Induction of multipotency in umbilical cord-derived mesenchymal stem cells cultivated under suspension conditions

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Abstract Due to the limitations in the clinical application of embryonic stem cells (ESC) and induced pluripotent stem cells, mesenchymal stem cells (MSCs) are now much more interesting for cell-based therapy. Although MSCs have several advantages, they are not capable of differentiating to all three embryonic layers (three germ layers) without cultivation under specific induction media. Hence, improvement of MSCs for cell therapy purposes is under intensive study now. In this study, we isolated MSCs from umbilical cord tissue at the single-cell level, by treatment with trypsin, followed by cultivation under suspension conditions to form a colony. These colonies were trypsin resistant, capable of self-renewal differentiation to the three germ layers without any induction, and they were somewhat similar to ESC colonies. The cells were able to grow in both adherent and

suspension culture conditions, expressed both the MSCs markers, especially CD105, and the multipotency markers, i.e., SSEA-3, and had a limited lifespan. The cells were expanded under simple culture conditions at the single-cell level and were homogenous. Further and complementary studies are required to understand how trypsin-tolerant mesenchymal stem cells are established. However, our study suggested non-embryonic resources for future cell-based therapy.

Keywords Umbilical cord-derived mesenchymal stem cell · Multipotency markers · Suspension cultivation · Poly-HEMA · Trypsin treatment

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Introduction

Pluripotent stem cells, with embryonic stem cells (ESCs) as a major group, are very important in cell therapy because of their ability to differentiate into various cell types. However, despite the differentiation capability of these cells, ethical concerns, potential tumorigenesis, and graft versus host disease are major challenges in the development and clinical application of these cells (Draper and Fox 2003; Solter 2005; Mertes and Pennings 2009; Knoepfler 2009).

Recently, another type of stem cells produced through genetic manipulation technologies has been introduced. These cells are called induced pluripotent stem cells (IPS) and are easily prepared. Such cells could be used in the screening of genetic disorders or in infectious diseases and do not cause immunological problems when grafted. However, the potential for tumorigenesis is still the main challenge in the development and clinical application of IPSs (Takahashi and Yamanaka 2006; Zhou et al. 2009; Condic and Rao 2008; Takahashi et al. 2007).

Mesenchymal stem cells (MSCs) are multipotent precursors that have the capacity to differentiate to various cell types under induction conditions. MSCs are attractive for therapeutic applications because they can secrete bioactive molecules with tropical and immunomodulatory properties. These cells can be isolated from several sources such as adipose tissue, dermis, peripheral blood, and bone marrow (Fraser et al. 2006; Cao and Dong 2005; Pittenger et al. 1999). Other important sources of MSCs are umbilical cord (UC), umbilical cord blood (UCB), placenta, and amnion fluid. Umbilical cord tissue, which is also called Wharton's jelly (WJ), is an appropriate source for the collection of MSCs. Being easily accessible without the need for any invasive procedures to obtain them is the most important advantage of this birth-associated tissue (Caplan 2009; Venugopal et al. 2011). However, because of the low MSC content of this tissue, they must be expanded *in vitro* before any application (Caplan 2009; Hass et al. 2011). In addition, the mesenchymal stem cells isolated from these sources are not capable of differentiating into all three embryonic layers (three germ layers) without cultivation under specific induction media. On the other hand, cell therapy application of MSCs has been hindered mostly because of limitations such as low proliferation rate, restricted life span, and gradual loss of their stem cell properties during *ex vivo* expansion. Hence, not only are the preparation of efficient MSCs and the acquisition of sufficient quantities of them very important, but also improvement and optimization of their culture conditions are very critical. Therefore, *in vitro* expansion of MSCs expressing primitive multipotency markers without genetic manipulation or cultivation under specific induction media would be an important strategy to improve the efficiency of MSCs-based cell therapy.

In this study, a type of UC-derived MSCs was isolated and characterized at the single-cell level, following treatment with trypsin and cultivation under suspension conditions. The colonies obtained under these conditions were clusters of cells that showed some unique properties such as trypsin resistance, self-renewability, and potential differentiation into the three germ layers, i.e., endoderm, ectoderm, and mesoderm without the requirement of any specific induction media. In addition, the appearance of the cells was similar to the ESC colonies and they were able to grow in both adherent and suspension cultures for various time lengths.

