

Simultaneous harvesting of endothelial progenitor cells and mesenchymal stem cells from the human umbilical cord

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Abstract. The human umbilical cord (UC) is usually discarded as biological waste. However, it has attracted interest as a source of cells including endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs), which have demonstrated enormous potential in regenerative medicine. The present study describes a convenient protocol that has been developed to sequentially extract these two cell types from a single UC. EPCs which had properties of progenitor cells were successfully isolated from the UC vein. These cells had cobble-shaped morphology and expressed Flt-1, KDR, VE-cadherin, von Willebrand factor and CD31 mRNA, in addition to CD73, CD105 and vascular endothelial growth factor receptor-2. In addition to absorbing fluorescent-labeled acetylated low density protein and binding to fluorescein isothiocyanate-UEA-I, they were able to form vascular tube-like structures on Matrigel. Typical fibroblast-like cells, which were isolated from the Wharton's jelly, were confirmed to be MSCs by their expression of CD73, CD90 and CD105, and their ability to differentiate into adipocytes and osteoblasts. Thus, the human UC-derived cells may be suitable for use in tissue engineering and cell therapy.

Introduction

Endothelial progenitor cells (EPCs) are a cell population participating in vessel formation in physiological and pathological processes. EPCs may be categorized as endothelial outgrowth cells (EOCs) or endothelial colony forming cells (ECFCs) (1). ECFCs do not express cell surface markers found on monocytes and macrophages whereas EOCs do (2). ECFCs proliferate in a relatively stable and rapid manner while EOCs have limited growth potential *in vitro*. Thus, ECFCs have the greater research value for scientific research and clinical applications. EPCs have been detected, characterized and isolated from bone marrow, peripheral blood, human umbilical cord (UC) and UC blood (3-5). We have previously collected ECFCs from human UC (unpublished data).

Mesenchymal stem cells (MSCs) are multipotent cells that can be induced to differentiate to adipocytes, osteocytes, chondrocytes and stromal cells under certain conditions; they are traditionally obtained from sources such as bone marrow, human UC and UC blood (6-8). They have wide applications in tissue regeneration and cellular therapy.

The accurate identification, characterization and isolation of EPCs and MSCs is crucial to their use. The disadvantages of invasive isolation, limited cell numbers, and ethical constraints when obtaining the two types of cells from human bone marrow, organs and foetuses has increased the use of birth-associated tissues as a source of EPCs and MSCs and for the evaluation of stem cells, and the UC has been the most popular source (9). The UC is a connection between the mother and fetus. It is currently a focus of considerable attention, not only because of the important role it plays during pregnancy, but also because of the many kinds of stem cells it contains (10). Usually, the UC has two arteries and a vein enveloped in loose Wharton's jelly (10). Since hematopoietic stem cells were first successfully harvested from UC blood, they have been successfully used for the treatment of hematopoietic diseases. Stem cell populations have also been found in other parts of the UC, including the endothelium, umbilical blood vessel adventitia and Wharton's jelly, which is composed of stromal cells and extracellular matrix (11). Numerous researchers have succeeded in isolating stem cells from UC (11,12).

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In our previous studies, we successfully isolated MSCs from whole human UC (13) and ECFCs from the UC vein (unpublished data). In the present study, a simple and convenient strategy to isolate two major cell types from a single UC at the same time was developed. The two cell types were isolated on the basis of the different sites they occupy in the UC and the selection of suitable media for culturing. Following the isolation of cobble-like cells from the UC vein, those cells were identified as EPCs with a high proliferative potential. From the Wharton's jelly, typical fibroblast-like cells were tested as potential MSC candidates. Their characteristics were consistent with those of MSCs (14,15). Given the extra-embryonic nature of the two cell types, they will be of great benefit in the field of tissue engineering and may be valuable for possible future cellular therapeutic applications.

Materials and methods

Isolation and culture of UC-EPCs and MSCs. Two types of stem cells in human UC were isolated on the basis of the different sites they occupy in the UC and the choice of suitable media for culturing them. This follows on from a previous study, in which we successfully applied a single enzyme approach to isolate MSCs from human UC (13), and observed that the majority of the MSCs were obtained from the Wharton's jelly, while EPCs were obtained from the surroundings of the umbilical cord vein. This study was conducted in accordance with the Declaration of Helsinki, and with approval from the Ethics Committee of the Affiliated Hospital of Jining Medical University (Jining, China). Written informed consent was obtained from the participants. Four replicates were performed.

