ARTICLE
Journal of Cellular Biochemistry 107:926–932 (2009)

Journal of Cellular Biochemistry

Cardiac Potential of Stem Cells From Whole Human Umbilical Cord Tissue

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ABSTRACT

We investigated the role of stem cells from human umbilical cord tissue in cardiomyocyte regeneration. The umbilical cord stem cells were initially characterized and differentiated in a myocardial differentiation medium containing 5-azacytidine for 24 h. Differentiation into cardiomyocytes was determined by expression of cardiac specific markers, like cardiac α-actin, connexin43, myosin, Troponin T, and ultrastructural analysis. In vivo, the transplanted umbilical cord stem cells were sprouting from local injection and differentiated into cardiomyocyte-like cells in a rat myocardial infarction model. Echocardiography revealed increasing left ventricular function after umbilical cord stem cell transplantation. These results demonstrate that umbilical cord stem cells can differentiate into cardiomyocyte-like cells both in vitro and in vivo. Therefore, human umbilical cord might represent a source of stem cells useful for cellular therapy and myocardial tissue engineering. Future studies are required to determine the molecular signaling mechanisms responsible for this phenomenon. J. Cell. Biochem. 107: 926–932, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: HUMAN UMBILICAL CORD; STEM CELLS; DIFFERENTIATION; CARDIOMYOCYTE

The human heart cannot regenerate significantly despite recent advances suggest there may be replicating cardiomyocytes or stem cells that are participating in the process of cellular maintenance and myocardial regeneration. Cardiac transfer of stem cells can have a favorable impact on tissue regeneration and contractile performance of the infarcted heart. A variety of cells including hemopoietic stem cells, skeletal myoblasts, mesenchymal stem cells (MSCs), and endothelial progenitol cells have been suggested to restore cardiac function after a myocardial infarction [Wu et al., 2006]. It has been demonstrated that MSCs have the potential to differentiate into cardiomyocytes both in vitro and in vivo [Hakuno et al., 2002; Kajstura et al., 2005]. However, MSCs are rare in adult human bone marrow and the number significantly decreases with age, which makes it necessary to search for alternative sources of these cells for autologous and allogenic use [Sethe et al., 2006]. Umbilical cords are readily available and easy to obtain and help to avoid several ethical and technical issues. Recent studies show that a population of MSC-like cells exists in human umbilical cord tissue and cord blood [Lee et al., 2004; Lu et al., 2006].

In this study, the stem cells were successfully isolated from whole human umbilical cord tissue by enzymatic digestion. After exposure to 5-azacytidine for 24 h, it can differentiate into cardiomyocte-like cells in vitro. When the cells were transplanted into the infarcted myocardium, the injected cells differentiated into cardiomyoctes and improved the cardiac function of the experimental rats. Thus, human umbilical cord-derived stem cells may be a new cell source for cellular therapy and may play an important role in tissue engineering myocardial constructs.

MATERIALS AND METHODS

CELL ISOLATION AND CHARACTERIZATION

With the consent of the parents, fresh umbilical cords were collected from normal full-term pregnancies according to the regulations of

Grant sponsor: Natural Science Foundation of Jiangsu Province, China; Grant number: BK2008076.
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Received 27 December 2008; Accepted 2 April 2009 • DOI 10.1002/jcb.22193 • © 2009 Wiley-Liss, Inc.
Published online 20 May 2009 in Wiley InterScience (www.interscience.wiley.com).

Chinese Academy of Medical Sciences and Peking Union Medical College Research Ethics Committee. And the stem cells were isolated immediately as described before [Wu et al., 2007a]. In brief, after removal of blood vessels, the cord was minced and treated with collagenase II and trypsin. The digested mixture was then passed through a 100 μm filter to obtain cell suspensions. Next, the dissociated cells were centrifuged and plated in culture flasks. The cells were serially passaged and expanded in an incubator at 37°C with 5% CO_2. The cultivated cells were characterized by flow cytometry and osteogenic, adipogenic differentiation.

