Human umbilical cord-derived mesenchymal stem cells differentiate into epidermal-like cells using a novel co-culture technique

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Abstract Human umbilical cord-derived mesenchymal stem cells (hUCMSCs) isolated from human umbilical Wharton's Jelly are a population of primitive and pluripotent cells. In specific conditions, hUCMSCs can differentiate into various cells, including adipocytes, osteoblasts, chondrocytes, neurocytes, and endothelial cells. However, few studies have assessed their differentiation into epidermal cells in vitro. To assess the potential of hUCMSCs to differentiate into epidermal cells, a microporous membrane-based indirect co-culture system was developed in this study. Epidermal stem cells (ESCs) were seeded on the bottom of the microporous membrane, and hUCMSCs were seeded on the top of the microporous membrane. Cell morphology was assessed by phase contrast microscopy, and the expression of early markers of epidermal cell lineage, P63, cytokeratin19 (CK19), and β 1-integrin, was determined by immunofluorescence, Western blot, and quantitative real-time PCR (Q-PCR) analyses. hUCMSC morphology changed from spindle-like to oblate or irregular with indirect co-culture with ESCs; they also expressed greater levels P63, CK19, and β 1-integrin mRNA and protein compared to the controls ($p < 0.01$). As compared to normal co-cultures,

indirect co-culture expressed significantly greater CK19 protein ($p < 0.01$). Thus, hUCMSCs may have the capability to differentiate into the epidermal lineage in vitro, which may be accomplished through this indirect co-culture model.

Keywords Human umbilical cord-derived mesenchymal stem cells · Differentiation · Epidermal-like cells · Microporous membrane · Indirect co-culture system

Introduction

Almost fifty years ago, Friedenstein (1961) first reported fibroblast-like cells isolated from the non-hematopoietic compartment of the bone marrow with osteogenic potential. This cell, termed mesenchymal stem cell (MSC), was defined as any cell that retains a high capacity for self-renewal throughout adult life. MSCs were known to be multipotent, differentiating into multiple lineages, including adipose tissue, bone and cartilage. More recently, MSCs have also been isolated from adipose tissue (Pittenger et al. 1999), peripheral blood (Zvaifler et al. 2000), cord blood (Rogers and Casper 2004), and Wharton's Jelly (Can and Karahuseyinoglu 2007).

Wharton's Jelly consists of the stroma of umbilical cords that is rich in proteoglycans and mucopolysaccharides. Primitive Wharton's jelly, surrounds the migrating embryonic blood island cells during their

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migration to the aorta-gonad mesonephros from the yolk sac region (Sadler 2004). Greater numbers of MSCs have been isolated from the umbilical cord (termed hUCMSCs) as compared to umbilical cord blood (Secco et al. 2008). hUCMSCs can be easily isolated from nearly all samples, even from those umbilical cords that have been stored for up to 48 h (Weiss et al. 2006). Within Wharton's jelly, hUCMSCs have been isolated from three relatively indistinct regions, the perivascular zone, the intervacular zone, and the subamnion; however, whether hUCMSCs isolated from different compartments represent different populations has yet to be determined (Karahuseyinoglu et al. 2007).