Methods and materials

Isolation of MSCs from umbilical cord tissue

After consent forms were received from parents, fresh human umbilical cords were obtained immediately after delivery. Samples were transferred in Hank's balanced salt solution (Invitrogen, Germany) supplemented with 100 U penicillin/

streptomycin (Gibco, Germany). Following removal of blood vessels and milking, the sample was cut into 2–5 mm³ pieces, washed with Dulbecco's modified Eagle's medium (DMEM; Gibco) and treated with collagenase type IV (2 mg/ml) for 16 h at 37 °C. Then, the sample was washed and incubated with 2.5 % trypsin (Invitrogen) for 30 min at 37 °C. The resulted suspension was filtered by 70 µm nylon mesh to remove aggregations and particles. Viable cells were counted using Trypan Blue exclusion dye technique, and suspended in α -MEM culture medium (Gibco) supplemented with 10 % fetal bovine serum (FBS; Gibco), 100 U penicillin/ml, and 100 mg streptomycin/ml (Gibco), then seeded in appropriate flasks. The cells were incubated in a 37 °C incubator containing 5 % CO₂ at 90 % humidity. Non-adherent cells were removed after 24 h. The culture medium was changed every 2–3 days, and at 80–90 % confluency, the cells were trypsinized and passaged to new flasks to expand the number of the cells.

UC-MSCs surface marker profiling by flow cytometry

For flow cytometry, the umbilical cord-derived mesenchymal stem cells (UC-MSCs) were trypsinized with 0.05 % trypsin/ethylenediaminetetraacetic acid (EDTA; Gibco), washed, and resuspended in phosphate-buffered saline (PBS). Human AB serum (Sigma, USA) was added to 1×10^5 cells to block FC receptors, then the cells were incubated with specific PE- or fluorescein isothiocyanate-conjugated (FITC)-conjugated anti-CD34, CD45, SSEA-3, CD105, CD90, and CD29 monoclonal antibodies for 20 min at 4 °C in a dark place. Afterwards, the cells were washed and resuspended in PBS. Positive and negative controls and immunoglobulin isotype controls were also evaluated in each run.

Differentiation studies

The differentiation potential of cells was examined on the third passage of the UC-MSCs. For induction of osteogenic differentiation, the UC-MSCs were plated in six-well plates at a density of 10,000 cells/cm² in triplicates. After 48 h, osteogenic differentiation medium containing dexamethasone, L-glutamin, ascorbate, and β -glycerophosphate was added to the test cells, and a supplemented α -MEM culture medium was added to the control cells. Medium refreshment was performed every 3–4 days for 21 days. Finally, the cells were washed with PBS, fixed with 4 % paraformaldehyde, and stained with Alizarin Red S 2 % to detect the presence of calcium deposits in osteocytes.

For induction of adipogenic differentiation, the UC-MSCs were plated in six-well plates at a density of 10,000 cells/cm² in triplicates. After 48 h, adipocyte differentiation medium containing recombinant human insulin, L-glutamin, dexamethasone, indomethacin, and IBMX (3-isobutylmethyl-xanthine)

was added to the cells. In the case of controls, a supplemented α -MEM culture medium was added to the cells. The culture media were replaced every 2–3 days for 10 days. Finally, the cells were washed with PBS, fixed with 4 % paraformaldehyde and stained with 1 % HCS lipidTOX™ green neutral lipid to detect the presence of neutral lipid vacuoles in adipocytes.

For induction of chondrogenic differentiation, the UC-MSCs were plated in six-well plates in micromass culture and high humidity levels in triplicates. After 2 h, chondrogenic differentiation medium was added to the test cells and a supplemented α -MEM culture medium was added to the other three wells (used as a control group). The culture media were changed every 2–3 days for 14 days. Finally, the cells were washed with PBS, fixed with 4 % paraformaldehyde and stained with Alcian Blue 1 % to detect sulfated proteoglycans accumulated in chondrocytes.

Long-term trypsinization to produce trypsin-tolerant umbilical cord mesenchymal stem cells

To induce stress conditions, UC-MSCs with 80 % confluency were detached with 0.25 % trypsin/EDTA (Gibco). Viable cells were counted with Trypan Blue dye exclusion technique and 6×10^5 cells were transferred to culture dishes containing fresh 0.25 % trypsin solution and placed in an incubator for 10 h. The cell suspensions were centrifuged at 500 rpm for 4 min to remove dead cells. Then, the viable cells were subjected to suspension culture.