Approximately 10^6 cells were incubated with phycoerythrin (PE) or isothiocyanate (FITC)-labeled monoclonal antibodies against CD13, CD31, CD38, CD44, CD45, CD90, CD105, and CD106 (all from Becton Dickinson, San Jose, CA). All incubations were performed at room temperature for 20 min. Control groups were incubated with FITC and PE-conjugated mouse IgG1 isotype antibodies. Finally, the cells were assayed in a flow cytometer (FACSort, Becton Dickinson). For osteogenic differentiation, the cultured cells from passages (P4–6) were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, dexamethasone (0.1 μmol/L), ascorbic acid (0.2 mmol/L), and β-glycerophosphate (10 mmol/L) (all Sigma) for 2 weeks. Then the cells were stained with von Kossa to reveal osteogenic differentiation. For adipogenic differentiation, the 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 from 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 DIFFERENTIATION PROTOCOL

To analyze in vitro cardiomyogenic differentiation, the isolated cells (passages 4–6) were incubated for 24 h in DMEM-F12 medium containing 5 μmol/L 5-azacytidine. Then the cells were washed and replaced with fresh DMEM-F12 containing 5% FBS, 10 ng/ml basic fibroblastic growth factor (b-FGF, R&D), 10 ng/ml PDGF (R&D), and incubated in a humidified incubator at 37°C with 5% CO_2. The cells were observed by phase-contrast microscopy daily. The medium was changed once in every 2 days and the cells were serially passaged. Four weeks later, the cells were suspended in DMEM-F12 medium at a concentration of 5×10^4/ml and were seeded in 12-well plates at a concentration of 50,000 cells per well.

IMMUNOCYTOCHEMISTRY AND IMMUNOFLUORESCENCE

For immunostaining, the cells grown on coverslips were fixed with 4% paraformaldehyde in PBS containing 0.1% Triton X-100 for 15 min at room temperature. Then the cells were incubated overnight at 4°C with primary antibody sarcomeric α-actin (dilution 1:100; Abcam) and cardiac connexin43 (dilution 1:50; Chemicon). After washing with PBS, cells were incubated with biotinylated rabbit anti-mouse IgG (Zhongshan, Beijing, China) as secondary antibody. Then horseradish peroxidase (HRP) conjugated with streptavidin was used as detection reagent and finally DAB substrates for peroxidase were used to visualize the antibody binding. For fluorescent immunostaining, the coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and placed in a petridish, 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. The primary antibodies that were used are: mouse anti-cardiac myosin (dilution 1:100; Chemicon), mouse anti-cardiac Troponin T (dilution 1:50; cTnT, Newmarker). The cardiomyogenic specific proteins were visualized by incubation with the primary antibodies and conjugated with FITC- or TRITC-conjugated secondary antibodies (Zhongshan). Then, the cells were viewed using a confocal microscope (Leica Microsystems, GmbH).

ULTRASTRUCTURAL ANALYSIS

For transmission electron microscopy of cultured cells, 4 weeks after differentiation, differentiated umbilical cord stem cells were washed three times with PBS. The initial fixation was done in PBS containing 2.5% glutaraldehyde for 10 min, then, the cells were scraped down from the flask. After centrifugation, the cells were embedded in epoxy resin. Ultrathin sections cut horizontally to the growing surface were double stained in uranyl acetate and lead citrate, and were viewed under a JEM-1200EX transmission electron microscope (Nihon).

MYOCARDIAL INFARCTION MODEL

Adult male Sprague–Dawley rats (8-week-old, $n = 30$) weighing about 260–280 g were used in this study. Thirty rats underwent ligation of the left coronary artery to produce myocardial infarction according the procedures described before. Two weeks later, the survived rats were randomized into cell transplantation group and PBS group. The umbilical cord stem cells at the fifth passage were harvested and labeled with fluorescent carbocyanine 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (CM-DiI) dye (Molecular Probes, Beijing, China). About 5×10^6 umbilical cord stem cells in 200 μl PBS were injected using a tuberculin syringe into the anterior and lateral aspects of the viable myocardium bordering the infarction. An equal volume of PBS was injected into the control animals. After cell transplantation, cyclosporine was administered sub-cutaneously (10 mg/kg) every day in the experimental rats.

IN VIVO DIFFERENTIATION ANALYSIS

Four weeks after cell transplantation, all surviving animals underwent the final functional assessment with transthoracic echocardiography. On the following day, the animals were euthanized with an overdose of ketamine and pentobarbital, and the hearts were removed and washed quickly in PBS and embedded in Tissue-Tek OCT compound (Sakura Fineteck, Torrance, CA), cryopreservated in liquid nitrogen. 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) was used to detect the incorporation of transplanted cells. Both the rhodamine and fluorescein filters were used for each image collected during the scanning process.

