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Mesenchymal progenitor cells in the human umbilical cord

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Abstract Mesenchymal progenitor or stem cells (MPCs) isolated from fetal blood, liver, and bone marrow are a population of multipotential cells that can proliferate and differentiate into multiple mesodermal tissues including bone, cartilage, muscle, ligament, tendon, fat, and stroma. The objective of this study was to isolate and characterize MPCs in the human umbilical cord. The suspensions of endothelial and subendothelial cells in cord vein were collected and cultured in M199 supplemented with 10% fetal bovine serum (FBS). Of 50 umbilical cord samples, 3 had numerous fibroblastoid cells morphologically distinguishable from endothelial cells. Fibroblastic cells displayed lack of expression of vWF, Flk-1, and PECAM-1, indicating the endothelial cell-specific marker. To investigate the differentiation potentials, the cells were cultured in adipogenic or osteogenic medium for 2 weeks. Fibroblast-like cells treated with adipogenic supplementation showed Oil red O-positive staining and expressed adipsin, FABP4, LPL, and PPARγ2 genes by reverse transcriptase polymerase chain reaction (RT-PCR). In osteogenic differentiation, alkaline phosphatase activity and calcium accumulation were detected. RT-PCR studies determined that Cx43, osteopontin, and Runx2 genes were expressed in the osteogenic cultures. Among three cell lines cultured continuously for passage 10, two had normal karyotypes; however, one retained a karyotype of mos 46,XY[19]/47, XY,+mar[3]. These observations suggest that MPCs are present in human umbilical cord and possess several typical traits of MPCs.

Keywords Umbilical cord · Mesenchymal stem or progenitor cells · Cell differentiation

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Introduction

Stem cells showing self-renewal and multipotentiality are divided into two major groups: embryonic and adult stem cells [1]. Adult stem cells demonstrate differentiation capacity limited to organs. However, recent studies indicate that tissue-specific stem cells are capable of differentiating into cells of other tissues. Interestingly, mesenchymal stem or progenitor cells (MPCs) in blood and tissue can be expanded to differentiate into several types of cells, including adipocytes, chondrocytes, osteocytes [2], myocytes [3], astrocytes, and neurons [4]. MPCs contain homogeneous fibroblast-like cells, which have a population doubling time of 33 h [5]. These cells do not express typical hematopoietic lineage markers (CD14, CD34, and CD45) by immunophenotype, but are consistently positive for CD13, CD28, CD33, CD44, CD105, CD166, and HLA class 1. MPCs generate the microenvironment essential for the maintenance, proliferation, and differentiation of hematopoietic stem cells [6].

Current clinical application of MPCs for treatment of osteogenesis imperfecta demonstrated impressive histological changes in trabecular bones, which indicate new dense bone formation [7]. Several animal experiments were conducted using MPCs for fracture healing, tendon repair, and cartilage regeneration, as well as other clinical applications in treating central nervous system diseases, hepatic failures, and myocardial infarctions [8–10]. Pereboeva et al. reported that MPCs possess key properties that ensure their employment as cellular vehicles and could be used to deliver either therapeutic genes or viruses to tumor sites [11]. These data support the potential utility in tissue engineering and gene therapy applications.

Cells with some features of MPCs were known to exist in bone marrow [2] as well as synovium [12], fetal lung [13], adipose tissue [14], peripheral blood [15, 16], cord blood [17, 18], and fetal blood [19]. Romanov and colleagues reported that MPC-like cells were present in the subendothelial layer of the human umbilical cord vein [20]. However, the presence of circulating MPCs has not been confirmed in the peripheral [21, 22] and cord blood [23]. In this study, we investigated whether fibroblastic cells isolated from human umbilical cord were capable of multilineage differentiation in vitro.