Suspension culture

There are different ways to subject MSCs to suspension culture such as using poly-[poly (2-hydroxyethyl methacrylate)] (HEMA)-coated dishes to prevent cell attachment. To coat culture dishes with the poly-HEMA, 500–550 mg poly-HEMA was dissolved in 50 ml ethanol and was added to the dish (e.g., 50 μ L per well for 96-well plates and 100 μ L per well for 24-well plates), incubated overnight under a biological safety hood, then exposed to ultraviolet (UV) light for sterilization. After long-term trypsinization, approximately 1×10^4 live cells were added to semisolid methylcellulose-based medium (Methocult H4100, StemCell Technologies) diluted in α -MEM culture medium containing 10 % FBS to a final concentration of 1 % and mixed by pipetting. Then, the cells were seeded in poly-HEMA-coated 24- and 96-well plates and incubated at standard incubation conditions of cell culture for 14 days to form colonies and clusters. Diluted methylcellulose-based medium was added to each well every 2 days. Not-trypsin-treated UC-MSCs and ESCs were considered as negative and positive controls, respectively, and cultured similarly.

Formation of cell clusters

Cluster formation was inspected within 2–14 days. After 8 days, clusters were observed with an estimated size of about 40 μ m. The clusters grew slowly until day 14, then their growth rate decreased and their size became approximately 150 μ m. These cells were named trypsin-tolerant umbilical cord mesenchymal stem cells (TTUC-MSCs). On day 10, the contents of the culture dishes were transferred to sterile Falcon tubes, washed with PBS for three times and viable clusters were counted using Trypan Blue dye exclusion technique. Clusters larger than 30 μ m, not stained with Trypan Blue, and showing the same round appearance as ESCs clusters were isolated using 30 μ m nylon mesh or glass micropipette for further proliferation and characterization. The cells of each cluster were separated following 10 min of treatment with trypsin or mechanically (by pipetting) and the cell number of each cluster was counted at various time points over a period of 14 days. Finally, appropriate numbers of clusters and TTUC-MSCs were separated and subjected to RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time PCR, immunocytochemistry (ICC), and western blot analyses for detecting multipotency markers.

TTUC-MSCs differentiation

Following formation of clusters within 8–10 days of suspended cultivation in poly-HEMA-coated culture dishes, single clusters containing TTUC-MSCs were picked up by glass micropipette and transferred to gelatin-coated culture dishes to attach and differentiate. For coating of culture dishes with gelatin, 0.1 % gelatin solution was added to dishes and incubated for 0.5–1 h under a biological safety hood. Then, the solution was aspirated and clusters were transferred to the dishes. α -MEM medium containing 10 % FBS was added to the cells and incubated for 7–8 days to evaluate their differentiation to the three germ layers (ecto, endo, and mesoderm layers). The medium was exchanged every 2–3 days and differentiation ability was analyzed by RT-PCR, ICC, and western blotting for detection of ecto-, endo-, and mesodermal markers.

Flow cytometry analysis of TTUC-MSCs

The obtained clusters of TTUC-MSCs were separated by 10 min of treatment with trypsin, or mechanically (by pipetting), washed with and resuspended in PBS. Human AB serum was added to the cells to block FC receptors. Then, the cells were incubated with monoclonal anti human CD105 and SSEA-3 antibodies for 20 min at 4 °C in a dark place. Immunoglobulin isotype control was also performed. The

incubated cells were then subjected to flow cytometry analysis.

RNA extraction and RT-PCR

After proliferation of ESCs (as positive control), not-trypsin-treated UC-MSCs (as negative control), TTUC-MSCs, and differentiated cells obtained following cultivation of TTUC-MSCs, or ESCs on gelatin-coated dishes, their total RNA was extracted using tripure reagent (Roche, Germany) according to the manufacturer's instructions. First-strand cDNA was generated using 2 µg of the isolated total RNA by a cDNA synthesis kit (Invitrogen, USA) according to the kit instructions. Appropriate primer sets for amplification of multipotency markers including (Oct3/4, Nanog, Sox2), ecto (MAP2), endo (AFP, GATA-6), and mesodermal (α -SMA) markers, in addition to β -actin were designed using Primer3 software. Nucleotide sequences and the amplicon size of the designed primers are listed in Table 1. PCR reactions were performed using Taq DNA polymerase (Roche).

