

Mesenchymal Stem Cells in the Wharton's Jelly of the Human Umbilical Cord

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ABSTRACT

The Wharton's jelly of the umbilical cord contains mucoid connective tissue and fibroblast-like cells. Using flow cytometric analysis, we found that mesenchymal cells isolated from the umbilical cord express matrix receptors (CD44, CD105) and integrin markers (CD29, CD51) but not hematopoietic lineage markers (CD34, CD45). Interestingly, these cells also express significant amounts of mesenchymal stem cell markers (SH2, SH3). We therefore investigated the potential of these cells to differentiate into cardiomyocytes by treating them with 5-azacytidine or by culturing them in cardiomyocyte-

conditioned medium and found that both sets of conditions resulted in the expression of cardiomyocyte markers, namely *N*-cadherin and cardiac troponin I. We also showed that these cells have multilineage potential and that, under suitable culture conditions, are able to differentiate into cells of the adipogenic and osteogenic lineages. These findings may have a significant impact on studies of early human cardiac differentiation, functional genomics, pharmacological testing, cell therapy, and tissue engineering by helping to eliminate worrying ethical and technical issues. *Stem Cells* 2004;22:1330–1337

INTRODUCTION

The umbilical cord contains two arteries and one vein, which are surrounded by mucoid connective tissue, and this is called the Wharton's jelly. The cord is covered by an epithelium derived from the enveloping amnion. The network of glycoprotein microfibrils and collagen fibrils in the Wharton's jelly has been previously studied [1]. The interlaced collagen fibers and small, woven bundles are arranged to form a continuous soft skeleton that encases the umbilical vessels [2]. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid [3], which forms a hydrated gel around the fibroblasts and collagen fibrils and

maintains the tissue architecture of the umbilical cord by protecting it from pressure [4]. The phenotypic stromal cells in the Wharton's jelly are fibroblast-like cells [5]. However, cells with the ultrastructural characteristics of myofibroblasts have been found [6]. Recently, Mitchell et al. [7] found that matrix cells from Wharton's jelly can be induced to form neurons and glia cells by treating with basic fibroblast growth factor and low-serum media plus butylated hydroxyanisole and dimethyl sulfoxide.

In this study, we demonstrate that mesenchymal cells from the umbilical cord, when expanded in culture, express adhesion molecules (CD44, CD105), integrin markers

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(CD29, CD51), and mesenchymal stem cell (MSC) markers (SH2, SH3) but not markers of hematopoietic differentiation (CD34, CD45). After exposure of these cells to cardiomyocyte-conditioned medium or 5-azacytidine, they expressed cardiac troponin-I and *N*-cadherin, indicating differentiation into cardiomyocytes. Under suitable culture conditions, these cells could also differentiate into osteogenic and adipogenic cells. Thus, human umbilical cord mesenchymal cells can be expanded in culture and induced to form several different types of cells. They may therefore prove to be a new source of cells for cell therapy, including targets such as stromal tissue and cardiac muscle. This will help to avoid several ethical and technical issues.

MATERIALS AND METHODS

Cell Culture

Institutional review board approval was obtained for all procedures. With the consent of the parents, fresh human umbilical cords were obtained after birth and stored in Hanks' balanced salt solution for 1–24 hours before tissue processing to obtain mesenchymal cells. After removal of blood vessels, the mesenchymal tissue was scraped off from the Wharton's jelly with a scalpel and centrifuged at 250 *g* for 5 minutes at room temperature and the pellet was washed with serum-free Dulbecco's modified Eagle's medium (DMEM). Next, the cells were centrifuged at 250 *g* for 5 minutes at room temperature and then treated with collagenase (2 mg/ml) for 16 hours at 37°C, washed, and treated with 2.5% trypsin for 30 minutes at 37°C with agitation. Finally, the cells were washed and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and glucose (4.5 g/l) in 5% CO₂ in a 37°C incubator.

Induction of Cardiogenic Differentiation

For 5-azacytidine treatment, the mesenchymal cells were incubated for 24 hours in serum-free DMEM containing 3 μ M 5-azacytidine. The medium was then changed to DMEM/10% FBS to prevent cell death due to prolonged exposure to 5-azacytidine, and the cells were maintained in DMEM/10% FBS for 3 days to 5 weeks. For treatment with cardiomyocyte-conditioned medium, conditioned medium was prepared by culturing cardiomyocytes, obtained from the hearts of 7-day-old rats, in DMEM supplemented with glucose (4.5 g/l) and 10% FBS for approximately 72 hours. The cell culture medium was then harvested and centrifuged at approximately 800 *g* for 10 minutes at room temperature, and, finally, the supernatant was filtered for use as conditioned medium for the mesenchymal cells.