To detect differentiation of transplanted umbilical cord stem cells, immunofluorescent staining for cardiac cell specific markers was performed using monoclonal mouse anti-cTnT (Newmarker),

Briefly, the frozen sections were fixed in acetone at 4°C for 10 min, blocked by blocking solution at room temperature for 20 min, and then incubated separately with antibodies overnight at 4°C. After a washing with PBS solution three times, sections were incubated with FITC-conjugated secondary antibodies (Zhongshan) for Troponin T.

EVALUATION OF LEFT VENTRICULAR FUNCTION

Left ventricular ejection fraction (LVEF) was assessed by echocardiography before transplantation, 2 and 4 weeks after cell transplantation according to the protocols described [Litwin et al., 1994]. Rats were anesthetized with an intraperitoneal administration of pentobarbital sodium at 30 mg/kg. Parasternal long- and short-axis views were obtained with both M-mode and two-dimensional echo images. LVEF (%) was calculated automatically by the echocardiography system. The measurements were averaged for at least three consecutive cardiac cycles and were made by an experienced technician who was blinded to the group identity. The echocardiographic data are presented as mean ± standard deviation. Comparisons between groups were made by Students *t*-test using SPSS statistical software. Results were considered statistically significant if $P < 0.05$.

RESULTS

MORPHOLOGY AND CHARACTERIZATION OF THE ISOLATED CELLS

When initially plated, the isolated cells appeared rounded in shape. After 48 h of plating, the cells were adherent, elongated, and spindle-shaped (Fig. 1A). The sub-cultured cells were shown in Figure 1B. The umbilical cord stem cells can be passaged more than 20 times without detecting signs of senescence in our laboratory. When the cells were cultured in osteogenic medium for 15 days, the morphology changed and was positive for von Kossa staining (Fig. 1C). These cells were also able to differentiate into adipocytes, as they accumulated different amounts of lipid vacuoles after cultivation in adipogenic medium (Fig. 1D). Flow cytometry results showed that the isolated cells highly expressed CD13, CD44, CD90, CD105, but not CD31, CD38, CD45, and CD106, similar to the FACS results of bone marrow-derived MSCs (Fig. 1E).

