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Characterization of human umbilical cord mesenchymal stem cells following tissue mass culture

L. M. Yang[1], Y. Liu[2], J. Zhao[2], L. M. Hao[2], K. X. Huang[3] and W. H. Jiang[2]✉

[1] Department of Nephropathy, the First Affiliated Hospital of Jilin University, Changchun 130021, Jilin Province, China
[2] Department of Histology and Embryology, Norman Bethune Medical College of Jilin University, Changchun 130021, Jilin Province, China
[3] Center of Morphology, Norman Bethune Medical College of Jilin University, Changchun 130021, Jilin Province, China

Corresponding author: Wenhua Jiang, Department of Histology and Embryology, Norman Bethune Medical College of Jilin University, No.126 Xinmin Street, Changchun, Jilin province, China. Tel: +86-13604321909, Fax: + 86-43188962607, Email: jiangwenhua468@163.com

Abstract

The human umbilical cord represents a promising resource of mesenchymal stem cells (MSCs). In order to improve our understanding of MSCs derived from human umbilical cord (UC-MSCs), we isolated UC-MSCs from human umbilical cord tissues through a direct culture approach. We performed a comprehensive characterization of these cells based on analyses of morphology, growth features, cell surface antigen markers and differentiation capacity. All these analyses validated their stem cell nature. The UC-MSCs presented a spindle-shaped morphology and could be subcultured for up to 15 passages without losing their cellular features. Moreover, these UC-SMCs presented an expression profile of cell surface antigens similar to other MSCs: positive for CD44, CD90, and CD105 expression and negative for CD34, CD31, and CD45 expression. Differentiation assays further validated the multipotency of UC-MSCs by inducing these cells into osteoblasts, adipocytes and functional hepatocytes. Our studies clearly demonstrated that UC-MSCs resemble other types of MSCs in many aspects and have a great potential to be applied in tissue engineering and regenerative medicine.

Key words: Umbilical cord, Mesenchymal stem cells, tissue mass culture, functional hepatocytes.

Introduction

Human mesenchymal stem cells (MSCs) may be useful in tissue regeneration, gene engineering and cell-based therapy because of their proliferative potential and multilineage differentiation capacity. MSCs can be derived from various tissues such as bone marrow, fat tissue, placenta, fetal lung and fetal liver (1, 2, 3, 4). However, clinical application of MSCs remains questionable due to concerns such as reduced potential of proliferation and differentiation with increased donor's age, uncomfortable collection procedures and the risk of teratoma formation after transplantation (5, 6, 7, 8). Umbilical cord, usually discarded as medical trash after delivery, is enriched with MSCs and could serve as a promising resource of MSCs due to its convenient availability and fewer ethical concerns (9, 10).

Wharton's jelly of human umbilical cords has been identified as a major source of MSCs by several studies (11, 12, 13, 14). It has been reported that these cells derived from human umbilical cords are different with hematopoietic multipotent stromal cells and express surface markers such as CD73, CD90, and CD105(9). Differentiation assays have further demonstrated that these cells can differentiate into chondrogenic, osteogenic, adipogenic, myogenic, pancreatic, neurogenic, and hepatogenic lineages with appropriate conditioned medium, indicating their multipotency (11, 15, 16, 17, 18).

In order to improve the understanding of MSCs derived from human umbilical cord (UC-MSCs) and push forward their potential application in stem cell research and clinical therapy, we refined a direct culture method to isolate and expand US-MSCs and performed a comprehensive characterization of these cells based on analyses of morphology, cell surface antigen markers and differentiation capacity.

Materials and methods

Source of umbilical cords

After informed consent and approval from the local Ethical Review Board at Jilin University, umbilical cords of babies born between 37 and 40 weeks of pregnancy were obtained from the Maternity Hospital of Changchun.

Isolation, culture and expansion of UC-MSCs

Umbilical cord was soaked in saline with 25 U heparin sodium and was minced to 1 mm^3 pieces after washing with D-Hank's buffer. Tissue pieces were cultured in DMEF/F12 medium supplemented with 10% fetal bovine serum (FBS). Culture flasks were incubated at 37°C with 5% CO_2. Medium was changed every 3–4 days, and the unattached tissues were discarded when confluence reached 60%. Attached tissues remaining in flasks were digested by using trypsin-EDTA solution. The trypan blue method revealed a 98% cell viability. Detached cells were then passaged at a ratio of 1:2–1:3. Cells at passage 3 (P3) appeared homogeneous under a microscope and were used for further experiments.