Flow Cytometry

After the mesenchymal cells were extracted from the umbilical cord, they were cultured in DMEM supplemented with

glucose (4.5 g/l) and 10% FBS for 5–7 days. Next, the cells were trypsinized and suspended in DMEM at a concentration of 5×10^6 /ml, and then a 1-ml sample was incubated for 45 minutes at 4°C with 150 μ l of various nonlabeled mouse anti-human antibodies followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibodies (10 mg/ml) for 1 hour at room temperature. Finally, they were washed twice with phosphate-buffered saline (PBS), pH 7.4; centrifuged; and fixed in 1.5 ml of 4% paraformaldehyde. Control samples were incubated with PBS instead of primary antibody. A FACScan machine (Becton, Dickinson, Franklin Lakes, NJ) was used to analyze antibody binding.

Induction of Adipogenic, Chondrogenic, or Osteogenic Differentiation

Cultured cells at passage 2 were incubated in adipogenic differentiation medium (DMEM and 1 g/ml glucose [DMEM-LG] containing 10% FBS, 50 μ g/ml of ascorbate-1 phosphate, 10^{-7} M dexamethasone, and 50 μ g/ml indomethacin [Sigma, St. Louis]) in chondrogenic differentiation medium (as cell pellets) (serum-free DMEM-LG containing insulin-transferrin-selenium (ITS) + premix [GIBCO, Carlsbad, CA] and 10 ng/ml transforming growth factor [TGF]- β 1 [Pepro Tech, Rocky Hill, NJ]), in osteogenic differentiation medium (DMEM-LG containing 10% FBS, 50 μ g/ml ascorbate-2 phosphate, 10^{-8} M dexamethasone, and 10 mM β -glycerophosphate), or in DMEM-LG supplemented with 10% FBS as a control. The medium was changed every 3 days, and the cells, after completion of differentiation had been established by morphology, were used for the histochemical staining and immunohistochemistry studies.

Immunocytochemistry

After the mesenchymal cells were extracted from the umbilical cord, they were cultured in DMEM supplemented with glucose (4.5 g/l) and 10% FBS for 5–7 days, and then various cell-differentiating factors were added for 3 days to 5 weeks. Staining was performed on fixed, nonpermeabilized monolayers of mesenchymal cells grown on coverslips. The cells were washed with PBS, fixed for 30 minutes at 37°C in 0.05% glutaraldehyde, and incubated for 25 minutes at room temperature with a 1:9 dilution of normal goat serum in PBS to block nonspecific binding of the primary antibody. The slides were then incubated for 75 minutes at 4°C with various nonlabeled mouse anti-human antibodies (10 μ g/ml) (Biosource International, Camarillo, CA) followed by FITC-coupled anti-mouse IgG antibodies (Vector, Burlingame, CA) for 1 hour at room temperature. The coverslips were mounted with mounting medium (Vector) and viewed with a confocal laser-scanning microscope (Leica) using a 100/1.30 oil-immersion

objective lens and an appropriate filter. In the negative controls, in which the primary antibodies were omitted, negligible immunofluorescence was seen.

Western Blotting

For immunoblotting on polyvinylidene difluoride (PVDF) membranes, cells from two 100-mm dishes per treatment group were pooled, rinsed briefly with PBS, and then lysed at room temperature for 10 minutes in 1 ml of PBS containing 1% sodium dodecyl sulfate, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, 10 µg/ml soybean trypsin inhibitor, and 0.5 mM dithiothreitol. After centrifugation for 20 minutes at 1,300 g and 4°C, the supernatant was removed and centrifuged at 6,000 g for 1 hour at 4°C. The final supernatant was used as the cytosolic fraction, and the pellet was used as the membrane fraction. Equal amounts (50 mg of protein) of the membrane fractions or cytosolic fractions were run on 12% polyacrylamide gels and transferred to PVDF membrane in transfer buffer (four parts 25-mM Tris/200 mM buffer, pH 8.0, and one part methanol). The membranes were then blocked for 2 hours at room temperature with 50 mM Tris HCl, 150 mM NaCl, 0.05% Tween-20 (tris-buffered saline with Tween-20 [TBST]), pH 7.0, containing 5% nonfat dry milk and then incubated overnight at 4°C with rabbit anti-human cardiac troponin I monoclonal antibody (1:500) (Serotec, Oxford, U.K.) or mouse anti-N-cadherin antibody (1:500) (Zymed, San Francisco) in TBST. After three washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody at a 1:1,000 dilution (Vector, Burlingame, CA), and bound antibody was detected by enhanced chemiluminescence (Amersham Life Sciences, Uppsala, Sweden).