Quantitative real-time PCR

The extracted RNAs were subjected to quantitative real-time PCR analysis to evaluate the expression level of multipotency markers. First-strand cDNA was generated as described above. Quantitative real-time PCR was performed in triplicate by SYBR Green Real-Time Master Mix (Takara, Japan) in Rotor gene 3,000 system (Corbett, Germany). The cycle threshold (Ct) was calculated automatically and normalization was carried out against the β -actin Ct value.

Western blot analysis

Western blot analysis was performed to evaluate the expression of the markers in protein level. Whole cell extract was isolated from ESCs, not-trypsin-treated UC-MSCs, TTUC-MSCs, differentiated TTUC-MSCs, and differentiated ESCs.

Denatured samples were run on 10–12 % sodium dodecyl sulfate polyacrylamide gel and transblotted to polyvinylidene difluoride membrane. Then, the membrane was incubated at room temperature for 2–3 h with proper dilution of primary rabbit antibodies against multipotency markers Oct3/4 (1:500, Sigma), Nanog (1:1,000, Millipore), Sox2 (1:100, Abcam), ecto- (MAP2, 1:1,000, Millipore), endo- (AFP, 1:100, Thermo Scientific and GATA-6, 1:1,000, Cell Signaling), and meso- (α -SMA, 1:500, Abcam) dermal markers, followed by washing and incubation with secondary horseradish peroxidase-conjugated goat-anti-rabbit antibody (1–1.5 h, while shaking at room temperature). Finally, DAB solution was used to visualize the expected protein bands on PVDF membrane.

Immunocytochemistry

After clusters had formed, the cells were transferred to cover slips. Then, the differentiated cells obtained following cultivation of TTUC-MSCs on gelatin-coated plates and clusters were fixed with 4 % (v/v) paraformaldehyde in 0.01 M PBS to perform immunocytochemistry. The samples were incubated with blocking buffer containing 5 % normal goat serum and 0.3 % Triton[®] X-100 for 1 h at room temperature. Then, the clusters were incubated with anti-SSEA-3 primary antibody (Abcam 1:100), anti-Oct3/4 primary antibody (1:100, Sigma), and anti-Nanog primary antibody (1:400, Millipore) in blocking buffer at 4 °C overnight. Other primary antibodies were used for ICC were MAP2 (1:100, Millipore), α -SMA (1:100, Abcam), and incubated for 2–3 h at room temperature. After washing three times with PBS containing 0.05 % triton X-100, the samples were incubated with proper FITC- or PE-conjugated secondary antibodies for 1–2 h at room temperature in a dark place. Counterstaining was performed by 4',6-diamidino-2-phenylindole (DAPI; 1:500, Roche). Finally, the samples were washed three times with PBS containing 0.05 % triton X-100 and observed under fluorescence microscope.

Table 1 The number of amplification cycles, sequences, and the amplicon size of the specific primers designed for assessing expression of multipotency and three germ layers markers

Markers	Forward primer	Reverse primer	Amplicon size (bp)	Cycles
Oct3/4	AAGCTGGAGCAAAACCCGGAGG	GGTCGTTTGCTGAATACCTTCCCA	162	35
Nanog	TACCTCAGCCTCCAGCAGATGCAA	TAGGTGCTGAGGCCCTTCTGCGT	163	35
Sox2	AAATACCGGCCCGCGGAAA	CCAGCCGTTTCATGTGCGCGTAA	175	35
SMA	TTGGCAATGAGCGCTTCCGC	TGGTGCCCCAGAGAGGACATT	168	35
GATA-6	GAGGGAATTCAAACAGGAAACG	CGAATACTTGAGCTCGCTGT	234	35
MAP2	AGCGCCAATGGATTCCCATA	AGAGCTGCAGTCTGGTCTTT	239	35
AFP	CCACTGTGTGCCAACTCAGTGA	TGCAGGAGGGACATATGTTTCA	209	35
B-actin	TTCTACAATGAGCTGCGTGTGG	GTGTTGAAGTCTCAAACATGAT	115	35

Results

UC-MSCs displayed fibroblastic morphology and expressed MSC-specific surface markers

Following disruption of umbilical cord tissue and isolation of UC-MSCs, their morphological characters were inspected under an inverted microscope. Microscopic observations confirmed the fibroblastic-like appearance of the isolated cells, which were similar to MSCs (Fig. 1a).