Proliferation measurement by the MTT method

Healthy UC-MSCs in the log phase of growth were obtained from P3, P5, and P7 and were seeded in 96-well plates. Cells were harvested after 1, 2, 3, 4, 5, 6, and 7 days, and cell proliferation was measured using a MTT kit (Sigma, USA) following the manufacturer's protocol. Growth curves were plotted based on the ab-

sorbance values (492 nm) and incubation time.

Cell surface antigen analysis

For cell surface antigen analysis, single cell suspensions at a concentration of 1×10^7 cells/ml were prepared and stained with fluorescein-conjugated antibodies including APC-CD90, FITC-CD31, FITC-CD34, FITC -CD 44, FITC-CD45, and PE-CD105 (Becton Dickinson, USA) for 30 min on ice. The dilution of antibodies was applied according to the manufacturer's protocols. Stained cells were subjected to flow cytometric analysis after washing with phosphate-buffered saline (FACS-Calibur, Becton Dickinson, USA).

Transmission electron microscopy

Single cell suspensions were prepared from UC-MSCs at passage 7 and were subjected to a double fixation using 2% glutaraldehyde solution for 12 h and 1% osmium tetroxide for 0.5 h. The samples were dehydrated and embedded before sectioning using a LKB II ultramicrotome. Sectioned samples were further stained with uranyl acetate and lead citrate before recording the ultrastructure using transmission electron microscopy (JEM-1200EX, Olympus, Japan).

Osteoblast differentiation

UC-MSCs were seeded in 3.5 cm^2 dishes at a density of 2×10^4 cells/well and were cultured for 12 h. Then, the medium was changed to osteoblast-inducing medium (1.0×10^{-8} M dexamethasone, 2.0×10^{-4} M ascorbic acid, 7.0×10^{-3} M β-glycerophosphate and 10% fetal calf serum (FCS) in DMEM/F12 culture medium). A half-medium change was carried out every 3–4 days according to the cell morphology. Cells were cultured for 3 weeks before staining using the Gomori method (16).

Adipocyte differentiation

UC-MSCs were seeded in 3.5 cm^2 dishes at a density of 2×10^4 cells/well and were cultured for 12 h. Then, the medium was changed to adipocyte-inducing medium (1.0×10^{-6} M dexamethasone, 10 mg/L insulin, 100 mg/L 1-Methyl-3-isobutylxanthine, 100 mg/L indomethacin, 50 mg/L ascorbic acid, 100 U/mL penicillin, 100 mg/L streptomycin and 10% FCS in DMEM/F12 culture medium). A half-medium change was carried out every 3–4 days according to the cell morphology. Cells were cultured for 6 weeks before fixation and staining with 50% isopropanol and oil red O solution (16). Cell images were obtained using a light microscope (Olympus, Japan).

Functional hepatocyte differentiation

UC-MSCs at passage 3 were seeded in culture flasks at a density of 1×10^6 cells/ml in DMEM/F12 medium containing 10% FBS, 20 μg/L hepatocyte growth factor (HGF), 10 μg/L basic fibroblast growth factor (bFGF) and 20 μg/L oncostatin M. No growth factors were added to the control groups. Medium was changed every 3–4 days. Cells were harvested at 7, 14, and 21 days after induction for further tests.

Immunochemistry

Alpha-fetoprotein (AFP) and albumin (ALB) (Dako, Denmark) expression was examined using an immunochemistry method described previously (17). AFP and ALB were diluted at ratios of 1:200 and 1:100, respectively. Percentages of positive cells were calculated based on 100 cells counted in 10 random chosen fields under a microscope.

Measurement of urea in cell supernatant

Cells were seeded in Petri dishes at a density of 1×10^6 cells/dish in serum-free induction medium containing 0.15 M NH_4Cl and cultured for 8 h. The supernatant was collected to determine the urea concentration by using an automatic biochemical analyzer (Hitachi 7180, Japan), through a urea assay (Shanghai Kehua Bioengineering Institute, Shanghai, China) according to the manufacturer's instructions.