Materials and methods

Culture of human umbilical cord vein

The research protocol was approved by the Ethical and Research Committees. The cell culture was prepared by the modified method of Marin et al. [24]. Briefly, umbilical cord was collected and processed within 4 h after normal delivery. The cord vein was cannulated on both sides and washed out with phosphate buffered saline (PBS) (Gibco BRL, Gaithersburg, Md., USA). The vessel was filled with 0.1% collagenase type I (Sigma, St. Louis, Mo., USA) and incubated at 37°C for 10 min. After gently massaging the cord, the suspension of endothelial and subendothelial cells was collected. Cells were washed with M199 (Gibco BRL, Gaithersburg, Md., USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah, USA) and 100 units/ml penicillin—100 μg/ml streptomycin (Gibco BRL, Gaithersburg, Md., USA). Cells were centrifuged for 10 min at 2000 rpm and seeded in a 0.5% gelatin-coated 25 cm^2 culture flask at 37°C under 5% CO_2 humid atmosphere. Nonadherent cells were removed on day 3 and the medium was changed every 3 days. Approximately 2 weeks later, cultured cells were placed into a new flask for expansion.

Characterization of endothelial and fibroblastic cells

Endothelial and fibroblastic cells in human umbilical cord vein were evaluated by immunocytochemical reaction. Both cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and incubated for 1 h with primary antibody: vWF (von Willebrand's factor; 1:100, Novocastra Ltd., Newcastle upon Tyne, UK), PECAM-1 (platelet/endothelial cell adhesion molecule-1; 1:50, Santa Cruz Biochemicals, Santa Cruz, Calif., USA), and Flk-1 rabbit polyclonal antibody (1:100, NeoMarkers, Fremont, Calif., USA). After rinsing with PBS, cells were incubated with biotinylated secondary antibody in Vecstatin Elite ABC Kit (Vector Laboratories, Burlingame, Calif., USA). DAB substrates for peroxidase (Vector Laboratories, Burlingame, Calif., USA) were used to visualize the antibody binding.

Adipogenic and osteogenic differentiation

To induce adipocyte differentiation, fibroblastic cells were cultured in α-MEM (Gibco BRL, Gaithersburg, Md., USA) supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 60 μM indomethacin, and 5 μg/ml insulin for 2 weeks. Osteogenic differentiation was induced by incubation with α-MEM supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerolphosphate, and 100 μM ascorbic acid for 2 weeks.

Accumulation of lipid, alkaline phosphatase, and calcium

Adipogenic differentiated cells were fixed in 10% formalin for 5 min and washed in PBS. After PBS was dried, cells were rinsed in 60% isopropanol and stained for 15 min in 0.18% Oil red O and 0.4% dextrin with 60% isopropanol. Cells were washed in 60% isopropanol and photographs of the cells were taken.

Cells induced by osteogenic formula were treated with alkaline phosphate staining and von Kossa staining. To evaluate the expression of alkaline phosphatase, cells were washed in alkaline leukocyte phosphatase (ALP) buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–Cl) and stained with solution containing naphthol As-T phosphate (1 mg/ml) and Fast red violet LB salt (0.1 mg/ml) for 30 min at 37°C. Cells were washed in PBS and photographed. With von Kossa's method, cells were washed in water and plated in 1% silver nitrate solution for 30 min. Cells were incubated in 2.5% sodium thiosulphate, rinsed in water, and counterstained with 1% safranin O.

Reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from differentiation-induced fibroblastoid and endothelial cells using TRIzol reagent (Gibco BRL, Gaithersburg, Md., USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed at 37°C for 1 h in the presence of oligo(dT) primer (BM, USA), RNase inhibitor (Promega, Madison, Wis., USA), and M-MLV reverse transcriptase (Promega, Madison, Wis., USA) and terminated at 90°C for 10 min. PCR assays were performed in 20 μl reaction volume using 2 μl cDNA, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, 20 pmol of each primer, and 0.4 U of AmpliTaq Gold DNA polymerase (PE Biosystems, Cambridge, UK). GenBank access number, product size, and forward and reverse sequence for each primer set were as follows: adipsin (NM001928, 271 bp, 5′-ggT CAC CCA AgC AAC AAA gT-3′, 5′-CCT CCT gCg TTC AAg TCA TC-3′), fatty acid binding protein 4 (FABP4; NM001442, 357 bp, 5′-TgC AgC TTC CTT CTC ACC TT-3′, 5′-Tgg TTg ATT TTC CAT CCC AT-3′), lipoprotein lipase (LPL;