Histochemical Staining

The medium was removed and the cells were washed twice with PBS, fixed for 10 minutes at room temperature in 3.7% paraformaldehyde, and washed twice with PBS. Cells treated with the adipogenic and chondrogenic formulas were stained with Oil red O or Toluidine blue to show adipogenic or chondrogenic differentiation, respectively. Immunohistochemical staining for human type II collagen was also used to demonstrate chondrogenic differentiation of the treated cells. The mesenchymal cells treated by the osteogenic formula were stained with alkaline phosphatase staining and von Kossa staining to reveal osteogenic differentiation.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNA was extracted from untreated (control) and treated cells using RNeasy Purification Reagent (Qiagen,

Valencia, CA), and then a sample (1 µg) was reverse transcribed with Mmlv reverse transcriptase (RT) for 30 minutes at 42°C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers designed from the published sequence of each cDNA as follows: peroxisome proliferator-activated receptor-γ2 (PPARγ2) (595 bp), antisense 5'-CCTATTGACCCAGA AAGCGATTC-3' and sense 5'-GCATTATGAGACATCCC-CACTGC-3'; osteopontin (330 bp), antisense 5'-CAGTGA CCAGTTCATCAGATTCATC-3' and sense 5'-CTAGGC ATCACCTGTGCCATACC-3'; GAPDH (352 bp), antisense 5'-TCACGCCACAAGTTTCCCGGA-3' and sense 5'-CAC CATCTTCCAGGAGCGAG-3'. PCR was performed for 30 to 35 cycles, with each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 55°C to 63°C for 30 seconds, and elongation at 72°C for 1 minute, with an additional 10-minute incubation at 72°C after completion of the last cycle. To exclude the possibility of contaminating genomic DNA, PCRs were also run without RT. The amplified cDNA was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

RESULTS

Characterization of Mesenchymal Cells in Wharton's Jelly

To determine whether stromal cells in the Wharton's jelly of the umbilical cord have multipotent potential, we extracted cells from human umbilical cords and cultured them in DMEM supplemented with glucose (4.5 mg/l) and 10% FBS. The results reported are representative of the results obtained using 30 umbilical cords. The cells demonstrated a fibroblast-like phenotype (Fig. 1A). Flow cytometric analysis showed that the cells expressed high levels of matrix markers (CD44, CD105), integrin markers (CD29, CD51), and MSC markers (SH2, SH3) but did not express hematopoietic lineage markers (CD34, CD45) (Fig. 1B).

Cardiogenic Differentiation of Mesenchymal Cells in Wharton's Jelly

To investigate the potential of umbilical cord mesenchymal cells to differentiate into cardiomyocytes, 5-azacytidine (3 µmol/l), a drug that has been used to trigger this change [8], was added to the culture medium. We stained the cells with antibodies against human cardiac troponin I or F-actin and examined the cells under confocal laser microscopy. As shown in Figures 2A–2D, cardiac troponin I was clearly more strongly expressed in the differentiated cells than in untreated cells. When umbilical cord mesenchymal cells were maintained in conditioned medium from primary rat cardiomyocyte cultures, the presence of N-cadherin expression was

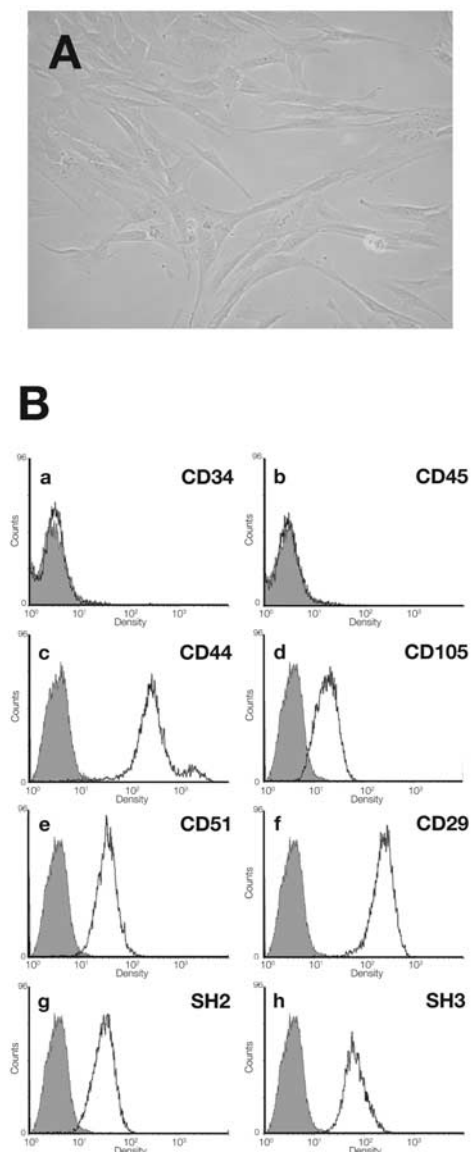


Figure 1. Characteristics of mesenchymal cells from Wharton's jelly. **(A):** Cell cultures 3 days after the initial seeding of umbilical cord mesenchymal cells. The cells have a fibroblast-like morphology. **(B):** Flow cytometric analysis of surface-marker expression on umbilical cord mesenchymal cells. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After one passage at approximately 1 week, the cells were analyzed by flow cytometry. The shaded area shows the profile of the negative control. The data shown are representative of those obtained in six different experiments.

detected by staining at the cell–cell junctions (Fig. 2F). Moreover, Western blots showed that newly extracted cells did not express *N*-cadherin or cardiac troponin I but that both were expressed in cells grown in conditioned medium. Cells grown in DMEM with 10% FBS for 5 weeks expressed lower amounts of *N*-cadherin and cardiac troponin I (Fig. 2G).