Previous studies have reported the *in vitro* differentiation of hUCMSCs into mesoderm-type cells, such as osteoblasts, chondrocytes, adipocytes, cardiomyocytes, skeletal muscle cells, and endothelial cells (Breymann et al. 2006; Conconi et al. 2006; Hoerstrup et al. 2002; Sarugaser et al. 2005; Schmidt et al. 2006; Wang et al. 2004; Wu et al. 2007) as well as cells of nonmesoderm lineages, including neurocytes (Wang et al. 2004; Fu et al. 2004; Ma et al. 2005; Mitchell et al. 2003). hUC blood-derived MSCs enhanced wound healing in mice by differentiating into keratinocytes (Luo et al. 2010). Although it is known that bone marrow-derived MSCs (BMSCs) will home to the injury sites and contribute to keratinocytes for skin regeneration *in vivo* after skin injury (Borue et al. 2004; Satoh et al. 2004; Badiavas et al. 2003) and differentiate into epidermal lineage in 3D organotypic co-culture (Ma et al. 2009), similar analyses have not been performed using hUCMSCs. In addition, Schneider et al. (2010a, b) have proposed that UCMSCs might support the regeneration of wounds due to their differentiation into myofibroblasts and extensive synthesis of growth factors as well as extracellular matrix (ECM) production and remodeling. However, the potential of hUCMSCs to differentiate into epidermal cells is unknown. Thus, the present study assessed the potential of hUCMSCs to differentiate into epidermal cells using a microporous membrane-based indirect co-culture system consisting of hUCMSC and epidermal stem cells (ESCs). Cell morphology was evaluated using phase contrast microscopy, and the expression of early markers of epidermal cell lineage, P63, cytokeratin19 (CK19), and β 1-integrin, was determined by immunofluorescence, Western blot, and quantitative real-time PCR (Q-PCR) analyses.

Materials and methods

Cell isolation and culture

The isolation of hUCMSCs was performed as previously described (Wang et al. 2004). In brief, the umbilical cord was cut into 1-cm pieces, and the vessels were removed. After the Wharton's Jelly was isolated, it was digested in a collagenase IV/DMEM solution (172 IU/mL, Worthington Biochemical Corporation, Lakewood Township, NJ, USA) for 24 h, and the enzyme reaction was stopped by the addition of 10 % fetal calf serum (FCS, Gibco Life Technologies, Carlsbad, CA, USA) serum-containing stem cell medium (DMEM/F12, 100 U/mL penicillin/ 100 μ g/ml streptomycin, and 2.5 ng/mL Amphotericin B) (all from Gibco). After centrifugation at $500\times g$ for 10 min, the cell pellet was resuspended and incubated in 2.5 % trypsin (Gibco) for 30 min at 37 °C. The enzyme reaction was stopped again by the addition of serum-containing stem cell medium followed by centrifugation. Finally, the cell pellet was resuspended in stem cell medium, and 1.0×10^5 cells were seeded in a T25 culture flask. Non-adherent cells were removed by initial medium change after 24 h. hUCMSCs were characterized by immunofluorescence analysis and flow cytometry as described below.

The isolation of human epidermal stem cells (ESCs) was performed as previously described (Rheinwald and Green 1975). Human neonatal foreskins from routine circumcisions were processed within 2 h of collection. Epithelial sheets were obtained after overnight incubation with 4 mg/mL Dispase (Gibco) at 4 °C, and basal keratinocytes were isolated by trypsinization at 37 °C, 5 % CO₂ for 5 min. Isolated keratinocytes were maintained in keratinocyte growth medium (KGM; Gibco). Collagen IV-coated dishes were prepared by coating 100-mm dishes with 20 mg/mL type IV collagen (Sigma, St. Louis, MO, USA) overnight at 4 °C; cells were separated according to their ability to adhere to type IV collagen. The primary epidermal cells from foreskins were plated onto type IV collagen-coated dishes. A portion of these cells was selected according to their ability of adhere within 10 min at 37 °C.

Indirect co-culture

Mitomycin C (10 mg/L; Roche, Branchburg, NJ, USA)-treated ESCs (1.5×10^5 cells per insert and 2.0×10^5

cells per well) were seeded and cultured on an inverted transwell cell culture insert for six-well plates (24-mm diameter with 0.4- μ m pores, Transwell; Corning Costar, Corning, NY, USA) at 37 °C, 5 % CO₂ for 6 h. hUCMSCs (1.0×10^5 /insert) were seeded inside the insert at the following step. After two days, the serum-containing stem cell medium was refreshed every two days (Scheme 1a). In the second group, Mitomycin C-treated epidermal stem cells (1.5×10^5 cells per well) were seeded and cultured in the well (24 mm diameter, Transwell; Corning Costar, Corning, NY, USA) at 37 °C, 5 % CO₂ for 6 h after which 1.0×10^5 hUCMSCs were seeded inside the insert. After two days, the serum-containing stem cell medium was refreshed every two days (Scheme 1b). In the control group, 1.0×10^5 hUCMSCs were seeded inside the wells. After two days, the serum-containing stem cell medium was refreshed every two days (Scheme 1c).