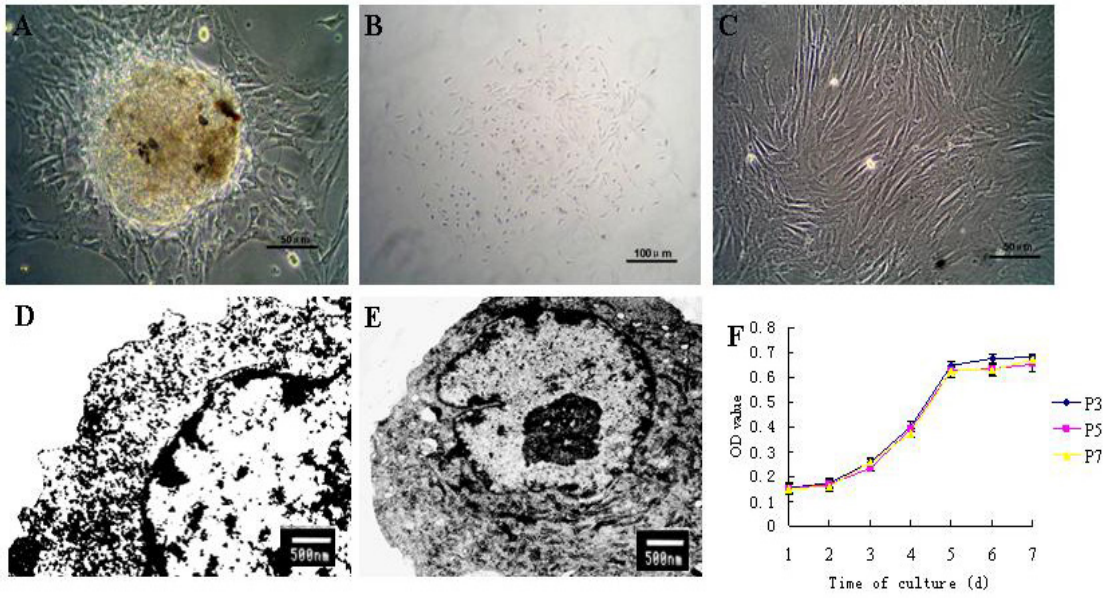

Figure 1. Primary culture of human UC-MSCs. The photos show the morphology of the primary cultured cells under an optical microscope at day 5 (A), day 8 (B) and day 14 (C). The cellular ultrastructure was determined by transmission electron microscopy for cells at passage 7 (D, E). The growth curves of UC-MSCs at passages 3, 5, and 7 were based on MTT tests (F). Cells were harvested at days 1, 2, 3, 4, 5, 6, and 7 for the MTT tests.