Adipogenic Differentiation of Mesenchymal Cells

Adipogenic differentiation, as seen by Oil red O–positive cells, was achieved by culturing the cells for 7 days in DMEM-LG containing ascorbate, dexamethasone, and indomethacin (Figs. 3B, 3C). When total RNA was isolated and analyzed by RT-PCR, expression of NADPH, used as an internal control, was the same in undifferentiated and differentiated cells, whereas the adipocyte marker PPAR γ 2 was not expressed in untreated cells but was expressed in adipogenic formula-treated cells (Fig. 3D).

Chondrogenic and Osteogenic Differentiation of Mesenchymal Cells

Chondrogenic differentiation of umbilical cord mesenchymal cells was achieved by pelleting the mesenchymal cells and incubating them for 21 days with serum-free medium supplemented with ITS + premix and 10 ng/ml of TGF- β 1. The cell pellets developed chondrogenic characteristics after treatment. Alcine blue staining of an aggrecan-rich extracellular matrix was evident in histological sections (Fig. 4A), and a type II collagen-rich extracellular matrix was demonstrated immunohistochemically (Fig. 4B). However, the control groups were also positive for Alcine blue and type II collagen staining (data not shown). Osteogenic differentiation of mesenchymal cells was seen as the formation of alkaline phosphatase–positive aggregates and von Kossa stain–positive nodules (Fig. 4C), and this was achieved after 28 days of culture in DMEM-LG containing 50 μ g/ml of ascorbate-2 phosphate, 10^{-8} M dexamethasone, and 10 mM β -glycerophosphate. When total RNA was isolated and analyzed by RT-PCR, expression of NADPH, used as an internal control, was the same in undifferentiated and differentiated cells, whereas osteopontin, an osteogenic marker, was not expressed in newly isolated cells but was expressed in osteogenic formula-treated cells (Fig. 4D).

DISCUSSION

MSCs were initially isolated from bone marrow by Friedenstein et al. [9] and then studied by other investigators [10–16]. Recently, MSCs have been found to have the potential to differentiate into muscle cells, adipocytes, osteocytes [16], and chondrocytes in culture [16–18]. After systemic injection, MSCs are incorporated into a variety of tissues, including bone [19, 20], muscle [21], lung [19, 20, 22], and epithelium [23]. Stem cells from bone marrow have also been shown to form cardiomyocytes [24–27]. The Wharton's jelly of the umbilical cord contains mucoid connective tissue and fibroblast-like cells. Using flow cytometric analysis, we found that mesenchymal cells isolated from umbilical cord, when expanded ex vivo, express matrix receptors (CD44, CD105) and integrin markers (CD29, CD51) but not hematopoietic