Cell morphology analysis using phase contrast and scanning electron microscopy

The morphologies of the ESCs and hUCMSCs were observed by phase contrast microscopy every day (Leica, Wetzlar, Germany). In addition, hUCMSC morphology was examined using a scanning electron microscope (SEM; S-3400 N; Hitachi, Tokyo, Japan). First, samples were fixed in 2.5 % glutaraldehyde for 1 h at room temperature. After the samples were washed with 0.1 M phosphate buffer, they were fixed again in 1 % osmium tetroxide solution. Next, the samples were dehydrated in ascending grades of ethanol, dried, and mounted on an aluminum stub using a double-sided carbon tape. The specimens were coated with platinum using an Ion Sputter Coater (E-1030; Hitachi) and examined at an acceleration voltage of 5 kV.

Immunofluorescence analysis

For immunofluorescence, cells were washed three times with phosphate-buffered saline (PBS) and pre-incubated in a permeabilization solution containing acetone and methanol (3:7) for 10 min. After washing in PBS three times, cells were incubated with blocking solution (Abcam, Cambridge, UK) for 30 min. The samples were then incubated with the following primary antibodies (diluted 1:5,000) for 2 h: CK19 (rabbit monoclonal, Abcam, Cambridge, UK), P63 (rabbit monoclonal, Abcam, Cambridge, UK) and β 1 integrin (mouse

monoclonal, Abcam, Cambridge, UK). After three washes with PBS, the cells were incubated with the secondary antibody (Abcam, Cambridge, UK) diluted in PBS with normal human serum for 1 h. After three washes with PBS, genomic DNA was then labeled with a DAPI solution in PBS for an additional 15 min at 37 °C. After the final washes, the cells were mounted with fluorescent mounting medium (Sigma). For ESC identification, immunofluorescence analysis was undertaken as previously described with primary antibodies specific for CK19, P63, and β 1-integrin (all from Abcam).

Western blot analysis

hUCMSCs were washed twice with PBS followed by protein extraction using the Phenol-Ammonium Acetate/Methanol method. 50 μ g Samples were separated with a 10 % SDS-PAGE and then transferred to a PVDF membrane. After blocking the membrane with 5 % skim milk in TBST for 30 min, the membranes were incubated with the following primary antibodies (diluted at 1:5,000) for 1.5 h at room temperature: monoclonal rabbit anti-human CK19 antibody (Abcam), monoclonal rabbit anti-human P63 antibody (Abcam), or mouse anti-human β 1-integrin antibody or mouse anti-human GAPDH (Abcam). The membranes were washed in TBST and incubated with HRP-conjugated secondary antibodies (ZSGB-Bio, Beijing, China) for 1 h, washed, and developed with electrogenerated chemiluminescence (ECL) western blotting detection reagent (Pierce Biotech, Rockford, IL, USA). Proteins were visualized using X-OMAT film (Kodak, USA).

Flow cytometry

For the staining of intracellular markers for flow cytometry analysis, hUCMSCs were trypsinized, resuspended in cold PBS containing 0.5 % BSA (MP Bio, Irvine, CA, USA) and then fixed with 4 % formaldehyde for at least 30 min. The cells were then washed twice with PBS, and the cell pellet was resuspended in 0.3 % saponin in PBS (saponin–PBS) at 4 °C for 30 min. After washing twice with PBS, the titred CK19 antibody (Abcam) was added in a volume of 50 μ l saponin–PBS and incubated for 60 min. The cell pellet was washed three times with saponin–PBS after which it was incubated with FITC-labeled secondary antibody (1:200; Abcam) for 40 min. The cell pellet was washed twice with saponin–PBS and