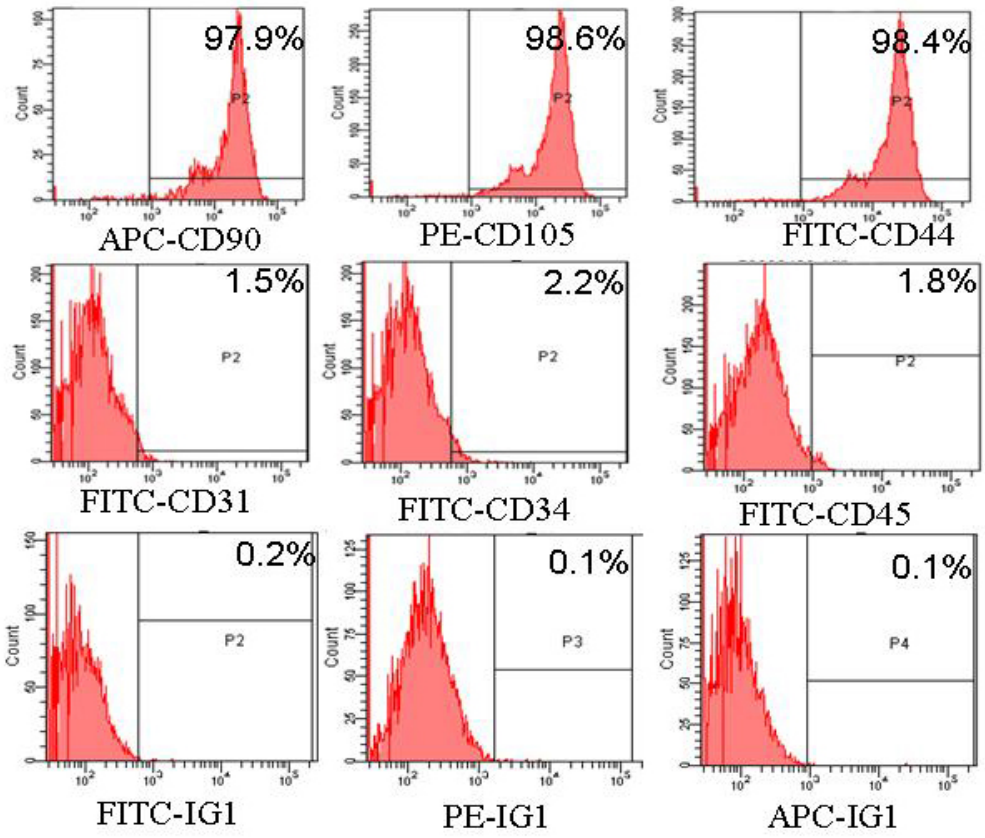

Figure 2. Flow cytometric analysis of cell surface antigens. Cells at passage 7 were stained with APC-CD90, FITC-CD31, FITC-CD34, FITC-CD44, FITC-CD45, and PE-CD105 for 30 min. The corresponding FITC-IgG1, PE-IgG1, and APT-IgG1 served as controls.

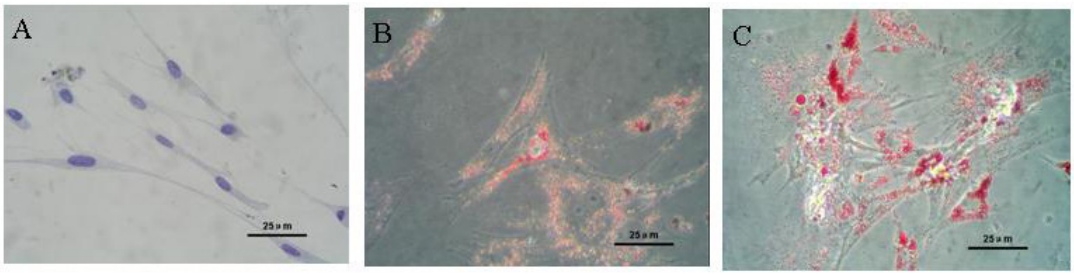

Figure 3. Induced differentiation into adipocytes. UC-MSCs at passage 7 were cultured in adipocyte-inducing medium and harvested at day 21 (B) and day 42 (C) for oil red O staining. UC-MSCs cultured with regular medium served as the control (A), presenting positive staining for hematoxylin staining in nuclei and negative staining for oil red O staining.

Periodic acid-Schiff (PAS) method

Glycogen expression was measured using the PAS method(18, 19).

Statistical analysis

All data were subjected to one-way ANOVA analysis using SPSS 11.0 software. $P < 0.05$ was defined as significant.

Results

Primary culture and passage of UC-MSCs

Five days after the umbilical cord tissue adhered to the flask in the initial culture setup, a single layer of cells stretched out from the tissue and grew around the tissue mass (Fig. 1A). Adherent cell colonies appeared 1 week after the initial culture setup (Fig. 1B). Most of the cells showed a long spindle-shaped or flat-shaped fibroblast-like morphology with high refraction and prominent nucleoli. Homogenous cells with spindle-shaped morphology presented in the culture 2 weeks after the initial culture setup (Fig. 1C). It took 10–14 days for primary cells to reach 70–80% confluence. Subsequently, the cells were trypsinized and passaged every 4–5 days. These cells could be subcultured for more than 20 passages and could maintain their regular growth rate and typical spindle-shaped morphology for up to 15 passages. However, reduced growth rate and altered morphology were observed after passage 15. Therefore, these cells were termed as UC-MSCs because of their proliferation capacity and multilineage differentiation potential confirmed in the following part of this report.

