed by centrifuging at 12000 × g for 15 minutes at 4°C. The upper colorless layer was transferred to a new microtube and RNA was precipitated using iso-propanol, washed with 77% ethanol and finally dissolved in RNase- free H2O. The RNA samples were treated with DNase I to remove DNA contamination. The purity and concentration of extracted RNA were determined spectrophotometrically.

Gene expression was assessed by qRT-PCR and using QuantiFast SYBR Green RT-PCR kit (QIAGEN, Germany). Briefly, 25 μL of the reaction mix was prepared in low-profile 0.2 mL microtube strips (Bio-Rad, Hercules, CA, USA) in the dark, by mixing RNase-free water with 12.5 μL 2× QuantiFast SYBR Green Master Mix, 1 μL forward and reverse primers of each gene, 3 μL of extracted RNA and 0.25 μL QuantiFast RT Mix. The experiment was performed
in quadruplicate using the real-time cycler (Biorad CFX96) system. After reverse transcription at 50°C for 10 minutes and then denaturation at 95°C for 5 minutes, the samples were subjected to 39 cycles of 10 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 62°C, and a final extension at 62°C for 5 minutes. To screen for the presence of transgene in the genomic DNA of transfected cells, a PCR-based assay was done using the primers for the Oct4-Sox2 minicircle-derived transgenes (Table 1). The PCR reaction was run with the following settings: 3 minutes at 94°C; 40 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 68°C; and a final extension step at 68°C for 7 minutes. The PCR products were verified on 1% (w/v) agarose gel.

2.7. Embryoid Body Formation. The induced cells seeded on gelatin-coated dishes were digested using collagenase type IV. The maturation to embryoid bodies (EBs) was achieved in ultra-low attachment culture dishes containing DMEM/F12 suspension medium containing 20% knockout serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 4.5 g/L L-glutamine and 0.1 mM 2-mercaptoethanol after 6 days. The EBs were enriched every day by replacing half of the medium with fresh medium. Spontaneous differentiation of induced cells into mesodermal, ectodermal and endodermal cells was then verified using RT-PCR method as described above. After six days of EBs development, the sphere-shaped EB structures were removed from the culture dishes. A GeneJET™ RNA Purification Kit was used for cellular RNA extraction, according to the manufacturer’s procedure. cDNA templates were synthesized from extracted RNAs using Tetro cDNA Synthesis Kit. Briefly, the 8 µL of extracted RNA was mixed with 2 µL Oligo (dT), 2 µL dNTP (10Mm) and 8 µL DEPC-treated water to make a final concentration of 20 µL. The mixture was incubated at 60°C for 10 minutes and then placed on ice for 2 minutes. The reaction mix was primed by mixing 8 µL 5X RT buffer, 2 µL RNase inhibitor, 2
μL reverse transcriptase and 8 μL DEPC-treated water to final concentration of 20 μL. The primed reaction mixture was blended with the prepared RNA and incubated at 45°C for 1 hour followed by incubation at 75°C for 15 minutes in the thermocycler. The Nanodrop system was utilized to determine the concentration of cDNA templates (Implen NanoPhotometer™ Germany). The level of the expression of GATA1, SOX1 and FIT1 genes was determined using RT-PCR. The reactions were all conducted in duplicates and a reaction sample without cDNA template served as negative control.

2.8. Statistical Analysis. A one-way ANOVA test (SPSS 20) was applied to determine the mean differences among groups. Student’s t-test was performed for group comparison. Differences were considered significant at \( P<0.05 \). Data were presented as the mean ± standard deviation (SD).

3. Results

3.1. Transfection. UC-MSCs were transfected with minicircle plasmid DNA twice over three days. The GFP expression was observed on fluorescent microscope 24 hours post-transfection (Figure 1). Flow cytometry analysis showed 18% GFP positive cells 48 hours later indicating that the transfection was successful (Figure 2).

