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In Vitro and In Vivo Differentiation of Human Umbilical Cord Derived Stem Cells Into Endothelial Cells

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Abstract The successful use of tissue-engineered transplants is hampered by the need for vascularization. Recent advances have made possible the using of stem cells as cell sources for therapeutic angiogenesis, including the vascularization of engineered tissue grafts. The goal of this study was to examine the endothelial potential of human umbilical cord-derived stem (UCDS) cells. UCDS cells were initially characterized and differentiated in an endothelial differentiation medium containing VEGF and bFGF. Differentiation into endothelial cells was determined by acetylated low-density lipoprotein incorporation and expression of endothelial-specific proteins, such as PECAM and CD34. In vivo, the transplanted UCDS cells were sprouting from local injection and differentiated into endothelial cells in a hindlimb ischemia mouse model. These findings indicate the presence of a cell population within the human umbilical cord that exhibits characteristics of endothelial progenitor cells. Therefore, human umbilical cord might represent a source of stem cells useful for therapeutic angiogenesis and re-endothelialization of engineered tissue grafts. J. Cell. Biochem. 100: 608–616, 2007.

Key words: human umbilical cord; stem cells; endothelial differentiation; tissue engineering

The successful use of tissue-engineered transplants is hampered by the need for vascularization. A solution would be to provide a preformed primitive vascular plexus within the transplant which could be used by pre-existing host vessels to form anastomoses, thereby accelerating vessel in-growth [Levenberg et al., 2005; Messina et al., 2005; Wu et al., 2006]. Endothelial cells are important participants in tissue repair and regeneration. Unfortunately, mature endothelial cells have limited proliferative capacities, which makes it necessary to search for alternative sources of these cells for autologous and allogenic use. Recent advances have created the possibility of using stem cells as cell sources for therapeutic angiogenesis, including the vascularization of engineered tissue grafts [Shen et al., 2003; Schmidt et al., 2004; Cao et al., 2005]. Oswald et al. [2004] established a protocol based on low-serum culture supplemented with vascular endothelial growth factor (VEGF) and showed that under these conditions, bone marrow-derived mesenchymal stem cells (MSCs) could acquire several features of mature endothelium and demonstrated an enhanced ability to form capillary structures, making these cells promising candidates for in vitro re-endothelialization of engineered tissue grafts and therapeutic

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angiogenesis in ischaemic diseases. However, the clinical use of bone marrow MSCs has presented problems, including pain, morbidity, and declined number or plasticity contribute to aging and age-related disease [Zhang et al., 2005]. This has led many researchers to investigate alternate sources for MSCs. Recent reports indicated that human umbilical cord as a novel source of multipotent stem cells. These cells display a fibroblast-like morphology, express mesenchymal markers, and have the potential to differentiate into osteogenic, adipogenic, chondrogenic, and cardiogenic cells [Romanov et al., 2003; Wang et al., 2004].

In the present study, we develop a method that can readily isolate and expand stem cells from human umbilical cord tissues, called human umbilical cord-derived stem (UCDS) cells. After characterization by flow cytometry and differentiation into osteogenic and adipogenic cells, the isolated UCDS cells were treated with VEGF and basic fibroblast growth factor (bFGF) in an in vitro model system for study of their differentiation and plasticity potential towards endothelial lineage. In addition, UCDS cells were transplanted into the hindlimb ischemia model of nude mice and showed to participate in the vessel network and differentiate into endothelial-lineage cells in vivo. To our knowledge, this is first report that UCDS cells can be differentiated into functional endothelial cells both in vitro and in vivo.