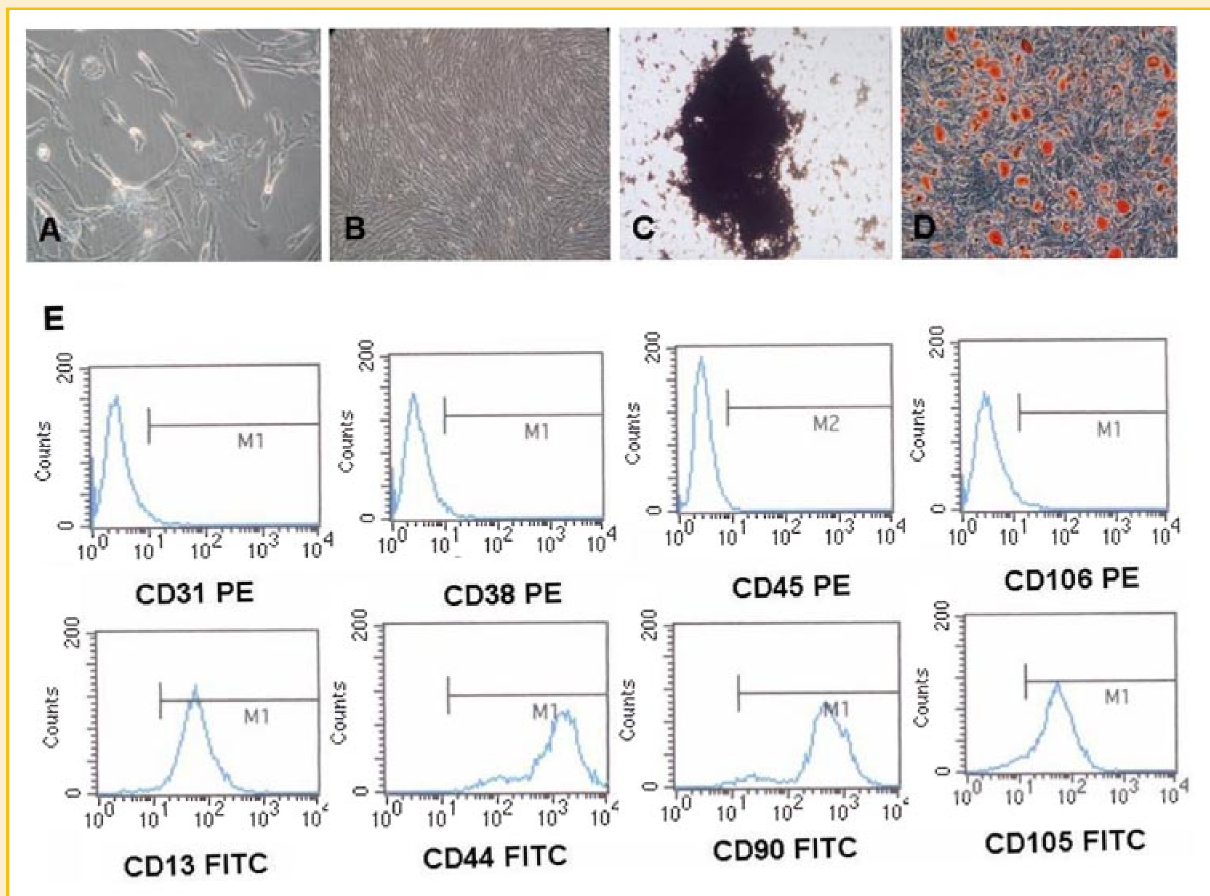

Fig. 1. Morphology and characterization of umbilical cord stem cells. A: Morphology of primary cultured umbilical cord stem cells. B: Morphology of sub-cultured stem cells, the cells were fibroblast-like and grew as a whirlpool. C: Von Kossa staining of the stem cells after osteogenic induction. D: Oil-red-O staining of the stem cells after adipogenic induction. E: Phenotypes of the umbilical cord stem cells. Cells were positive for CD13, CD44, CD90, and CD105, but not CD31, CD38, CD45, and CD106. Data are representative of several independent experiments.

CHARACTERIZATION OF IN VITRO DIFFERENTIATION

To characterize the myocardiogenic differentiation potential of umbilical cord stem cells induced by 5-azacytidine, phase-contrast photography and immunostaining with anti-sarcomeric α-actin, cardiac connexin43, ardiac myosin, and cTnT antibodies were performed on differentiated cells. Umbilical cord stem cells showed a fibroblast-like morphology before 5-azacytidine treatment, and after 5-azacytidine treatment, the morphology of the cells gradually changed. Approximately 30% of the cells gradually increased in size, formed a ball-like appearance after induction for 2 weeks (Fig. 2A). But, we have not found beating cells during the 6 week differentiation process (Fig. 2B). DAB-immunostaining (Fig. 2C,D) showed 4 weeks after 5-azacytidine treatment, differentiated cells had significant DAB signal and were strongly stained with anti-sarcomeric α-actin and cardiac connexin43. Fluorescent immunostaining revealed that cardiac specific anti-myosin and cTnT were strongly expressed in differentiated umbilical cord stem cells (Fig. 2E,F). In the non-induced negative controls, in which the primary antibodies were omitted, negligible immunofluorescence was seen.

ULTRASTRUCTURAL ANALYSIS

Representative transmission electron microscopy photographs are shown in Figure 3. Immature differentiated cells at 2 weeks after induction showed myofilaments, but their alignment was intricate (Fig. 3A). After upto 1 month of induction, the differentiated umbilical cord stem cells filled with well-aligned myofilaments and revealed typical striation and pale-staining pattern of the sarcomers (Fig. 3B,C). These findings indicated that differentiated cells had a cardiomyocyte-like ultrastructure.