BT006726, 717 bp, 5′-gTC CgT ggC TAC CTg TCA TT-3′, 5′-AgC CCT TTC TCA AAg gCT TC-3′), peroxisome proliferative activated receptor, gamma 2 (PPARγ2; NM015869, 351 bp, 5′-gCT gTT ATg ggT gAA ACT CTg-3′, 5′-ATA Agg Tgg AgA TgC Agg CTC-3′), connexin43 (Cx43; AF151980, 711 bp, 5′-ggA ggg AAg gTg Tgg CTg TC-3′, 5′-ggC Agg gCT CAg CgC ACC AC-3′), osteopontin (X13694, 373 bp, 5′-CTA ggC ATC ACC TgT gCC ATA CC-3′, 5′-CAg TgA CCA gTT CAT CAg ATT CAT C-3′), runt-related transcription factor 2 (Runx2; NM004348, 289 bp, 5′-CCC CAC gAC AAC CgC ACC AT-3′, 5′-CAC TCC ggC CCA CAA ATC-3′), and β-actin (NM001101, 512 bp, 5′-TCA TgT TTg AgA CCT TCA A-3′, 5′-gTC TTT gCg gAT gTC CAC g-3′). The reaction products were analyzed by electrophoresis with 2% agarose or 8% polyacrylamide gel and visualized with ethidium bromide.

Cytogenetic analysis

Standard karyotypic analysis was performed. Briefly, cultured cells at passages 10 were incubated in growth medium with 0.1 μg/ml of colcemid for 3 h, trypsinized, resuspended in 0.075 M KCl, and incubated for 20 min at 37°C, then fixed in 3:1 methanol/acetic acid.

Results

Characterization of endothelial and fibroblastic cells

Human umbilical cord vein cells in M199 containing 10% FBS grew as a confluent monolayer with cobblestone morphology (Fig. 1a). Characterization studies revealed that cultured cells were positive for vWF, PECAM-1, and Flk-1 indicating that the cells were endothelial cells (Fig. 1b–d). However, in 3 flasks of 50 umbilical cord samples (6%), fibroblast-like cells, which were morphologically distinguishable from adherent endothelial cells, were shown after 1 week of culture (Fig. 1e). Fibroblastic cells maintained uniform morphology and occupied the whole flask surface after 3 weeks. Cytochemistry analysis showed that they were negative for vWF, PECAM-1, and Flk-1 (Fig. 1f–h).

Adipogenic differentiation of fibroblastic cells

To confirm whether fibroblast-like cells are capable of differentiating to multiple cell type, passages 1 and 2 were treated with adipogenic and osteogenic supplementation and archived during 2 weeks. Treatment of the adipogenic medium resulted in adipocyte formation, which was identified by the Oil red O staining. Over 35% of induced cells produced lipid droplets (Fig. 2a,b). As a positive control, MPCs derived from bone marrow of rats indicated the accumulation of neutral lipid vacuoles (Fig. 2c). Nontreated endothelial cells, as negative controls, were not stained (Fig. 2d). In RT-PCR analysis, the adipsin, FABP4, LPL, and PPARγ2 genes were expressed in adipogenic formula-treated cells, while they were not expressed in the corresponding nontreated endothelial cells (Fig. 3a).