MATERIALS AND METHODS

Isolation and Cultivation of UCDS Cells

With the consent of the parents, fresh umbilical cords were collected from normal full-term pregnancies according to the regulations of Chinese Academy of Medical Sciences and Peking Union Medical College Research Ethics Committee and the UCDS cells were isolated immediately. After removal of blood vessels, the cord was washed extensively with D-Hanks' balanced salt solution. Then, the tissue was minced into 1-mm^3 pieces with sharp scissors and the tissue pieces were treated with 0.075% collagenase II (Sigma) for at least 2 h at 37°C with agitation, then, further digested with 0.25% trypsin (Sigma) at 37°C for 30 min. Fetal bovine serum (FBS) was added to the mesenchymal tissues to neutralize the excess trypsin. The digested mixture was then passed through a 100-μm filter to obtain cell suspensions. Next, the dissociated cells were centrifuged at 2,000 rpm for 18 min at room temperature and washed three times. Finally, the cells were plated on uncoated 25-cm^2 culture flasks (Beckon Dickinson) and allowed to proliferate in Dulbecco's modified Eagle's medium with low glucose (DMEM-LG/F-12, DF12; Gibco), supplemented with 10% FBS (HyClone, Logan, UT), 10 ng/ml epidermal growth factor (EGF; Pepro Tech), 100 U penicillin/streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (Gibco). Medium was replaced at 48 h and every 2 or 3 days following. Daily progress was monitored by phase-contrast microscopy. The cells were serially passaged and expanded in a humidified incubator at 37°C with 5% CO_2. Only cells cultured from passage 4 to 8 were used for experiments.

Characterization of UCDS Cells Phenotype

The cell surface markers of UCDS cells were analyzed by flow cytometry. After trypsinization, the detached cells were washed and resuspended in phosphate buffered saline (PBS). Approximately 10^6 cells were incubated with monoclonal antibodies against phycoerythrin (PE)-conjugated CD13, CD29, CD34, CD44, CD166 and HLA-DR, fluorescein isothiocyanate (FITC)-conjugated CD31, CD45, CD90, CD106, CD117, HLA-ABC (All from Becton Dickinson, San Jose, CA). All incubations were performed at room temperature for 20 min. Control samples were incubated with FITC and PE-conjugated mouse IgG1 isotype antibodies. After incubation, cells were washed with PBS containing 0.1% BSA. Finally, the cells were assayed in a FACS flow cytometer (Becton Dickinson).

Differentiation Into Osteocytes and Adipocytes

For osteogenic differentiation, the cultured UCDS cells from passages (P4–6) were incubated in DMEM-LG supplemented with 10% FBS, dexamethasone (0.1 μmol/L), ascorbic acid (0.2 mmol/L), and β-glycerophosphate(10 mmol/L) (all Sigma). The medium was replaced twice a week. After day 2 or 3 weeks in differentiation medium, cell colonies displayed bone-like nodular aggregates of matrix mineralization. Then the cells were stained with von Kossa to reveal osteogenic differentiation. For adipogenic differentiation, the UCDS cells were induced for 3 weeks in DMEM supplemented with 10% FBS, hydrocortisone (0.5 μmol/L), isobutylmethylxanthine (0.5 mmol/L), and

indomethacin (50 μg/ml) (all Sigma). At the end of the culture, the cells were fixed in 10% formalin for 10 min and stained with fresh Oil red-O solution (Sigma) to show lipid droplets in induced cells.

In Vitro Endothelial Differentiation of UCDS Cells

To analyze in vitro endothelial differentiation, a 24-well cell culture plate was coated with Matrigel (8.8 mg/ml; BD Bioscience Biotech.) in each well. UCDS cells were suspended in endothelial differentiation medium at a concentration of 5×10^4/ml and 1 ml of cell suspension was added to each well. Differentiation medium contained medium199, 50 ng/ml VEGF (R&D), 10 ng/ml b-FGF (R&D), and 2% FBS. Cultures were incubated in a humidified incubator at 37°C with 5% CO_2 for 2–3 days observed by phase-contrast microscopy.