Flow cytometry analysis of the UC-MSCs with specific antibodies confirmed the presence of MSC markers (including CD105, CD90, and CD29) and the absence of hematopoietic stem cell markers (including CD34 and CD45). Furthermore, the absence of SSEA-3 antigen on the surface of the isolated cells was also confirmed (Fig. 1b). Taken together, flow cytometry confirmed the MSC characteristics of the UC-MSCs at the third passage.

UC-MSCs were able to differentiate to osteocytes, adipocytes, and chondrocytes

After isolation and proliferation of the UC-MSCs, osteogenic differentiation was induced. Twenty-one days after induction, the Alizarin Red S staining of the cells confirmed their

osteogenic differentiation (Fig. 1c). In addition, the adipogenic differentiation capacity of the isolated cells was confirmed following staining of intra cytoplasmic lipid droplets by HCS lipidTOXTM green neutral lipid stain (Fig. 1d). Finally, cultivation of the cells in the chondrogenesis induction media, followed by their staining with Alcian Blue revealed the accumulation of sulfated proteoglycans which confirmed the chondrogenesis differentiation capacity of the UC-MSCs (Fig. 1e).

Overall, these findings and their comparison with the results obtained following cultivation of the UC-MSCs only in α -MEM as negative controls, which did not show any sign of differentiation, (C^{*}-E^{*}), confirmed the MSCs identity of the isolated cells.

TTUC-MSCs formed round colony with small cells similar to ESCs colony

The colony forming potential of the UC-MSCs was examined using passages 3–5. Following 10 h of treatment with trypsin solution and elimination of dead cells, viable cells were subjected to flow cytometry for detection of CD105 and SSEA-3 surface markers. The expression of SSEA-3 on the surface of UC-MSCs was very low or not detectable. However, after long-term treatment with trypsin, the cells showed increased levels of SSEA-3 (Fig. 2a). In addition, according to Fig. 2a, a

Fig. 1 Characterization of human umbilical cord matrix stem cells (UC-MSCs). **A**. Fibroblastic-like morphology of UC-MSCs after 5 days from third passage. **B**. Flow cytometry analysis showed expression of CD105, CD90, CD29, and no expression of CD34, CD45, and SSEA-3. **C**. Differentiation potential of UC-MSCs. UC-MSCs were cultured in osteogenic differentiation medium and stained with Alizarin Red S 2 %. Osteogenesis was observed by detection of calcium mineralization. **D**.

Accumulated neutral lipids stained with 1 % HCS lipidTOXTM green neutral lipid indicated adipogenic differentiation of UC-MSCs. **e** Deposition of sulfated proteoglycans was tested by staining with Alcian Blue 1 % which confirmed chondrogenic differentiation capacity of UC-MSCs. Negative controls for osteogenic (C^{*}), adipogenic (D^{*}), and chondrogenic lineages (E^{*})

Fig. 2 Characterization of TTUC-MSCs. **A** Flow cytometry analysis showed that long-term trypsinization-induced expression of SSEA-3 on UC-MSC and about 11 % of cells were double positive for CD105 and SSEA-3. These cells were named TTUC-MSCs. **B** TTUC-MSCs formed round colony in suspension culture on day 10. **C** Embryonic stem cells (ESCs) colony. **D** Not-trypsin-treated UC-MSCs did not form colony or their colony did not have desired criteria. **E** Viability evaluation by Trypan Blue exclusion dye technique indicated that the number of viable TTUC-MSCs in colonies decreased after 12 days of suspension culture. **F** Clusters of TTUC-MSCs were dual positive for CD105 and SSEA-3 surface markers. **G** RT-PCR analysis of TTUC-MSCs; **M** 100 bp DNA marker, **I** not-trypsin-treated UC-MSCs as negative control, **2** ESCs as