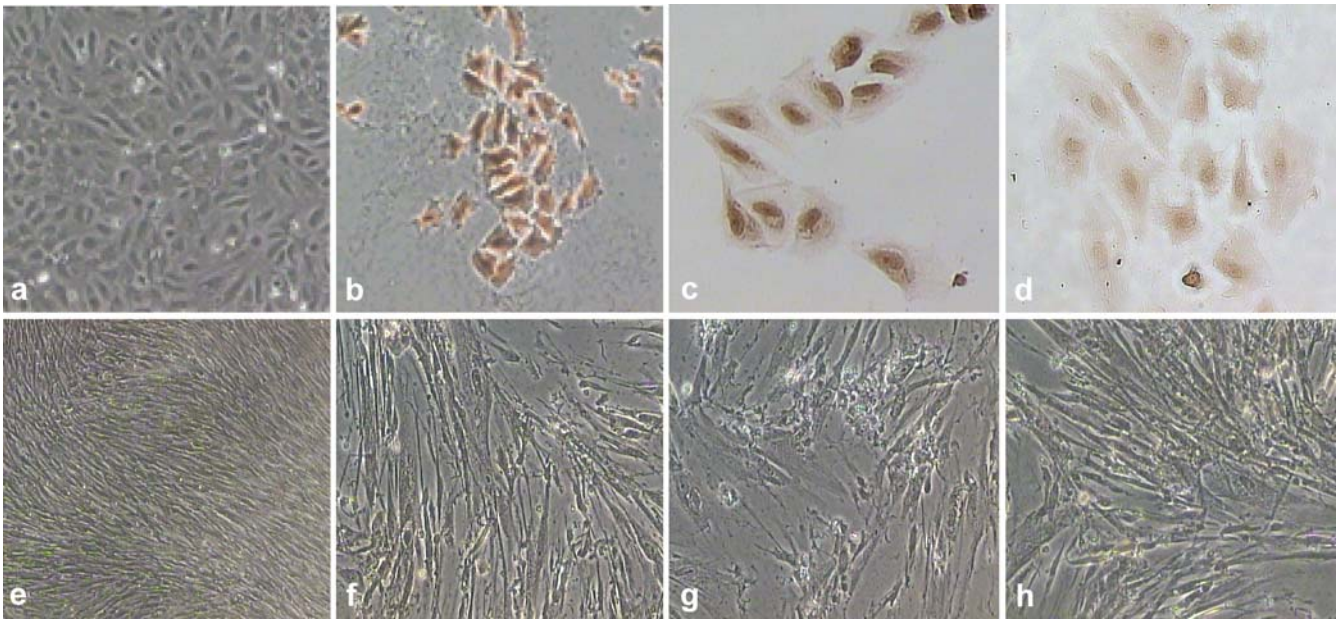

Fig. 1 Morphology and immunophenotyping of endothelial cells and fibroblastic cells in human umbilical cord vein. Cells have typical endothelial morphology (**a**), but fibroblast-like cells in 3 of 50 cultured cords were shown (**e**). Endothelial cells were positive for vWF (**b**), Flk-1 (**c**), and PECAM-1 (**d**), but fibroblastoid cells were negative for vWF (**f**), Flk-1 (**g**), and PECAM-1 (**h**).