Immunofluorescence

Matrigel with differentiated UCDS cells was dispersed with dispase and the retrieved cells were allowed to attach to the gelatin-coated glass coverslips for 24 h. Differentiated UCDS cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and stained for surface markers. The coverslips were placed with cells side up in a petri dish, rinsed with PBS, and then incubated with blocking buffer (1% BSA in PBS) for 30 min at 37°C to minimize non-specific adsorption of the antibodies to the coverslip. PECAM (CD31) and CD34 were visualized by incubation with mouse anti-human PECAM and CD34 antibodies (BD PharMingen) and conjugated with FITC-conjugated antibodies (Zhongshan, Beijing, China). Then, the cells were viewed using a confocal microscope (Leica Microsystems, GmbH).

Acetylated Low-Density Lipoprotein Uptake

To further verify that exposure to VEGF and bFGF resulted in cellular differentiation toward an endothelial-like phenotype, uptake of DiI-labeled acetylated low-density lipoprotein (ac-LDL), a function associated with endothelial cells, was determined. Cells were incubated with 10 μg/ml DiI-acLDL (Molecular Probes) at 37°C for 4 h and analyzed by fluorescence microscopy.

Hindlimb Ischemia Model

The endothelial potential and neovascularization capacity of UCDS cells was investigated in a murine ischaemic hindlimb model. All procedures were performed on male athymic nude mice (7–8 weeks, 16–20 g; Institute of Experimental Animal, Beijing, China) according to Peking Union Medical College Animal Care and Use Committee guidelines. Mice were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally. Left femoral artery, great saphenous artery, iliac circumflex artery/vein, and muscular branch were ligated and cut to induce left hindlimb ischemia. Twenty-four hours later, UCDS cells (1×10^6 cells in 60 μl volume) were injected in six sites in the left adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site.

In Vivo Endothelial Differentiation Analysis

Four weeks after DiI-labeled UCDS cells transplantation, mice were euthanized with an overdose of pentobarbital, and ischaemic tissue was obtained. Multiple frozen sections of 5-μm thick were prepared and examined under fluorescence microscopy and frozen sections of 100 μm were sequentially scanned using a confocal microscope. Bandeiraea simplicifolia lectin 1 (BS-1 lectin, Vector Lab) and CD34 (BD PharMingen) were used to detect the differentiated UCDS cells. Both the rhodamine and fluorescein filters were used for each image collected during the scanning process.

Laser Doppler Perfusion Image

Laser Doppler perfusion image (LDPI) (Lisca AB, Linkoping, Sweden) was used to record the blood flow of hindlimbs that underwent no self-amputation after operation. Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed ventral side up on a piece of black paper. The hindlimbs were gently immobilized and scanned using a laser Doppler imager under room temperature (24–28°C). Pre-surgery scans were performed (three times) 1 day before surgery to determine if flow was similar in both limbs. Post-surgery measurements were done three times. The ambient light of operating surroundings was in faint without any obvious airflow around. In some instances, the initial scan varied significantly from the subsequent scans and was not included in the data analysis. In such cases, scans were performed until the

blood flow measurements were stable for three scans. The stored images were subjected to computer-assisted quantification of blood flow. In these digital color-coded images, red hue indicates regions with maximum perfusion; medium perfusion values are shown in yellow; lowest perfusion values are represented as blue. To minimize data variables due to ambient light and temperature, the LDPI index was expressed as the ratio of ischemic to non-ischemic limb blood flow [Li et al., 2006; Zhou et al., 2006].

RESULTS

Cell Morphology

The cells isolated by collagenase digest showed heterogeneity during the first 3 days. When initially plated, the UCDS cells appeared rounded in shape. After 48 h of plating, the cells were adherent, elongated, and spindle-shaped (Fig. 1A). When the medium was changed, the suspending cells were removed and the sub-cultured cells were much pure and fibroblast-like (Fig. 1B). The primary culture cells reached confluence about 1 or 2 weeks later, the cells sub-cultured at a ratio of 1:3 reached confluence 2 or 3 days later.

Osteogenic and Adipogenic Differentiation

The ability of UCDS cells to differentiate into osteocytes and adipocytes was tested. When cultured in osteogenic medium for 15 days, the morphology changed, began to mineralize their matrix, and was positive for Kossa staining (Fig. 1C). They were also able to differentiate into adipocytes, as cells accumulated different amounts of lipid vacuoles after cultivation in adipogenic medium (Fig. 1D).