Growth curves for cells at passages 3, 5, and 7 were established to examine the proliferation potential of UC-MSCs. The three batches of cells showed a similar growth pattern: cells entered into the log phase of growth within 24–36 h and kept proliferating for 5 days before reaching the plateau phase (Fig. 1F). These results suggested that the UC-MSCs isolated in this study could maintain a stable proliferation capacity for many passages.

Cell surface antigen profile of UC-MSCs

MSCs express various patterns of cell surface an-

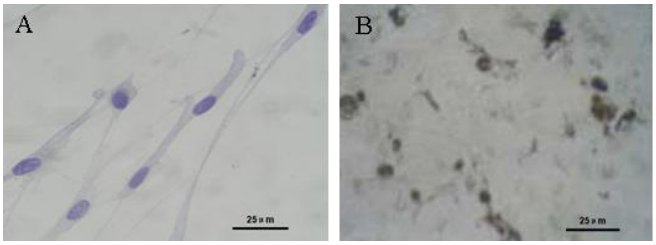

Figure 4. Induced differentiation into osteoblasts. UC-MSCs at passage 7 were cultured in osteoblast-inducing medium and harvested at day 21 for alkaline phosphatase staining (B). UC-MSCs cultured with regular medium served as the control (A), presenting positive staining for hematoxylin staining in nuclei and negative staining for alkaline phosphatase staining.

tigen proteins depending on the tissue source and the donor age (19, 20). Therefore, it is difficult and sometimes controversial to define MSCs by cell surface protein markers. However, some common antigen markers were observed more frequently than other markers in MSCs. CD105, CD90, and CD44 are the top three positive MSC markers among published reports. CD34 and CD45 are the first and the third most frequently reported negative markers. CD31 has also been reported as a negative marker in some studies (19). In order to define the cellular features of the UC-MSCs in our study, all of the above-mentioned cell surface markers were analyzed for the UC-MSCs at passages 3, 5, and 7 by flow cytometric analysis with antibodies against these marker proteins. The flow cytometric results showed that cells at these three passages presented a similar expression profile: strong expression of CD105, CD90, and CD44; and negative expression of CD34, CD31, and CD45 (Fig. 2).

Cellular ultrastructure of UC-MSCs

UC-MSCs at passage 7 were examined by transmission electron microscopy. Most of the cells showed small protrusions, a large and irregular nucleus, a visible nucleolus, abundant euchromatin, less heterochromatin, less cytoplasm and scattered ribosome, suggesting their G0/G1 properties, which is consistent with the cell cycle analysis results (Fig. 1D). A small number of cells showed a smaller nucleus, rough endoplasmic reticulum and mitochondria, suggesting the proliferative state of the cells (Fig. 1E).

Differentiation into adipocytes

After 14 days of culturing in adipocyte-inducing medium, cells changed from a long-spindle shape to a shorter morphology with the formation of lipid droplets, which turned red after oil red O staining, showing a positive marker for adipocyte lineages. After 21 days of culturing, more lipid droplets formed and distributed around the nucleus (71.2±4.8%) (Fig. 3B). After 42 days, lipid droplets were observed in most of the cells (85.9±3.7%) (Fig. 3C). While, the control cells cultured in non-inducing medium remained lipid droplet-free and were negative for oil red O staining (Fig. 3A).

Differentiation into osteoblasts

After 14 days of culturing in osteoblast-inducing medium, the cellular activity of alkaline phosphatase, a common marker for osteogenic differentiation, was still weak. After 21 days of culturing, strong alkaline phosphatase activity and the presence of black staining in the cytoplasm were observed (86.7±3.9%) (Fig. 4B). In contrast, the control cells cultured in non-inducing medium did not show any alkaline phosphatase activity (Fig. 4A).