Human UCs were obtained from cesarean section births following full-term pregnancy, and were then kept at 4°C in DF12 (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) and treated within 24 h. The UCs were obtained from the General Hospital of Chinese People's Liberation Army (Beijing, China). The mothers of all newborns provided written informed consent. At the beginning of the experiment, samples were flushed with phosphate-buffered saline (PBS, pH 7.0) containing 2% gentamicin (Thermo Fisher Scientific, Inc.) twice. The umbilical vein cavity was also flushed with PBS to remove the residual blood. Following the ligation of one end of the cord with a surgery line, 5-10 ml 0.1% collagenase type II (Gibco; Thermo Fisher Scientific, Inc.) was injected into the umbilical vein cavity. With dual-port ligation, the cord was placed in PBS for 1 h at 37°C. The digested umbilical vein was then fully washed and the digested cells were collected by centrifugation at 724 x g for 10 min. The resuspended cells were cultured in fibronectin-coated T₇₅ culture flasks (Corning Incorporated, Corning, NY, USA) containing 12 ml complete EGM-2 medium supplemented with 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland) at a density of 2x10⁴ cells/cm². The cells were incubated in a humidified incubator at 37°C under 5% CO₂. Approximately 6 days later, non-adherent cells were removed and the adherent cells were cultured; these were considered to be EPCs.

After the digested cells had been obtained, the UC vessels were cleared off, and the remainder of the cord was incised into cubes 1-2 cm³ in size. The cubes were incubated in 0.1%

collagenase type II for 1 h at 37°C. The digested cubes were collected. After passing them through a 100- μ m nylon strainer, the filtered cells were centrifuged at 322 x g for 10 min. The resuspended cells were seeded in T₇₅ flasks containing 12 ml DF12 medium with 10% FBS, supplemented with 100 U penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a density of 2x10⁴ cells/cm². All flasks were incubated at 37°C with 5% CO₂. After 3 days, the adherent cell population was cultured. This population was considered to comprise MSCs. Four replicates were performed.

EPCs or MSCs were passaged at a ratio of 1:3 at subconfluence using 0.05% trypsin/EDTA (Thermo Fisher Scientific, Inc.). For the following experiments, all cells were used at passage 3.

Proliferative potential of UC-EPCs and MSCs. Firstly, colony-forming assays of the two types of cells were conducted. The colony-forming assays were performed as previously described (13). The numbers of colonies that contained >50 cells were counted. Subsequently, a growth kinetics assay was conducted. Adherent cells were harvested with 0.25% trypsin/EDTA digestion when they had grown to 80% confluence. Mononuclear EPCs and MSCs were separately seeded to 24-well plates at 2x10⁴ cells/cm² in 0.5 ml EGM-2 medium (EPCs) or DF-12 medium (MSCs), which was replaced every 3 days. The procedure was performed as previously described (13). Four replicates were performed.

Finally, cell cycle analysis was performed. The two types of cells, which were in the logarithmic phase, were fixed with precooled 75% ethanol at 4°C for 1 h after trypsinization. Cells were incubated with 50 μ g/ml of propidium iodide (PI) for 5 min at 4°C protected from light and then analyzed using CellQuest software (version 5.1) and a FACSCalibur system (both BD Biosciences, Franklin Lakes, NJ, USA). Four replicates were performed.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of UC-EPCs and MSCs. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA synthesis was performed using the MLV RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) for 50 min at 37°C. In order to evaluate the expression of human von Willebrand factor (vWF), Fms-related tyrosine kinase 1 (Flt-1), CD31, vascular endothelial (VE)-cadherin and kinase insert domain receptor (KDR) in the EPCs, primers (details shown in Table I) were used as previously described (16-19). To detect the expression of fatty acid binding protein 4 (FABP4), peroxisome proliferator-activated receptor γ (PPAR- γ), alkaline phosphatase (ALP) and osteocalcin in UC-MSCs, primers (details shown in Table II) were used as previously described (13). PCR was performed using a Mini Cyclor (MJ Research; Bio-Rad Laboratories, Inc., Hercules, CA, USA). β -actin was used as a reference. The PCR conditions were 5 min at 95°C, then 45 cycles of 95°C for 30 sec, 60-65°C for 30 sec and 72°C for 30 sec, then a final extension for 10 min at 72°C. The expression of genes was detected using an Applied Biosystems 7500 Real-time PCR system (Thermo Fisher Scientific, Inc.) and SYBR Green Master mix (Beijing TransGen Biotech Co., Ltd., Beijing, China). The amplified samples were run on a 1%