Flow-Cytometric Characterization

To characterize the phenotypes of UCDS cells, flow cytometry was performed. The results showed that UCDS cells were positive for CD13, CD29, CD44, CD90, CD166, and HLA-ABC, in addition, no expression of the hematopoietic and endothelial lineage markers (CD31, CD34, CD45, CD106, and CD117) or HLA-DR was observed (Fig. 2).

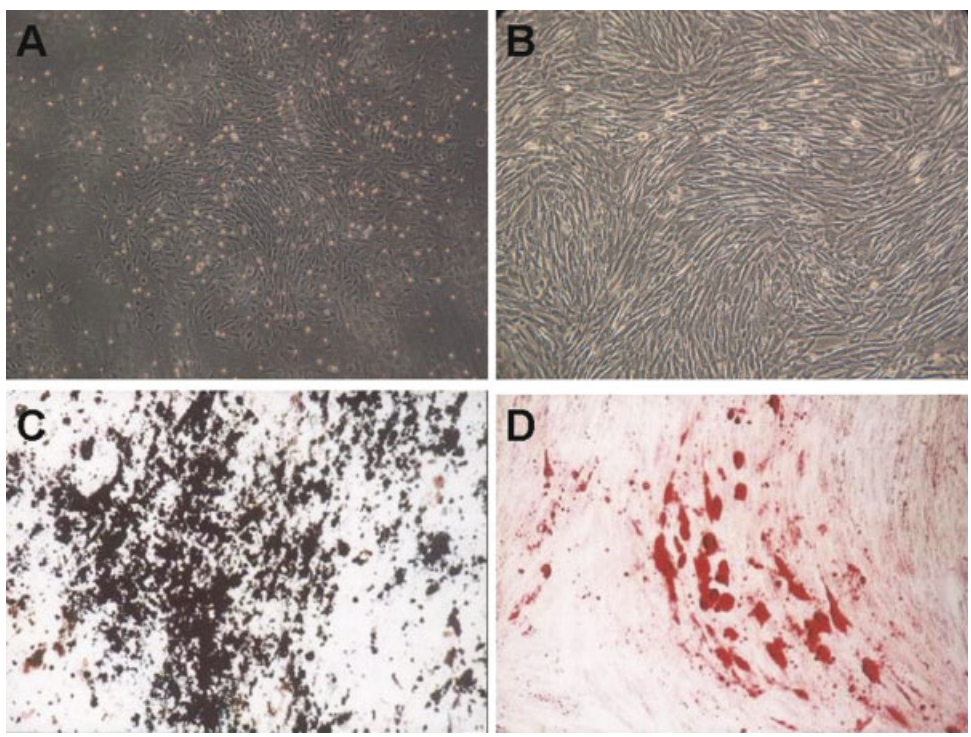

Fig. 1. Morphology and differentiation ability of UCDS cells. **A**: Morphology of primary cultured UCDS cells. **B**: Morphology of sub-cultured UCDS cells, the cells were fibroblast-like and grew as a whirlpool. **C**: Von Kossa staining of the UCDS cells after osteogenic induction. **D**: Oil-red O staining of the UCDS cells after adipogenic induction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