Differentiation into functional hepatocytes

UC-MSCs did not change their morphology until 7–8 days after hepatocyte induction (Fig. 5B). Spindle-shaped cells started to become polygonal, elliptical and round. After 12–14 days, more cells with a polygonal or elliptical shape were observed (Fig. 5D). Most cells

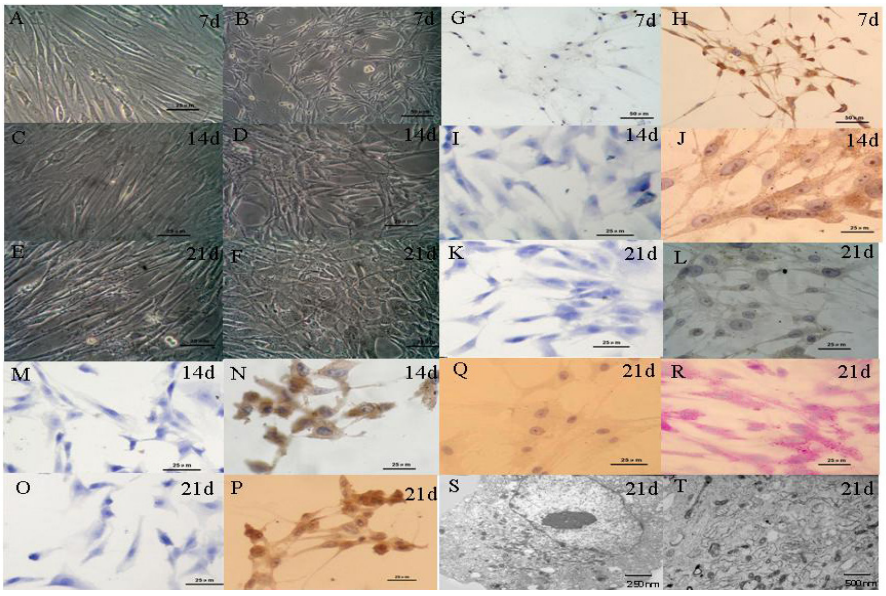

Figure 5. Induced differentiation into hepatocytes. UC-MSCs at passage 3 were cultured in hepatocyte-inducing medium. Images to monitor cell morphology changes were taken at day 7 (B), day 14 (D) and day 21 (F) after induction, respectively. UC-MSCs cultured with regular medium served as the control and were analyzed at the same time points as those for the induction group (A, C, E). AFP expression is shown for the control group (G, I and K) and for the induction group (H, J and L). ALB expression is shown for the control group (M and O) and for the induction group (N and P). Glycogen synthesis is shown for the control group (Q) and for the induction group (R). Red dots indicate glycogen granules. Cellular morphology was revealed by transmission electron microscopy for cells at day 21 in the control group (S) and in the induction group (T). Time points for sampling are labeled in each image.

showed a polygonal and round epithelial-like shape 20–21 days after induction (Fig. 5F). In contrast, the cells in the control group retained their fibroblast-like morphology (Fig. 5A, 5C, 5E). Consistent with the morphological change, conditioned medium induced the strongest AFP expression at day 7 (81.1±2.2%) (Fig. 5H), followed by a reduced expression at day 14 (38.1±2.1%) (Fig. 5J). AFP expression was undetected on day 21 (Fig. 5L). Cells cultured in regular medium appeared the same as the control cells (Fig. 5G, 5I, 5K). In addition, other markers of functional hepatocytes were also detected during the differentiation process. Induced expression of ALB was observed at differentiation day 14 (Fig. 5N) and day 21 (Fig. 5P). Cells cultured in regular medium appeared the same as the control cells (Fig. 5M, 5O). Increased urea synthesis was also observed and the peak value occurred at differentiation day 21 (Table 1).

PAS-positive cells (69.3±3.8%) (Fig. 5R) appeared in the conditioned medium but not in the control medium at day 21 after induction (Fig. 5Q), indicating active glycogen synthesis, a defined function of hepatocytes.

Examination by transmission electron microscopy showed that cells at day 21 after hepatocyte differentiation presented with an enlarged cell size, a round nucleus and plenty of mitochondria, rough endoplasmic reticulum (RER) and Golgi bodies, indicating active cell metabolism (Fig. 5S). Expansion of the RER suggested active protein synthesis (Fig. 5T). In contrast, cells cultured in control medium only showed basic organelles (Fig. 1D, 1E).

Table 1. Urea secretion (mM) after hepatocyte induction (n=6, $\bar{x}$ ± s).