Table I. Primers for human endothelial progenitor cells.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
CD31	GCTGTTGGTGGAAAGGAGTGC	GAAGTTGGCTGGAGGTGCTC
KDR	CAACAAAGTCGGGAGAGGAG	ATGACGATGGACAAGTAGCC
Flt-1	AGCAAGTGGGAGTTTGC	AGGTCCCAGATGAATGC
VE-Cadherin	AAGACATCAATGACAACCTCC	CCTCCACAGTCAGGTTATACC
vWF	GAGGCTGAGTTTGAAGTGC	CTGCTCAGCTCATCCAC

KDR, kinase insert domain receptor; Flt-1, Fms-related tyrosine kinase 1; VE, vascular endothelial; vWF, human von Willebrand factor.

Table II. Primers for human mesenchymal stem cells.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
FABP4	GTCACAGCACCTCCT	AGCCCACTCCTACTTCTT
PPAR- γ	AAGCCAACACTAAACCACA	GAAATGCTGGAGAAGTCAA
ALP	CCTGGGAGACAAAGCAATAA	TCCTGGGTAGCTGGGACTA
Osteocalcin	GAGGGCAGCGAGGTAGTGAAG	CCTGAAAGCCGATGTGGTC

FABP4, fatty acid binding protein 4; PPAR- γ , peroxisome proliferator activated receptor γ ; ALP, alkaline phosphatase.

agarose gel with ethidium bromide and photographed. Four replicates were performed.

Flow cytometric analysis of UC-EPCs and MSCs. Cells were harvested and washed prior to suspension in PBS for flow cytometry. EPCs (1×10^5 cells/well) were respectively labeled with eight different antibodies, namely, fluorescence isothiocyanate (FITC) conjugated mouse anti-human CD90 (cat. no. 555595) and HLA-DR (cat. no. 555560), phycoerythrin (PE) conjugated mouse anti-human CD31 (cat. no. 560983), CD34 (cat. no. 555822), CD73 (cat. no. 550257), CD105 (cat. no. 560839) and vascular endothelial growth factor receptor (VEGRF) 2 (cat. no. 560872), and peridinin chlorophyll protein complex (PerCP) conjugated mouse anti-human CD45 (cat. no. 564106). MSCs (1×10^5 cells/well) were separately stained using the aforementioned antibodies with the exception of PE-conjugated mouse anti-human CD31 and VEGRF-2. Mixtures were placed in the dark for 15 min at room temperature. All of the monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Cells were analyzed by flow cytometry as described above. Four replicates were performed.

Fluorescent-labeled acetylated low-density protein (Ac-LDL) uptake and lectin binding assay of UC-EPCs. EPCs were placed in 24-well plates at a density of 1×10^5 cells/cm² in 1 ml DF-12 medium with 10 μ g/ml 1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbocyanine perchlorate-labeled Ac-LDL (DiL-Ac-LDL; Molecular Probes; Thermo Fisher Scientific, Inc.) at 37°C for 24 h. Cells were then fixed with 4% paraformaldehyde for 20 min and incubated in PBS containing 10 μ g/m plant lectin from *Ulex europaeus* conjugated with FITC (FITC-UEA-1; Sigma-Aldrich; Merck KGaA) at room

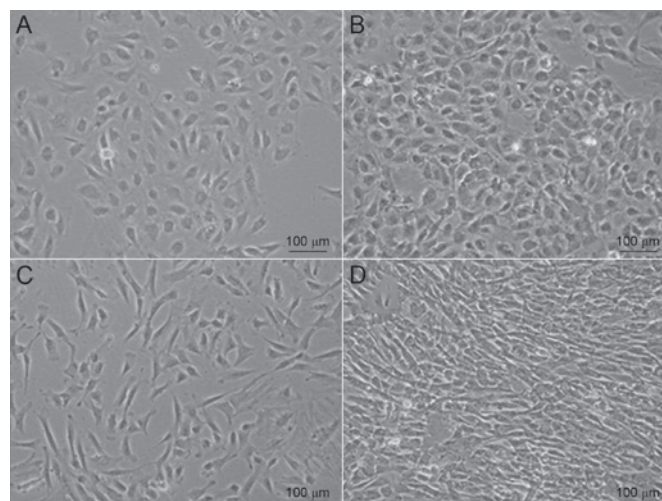


Figure 1. Morphology of mononuclear cells isolated from human UC. The human UC-derived endothelial progenitor cells were cultured in complete EGM-2 medium for (A) 72 h and (B) 7 days. The cells exhibited a typical cobblestone appearance. The human-derived mesenchymal stem cells were cultured in DF12 medium with 10% fetal bovine serum for (C) 72 h and (D) 7 days. The cells exhibited a fibroblast-like morphology. Scale bars, 100 μ m. UC, umbilical cord.