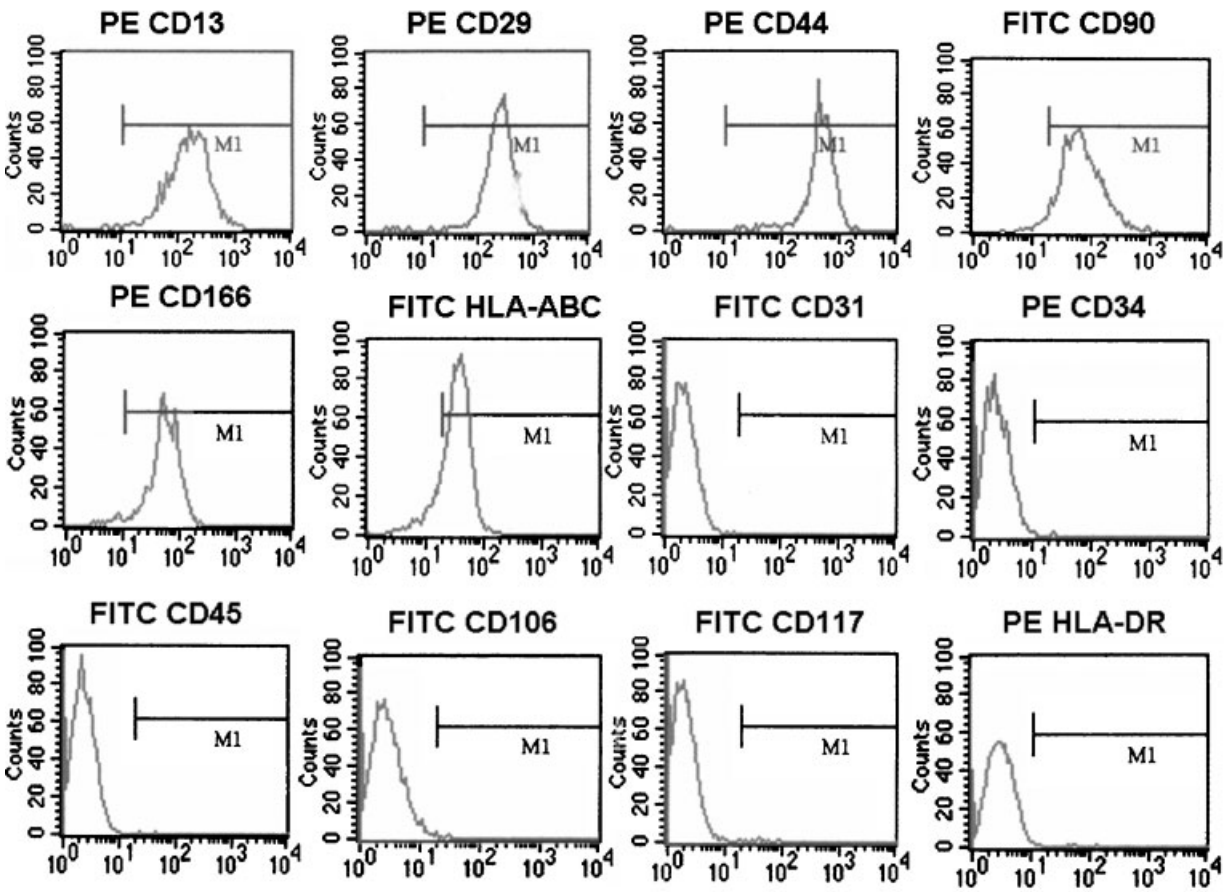

Fig. 2. Phenotypes of the UCDS cells. UCDS cells were labeled with PE- or FITC-conjugated antibodies and examined by means of flow cytometory. Cells were positive for CD13, CD29, CD44, CD90, CD166, and HLA-ABC, but not CD31, CD34, CD45, CD106, CD117, and HLA-DR. Data are representative of several independent experiments.

Endothelial Differentiation of UCDS Cells In vitro

To induce UCDS cells into functional endothelial cells in vitro, UCDS cells were cultured with 50 ng/ml VEGF, 10 ng/ml b-FGF on the Matrigel coated coverslips. Light microscopy observation over a period of 48 h shows that vascular network was formed by UCDS cells after incubation with endothelial differentiation medium (Fig. 3A).

The uptake of DiI-labeled ac-LDL is a specific marker for endothelial cells in vitro. Immunofluorescence revealed that the induced cells were positive for ac-LDL uptake (Fig. 3B). Further, immunofluorescence studies confirmed their endothelial phenotype with expression of known endothelial cell markers including PECAM, CD34, (Fig. 3C,D) and 30–50% UCDS cells were positively stained after cultured in endothelial differentiation medium containing VEGF and bFGF. In addition, different staining from cells in the same area of culture showed the induced cells which were positive for ac-LDL uptake (Fig. 3E,G) were also positively stained with PECAM (Fig. 3F) and CD34 (Fig. 3H).