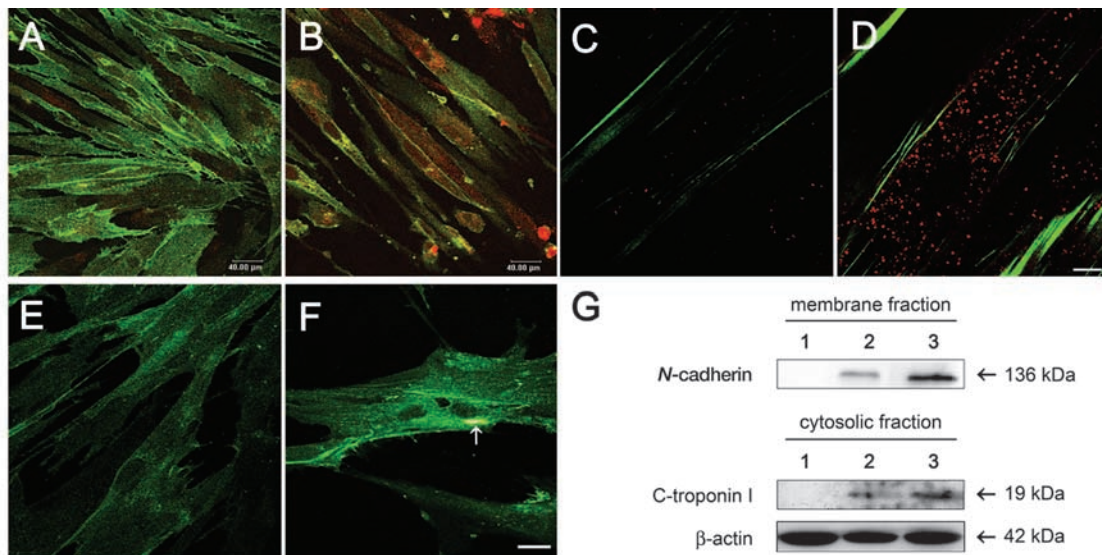


Figure 2. Induction of cardiac troponin I and *N*-cadherin expression. (A, C): Expression of cardiac troponin-I (red) and F-actin filaments (green) in untreated mesenchymal cells. (B, D): Expression of cardiac troponin I and F-actin filaments in mesenchymal cells incubated with serum-free DMEM containing 3 μ mol/l 5-azacytidine for 24 hours and DMEM/10% FBS for 21 days. (E): Immunofluorescence staining of *N*-cadherin in untreated umbilical cord mesenchymal cells. (F): *N*-cadherin expressed in the junctions between mesenchymal cells (arrow) cultured in cardiomyocyte-conditioned medium for 5 weeks. A and B, bar = 40 μ m; C and D, bar = 10 μ m; E and F, bar = 20 μ m. (G): Western blot showing *N*-cadherin and cardiac troponin I expression in, respectively, the membrane and cytosolic fraction of untreated and conditioned medium-treated umbilical cord mesenchymal cells. Lane 1: newly extracted mesenchymal cells; lane 2: control cells cultured in DMEM supplemented with 10% FBS for 5 weeks; lane 3: cells grown in rat cardiomyocyte-conditioned medium for 5 weeks. β -actin was used as a loading control. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

lineage markers (CD34, CD45). Interestingly, these cells also express significant levels of some MSC markers (SH2, SH3) (Fig. 1). These results suggest that stroma cells from Wharton's jelly are similar to MSCs.

We investigated the potential of these cells to differentiate into cardiomyocytes by treating them with 5-azacytidine or maintaining them in cardiomyocyte-conditioned medium and found that both treatments resulted in expression of the cardiomyocyte markers *N*-cadherin and cardiac troponin I (Fig. 2). Other cardiomyocyte-related markers, such as connexin 43, α -actinin, and desmin, were also expressed after 5-azacytidine treatment for 3 weeks (data not shown). Troponin I, the inhibitory subunit of the troponin complex, is involved in cardiac muscle contraction, whereas F-actin is an essential cell cytoskeletal protein that maintains the structure of the cardiac cell. Under confocal laser microscopy, cardiac troponin I was clearly more strongly expressed in the differentiated cells than in untreated cells (Fig. 2), suggesting that the cells differentiated into cardiomyocytes rather than skeletal cells, whereas F-actin expression in the differentiated cells confirmed that the differentiated cells exhibited a specific cell-attachment pattern similar to that of cardiomyocytes.

Undifferentiated cells can respond to signals from their host tissue microenvironment and differentiate to produce

progeny with the phenotypic characteristics of the mature cells of that tissue. *N*-cadherin is localized in the adherens junction and is important for the assembly of the adherens junction in cardiomyocytes [28, 29]. When umbilical cord mesenchymal cells were maintained in conditioned medium from primary rat cardiomyocyte cultures, Western blots showed that the newly extracted cells did not express *N*-cadherin or cardiac troponin I but that both markers were expressed in cells grown in conditioned medium and, to a lesser extent, those grown in 10% FBS (Fig. 2E). This suggests that FBS provides some differentiating factors, but not to the same extent as cardiomyocyte-conditioned medium. However, it should be noted that there has been no report up to the present that has described MSCs from adult bone marrow and adipose tissue as able to spontaneously become cardiomyocytes in the control medium. It is possible that MSCs from Wharton's jelly are earlier-stage cells than MSCs from adult bone marrow and adipose tissue. Thus, there may be a fundamental difference between these cells from Wharton's jelly and the adult MSCs from bone marrow and adipose tissue.

It has been found that after 5-azacytidine treatment for 2 weeks, murine bone marrow stromal cells can be differentiated into cardiomyocytes, which formed myotube-like struc-