positive control, and **3** TTUC-MSCs. RT-PCR was performed for Oct3/4, Nanog, and Sox2 pluripotency markers. β -actin was used for normalization. **H** Quantitative real-time PCR analysis of the TTUC-MSCs indicated upregulated expression of Oct3/4, Nanog, and Sox2 compared with not-trypsin-treated UC-MSCs. **I** Western blot analysis of the TTUC-MSCs; **I** not-trypsin-treated UC-MSCs as negative control, **2** ESCs as positive control, and **3** TTUC-MSCs. **J(I)** and **J(II)** Immunocytochemistry of TTUC-MSCs clusters showed expression of SSEA-3; DAPI was used for nuclear staining. **K(I)** and **K(II)** TTUC-MSCs clusters expressed Oct3/4 followed by counterstaining with DAPI. **L(I)** and **L(II)** Clusters were positive for Nanog, DAPI staining for nuclear staining

small percentage of the cells were CD105/SSEA-3 double positive after long-term trypsinization. Then, for cluster formation, the viable cells were cultured in Methocult medium diluted to 1 % final concentration in α -MEM medium containing 10 % FBS.

The culture dishes were investigated for cluster formation by invert microscope within 2–14 days. After 10 days, some round clusters were observed (Fig. 2b) that were similar to clusters formed by ESCs which were cultivated as positive controls (Fig. 2c). The clusters consisted of several small round cells (TTUC-MSCs) compared with not-trypsin-treated UC-MSCs which were cultivated as negative controls and were not able to produce such colonies (Fig. 2d). On the other hand, those cells that were CD105/SSEA-3 double-positive generated cell clusters under suspension culture conditions. The not-trypsin-treated UC-MSCs negative controls rarely generated cell clusters, and in the case of cluster formation, they did not have enough size or the desired criteria. The clusters of the TTUC-MSCs grew slowly until the 14th day, then their growth rate decreased and their size reached approximately 150 μ m. Those clusters which were larger than

30 μ m, Trypan Blue staining negative and have round appearance like embryonic stem cells. Clusters were isolated by 30 μ m nylon mesh or glass micropipette. Following 10 min of treatment with trypsin, or mechanically (by pipetting), the TTUC-MSCs were segregated from each other and the cell number of each cluster was counted at various time points over 14 days. Cell counting showed an increasing number of the TTUC-MSCs in each colony as time passed, but the percentage of live TTUC-MSCs in clusters decreased in the final days and they did not form further clusters in the next passages. In other words, the number and size of live TTUC-MSCs in each cluster did not increase after 10 days (Fig. 2e). Therefore, other analysis such as flow cytometry, RT-PCR, quantitative real-time PCR, ICC, and western blotting were performed on clusters that formed until the 10th day.

The TTUC-MSCs were CD105/SSEA-3 double positive

After colony formation in suspension conditions and segregation of TTUC-MSCs from each other, flow cytometry analysis was used for detection of surface antigens. Results showed

that the TTUC-MSCs not only expressed CD105, a specific marker for MSCs, but also SSEA-3, a multipotency marker, in dual form (Fig. 2f).

CD105/SSEA-3 double-positive cells expressed multipotency markers Oct3/4, Nanog, and Sox2

RT-PCR evaluation of the total RNA extracted from ESCs (positive control), not-trypsin-treated UC-MSCs (negative control), and TTUC-MSCs indicated the expression of multipotency markers Oct3/4, Nanog, and Sox2 (Fig. 2g).

Upregulation of Oct3/4, Nanog, and Sox2 in TTUC-MSCs

Quantitative real-time PCR analysis of the TTUC-MSCs indicated increased expression levels of multipotency markers including Oct3/4, Nanog, and Sox2 when compared with the not-trypsin-treated UC-MSCs (Fig. 2h).

Furthermore, western blot analysis of the TTUC-MSCs lysate using specific antibodies against the multipotency markers showed a single band of about 43 kDa corresponding to Sox2, a band of about 39 kDa corresponding to Nanog, and a band of about 40 kDa corresponding to the Oct3/4 markers. No corresponding protein band was detected in lanes containing the not-trypsin-treated UC-MSCs lysate (Fig. 2i).

For further confirmation, immunocytochemistry staining of the clusters indicated the expression of SSEA-3 marker (II in Fig. 2j) in combination with DAPI for nuclear staining (I in Fig. 2j). Results of ICC staining showed that these colonies were positive for Oct3/4 (II in Fig. 2k) and Nanog (II in Fig. 2l) markers. DAPI was used for nuclear staining (I in Fig. 2k and I in Fig. 2l). The TTUC-MSCs saved their ability to form a colony in subsequent passages and showed similar properties in the next generation.