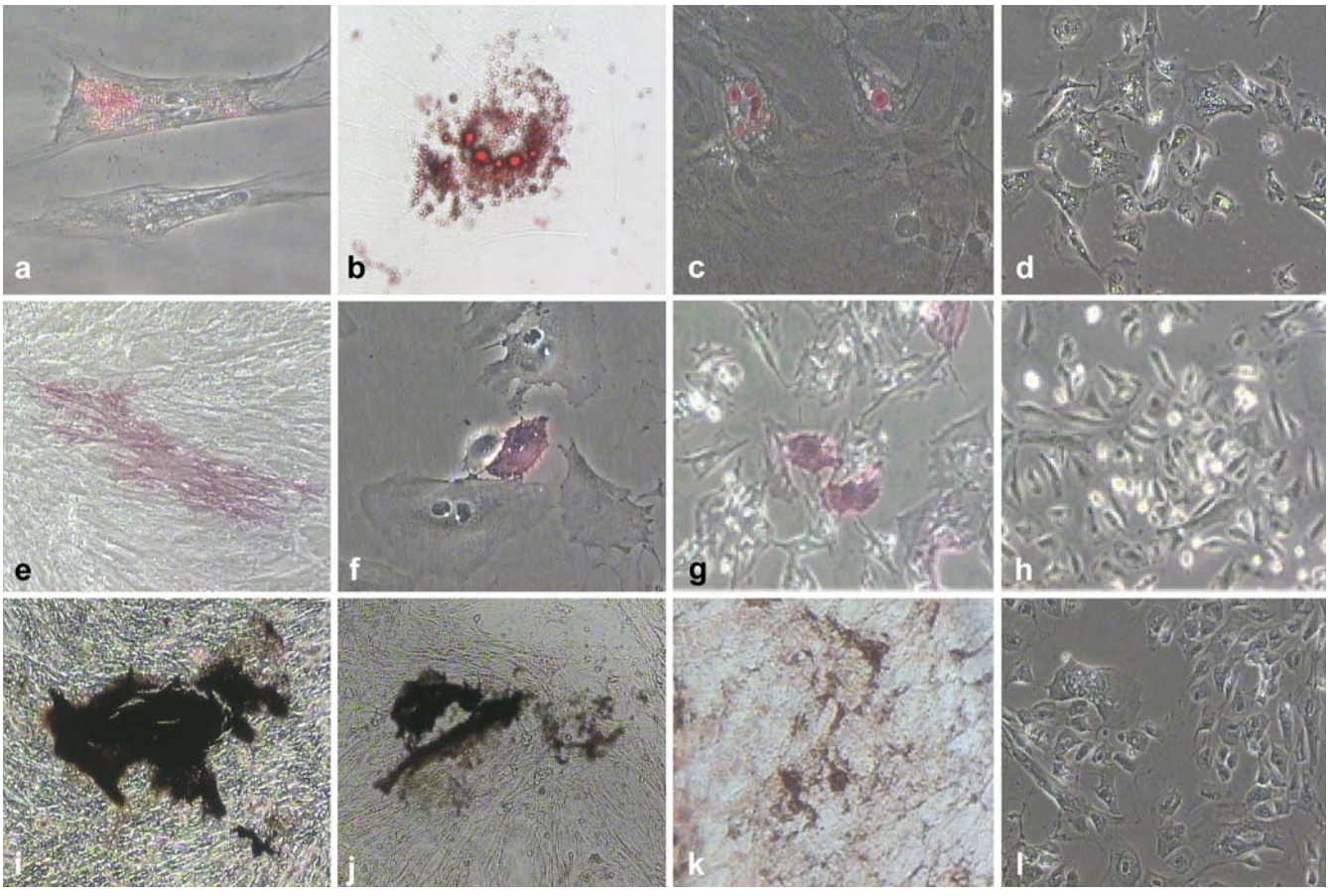

Fig. 2 Adipocyte and osteocyte differentiation of fibroblast-like cells. After incubation for 2 weeks followed the addition of differentiation medium, cells were stained by Oil red O (**a–d**), alkaline phosphatase (**e–h**), and silver nitrate (**i–l**). Adipogenic differentiated cells (**a,b**) and osteogenic differentiation cells (**e,f,i,j**) were positively stained. Bone marrow cells in rat were used as a positive control (**c,g,k**). Nontreated endothelial cells, negative control, were not stained (**d,h,l**).

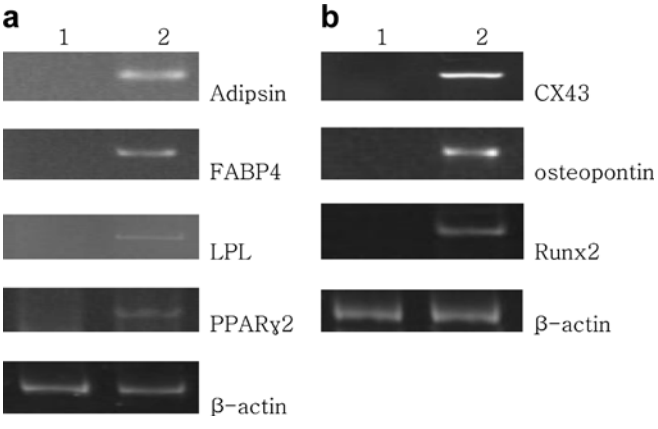

Fig. 3 RT-PCR of adipogenic- and osteogenic-specific genes. Total RNA was extracted and analyzed with indicated primers. *Lane 1* indicated endothelial cells as a negative control and *lane 2* showed fibroblast-like cells grown in adipocyte- (**a**) and in osteocyte-induced medium (**b**). β-actin was used as an internal control.