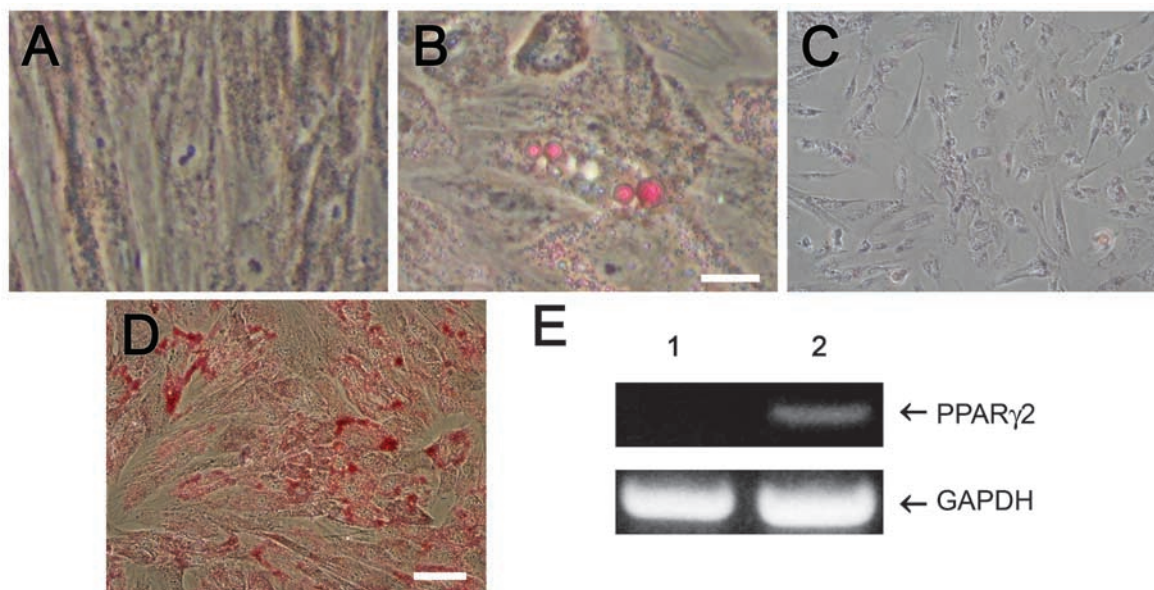


Figure 3. Adipogenic differentiation of umbilical cord mesenchymal cells. **(A):** Control cells. **(B):** Cells in the adipogenic induction group showing varying degrees of staining for Oil red O at 7 days of culture (bar = 10 μ m). **(C):** Control cells (bar = 25 μ m). **(D):** Cells in the adipogenic induction group showing varying degrees of staining for Oil red O at 14 days of culture (bar = 25 μ m). **(E):** RT-PCR analysis of untreated (lane 1) and adipogenic formula-treated (lane 2) cell cultures. Total RNA was analyzed by RT-PCR for PPAR γ 2 (595 bp) and GAPDH (352 bp) mRNA. Abbreviations: PPAR γ 2, peroxisome proliferator-activated receptor- γ 2; RT-PCR, reverse transcription polymerase chain reaction.

tures and began spontaneously beating [30]. However, in our study, after 5-azacytidine treatment or culture in cardiomyocyte-conditioned media, the cells connected with adjoining cells but did not form myotubes or start spontaneous contraction. The differentiation differences may possibly be explained by the fact that the cells used in this study are derived from a different species; namely, they are human rather than mouse.

Under various culture conditions, these cells were able to also differentiate into the chondrogenic, osteogenic, and adipogenic lineages. Adipogenic differentiation was seen as Oil red O-positive cells and was also analyzed by RT-PCR, in which the adipocyte marker PPAR γ 2 was expressed in the adipogenic formula-treated cells (Fig. 3). Chondrogenic differentiation of umbilical cord mesenchymal cells was achieved by pelleting the mesenchymal cells and then incubating them with serum-free medium containing chondrogenic induction factors. The cell pellets expressed chondrogenic characteristics both in the control and after treatment with chondrogenic induction formula. RT-PCR analysis of chondrogenic markers Col2a1 and Col10a1 showed that these were also expressed in the newly extracted mesenchymal cells (data not shown). Because umbilical cord is a supporting structure that contains an abundant amount of hyaluronan, the mesenchymal cells from Wharton's jelly

might have early chondrogenic characteristics in order to give stiffness to the cord. Osteogenic differentiation of mesenchymal cells was seen as the formation of alkaline phosphatase-positive aggregates and mineral deposition staining (Fig. 4). Osteopontin, an osteogenic marker, although not expressed in newly isolated cells, was expressed in chondrogenic or osteogenic formula-treated cells (Fig. 4E). Taking all these results together, the evidence suggests that multipotent cells exist in the umbilical cord.

The Wharton's jelly contains colony-stimulating activity [31] and high levels of insulin-like growth factor (IGF)-I and IGF-I-binding proteins [32], epidermal growth factor, TGF- α , and their common receptor genes [33]. Cultured human MSCs spontaneously produce, or can be induced to produce, cytokines for the support of hematopoietic cells [34, 35]. Thus, umbilical cord mesenchymal cells may have the characteristic ability of bone marrow MSCs to synthesize these same cytokines for the support of hematopoietic cells. However, the possible existence of hematopoietic cells in the umbilical cord needs to be investigated.