temperature for 1 h. Cells were observed under an inverted microscope (Olympus IX73; Olympus Corporation, Tokyo, Japan). Four replicates were performed.

Matrigel assay of UC-EPCs. Matrigel basement membrane matrix (BD Biosciences) was used according to the manufacturer's recommended protocol to evaluate the tube formation capability of the EPCs *in vitro*. A 300- μ l quantity of Matrigel

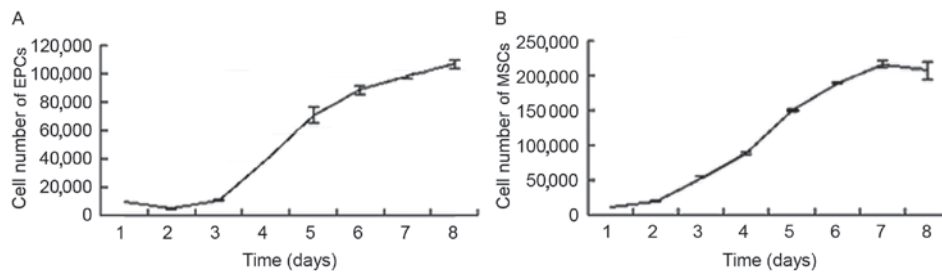


Figure 2. Growth curves of cultured human UC-derived (A) EPCs and (B) MSCs. Each data point represents the mean of three separate wells. EPC, endothelial progenitor cell; MSC, mesenchymal stem cell.

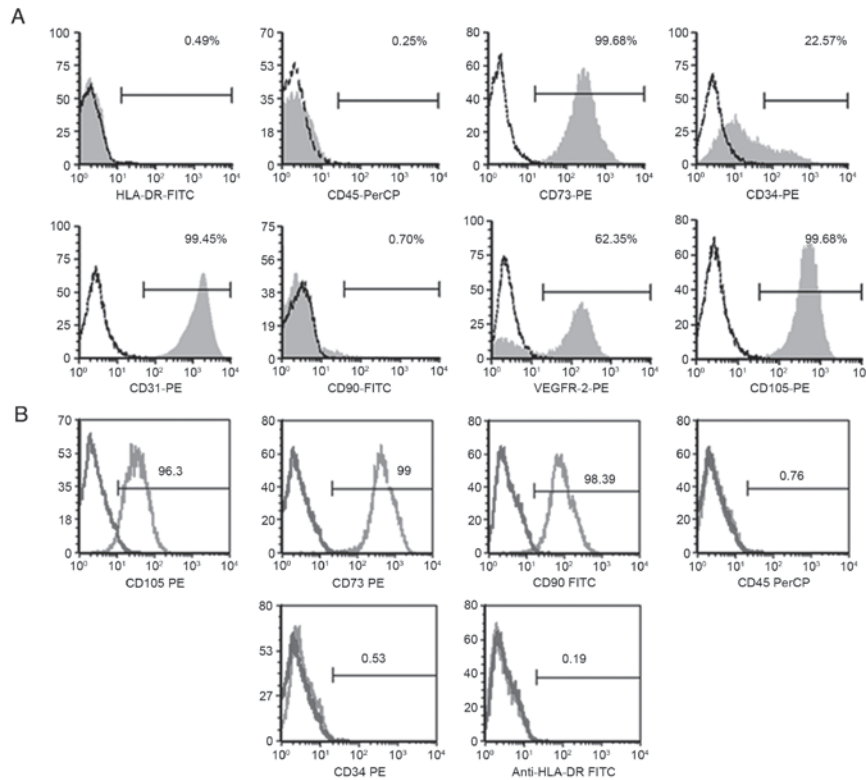


Figure 3. Human umbilical cord cell phenotypes as determined by flow cytometric analysis. Phenotypes of (A) EPCs and (B) MSCs. The EPCs were positive for CD31, CD34, CD73, VEGFR-2 and CD105 and negative for CD45, CD90 and HLA-DR. The MSCs were positive for CD73, CD90, CD105, and negative for CD34, CD45 and HLA-DR. EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; VEGFR-2, vascular endothelial growth factor receptor 2.