Osteogenic differentiation of fibroblastic cells

The results of alkaline phosphatase stain were evident by the significant increase in alkaline phosphatase activity after fibroblastic cells treated with osteogenic supplementation over 2 weeks (Fig. 2e,f). von Kossa staining for calcium accumulation revealed a positive reaction in fibroblastic cells (Fig. 2i,j). MPCs derived from bone marrow of rats were indicated by the accumulation of alkaline phosphatase (Fig. 2g) and calcium (Fig. 2k). Nontreated endothelial cells were not stained with alkaline phosphatase (Fig. 2h) and silver nitrate (Fig. 2l). RT-PCR studies determined that Cx43, osteopontin, and Runx2 were expressed in the osteogenic cultures, whereas they were not in the corresponding nontreated cells (Fig. 3b).

Cytogenetic analysis

Passage 10 fibroblastic cells maintained normal proliferation and an undifferentiated status. To investigate chromosomal aberrations, karyotypic analyses of three cell lines were performed. Two cell lines had normal karyotypes (46,XY) on 20 cells, but one had a karyotype of mos 46,XY[19]/47,XY,+mar[3] (Fig. 4).

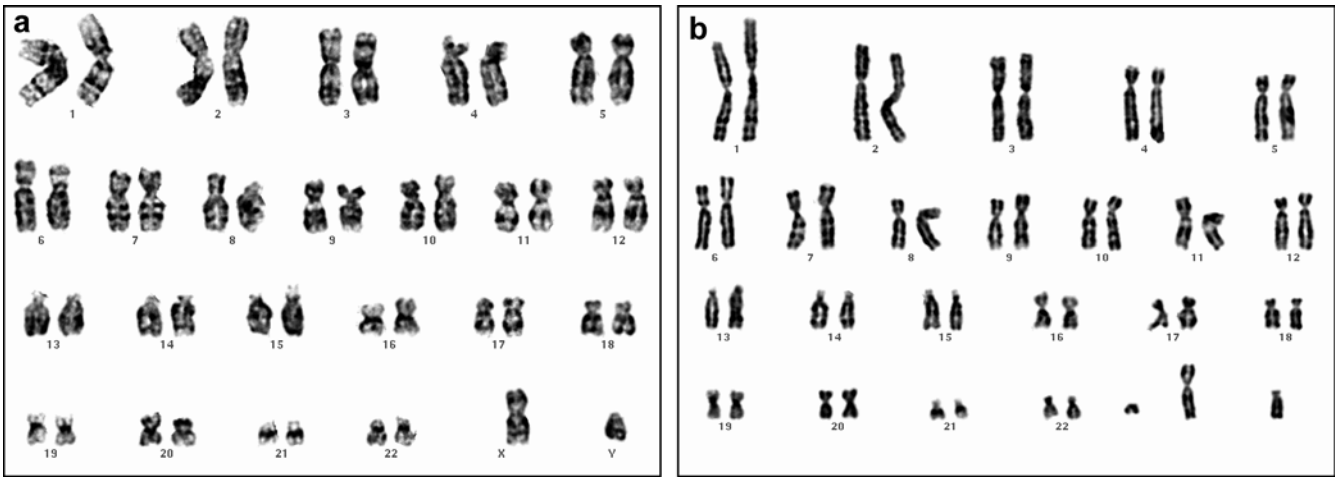

Fig. 4 Karyotypic analyses of fibroblastic cells in human umbilical cord. During culture expansion at passages 10, one cell line presented a cytogenetic mosaicism of 46,XY[19] (**a**) and 47,XY+mar[3] (**b**).