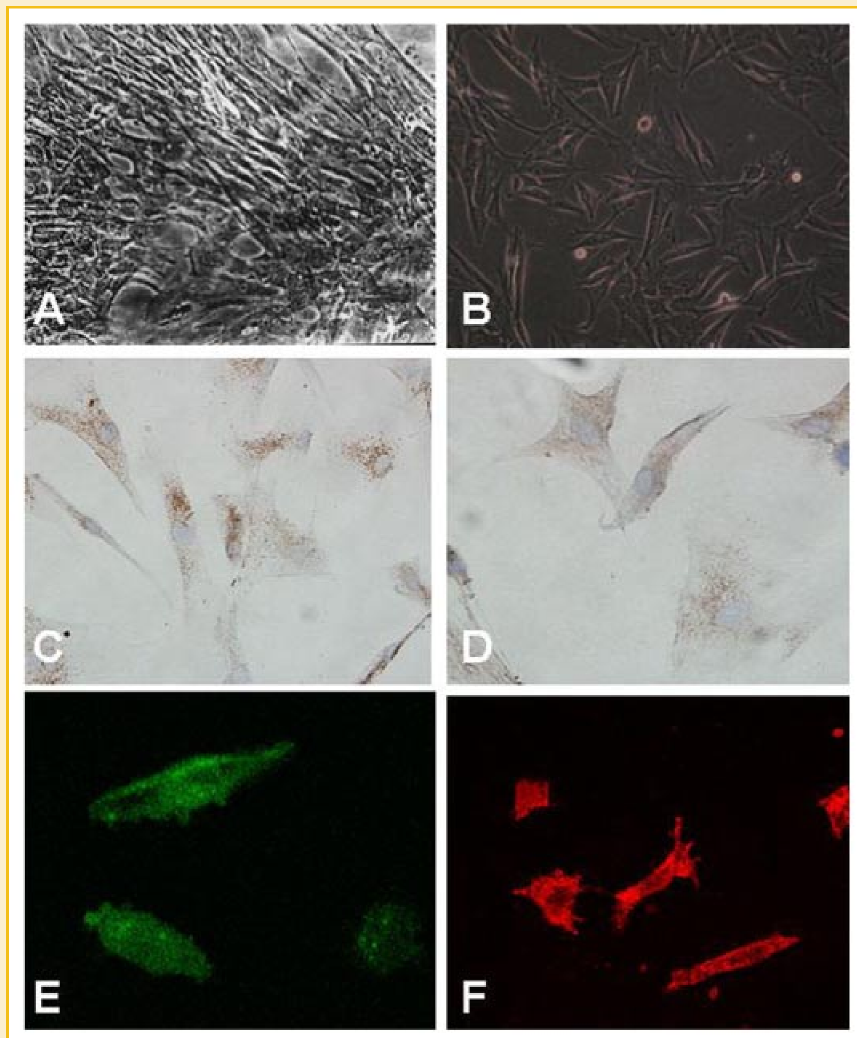

Fig. 2. In vitro differentiation of umbilical cord stem cells. A: Two weeks after treatment, some cells gradually increased in size and formed a ball-like or stick-like appearance. B: The morphology of the differentiated cells grown on coverslips 4 weeks after treatment. The DAB-immunostaining of the differentiated cells with anti-sarcomeric α-actin (C) and anti-cardiac connexin43 (D) 4 weeks after 5-azacytidine treatment, differentiated cells have significant DAB signal and are strongly positive. Fluorescent immunostaining for anti-cardiac myosin (E), anti-cTnT (F) revealed that these cardiomyocyte markers were strongly expressed in differentiated umbilical cord stem cells.

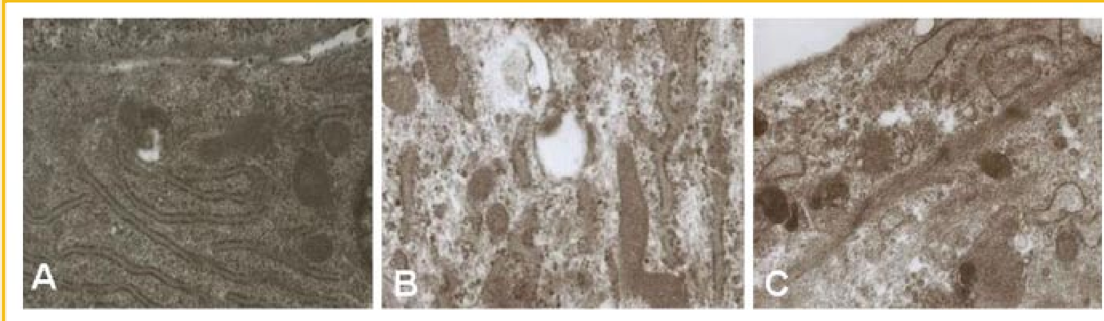

Fig. 3. Transmission electron microscopy of cardiomyocyte-like cells. After 2 weeks of induction with 5-azacytidine, some cells showed myofilaments, but their alignment are intricate (A). After upto 1 month of induction, the differentiated umbilical cord stem cells filled with well-aligned myofilaments and revealed typical striation and pale-staining pattern of the sarcomers (B,C).