Differentiated cells derived from TTUC-MSCs were positive for markers of the three germ layers markers

RT-PCR analysis of the TTUC-MSCs which were differentiated in the gelatin-coated dishes revealed expression of the specific markers for the three germ layers including ecto- (MAP2), endo- (AFP, GATA-6), and meso- (α -SMA) dermal markers compared to the not-trypsin-treated UC-MSCs (Fig. 3a).

Western blot analysis was also used to further confirm expression of the specific markers of the three germ layers. As is shown in Fig. 3b, expression of the ecto- (MAP2), endo- (AFP and GATA-6), and meso- (α -SMA) dermal markers by the differentiated TTUC-MSCs was confirmed by revealing a band of about 70 kDa for MAP2, 42 kDa for α -SMA, 55 kDa for GATA-6, and 70 kDa for AFP. This was also observed in the case of differentiated ESCs. However, the not-trypsin-treated negative control UC-MSCs did not express any of

the markers. Potential expression of MAP2 and α -SMA in differentiated TTUC-MSCs was tested by ICC technique. According to I and II in Fig. 3c and I and II in Fig. 3d, these cells were positive for MAP2 and α -SMA.

Discussion

ESC and IPS have an extraordinary importance in cell therapy because of their ability to differentiate into a variety of cell types. In spite of the advantages of these stem cells, they are not practical in clinical use due to many problems (Draper and Fox 2003; Solter 2005; Mertes and Pennings 2009; Knoepfler 2009; Takahashi and Yamanaka 2006; Zhou et al. 2009; Condit and Rao 2008; Takahashi et al. 2007). Recently, another type of stem cells, i.e., mesenchymal stem cells, has been the focus of intensive investigations because of their advantages (Pittenger et al. 1999; Venugopal et al. 2011). However, MSCs are not capable of differentiating into all three embryonic layers without induction (Kuroda et al. 2011; Zhang et al. 2009, 2012a, b; Spees et al. 2003). There are several strategies to enhance the efficiency of MSCs for cell therapy including the improvement of MSCs microenvironment, their genetic manipulation and preconditioning techniques (Zhang et al. 2012a, b; Francis and Wei 2010). It is noteworthy that although these strategies can improve the efficiency and survival of the MSCs, they do not affect the multipotency of the MSCs. MSCs can be grown in suspension or semisuspension and non-adherent conditions while retaining their properties such as stemness and increased immunomodulatory capacities during proliferation (Stolzing et al. 2012; Chen et al. 2012; Reynolds and Weiss 1992; Higuchi et al. 2012). Quiescent stem cells are available in various tissues to replace damaged cells. These stem cells are mobilized under different stresses and damage (Hong et al. 2009; Qiu et al. 2009). Therefore, stress may be a useful stimulator to propagate or increase the number and efficacy of stem cells for engraftment.

In this study, a subpopulation of UC-MSCs was isolated which we consider to have unique beneficial properties such as stress resistance, high capacity of colony formation in suspension media, expression of multipotency markers, and the ability to express the gene of the three germ layers. It is very interesting that these cells expanded and differentiated spontaneously; that is, without the addition of any specific inducers such as various cytokines or genetic manipulation, and occurring only following long-term trypsinization of the UC-MSCs. This simple method is neither costly nor time consuming. Here, we isolated MSCs from umbilical cord tissue. Today, using UC-MSCs is very common because of their easily accessibility and the absence of ethical concerns about them. In comparison to other sources of MSCs, UC-MSCs exhibit a higher proliferation capacity and show significantly faster population doubling times than bone marrow-derived MSCs

Fig. 3 In vitro differentiation of TTUC-MSCs. Following formation of clusters in suspension conditions, TTUC-MSCs and ESCs clusters were picked up by glass micropipette, transferred onto gelatin-coated culture dishes to attach and differentiate to the three germ layers for 7 days. **A.** RT-PCR analysis of differentiated cells; **M** 100 bp DNA marker, **1** not-trypsin-treated UC-MSCs as negative control, 2 differentiated cells from ESCs as positive control, and 3 differentiated cells from TTUC-MSCs. MAP-2 (ectodermal), α -SMA (mesodermal), and AFP and GATA6 (endodermal) markers were analyzed with RT-PCR. β -actin was used for

normalization. **B.** Western blot analysis of differentiated cells for MAP-2 (ectodermal), α -SMA (mesodermal), and AFP and GATA6 (endodermal) markers; **1** not-trypsin-treated UC-MSCs as negative control, 2 differentiated cells from ESCs as positive control, and 3 differentiated cells from TTUC-MSCs. **C (I)** and **(II)** ICC staining of differentiated cells for α -SMA indicated that the cells expressed this mesodermal marker (DAPI was used for nuclear staining). **D (I)** and **(II)** Differentiated cells were positive for MAP-2 (counterstained with DAPI)