3.2. iPSCs development through OSKM-plasmid application. In this experiment, negative control cells transfected with the empty EGFP vector showed no changes in morphology and kept growing as a monolayer. In contrast, the non-transfected UC-MSCs, the minicircle plasmid-transfected cells were found to form some small aggregates (Figure 3) of tightly packed
Structures form numerous undifferentiated colonies at 7 to 10 days post-transfection. When the colonies were cultured on gelatin-coated 6-well plates and in iPSCs medium supplemented with 20% KSR and bFGF for up to four weeks, the cells showed ES morphology, which was distinct from the fibroblast-like MSCs used as the parent cells (Figure 4). Further analysis demonstrated alkaline phosphatase (AP) activity (Figure 5) as well as expression of pluripotency markers such as OCT4 endogenes (Figure 6) in the selected colonies after 28 days post-transfection (Figure 5). Quantitative expression analysis for endogenous markers in the iPSCs at days 5, 10 and 15 post-transfection showed reduction of un-differentiation markers expression with time, which was associated with disappearance of minicircle DNA, indicating that transfected MSCs are independent exogenous markers (Figure 7). PCR assay using the specific primers for the contiguous plasmid genes Oct4 and Sox2 showed no evidence of transgene integration into the host genomic DNA (Figure 8).

3.3. Embryoid Body (EBs) Formation. The pluripotency of iPSCs colonies developed in this study was determined formation of EBs. Using the typical ESC differentiation protocol, spherical EBs were formed eight days after culturing in the suspension medium (Figure 9). Marker genes expression for each of the three cell lineages, ectoderm (SOX1), mesoderm (FIT1), and endoderm (GATA4) using RT-PCR assay verified that spontaneous differentiation of induced cells into the 3 germ layers cells was completed successfully (Figure 10).
4. Discussion

Induction of pluripotency in variant somatic cells can be fulfilled by ectopic expression of transcription factors [20]. Although, many studies utilized fibroblasts cells as the initial cell population for reprogramming research [21, 22], these cells need several weeks to expand from a single biopsy. In our study we showed that iPSCs can be developed from MSCs isolated from an umbilical cord. Deriving iPSCs from UC-MSCs in a feeder layer-free condition eliminates the probable variability initiated by the presence of the feeder cells. This study represents the first report on the establishment of human UC-MSC-derived iPSCs utilizing a polycistronic (minicircle) plasmid method. In comparison to established plasmid techniques [23, 24], reprogramming using a polycistronic vector presents many advantages [25, 26]. One of the advantages is the presence of CMV active promoter, which is very cooperative in the silencing exogenous genes at the time of induction of pluripotency [27]. In our methodology, we used STEMcircles™-LGNSO plasmid encoding for the most common transcription factors Lin28, Nanog, Sox2 and Oct4 that were required for reprogramming of human somatic cells to stem cells, and which were designed by Dr. Joseph Wu and colleges at Stanford University [28-30]. One of the most significant differences between minicircle plasmid and standard vectors is that the minicircle does not include the bacterial site of replication. Other benefits of minicircle are in its transcription as a single long mRNA for all 5 genes. The splitting of polycitrons is managed by 2A peptide sequences, which occur at specific sites [31]. The products of the split are translated into whole proteins. The translation of the RNA into proteins occurs in the cytoplasm, leaving the host genome intact and unaltered. The most important reason for the application of minicircle plasmid vector in this study was its ability to be transfected into somatic cells without the need for viral packaging and the subsequent removal of the cells without the use of a drug
selection method. These characteristics make the minicircle plasmid an appropriate vector for the transfection of desired transcription factors into UC-MSCs.

Our technique was a technically simple process, utilizing a single minicircle plasmid containing four transcription factor genes and characterized by a slight drop in plasmid vector expression during cell proliferation. In this study, we performed a variety of characterization methods to include morphological assessment of the iPSCs using a lighted microscope, ALP staining and qRT-PCR analysis for pluripotency marker detection. The iPSCs were also cultured for a period of one to two months to explore their ability to undergo long-term self-renewal. In addition, in vitro differentiation tests such as EB colony formation were conducted to reveal the developmental potential of the iPSCs. Therefore, the methodology used in this study was an integration-free reprogramming technique producing iPSCs without expression of transgenes or vectors. This approach can considerably reduce the risks of insertional mutagenesis or reactivation of transgene expression, which may otherwise cause tumor formation. Moreover, the gradual reduction of undifferentiated marker expressions associated with the disappearance of minicircle DNA demonstrated that the transfected MSCs are independent of exogenous marker expressions. The qRT-PCR assay showed that the four exogenous factor expressions are temporally required for several days, at which time the transfected cells genome prepares for conversion into a pluripotent state. Also unique relative to this method of cell programming was the downregulation of exogenous markers and reactivation of endogenous pluripotency markers. This was confirmed by immunocytochemistry analysis.