Discussion

MPCs characterized by the capacity for self-renewal and differentiation into multiple lineages have been investigated over the last few years. Recent data have shown that synovium, fetal lung, adipose tissue, peripheral blood, cord blood, and fetal blood in addition to bone marrow are sources of MPCs. However, the presence of circulating MPCs in peripheral and cord blood is controversial. The purpose of this study was to isolate and characterize MPCs resident in human umbilical cord vein.

We found fibroblastic cells with spindle-shaped morphology in 6% of the umbilical cord cultures. In contrast to cultured endothelial cells, fibroblastic cells did not express typical endothelial lineage markers: vWF, Flk-1, and PECAM-1. Morphology and characterization studies indicated that fibroblast-like cells differed significantly from endothelial cells. To serve as MPCs, fibroblastic cells were differentiated into adipogenic and osteogenic lineages. Cells in adipogenic medium accumulated lipid droplets, as assessed by Oil red O staining. In RT-PCR, the adipsin, FABP4, LPL, and PPARγ2 gene were significantly expressed in the treated cells. Also, fibroblast-like cells could differentiate into osteogenic lineage as assessed by the increase of alkaline phosphatase activity, the calcium accumulation used as an early marker of osteocytic differentiation, and the upregulation of mRNA for Cx43, osteopontin, and Runx2. Alkaline phosphatase activity is present at baseline levels in undifferentiated cells and at significantly increased levels in osteocyte-induced cells prior to calcium deposit [2]. Based on this capacity to differentiate, fibroblast-like cells can be considered true MPCs. Our data agreed with the findings of Romanov et al. who recently identified MPC-like cells in the subendothelial layer of the human umbilical cord vein [20].

In view of gene expression analysis, adipsin is expressed later during the differentiation of adipocytes. LPL and FABP4 are the essential genes in the metabolism of fatty acid. LPL is a gene expressed early in the adipocyte differentiation pathway [25]. The induction of LPL may provide energy for marrow stromal cell differentiation by enhancing the hydrolysis of extracellular triglycerides for cells in the early adipocyte pathway [26]. PPARγ2 is an early response gene that is involved in the commitment to the adipocyte pathway and appears to be more adipose specific than PPARγ1 [27]. Cx43 in osteoblasts mediates gap junction intercellular communication that is critically important in osteogenesis and normal osteoblast function [28]. Osteopontin, a noncollagenous phosphoprotein, is originally isolated from bone matrix. Expression of this protein is found in various cells and tissues, including osteoblasts, osteoclasts, and macrophages [29]. Runx2 (CBFA1/AML3/PEBP2αA) is the major regulator of osteoblast differentiation and has therefore been regarded largely as a bone-specific transcription factor [30].

MPCs have great therapeutic potentials because of their ability to differentiate into multiple tissues and to self-renew at a high proliferative rate. They enhance engraftment of donor hematopoietic cells after cotransplantation in animal models and migrate into areas of muscle degeneration to undergo myogenic differentiation in immunodeficient mice [31]. MPCs may be useful cells for cartilage engineering aimed at surgical repair of severe congenital tracheal anomalies [32]. MPCs can foster expression of suicide genes or support replication of adenoviruses as potential anticancer therapeutic payloads [11].

For potential uses of stem cells as biologic research tools and therapeutic agents, it is important to confirm normal chromosomes with no structural aberrations. In cytogenetic analysis, we observed that one cell line presented mosaicism. A number of studies have specifically explored the development of chromosome abnormality in cultured vascular endothelial cells [33, 34]. Genetic mutations may place a higher limit on the useful long-term culture of stem cells.

Though the frequency of MPCs in umbilical cord is very low, MPCs from endothelial cells are easily isolated

and have high prolific potential in cultures. Also, MPCs in umbilical cord vein are capable of differentiating into a wide variety of cell types in vitro. MPCs from the umbilical cord may make it possible to apply stem cell technology to a variety of valuable experimental and clinical needs.

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