basement membrane matrix was added to each well of a precooled 24-well plate and incubated at 37°C for 30 min. Monolayer cells (2×10^4) suspended in 300 μ l DF-12 medium were added to the Matrigel basement membrane matrix. After 24 h of normal culture, cells were assessed using an inverted microscope. Four replicates were performed.

Differentiation of UC-MSCs. Adipogenic and osteogenic differentiation of the MSCs was carried out as previously described (15). To induce adipocyte differentiation, adipogenic medium consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 100 μ g/ml 1-methyl-3-isobutyl-xanthine (Sigma-Aldrich; Merck KGaA), 50 μ g/ml ascorbic acid (Sigma-Aldrich; Merck KGaA) and 10^{-6} M dexamethasone (Sigma-Aldrich; Merck KGaA) was used. After 21 days, the cells were fixed in 10% formalin (Merck KGaA) for 30 min, then

stained with fresh 0.6% Oil Red O solution (Sigma-Aldrich; Merck KGaA). DMEM supplemented with 10% FBS, 7.0×10^{-5} M β -glycerophosphate (Sigma-Aldrich; Merck KGaA), 2.0×10^{-6} M ascorbic acid, and 10^{-8} M dexamethasone was used to induce osteogenic differentiation. After 14 days, the cells were fixed in 10% formalin (Merck KGaA) for 30 min, then stained with fresh 2% Alizarin Red (Sigma-Aldrich; Merck KGaA). Four replicates were performed.

Results

Isolation and culture of UC-EPCs and MSCs. Two types of stem cells were isolated from human UCs using a convenient protocol in the present study. After being cultured for 3-7 days, a few cells from the umbilical vein started to adhere to the well (Fig. 1A). These appeared to have a typical cobblestone appearance when observed under a microscope. After 7-10 days, the

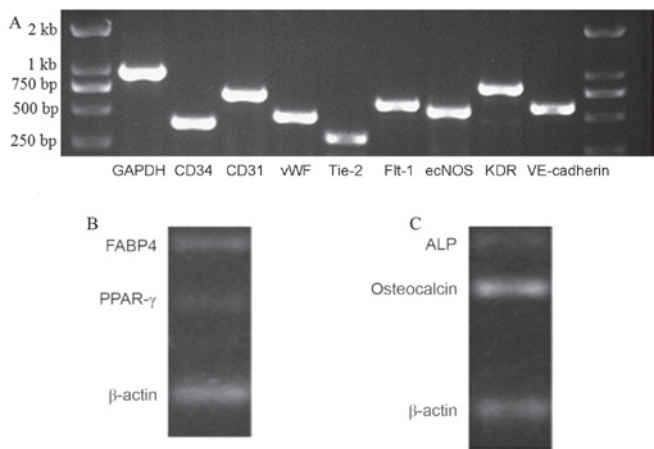


Figure 4. Reverse transcription polymerase chain reaction analysis of EPCs and differentiated MSCs from human umbilical cord. (A) EPC/endothelial cell RNAs vWF, Flt-1, CD31, VE-cadherin and KDR were expressed by the UC-EPCs. The expression of GAPDH was used as a reference. (B) These cells expressed FABP4 and PPAR- γ RNA. (C) Osteogenic medium was used to induce osteogenic differentiation. These cells expressed ALP and osteocalcin RNA. EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; vWF, von Willebrand factor; Flt-1, Fms-related tyrosine kinase 1; KDR, kinase insert domain receptor; VE, vascular endothelial; ecNOS, endothelial constitutive nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells became confluent and exhibited colony growth (Fig. 1B). When these cobblestone-like cells had reached >80% confluence in the T₇₅ flask, 2.5×10^6 cells (n=15) were obtained. By contrast, primary cells from Wharton's jelly required a shorter time to adhere to the well. Adherent cells were observed within 24-48 h (Fig. 1C). After 2-3 weeks, the cell morphology changed to become more homogeneous, as the fibroblast-like cells gradually increased while the polygonal cells decreased (Fig. 1D).