Scheme 1 **a** Novel co-culture group. New strategy to indirectly co-culture hUCMSCs with epidermal stem cells using porous membrane techniques. **b** Normal co-culture group. Normal way

to co-culture hUCMSCs with epidermal stem cells using porous membrane techniques. **c** Control group. HUCMSCs culture

resuspended in 500 μ L PBS + 0.5 %BSA. FACS analysis was performed on FACSCantoII (BD Biosciences, San Jose, CA USA), and data were analyzed by using Flow Jo software (Version 7.5). For hUCMSC identification, flow cytometry was undertaken as previously described with primary antibodies specific for CD34, CD45, CD31, HLADR, CD29, CD44, CD73, HLA1, and CD105 (all from Abcam).

Real-time quantitative-PCR

Total RNA was extracted from the hUCMSCs (1.0×10^4 cells) using Trizol-100 (Invitrogen, Carlsbad, CA, USA) and the RNApure total RNA fast isolation kit (Biotek, Beijing, China). Real-time Q-PCR was performed using the BioEasy SYBR Green I real time PCR kit (BioerTechnology Co., Ltd., Hangzhou, China) PCR conditions were as

follows: 45 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s. PCR products were quantified by measuring the fluorescence intensity at the end of each amplification cycle using a LineGene K real-time PCR instrument (Bioer Technology Co., Ltd.). For each sample, real-time Q-PCR analysis was repeated in three independent experiments to ensure the reproducibility of results. Primers used for real-time Q-PCR included the following: CK19, sense 5'-TGAAAGCTGCCTT GGAAGACA-3' and antisense, 5'-GCCGCTGGTAC TCCTGATTCT-3'; Δ NP63, sense, 5'-CCTCGTCCA CCAGTCCCTATAAC-3' and antisense, 5'-TGCTCG ACTGCTGGAAGGA-3'; and β -actin, sense, 5'-TGA CGTGGACATCCGCAAAG-3' and antisense, 5'-CT GGAAGGTGGACAGCGAGG-3'. Analysis of the dissociation curves confirmed the primer specificity (data not shown).

Statistical analysis

The continuous data were presented as mean $\pm$ standard deviation in the bar chart. Independent two samples t-tests were performed to compare the differences between the two treatment groups. All statistical assessments were two-sided. A p value < 0.05 was considered statistically significant. Data were analyzed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

Characterization of isolated hUCMSCs and ESCs

Surface marker expression in hUCMSCs and ESCs was analyzed by flow cytometry and immunofluorescence analysis, respectively. As shown in Fig. 1a, hUCMSCs expressed CD29, CD44, CD73, HLA-1, and CD105, but not CD34, CD45, CD31, and HLA-DR. In addition, expression of CK19 and β 1-integrin, but not P63 was confirmed in ESCs (Fig. 1b).

Microscopic analysis of indirect co-cultures of ESCs with hUCMSCs on either side of a transwell insert

hUCMSCs and ESCs were cultured on either sides of a transwell insert. Although phase contrast images seem to reveal both cell types on either side of the membrane (Fig. 2), there was a 10- μ m membrane separating the cultures. Moreover, hUCMSC cellular bodies, such as lamellipodia, were observed within the membrane pores by scanning electron microscopy (Fig. 3).

To determine if the indirect co-cultures could alter hUCMSC morphology, phase contrast microscopy of hUCMSCs grown in the absence or presence of ESCs was undertaken (Fig. 4). hUCMSCs cultured alone had a fibroblast-like morphology (Fig. 4a). However, after co-culture with ESCs for 10 days, some hUCMSCs adopted an oblate or irregular appearance (Fig. 4b).

P63, CK19, and β 1-integrin mRNA and protein expression in hUCMSCs co-cultured with ESCs

The effect of indirect co-culture on the expression of early markers of epidermal cell lineage, including P63,

CK19, and β 1-integrin, by hUCMSCs was determined by immunofluorescence, Western blot and Q-PCR analyses (Fig. 5). After co-culture, some hUCMSCs were positive for P63 (Fig. 5A-1) and CK19 (Fig. 5B-1) expression; however, neither were expressed in the control group (Fig. 5A-2 and 5B-2, respectively). hUCMSCs in the co-culture and control group expressed β 1-integrin (Fig. 5C-1 and 5C-2). Western blot analysis confirmed that co-cultured hUCMSCs expressed CK19 and P63 as compared to the control group which was non-expression of both markers, while β 1-integrin levels were similar (Fig. 5b).