Group	Culture time (d)		
	7	14	21
Control group	0.77±0.02	0.74±0.03	0.76±0.02
Experimental group	2.32±0.25	2.41±0.30	10.52±1.30*

** P<0.01 vs. 7 d and 14 d

Discussion

MSCs can be isolated from Wharton's jelly and the umbilical vein through different approaches such as enzymatic digestion and direct tissue culture (9, 10). It has been reported that direct tissue culture, a simple and fast approach with better cell viability and low contamination, is superior to enzymatic digestion (16, 21). Thus, we adopted the direct tissue culture approach and successfully obtained UC-MSCs for expansion and passaging. These cells could be subcultured for more than 15 passages with a stable growth rate and unchanged typical spindle-shaped morphology. Moreover, the multiple lineage differentiation potential still remained in the cells at later passages, e. g. passage 7. These results clearly demonstrated the self-renewal and proliferation features, the two definitive stem cell features, of these UC-SMCs.

How to define MSCs is still controversial (22). MSCs can express multiple cell surface antigens; however, none of them is unique for MSCs, since many of these antigens are also expressed in stromal cells, endothelial cells and epidemic cells (23). Several studies have suggested that MSCs express CD29, CD44, CD59, CD71, and SH2 but do not express CD3, CD14, CD34, CD38, CD45, CD56, CD117, and HLA-DR (23, 24, 25, 26). In order to define the expression profile of cell surface markers for the UC-SMCs isolated in our study, several of the most frequently reported positive and negative markers were analyzed. The results showed that these cells express CD105, CD90, and CD44 but do not express CD34, CD45, and CD31. A thorough review of the published profiles of cell surface markers for various MSCs found that CD105, CD90, and CD44 are the most frequently observed positive MSC markers. While, CD34 and CD45 are the first and the third most frequently reported negative markers (19). Our results showed that the UC-MSCs in our study represented a typical type of MSCs in terms of cell surface antigen expression. Moreover, CD34, CD45, and CD31 are well-known makers for hematopoietic, leukocyte and endothelial lineages, respectively. The negative expression of these markers indicated a high purity of our UC-MSCs and excluded a possibility of contamination in our cultures with MSCs derived from other lineages such as hematopoietic stem cells.

Differentiation potential is one of the two definitive features for stem cells. Therefore, differentiation assays to determine their potential of producing multilineage cells are required to define stem cells. These functional tests are especially necessary for defining MSCs due to their lack of unequivocal cell surface markers(22). Thus, we conducted a series of differentiation assays for the UC-MSCs and showed that these cells could be successfully differentiated into osteoblasts, adipocytes and hepatocytes by using the appropriate induction medium. Consistent with previous studies (11, 12, 13, 27), conditioned medium with dexamethasone induced osteogenic differentiation of the UC-SMCs, characterized by secretion of alkaline phosphatase, a functional marker for osteoblast differentiation. Dexamethasone, insulin, indomethacin and isobutylmethylxanthine have been used to induce adipocyte differentiation of MSCs in monolayer culture (11, 12, 28, 29). Here, we induced the formation of lipid droplets in the cells, a typical feature of adipose cells, by using conditioned medium containing dexamethasone and insulin. Furthermore, we could induce the differentiation of hepatocytes from these UC-MSCs by using conditioned medium containing several reported hepatocyte induction cytokines such as HGF, bFGF and oncostatin M (30, 31, 32, 33, 34). Further characterization confirmed that the differentiated cells had typical hepatocyte morphology: a polygonal or round shape, a large and round nucleus and the presence of binucleate cells. Cellular ultrastructure analysis indicated the abundance of organelles in these cells, suggesting a hepatocyte-like hypermetabolic activity. Moreover, these cells could express AFP and ALB as well as synthesize glycogen and urea, functionally resembling hepatocytes. Overall, successful differentiation into multilineage cells including osteoblasts, adipocytes and hepatocytes validated the multipotency, one definitive stem cell feature, of these UC-SMCs.

In short, our study confirmed the existence of MSCs

in umbilical cords by characterizing their morphology, cellular ultrastructure, cell surface antigen profile and differentiation potential to cells of multiple lineages. Given its convenient availability and little ethical controversy, the umbilical cord could be a very promising substitute for bone marrow as the source of MSCs used in tissue engineering and regenerative medicine applications.

Acknowledgements

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