To quantitatively evaluate the proliferative potential of the cells, three indicators were examined: Growth kinetics, cell cycle and colony-forming ability. The growth curves for the EPCs and MSCs were consistent with the basic law of cell growth. All cells maintained a normal growth state. The EPC doubling time was calculated as 43.55 ± 5.22 h (Fig. 2A) in the logarithmic growth phase, while the doubling time of MSCs was calculated as 24.03 ± 0.27 h (Fig. 2B). The DNA content of the MSCs and EPCs was analyzed to evaluate the cellular cycle using PI. Flow cytometric analysis demonstrated that the majority of the EPCs and MSCs were in the G₀/G₁ phase, accounting for 75.58 and 85.35% of the whole cell population, respectively. The results of cell cycle analysis for EPCs and MSCs were consistent with their stemness potencies. Moreover, the capacity to form colonies is one of the characteristics of stem cells. In the colony-forming experiment, EPC colony counts were ~36 per 1,000 single cells seeded on the cell culture dishes, whereas MSC colony counts were ~55 per 1,000 single cells. Thus, EPCs and MSCs both underwent relatively stable and rapid proliferation *in vitro*; however, the proliferation capacity of MSCs was greater than that of EPCs.

Flow cytometric analysis of UC-EPCs and MSCs. Results of the flow cytometric analysis for EPCs showed that cells were strongly positive for CD31, CD73, CD105 and VEGFR-2, and

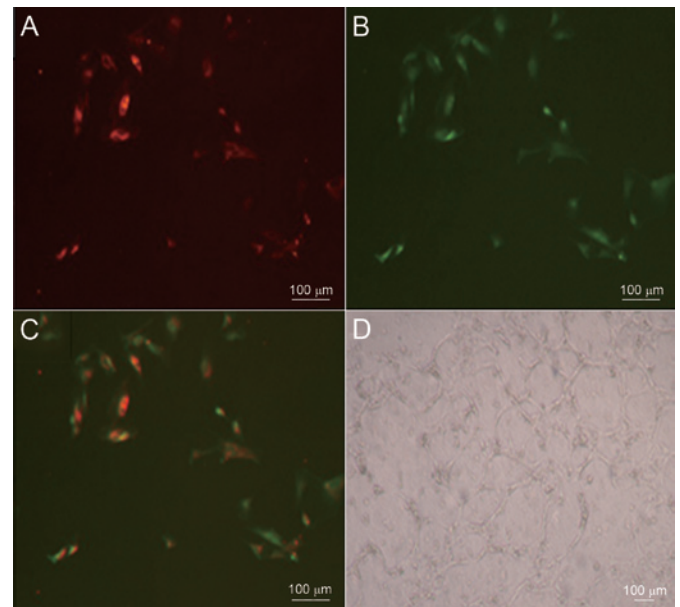


Figure 5. Human umbilical cord-derived EPCs presented the ability to take up DiI-ac-LDL and bind with FITC-UEA-I. Following culture for 24 h, the adherent cells (A) took up DiI-ac-LDL (red fluorescence) and (B) bound to FITC-UEA-I (green fluorescence). (C) The double-positive stained cells appeared yellow in color. (D) These EPCs readily formed tube-like structures when plated in 24-well plates on Matrigel. Scale bars, 100 μ m. EPC, endothelial progenitor cell; DiI-ac-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein; FITC-UEA-I, fluorescein isothiocyanate-conjugated plant lectin from *Ulex europaeus*.

weakly expressed the hematopoietic lineage CD34 (which decreased during passaging), but they were negative for CD45, CD90 and HLA-DR (Fig. 3A). When cell surface expression antigens of MSCs were analyzed, flow cytometry showed that these cells were positive for CD73, CD90 and CD105, and negative for CD34, CD45 and HLA-DR (Fig. 3B).

Identification of UC-EPCs. Total RNA of the UC-EPCs was obtained for RT-PCR analysis. The cells were characterized using RT-PCR to detect the expression of cell markers (Fig. 4). The endothelial progenitor cell/endothelial cell RNAs vWF, Flt-1, CD31, VE-cadherin and KDR were all expressed by the UC-EPCs. The expression of GAPDH was used as a reference (Fig. 4). These adherent cells were capable of taking up DiI-ac-LDL and binding to FITC-UEA-I (Fig. 5A-C). When these EPCs were seeded on a Matrigel basement membrane matrix in order to investigate whether they had true endothelial cell potential, the formation of vascular tube-like structures was observed after 24 h (Fig. 5D).