After exposure of these cells to 5-azacytidine or to cardiomyocyte-conditioned medium, the cells expressed both cardiac troponin I and N-cadherin. These changes were demonstrated by both immunofluorescent labeling and Western immunoblotting for cardiomyocyte markers. Because

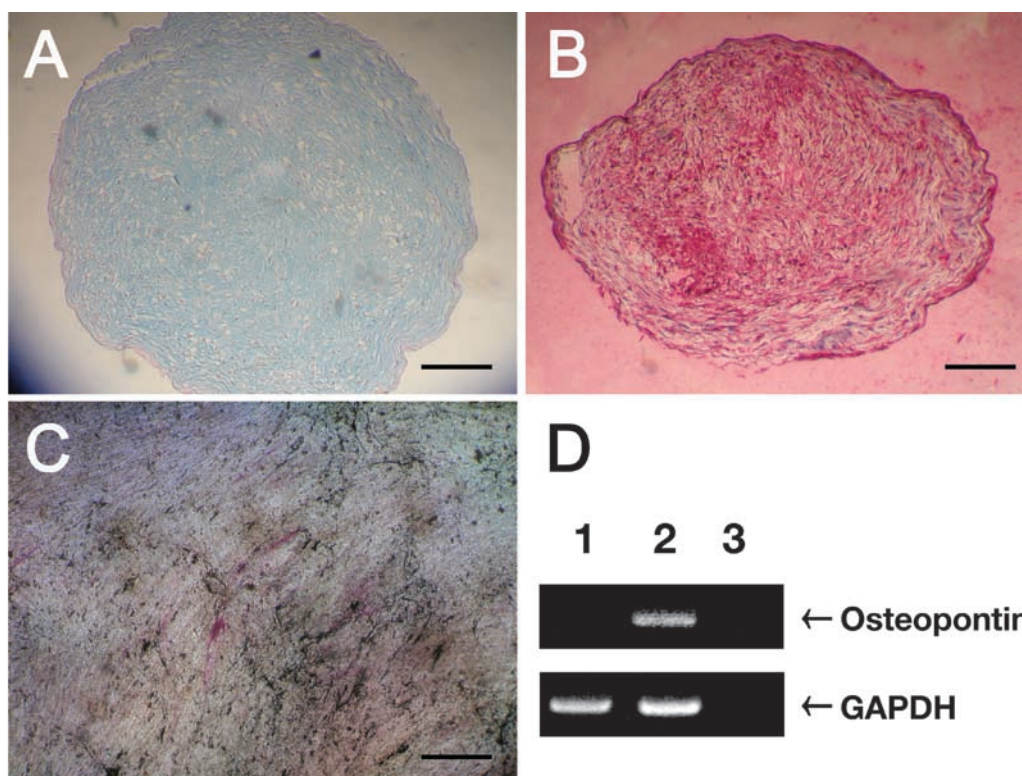


Figure 4. Chondrogenic and osteogenic differentiation of umbilical cord mesenchymal cells. **(A):** Cells stained with Alcine blue after chondrogenic induction for 21 days. Bar = 50 μ m. **(B):** Cells were treated as in **(A)** and then stained with anti-type II collagen antibody. Bar = 50 μ m. **(C):** Cells after osteogenic induction for 28 days showing varying degrees of positive staining for alkaline phosphatase and von Kossa technique show the presence of mineral associated with the matrix. Bar = 8 μ m. **(D):** RT polymerase chain reaction analysis of untreated cell cultures (lane 1), osteogenic formula-treated cell cultures (lane 2), and non-RT control (lane 3). Total RNA was analyzed by osteopontin (330 bp) and GAPDH (352 bp) mRNA. Abbreviation: RT, reverse transcriptase.

umbilical cord mesenchymal cells have been used in cardiovascular tissue engineering [36], umbilical cord mesenchymal cells may be a good source for such use in cardiomyogenic differentiation. Our results also show that these cells can be easily isolated and expanded in culture and induced to differentiate, and this can result in the expression of phenotypes from a variety of lineages. These cells may therefore prove to be a new source of cells for cellular therapies involving stromal tissue and, potentially, cardiac muscle repair. This will avoid the ethical and technical issues involved in the use

of cells from other origins. These findings may have a significant impact on the study of cell therapy, early human cardiac differentiation, functional genomics, pharmacological testing, and tissue engineering and potentially help to avoid worrying ethical issues.

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REFERENCES

- 1 Meyer FA, Laver-Rudich Z, Tanenbaum R. Evidence for a mechanical coupling of glycoprotein microfibrils with collagen fibrils in Wharton's jelly. *Biochim Biophys Acta* 1983;755:376–387.
- 2 Vizza E, Correr S, Goranova V et al. The collagen skeleton of the human umbilical cord at term: a scanning electron microscopy study after 2N-NaOH maceration. *Reprod Fertil Dev* 1996;8:885–894.
- 3 Sobolewski K, Bankowski E, Chyczewski L et al. Collagen and glycosaminoglycans of Wharton's jelly. *Biol Neonate* 1997;71:11–21.
- 4 Sakamoto T, Ono H, Saito Y. Electron microscopic histochemical studies on the localization of hyaluronic acid in Wharton's jelly of the human umbilical cord [in Japanese]. *Nippon Sanka Fujinka Gakkai Zasshi* 1996;48:501–507.