Induction of pluripotency under feeder layer-free condition is a recent development and has been achieved to-date with only a few types of cells [32-34]. In this study, the viral-free
transfection technology was successfully performed under a feeder layer-free condition to derive iPSCs from UC-MSCs. The resulting iPSCs expressed several pluripotency markers, contributed to EBs formation, and could also differentiate into all three germ layers, suggesting marked potential in using UC-MSCs reprogramming in future regenerative medicine [35]. However, there is need to optimize of the technique to ensure the production of iPSCs are efficient and safe for clinical applications.

Conflict of Interests

The authors declare that there is competing interests.

Acknowledgements

The authors are grateful to members of Stem Cell lab, Universiti Putra Malaysia for their continued support. This study was funded by the Ministry of Innovation, Science and Technology, Malaysia.

References


Table 1. Primers used to amplify the target genes

<table>
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<th>Primer</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
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<tr>
<td>Nanog-For</td>
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<td>269</td>
</tr>
<tr>
<td>Nanog-Rev</td>
<td>AGGCTCCAACCATACTCC</td>
<td></td>
</tr>
<tr>
<td>Sox2-For</td>
<td>CTCCCATTTCCTCGTTT</td>
<td>258</td>
</tr>
<tr>
<td>Sox2-Rev</td>
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<tr>
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<td>β-actin-Rev</td>
<td>TGGATGGGCGACATACATGGC</td>
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Figure 1. The comparison of transfection efficiency in different media condition. The capture shows the GFP expression evaluation using florescent microscope 24 hr after transfection with 150 µl of LTX/p DNA complex in cells with media (A), x 150 µl of LTX/p DNA complex in cells without media (B), 100 µl of LTX/p DNA complex in cells with media (C) and 100 µl of LTX/p DNA complex in cells without media (D) (40X magnification).
Figure 2. Flow cytometry results. These images show the analysis of GFP expressing MSCs transfected with 150 μl of the complex 4 days after transfection (18%). Non-transfected MSCs were used as negative control (0.08%).
Figure 3. Progressive Generation of iPSCs Colonies. The images show the distribution and morphology of UC-MSCs cultures 0 day (A), 2 days (B), 4 days (C) and 7 days post-transfection (D). Negative Control cells transfected with empty EGFP plasmid (40X magnification).
Figure 4. iPSCs colony formation. Undifferentiated colonies generated from transfected cells 7 days (Left image) to 10 days (Right image) post-transfection in the absence of feeder layer were different from the morphology of non-transfected UC-MSCs (10X magnification).
Figure 5. Alkaline phosphatase staining for expression of iPSCs. Representative image of two colonies stained with alkaline phosphatase exhibited the colony-developing capability of the transfected cells in the absence of feeder layer 12 days post transfection (20X magnification).
Figure 6. The Immunostaining result for endogenous pluripotency marker (Oct4). This image exhibits a colony of iPSCs stained with anti-Oct4 antibody (green), the nuclei of Oct4-positive iPSCs showed red staining (PI).
Figure 7. The comparison of Lin28, Oct4, Nanog and Sox2 exogenes log copy numbers in different days. The graphs show the qRT-PCR analysis of transgenic defined factors expression levels in iPSCs.
Figure 8. Genomic integration screening. This figure demonstrates that induced PSCs did not have the integration of plasmid vector. beta-actin was loaded as the positive control.
Figure 9. EBs-mediated differentiation of MSC iPSCs. Differentiation of EBs containing tight clusters of differentiating cells was observed at early stage by day 7 (A) and more differentiated by day 10 (B). Images were photographed by a 10X objective.
Figure 10. RT-PCR study of the expression of differentiation markers in EBs. 

In vitro-differentiated iPSCs revealed gene expression from RT-PCR. Undifferentiated iPSCs confirmed the gene expression of three main embryonic germ layers (endoderm, GATA4; mesoderm, FIT1; and ectoderm, SOX1). Housekeeping gene β-actin was used as the positive control. Lanes 1, 2: FIT1 (485 bp), Lanes 3, 4: SOX1 (320 bp), Lanes 5, 6: GATA4 (159 bp), Lanes 7, 8: blank, Lane 9: β-Actin (96 bp) and Lane 10: DNA Ladder 1Kb. Lanes 1, 3, 5: Undifferentiated, Lanes: 2, 4, 6: Differentiated.