Differentiation of UC-MSCs. An adipogenic medium was used to induce adipocyte differentiation. After 5 days, the cells had begun to store lipid drops in the cytoplasm, which was considered a sign of adipogenic differentiation. After 21 days, when the cells were stained with Oil Red O solution, red staining of the cells was observed, which confirmed that cells had changed into adipose cells (Fig. 6A). In addition, these cells expressed FABP4 and PPAR- γ RNA, which is consistent with the adipocyte phenotype (Fig. 4B) (20,21).

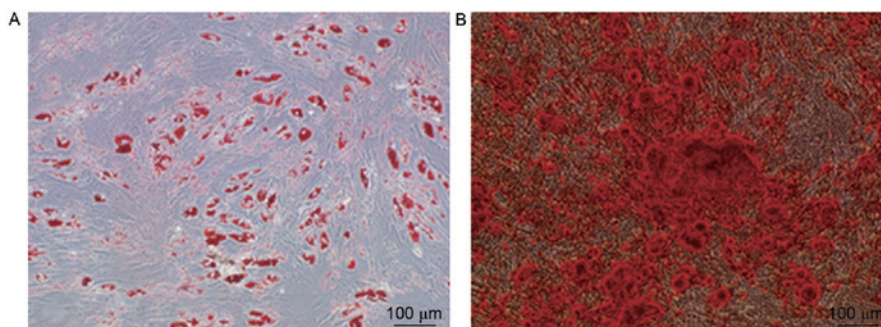


Figure 6. Differentiation potential human umbilical cord-derived MSCs. (A) MSCs from passage 3 were plated in adipogenic medium. After 3 weeks, the MSCs could be stained with oil red O. (B) After plating in osteogenic medium for 3 weeks, the cells showed calcium deposition as indicated by Alizarin Red S staining. Scale bars, 100 μ m. MSC, mesenchymal stem cell.

An osteogenic medium was used to induce osteogenic differentiation. The cells underwent a series of changes on the culture plates: Deposits formed on day 3 and gradually increased in size until day 21. It was confirmed that cells had changed into osteoblasts because they appeared red when treated with 2% Alizarin Red S (Fig. 6B) and were shown to express ALP and osteocalcin RNA (Fig. 4C).

Discussion

Recent studies have identified the human UC as a novel source of multipotent stem cells (22-24). There is a high demand for MSCs and EPCs, which are applied in the treatment of patients with hematologic disorders, malignancies, inherited immunodeficiency diseases, and even metabolic diseases (3,5,6,10-12). Although there have been some studies concerning the simultaneous collection of mesenchymal stem cells, hematopoietic cells or endothelial cells from the UC or the associated blood, the achievements have been limited (25,26). The present study describes a strategy to isolate MSCs and EPCs from a single UC according to their different locations within the cord.

EPCs have been isolated and expanded *ex vivo* from UC blood or Wharton's jelly in previous research (20,21). However, where the majority of EPCs reside in the UC is unknown. We considered that they may be distributed around the UC vein. EPCs were successfully isolated from the UC vein in the present study. The isolated EPCs were demonstrated to have the properties of progenitor cells in proliferative potential assays, including growth curve, cell cycle and colony-forming assays. The cells had a cobble-like morphology, and expressed Flt-1, KDR, VE-Cadherin, CD31 and vWF RNAs. They were strongly positive for CD31, CD73, CD105 and VEGFR-2, but were negative for CD45, HLA-DR and the mesenchymal marker CD90. In addition to absorbing DiI-ac-LDL and FITC-UEA-I, they were able to form vascular tube-like structures on Matrigel.

In the present study, the MSCs taken from the UC Wharton's jelly had a spindle-shaped morphology, resembling that of MSCs isolated from bone marrow. Flow cytometric analysis showed that the percentage of cells expressing CD73, CD90, CD105, CD34, CD45 HLA-DR was consistent with previously reported data (27,28). These MSCs were able to differentiate into adipocytes that accumulated lipid vacuoles and osteoblasts that were stained with Alizarin Red S *in vitro*. They expressed

adipocyte/osteoblast-specific RNAs that were consistent with previous reports for bone marrow MSCs (29).

In conclusion, the present study describes an economical and commercially viable option for the harvesting of EPCs and MSCs for use in stem cell research and cell replacement therapy.

Acknowledgements

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