To determine if the increased CK19 and P63 protein level in co-cultured hUCMSCs was due to increased gene expression, Q-PCR analysis was undertaken. As shown in Fig. 5c, P63 and CK19 mRNA expression significantly increased after hUCMSC co-culture as compared to the control group ($p < 0.01$). Again, no changes in β 1-integrin mRNA levels were observed.

Increased CK19 expression after indirect co-culture on either side of the transwell membrane

To determine if indirect co-culture on either side of a transwell membrane differed from normal co-cultures, CK19 expression was evaluated in both groups using flow cytometry. As shown in Fig. 6, the proportion of CK19-positive cells was greater in the group co-cultured on either side of the transwell as compared to those co-cultured using the normal method (54.3 and 11.4 %, respectively; $p < 0.01$).

Discussion

As with bone marrow stromal cells and other mesenchymal cells, hUCMSCs expressed mesenchymal cell markers, including CD10, CD13, CD29, CD44, CD90, and CD105, but were negative for markers of the hematopoietic lineage. hUCMSCs have the advantages of being less controversial (derived from abandoned umbilical cords after delivery) as well as relatively simple to isolate. Moreover, hUCMSCs have extensive differentiation potential to multiple mesenchymal lineages under controlled conditions and are considered to be at an earlier stage as compared to MSCs derived from adult fat or bone marrow (Wang et al. 2004; Wu et al. 2007). Therefore, the present study sought to determine if hUCMSCs can differentiate into epidermal

Fig. 1 Characterization of isolated hUCMSC and ESC marker expression. Surface marker expression in hUCMSCs was analyzed by flow cytometry (**a**). Surface marker expression of

ESCs was determined by immunofluorescence analysis of CK19, P63, and β 1-integrin (**b**)

lineage cells in vitro using an indirect co-culture method with ESCs.

There are several methods to induce MSC differentiation, such as use of specific induction media as well as

direct or indirect co-culture. For example, differentiation into mesenchymal lineages by CD133(+) hUC blood cells required direct cell–cell interaction with mesenchymal cells (Park et al. 2009). In some cases,

Fig. 2 Co-culture of ESCs with hUCMSCs on a transwell insert. Both cells were cultured on both sides of the membranes, one on one side and the other one on the other side of the membrane. **a** ESCs seeded on the porous membrane of the

transwell insert. **b** hUCMSCs co-cultured with ESCs on the porous membrane of the transwell insert for 2 days. hUCMSCs (white arrow), epidermal stem cells (black arrow) and micropores (yellow arrow). (Color figure online)

Fig. 3 Scanning electron micrographs of the porous membrane after hUCMSC and ESC co-culture. hUCMSC and ESC co-culture on both sides of the microporous membrane were

analyzed by scanning electron microscopy **a** Membrane micro-pore (square) $\times 1,500$. **b** Micropores containing cellular bodies such as lamellipodia (white arrow) $\times 5,000$

Fig. 4 Phase contrast microscopy of hUCMSCs after co-culture with ESCs. **a** Spindle-like fibroblastic hUCMSCs (yellow arrow) in the control group. **b** Oblate or irregular

hUCMSCs in the novel co-culture group after 10 days. $\times 200$ (Scale bar: 100 μm). (Color figure online)

stem cells are induced to differentiate only in an in vivo microenvironment. For example, hUCMSCs exposed to an in vivo muscle environment expressed dystrophin and formed myotubes in vitro; however, this differentiation potential was not observed in those directly co-

cultured with myoblasts (Nunes et al. 2007). Within specific microenvironments or so-called ‘stem cell niches’, bone marrow-derived MSCs proliferate and differentiate into specific types of specialized mature cells to ameliorate tissue injury and eventually replace