- 5 McElreavey KD, Irvine AI, Ennis KT et al. Isolation, culture and characterisation of fibroblast-like cells derived from the Wharton's jelly portion of human umbilical cord. *Biochem Soc Trans* 1991;19:29S.
- 6 Kobayashi K, Kubota T, Aso T. Study on myofibroblast differentiation in the stromal cells of Wharton's jelly: expression and localization of alpha-smooth muscle actin. *Early Hum Dev* 1998;51:223–233.
- 7 Mitchell KE, Weiss ML, Mitchell BM et al. Matrix cells from Wharton's jelly form neurons and glia. *STEM CELLS* 2003;21:50–60.
- 8 Wang JS, Shum-Tim D, Galipeau J et al. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. *J Thorac Cardiovasc Surg* 2000;120:999–1005.
- 9 Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;4:267–274.
- 10 Mets T, Verdonk G. In vitro aging of human bone marrow derived stromal cells. *Mech Ageing Dev* 1981;16:81–89.
- 11 Owen ME, Friedenstein AJ. Cell and molecular biology of vertebrate hard tissues. *Ciba Found Symp* 1998;132:42–60.
- 12 Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991;9:641–650.
- 13 Clark BR, Keating A. Biology of bone marrow stroma. *Ann NY Acad Sci* 1995;770:70–78.
- 14 Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 1997;64:278–294.
- 15 Zohar R, Sodek J, McCulloch CA. Characterization of stromal progenitor cells enriched by flow cytometry. *Blood* 1997;90:3471–3481.
- 16 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–147.
- 17 Lennon DP, Edmison JM, Caplan SI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol* 2001;187:345–355.
- 18 Sekiya I, Vuorio JT, Larson BL et al. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc Natl Acad Sci U S A* 2002;99:4397–4402.
- 19 Pereira RF, Halford KW, O'Hara MD et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. *Proc Natl Acad Sci U S A* 1995;92:4857–4861.
- 20 Pereira RF, O'Hara MD, Laptev AV et al. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A* 1998;95:1142–1147.
- 21 Ferrari G, Cusella-De Angelis G, Coletta M et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 1998;279:1528–1530.
- 22 Sandhu JS, Clark BR, Boynton EL et al. Human hematopoiesis in SCID mice implanted with human adult cancellous bone. *Blood* 1996;88:1973–1982.
- 23 Spees JL, Olson SD, Ylostalo J et al. Differentiation, cell fusion and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. *Proc Natl Acad Sci U S A* 2003;100:2397–2402.
- 24 Fukuda K. Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. *Artif Organs* 2001;25:187–193.
- 25 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001;410:701–705.
- 26 Alvarez-Dolado M, Pardo R, Garcia-Verdugo JM et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;425:968–973.
- 27 Hakuno D, Fukuda K, Makino S et al. Bone marrow-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. *Circulation* 2002;105:380–386.
- 28 Volk T, Geiger B. A 135-kD membrane protein of intercellular adherens junctions. *EMBO J* 1984;3:2249–2260.
- 29 Volk T, Geiger B. A-CAM: a 135-kD receptor of intercellular adherens junctions, II. Antibody-mediated modulation of junction formation. *J Cell Biol* 1986;103:1451–1464.
- 30 Makino S, Fukuda K, Miyoshi S et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 1999;103:697–705.
- 31 Irvine AE, Morris TC, Kennedy H et al. Human umbilical cord conditioned medium: a stimulus for human CFU-G. *Exp Hematol* 1984;12:19–24.
- 32 Palka J, Bankowski E, Jaworski S. An accumulation of IGF-I and IGF-binding proteins in human umbilical cord. *Mol Cell Biochem* 2000;206:133–139.
- 33 Rao CV, Li X, Toth P et al. Expression of epidermal growth factor, transforming growth factor-alpha and their common receptor genes in human umbilical cords. *J Clin Endocrinol Metab* 1995;80:1012–1020.
- 34 Majumdar MK, Thiede MA, Mosca JD et al. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells and stromal cells. *J Cell Physiol* 1998;176:57–66.
- 35 Cheng L, Qasab P, Vanguri P et al. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34⁺ hematopoietic progenitor cells. *J Cell Physiol* 2000;184:58–69.
- 36 Kadner A, Hoerstrup SP, Tracy J et al. Human umbilical cord cells: a new cell source for cardiovascular tissue engineering. *Ann Thorac Surg* 2002;74:S1422–S1428.