CHARACTERIZATION OF IN VIVO DIFFERENTIATION

To determine the myocardial potential and whether umbilical cord stem cells could participate in the revascularization of ischemic myocardium in vivo, cultured stem cells were injected into the anterior and lateral aspects of the viable myocardium bordering the infarction of the experimental rats 2 weeks after induction of ischemia. Transplanted human umbilical cord stem cells marked with DiI were identified in tissue sections by red fluorescence. In contrast, the vasculature of the experimental rats, stained by BS-1 lectin was identified by green fluorescence in the same tissue sections and merge of them showed the incorporation of the transplanted cells into murine vasculature (Fig. 4A–C). Moreover, immunofluorescence staining of anti-cTnT antibody in ischemic myocardium 4 weeks after transplantation showed some DiI-labeled stem cells were differentiated into cardiomyogenic cells (Fig. 4D–F). Thus, locally transplanted umbilical cord stem cells were incorporated into foci of neovascularization and differentiated into cardiomyocyte-like cells in ischemic myocardium.

ASSESSMENT OF CARDIAC FUNCTION

There was no significant difference in baseline values between the two groups. The data analysis of the functional outcome over the 4-week study period showed an significant improvement of LVEF at 2 and 4 weeks post-transplantation in the cell-treated group compared with

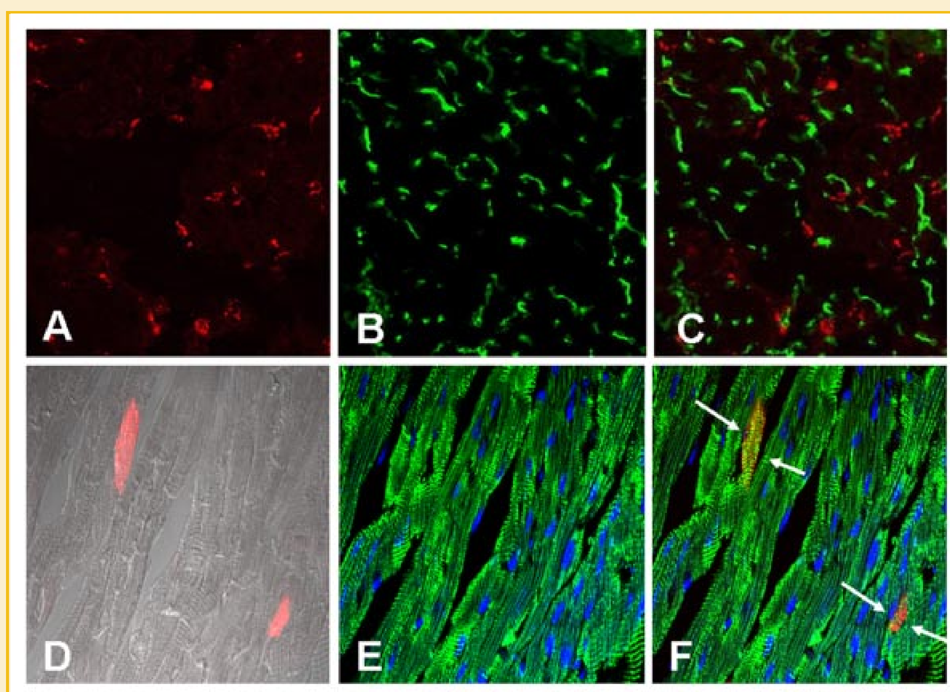

Fig. 4. Transplanted umbilical cord stem cells participated in the vessel network and differentiated into cardiomyogenic cells in vivo. The DiI-labeled umbilical cord stem cells (A) were sprouting from local injection. Murine capillary network was stained by BS-1 lectin (B), and merge of them showed the incorporation of umbilical cord stem cells into murine vasculature (C). Immunofluorescence staining of anti-cTnT antibody (E) in ischemic myocardium 4 weeks after ischemia showed some DiI-labeled stem cells (D) were differentiated into cardiomyocyte-like cells (arrows, F).

baseline (63.2 ± 8.3%; 68.4 ± 15.2%, 46.8 ± 12.2%, $P < 0.05$, respectively). The LVEF was also improved in control PBS group at the time point after the MI model was established (53.8±10.4%; 53.2 ± 13.4% vs. 47.6 ± 9.6%), but the difference were not significant ($P > 0.05$, respectively). Most importantly, the LVEF in cell treated group at 2 and 4 weeks post-transplantation was significantly improved compared with the control group ($P < 0.05$, respectively).