Endothelial Differentiation of UCDS Cells In vivo

To determine the endothelial potential and whether UCDS cells could participate in the revascularization of ischaemic tissues in vivo, cultured UCDS cells were injected into the left adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site of nude mice 24 h after induction of ischemia by the ligation of the left femoral artery. Transplanted human UCDS cells marked with DiI were identified in tissue sections by red fluorescence. In contrast, the mouse vasculature, stained by

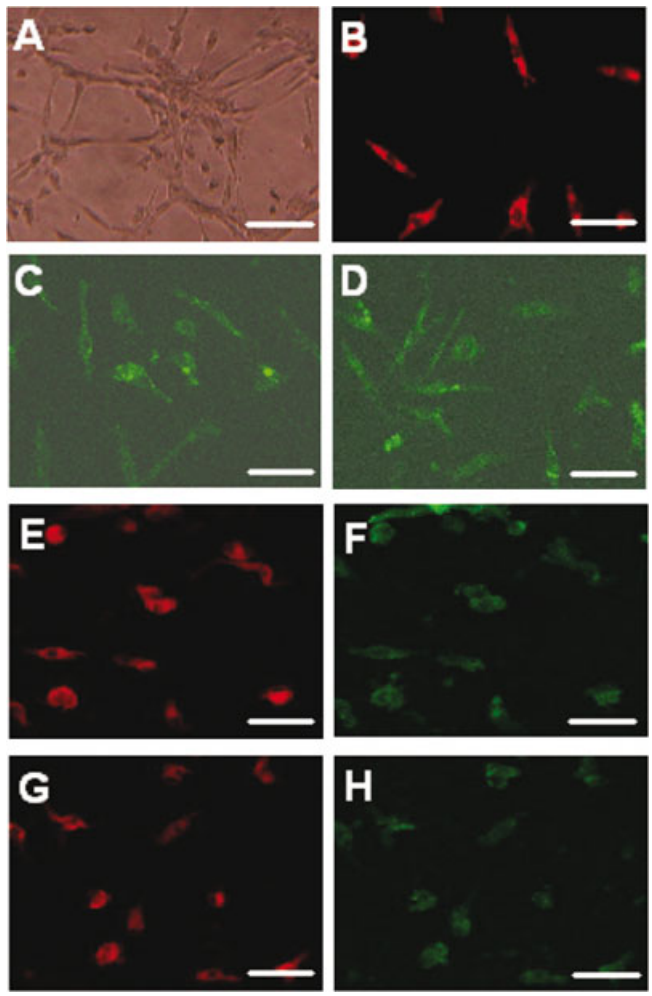

Fig. 3. Endothelial differentiation of UCDS cells in vitro. **A**: Morphological changes during endothelial differentiation of UCDS cells at 24 h. Differentiation into endothelial cells was demonstrated by ac-LDL incorporation (**B**), PECAM (**C**), and CD34 (**D**). Different staining from cells in the same area of culture were shown, uptake of ac-LDL (**E**, **G**) were also positively stained with PECAM (**F**) and CD34 (**H**). Bar indicates 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

premortem administration of BS-1 lectin, was identified by green fluorescence in the same tissue sections and merge of them showed the incorporation of UCDS cells into murine vasculature (Fig. 4A–C). Moreover, immunofluorescence staining of anti-human CD34 antibody in ischaemic hindlimb muscle 28 days after ischemia showed some DiI-labeled UCDS cells were differentiated into endothelial-lineage cells (Fig. 4D–F). Thus, locally transplanted UCDS cells were incorporated into foci of neovascularization and differentiated into mature endothelial cells in ischaemic hindlimb. In addition, LDPI analysis showed marked improvement of perfusion in UCDS cell-treated mice (Fig. 5B,D) compared to control medium (Fig. 5A,C).