(Caplan 2009; Fong et al. 2011; Venugopal et al. 2011; Troyer and Weiss 2008; Lu et al. 2006). Since in our study, the UC-MSCs were expanded after long-term treatment with trypsin and then cultivated on poly-HEMA-coated plates for suspended culture to colony formation, and then, these colonies were subjected to serial dilution for formation of a colony derived from single cells, therefore it seems the isolated UC-MSCs are homogenous. The mechanisms behind the production of trypsin-tolerant cells are not clear. However, long-term treatment with trypsin may cause reprogramming of the cells. Trypsin is a member of the serine proteases family that reacts with PARs and might affect expression of some growth factors such as granulocyte-macrophage colony-stimulating factor and IL-6, in addition to growth factor receptors such as platelet-derived growth factor receptor. Influencing the signaling pathways might ultimately affect culturing conditions (Wakita et al. 1997; Ball et al. 2012; Rasmussen et al. 2012). Trypsin also might enhance the multipotency of UC-MSCs through alteration of antigenic properties such as STRO-1, resulting in immunomodulatory effects on UC-MSCs (Stewart et al. 1999; Gronthos et al. 2003; Lokhov and Balashova 2010).

In our study, major ES/iPS-associated multipotency markers, such as Oct3/4, Nanog, and Sox2, were expressed uniquely in TTUC-MSCs but not in UC-MSCs. Since

expression of the above-mentioned ES markers is associated with methylation changes, it would be valuable to examine changes in general methylations patterns of TTUC-MSCs compared to UC-MSCs.

Recently, Kuroda et al. expanded a unique type of MSCs that had similar characteristics to the cells isolated in our study. They isolated the MSCs from bone marrow-derived mesenchymal stem cells (BM-MSCs), adult human skin fibroblasts, and native bone marrow aspirates and exposed them to different stress conditions including poor nutrition, low serum, low O₂, repetitive trypsin treatment, and long-term trypsin incubation (LTT). Among these conditions, cell clusters formed under LTT-expressed multipotency markers. They called these cells multilineage differentiating stress enduring (Muse) cells. However, in contrast to our study, they also collected Muse cells directly from adult hBM aspirates by isolating them as SSEA-3/CD105 double-positive cells by FACS and MACS techniques (Kuroda et al. 2010). These double-positive cells were further subjected to single-cell suspension culture after limiting dilution without LTT. Kuroda et al. used ICC to confirm expression of the pluripotency and the three germ layers protein markers, but here we also used western blotting for evaluation of the markers. More recently, Roy et al. identified a unique

population of somatic cells with extensive lineage plasticity from human breast tissue. They named these cells endogenous plastic somatic (ePS). These cells expressed high levels of pluripotency markers such as Sox2, Nanog, and Oct3/4 as did ESC and IPS. However, they had an epithelial nature and were different from MSCs. Similar to ESCs and other stem cells, ePS cells show various cell fates by an instructive microenvironment. Of particular note is that a single-cell-derived clone of ePS, similar to those in our study, was able to differentiate to the three germ layers. Nevertheless, it must be noted that their methods for obtaining the cells were invasive and labor intensive (Roy et al. 2013).

In summary, in this study, we introduced a type of UC-MSCs with several advantages such as the ability to form clusters in suspension cultures; the expression of multipotency markers; the capacity of self-renewal; stress tolerance; and the expression of endodermal, ectodermal, and mesodermal markers without any induction.

However, further and complementary studies are required including characterization and identification of other multipotency markers, potential tumorigenesis of the TTUC-MSCs in vivo, potential TTUC-MSCs-based cell therapy applications in tissue injuries and regenerative medicine, and an understanding of the molecular mechanisms underlying the trypsin-induced pluripotency of UC-MSCs. These unique UC-MSCs may be used as a non-embryonic resource for MSC-based cell therapy in the future.

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