DISCUSSION

Myocardial ischemia associated with coronary artery disease and subsequent heart failure is the leading cause of morbidity and mortality. Despite the enormous advances in the understanding and treatment of heart failure, that have taken place during the past years, this condition remains a serious, and in fact, a growing problem worldwide. Recent advances suggest cardiac transfer of stem cells can have a favorable impact on tissue regeneration and contractile performance of the infarcted heart [Orlic et al., 2001; Fraser et al., 2004; Amado et al., 2005; Dawn et al., 2005]. Despite the fact that bone marrow-derived stem cells represent the main available source for cell therapies, the use of bone marrow-derived cells is not always acceptable because of the significant decrease in cell number and proliferation/differentiation capacity with age [Sethe et al., 2006]. In addition, obtaining the therapeutic quantity of bone marrow requires general anesthesia and hospitalization. In this connection, most attention should be paid to tissues containing cells with higher proliferative potency, capability of differentiation, and low risk of contamination.

In the present study, we showed that stem cells could be readily isolated from the whole human umbilical cord tissue and the cells could be differentiated into osteogenic and adipogenic cells in vitro. Flow cytometry results showed that the isolated cells highly expressed CD13, CD44, CD90, CD105, but not CD31, CD38, CD45, and CD106, similar to the FACS results of bone marrow-derived MSCs. These results suggest that the umbilical cord stem cells are a crowd of undifferentiated stem cells that are different from hematopoietic stem cells.

Cardiomyogenic potential of bone marrow and umbilical cord vein-derived MSCs has been previously described [Makino et al., 1999; Hakuno et al., 2002; Kuramochi et al., 2003; Kadivar et al., 2006]. In this study, we showed the stem cells we isolated from whole umbilical cord tissue by enzymatic digestion also have the potential to differentiate into cardiomyogenic cells. In vitro, after treatment with DNA demethylation agent 5-azacytidine, the umbilical cord stem cells increased in size, formed a ball-like appearance. But, we have not found beating cells during the 6 week differentiation process. Immunostaining showed that the differentiated cells were strongly positive for cardiac α-actin, connexin43, myosin, and cTnT. Furthermore, ultrastructural analysis of longitudinal sections of the differentiated umbilical cord stem cells revealed well-aligned myofilaments. These results suggest that umbilical cord stem cells differentiate into cardiomyocyte-like cells. But, beating cells were not observed during the 6-week differentiation period. Therefore, the exact myocardial differentiation mechanisms warrant further study.

The differentiation potential of umbilical cord stem cells into cardiomyogenic cells was also supported by in vivo transplantation experiment. When transplanted into ischemic myocardium of experimental rats, they contributed to neovascularization as a result of a direct incorporation of the cells into the ischemic myocardium, 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 umbilical cord stem cells were differentiated into cardiomyogenic cells. Thus, locally transplanted umbilical cord stem cells were incorporated into foci of neovascularization and differentiated into cardiomyogenic cells in ischemic myocardium. Finally, in vivo experiments showed a marked improvement of cardiac function after transplantation strongly supporting the therapeutic potential of umbilical cord stem cells in ischemic diseases. The favorable functional effects are probably related to myocardial differentiation of umbilical cord stem cells within infracted myocardium, increase of capillary and arteriole density, and secretion of angiogenic factors.

Previously, we have demonstrated that human umbilical cord-derived stem cells can be differentiated into endothelial cells and have the great use in therapeutic angiogenesis and revascularization of engineered tissue grafts [Wu et al., 2007b]. The results of the current study demonstrate these cells can be differentiated into cardiomyocytic-like cells and represent an attractive cell source for clinical therapies like cellular therapy and tissue engineering of myocardial constructs. These findings also suggest that these cells are able to migrate, colonize, and survive in the infarcted myocardium and the ischemic myocardium or 5-azacytidine can supply the proper conditions for cardiomyogenic differentiation of stem cells.

In summary, our study provides encouraging evidence that human umbilical cord stem cells have the potential to differentiate into cardiomyocyte-like cells both in vitro and in vivo. Umbilical cords are easy to obtain and the 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 umbilical cord stem cells represent a promising cell source for future routine therapeutic applications. However, many issues require resolution before the safe application of these cells in the clinical setting, to take advantage of these cells for cell therapy applications will require a complete understanding of how the maintenance and differentiation of these cells are regulated both in vitro and in vivo and this provides an exciting arena for continued research.

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