Fig. 5 P63, CK19, and β 1-integrin expression in hUCMSCs co-cultured with ESCs. **a** P63 (top panels), CK19 (middle panels), and β 1-integrin (bottom panels) was assessed by immunofluorescence analysis in control cultures (right panels) and novel co-cultures (left panels). $\times 200$ (Scale bar: 100 μ m). **b** P63, CK19, and β 1-integrin expression in hUCMSCs in the

control and novel co-culture groups was also determined by Western blot analysis using GAPDH as the loading control. **c** P63, CK19, and β 1-integrin expression in hUCMSCs in the control and novel co-culture groups was also determined by Q-PCR analysis. Data were from three independent experiments performed in duplicate. $p < 0.01$

the injured tissue (Sun et al. 2007; Scadden 2006). In conventional indirect co-culture using a transwell system, the medium has to be refreshed every few days. Therefore, the microenvironment is not very stable. The present study attempted to create an artificial niche using indirect co-cultures of hUCMSCs and ESCs on either side of a transwell in order to induce hUCMSC differentiation into an epidermal lineage. This system allows for continuous conditioning of the medium of ESCs on the bottom of the porous membrane, which may provide a relatively stable microenvironment.

In the present study, hUCMSCs could be induced to differentiate into epidermal-like cells after indirect co-culture with ESCs on either side of a transwell as determined by increased CK19, P63, and β 1-integrin expression. This is consistent with Pedrosa et al. (2011)

in which hUCblood hematopoietic stem cells (CD34 +) differentiated into endothelial cells after 3-D co-culture with CD34 + -derived endothelial cells. This differentiation was induced through a combination of soluble and insoluble factors (Pedrosa et al. 2011). Although ESCs may provide soluble factors which induce hUCMSC differentiation into epidermal lineage, the cell types may have directly interacted in this co-culture system as lamellipodia were observed within the pores of the membrane. Further studies using membranes with different pore sizes will help determine whether the effects of ESC co-culture on hUCMSC differentiation were through a soluble factor or direct interactions.

As compared to normal co-cultures (Schema 1b), the co-cultures on either side of the transwell had a greater proportion of CK19-positive cells. CK19, P63,

Fig. 6 Increased CK19 expression with the novel co-culture. After normal or novel co-culture of hUCMSCs and ESCs. CK19 expression was determined by flow cytometry. **a** Red line, CK19 in the normal co-culture group. Green line, CK19 in the novel co-culture group. Blue area indicated the cells with fluorescence under 10, the negative control. **b** Normalized ratio of CK19-positive cells in the normal and novel co-culture groups. Values represent mean $\pm$ standard deviation of three independent experiments, $p < 0.01$. (Color figure online)

and $\beta 1$ -integrin are three important early epidermal lineage markers. P63 is used to identify ESCs (Pellengrini et al. 2001), while CK19 and $\beta 1$ -integrin are relatively specific markers of ESCs (Jones and Watt 1993; Michel et al. 1996). Furthermore, lack of $\beta 1$ -integrin drastically affects the keratinocyte differentiation in vitro (Bagutti et al. 2001), and high $\beta 1$ -integrin expression and adhesiveness are essential for maintaining keratinocytes in the stem cell compartment (Zhu et al. 1999). Because P63, CK19, and integrin- $\beta 1$ are considered early markers of epidermal cell lineage, further studies assessing late differentiation markers will be undertaken. In addition, further studies will determine whether hUCMSCs have the multipotency characteristic of adult epidermal stem cells (Oshima et al. 2001; Taylor et al. 2002) and

continue to differentiate into terminal mature epidermal cells with hemidesmosomes or desmosomes.

In conclusion, hUCMSCs co-cultured with ESCs on either side of a transwell membrane were induced to differentiate into epidermal-like cells, expressing P63, CK19, and integrin- $\beta 1$. Further in vivo studies are necessary to determine the effects of these differentiated cells on injured skin.

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