DISCUSSION

Endothelial cell-based repair has been targeted in many applications: for the vascularization of tissue-engineered grafts, for the formation of blood vessels, and for the treatment of ischaemic tissues [Hu et al., 2003; Nowak et al., 2004; Yang et al., 2004]. To date, most approaches utilizing vascular-derived cells present certain shortcomings. Cell harvesting necessitates the sacrifice of intact vascular structures of donor organisms requiring greater operational technical expertise on the part of the surgeon and increasing patient trauma. Additionally, endothelial cells from adult blood vessels quickly proliferate in vitro, but their proliferative activity and their specific function are gradually lost, thus, their clinical

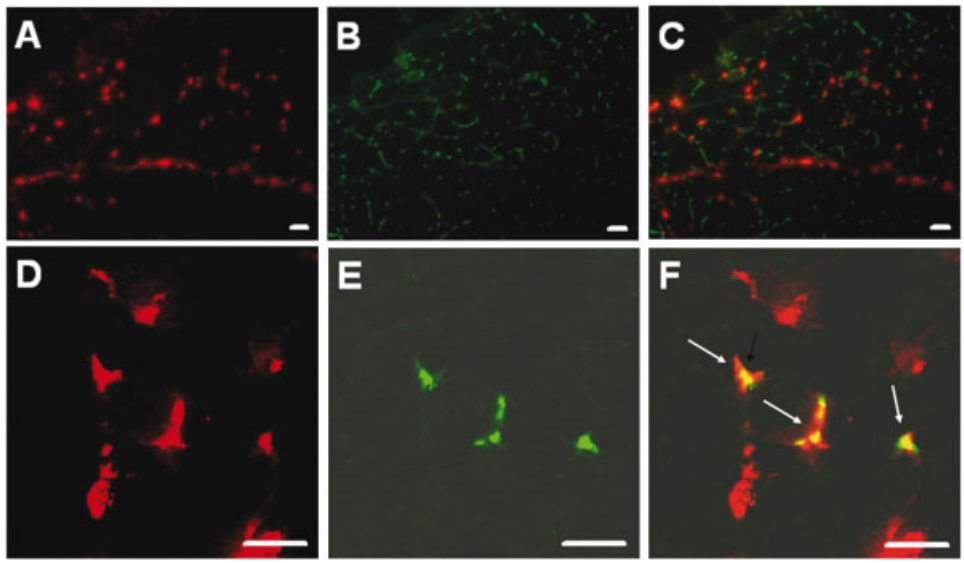

Fig. 4. Transplanted UCDS cells participated in the vessel network and differentiated into endothelial-lineage cells in vivo. The DiI-labeled UCDS cells (**A**) were sprouting from local injection. Murine capillary network was stained by BS-1 lectin (**B**), and merge of them showed the incorporation of UCDS cells into murine vasculature (**C**). Immunofluorescence staining of anti-human CD34 antibody (**E**) in ischaemic hindlimb muscle 28 days after ischemia showed some DiI-labeled UCDS cells (**D**) were differentiated into endothelial-lineage cells (arrows, **F**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

use is limited [Prasad Chennazhy and Krishnan, 2005].

Previous research has demonstrated that MSCs from adult tissues, under controlled microenvironments and exposure to appropriate induction factors in vitro, have the ability to differentiate into a variety of cell types, including endothelial cells [Pittenger et al., 1999; Ballas et al., 2002; Jiang et al., 2002]. Although addition of VEGF was essential for MSCs to

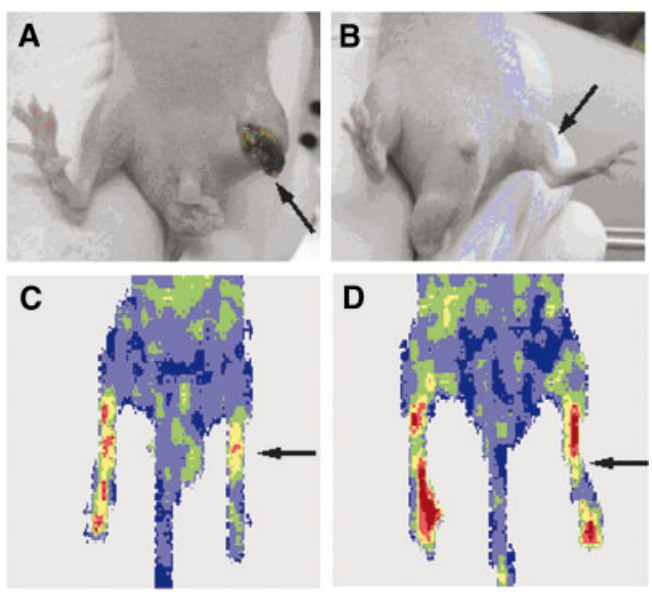

Fig. 5. Representative photographs of control medium (**A**) or UCDS cell-treated (**B**) ischaemic hindlimbs on Day 28 are shown. Representative photographs of LDPI in control medium-treated mice (**C**) or UCDS cell treated group (**D**). In color-coded images, high blood flow is depicted in red, while low perfusion is displayed as blue to dark. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

differentiate into endothelial cells, the precise mechanism by which these cells and their environment control differentiation is still not fully understood [Oswald et al., 2004].

Recent advances indicate that human umbilical cord as a novel source of multipotent stem cells [Wang et al., 2004]. In the present study, we showed that UCDS cells could be readily isolated from the human umbilical cord and the cells could be differentiated into osteogenic and adipogenic cells in vitro. FACS analysis showed that UCDS cells were positive for CD13, CD29, CD44, CD90, CD166, and HLA-ABC, in addition, no expression of the hematopoietic, endothelial lineage markers (CD31, CD34, CD45, CD106, and CD117) and HLA-DR were observed. The phenotype is similar to the MSCs we isolated from bone marrow. These results suggest that the UCDS cells are a crowd of undifferentiated stem cells that are different from hematopoietic stem cells.

The appearance of the mature endothelial cell markers PECAM and CD34 in UCDS cells in the presence of VEGF and bFGF demonstrated that these cells were capable of differentiating into endothelial cells in vitro. The uptake of ac-LDL indicated that they had developed endothelial cell function. The differentiation potential of UCDS cells into endothelial cells was also supported by in vivo transplantation experiment. When transplanted into ischaemic hindlimb of nude mice, they contributed to neovascularization as a result of a direct incorporation of the cells into the ischaemic limb, although other mechanisms cannot be ruled out, such as stimulation of proangiogenic factors, local stimulation of angiogenesis, or chemoattraction of endogenous stem/progenitor cells. Moreover, immunofluorescence staining showed some DiI-labeled UCDS cells were differentiated into endothelial-lineage cells. Thus, locally transplanted UCDS cells were incorporated into foci of neovascularization and differentiated into mature endothelial cells in ischaemic hindlimb. Finally, in vivo experiments showed a marked improvement of perfusion in the ischaemic hindlimb of nude mice strongly supporting the therapeutic potential of UCDS cells in ischaemic diseases.

The results of our study demonstrate that human UCDS cells represent an attractive cell source for clinical therapies like neovascularization of engineered tissue grafts and treatment of ischaemic diseases. These findings also suggest that VEGF or ischaemic environment can supply the proper conditions for endothelial differentiation of UCDS cells.

In summary, our work demonstrated that UCDS cells were capable of differentiating into endothelial cells both in vitro and in vivo. Umbilical cords are easy to obtain in segments between 20 and 30 cm length, which allows for the generation of a sufficient cell number in a relatively short period of time. In addition, UCDS cells can be easily extracted and cryopreserved, allowing for individuals to store their own samples for possible future autologous use even if there were no immediate indication that stem cell therapy would be required, suggesting human UCDS cells represent a promising cell source for tissue engineering and future routine therapeutic angiogenesis applications. Additional research is needed to determine whether genic mutations occur in the process of cell culture as well as the basic mechanisms of endothelial differentiation.

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