Acta Biochim Biophys Sin 2014, 46: 1056–1065 | © The Author 2014. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmu100.
Advance Access Publication 5 November 2014

Original Article

The immunologic and hematopoietic profiles of mesenchymal stem cells derived from different sections of human umbilical cord

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Mesenchymal stem cells (MSCs) have been widely used in allogeneic stem cell transplantation. We compared immunologic and hematopoietic characteristics of MSCs derived from whole human umbilical cord (UC), as well as from different sections of UCs, including the amniotic membrane (AM), Wharton's jelly (WJ), and umbilical vessel (UV). Cell phenotypes were examined by flow cytometry. Lymphocyte transformation test and mixed lymphocyte reaction were performed to evaluate the immuno-modulatory activity of MSCs derived from UCs. The mRNA expression of cytokines was detected by real-time polymerase chain reaction. Hematopoietic function was studied by co-culturing MSCs with $CD34^+$ cells isolated from cord blood. Our results showed that MSCs separated from these four different sections including UC, WJ, UV, and AM had similar biological characteristics. All of the MSCs had multi-lineage differentiation ability and were able to differentiate into osteoblasts, adipocytes, and chondrocytes. The MSCs also inhibited the proliferation of allogeneic T cells in a dose-dependent manner. The relative mRNA expression of cytokines was examined, and the results showed that UCMSCs had higher interleukin-6 (IL6), IL11, stem cell factor, and FLT3 expression than MSCs derived from specific sections of UCs. $CD34^+$ cells had high propagation efficiencies when co-cultured with MSCs derived from different sections of UCs, among which UCMSCs are the most efficient feeding layer. Our study demonstrated that MSCs could be isolated from whole UC or specific sections of UC with similar immunomodulation and hematopoiesis supporting characteristics.

Keywords mesenchymal stem cells; umbilical cord; immunomodulation; hematopoiesis; cytokine

Received: May 8, 2014 Accepted: September 22, 2014

Introduction

Mesenchymal stem cells (MSCs) are derived from the mesoderm early in development and widely exist in a variety of tissues and organs. MSCs have great therapeutic potential with a high degree of self-renewal and multi-lineage differentiation ability [1]. In addition, a large number of studies have shown that MSCs have low immunogenicity and multi-lineage differentiation ability, as well as immunomodulatory functions [2]. Since MSCs are relatively easy to be isolated and cultured *in vitro*, they are widely used in clinic to treat self-repair related and other intractable diseases, including spinal cord injury, cerebral palsy, amyotrophic lateral sclerosis, myocardial infarction, diabetes, and cirrhosis of the liver [3–6].

As immunomodulators and hematopoietic supporting cells, MSCs express specific antigens, including CD44 (transparent lipid salt receptor), CD73 (SH-3, 4), CD90 (Thy-1), CD105 (SH-2/endoglin), CD166 (ALCAM), and HLA-ABC. They also have weak expression of CD106 (VCAM-1) and TRA-1-60 (embryonic stem cell marker) [7]. In addition, MSCs do not express CD14 (monocyte and macrophage marker), CD31 (endothelial cell marker), CD34 (hematopoietic and endothelial cell marker), and CD45 (white cell marker), nor do they express CD80, CD86, CD40, and CD40L, which are co-stimulatory molecules required in the activation of effect T cells. Due to the lack of HLA class II antigen, MSCs can escape immune recognition by $CD4^+$ T cells [8], and thus MSCs are potential candidates in treating immune rejection and autoimmune diseases such as systemic lupus erythematous, systemic sclerosis, and Crohn's disease [9–12].

MSCs were first discovered in bone marrow, but the number of MSCs is extremely small. In addition, isolation of MSCs from bone marrow has a high susceptibility of viral contamination. Moreover, the cell number and capacity of

proliferation and differentiation are significantly reduced with increasing age [13]. Recently, many studies have shown that the umbilical cord (UC) contains large amount of MSCs, which are highly proliferative [14]. Therefore, MSCs derived from UC may be a potential alternative for MSCs derived from bone marrow.

The human UC is about 40 g in weight, 60–65 cm in length and 1.5 cm in diameter [15,16], and it is covered by a monolayer or multilayer of umbilical epithelial cells. In addition, the UC is supplied by two arteries and one vein, and is surrounded by mucous connective tissue [17,18]. Researchers have isolated MSCs from whole UC [19], Wharton's jelly (WJ) [20], human umbilical vein endothelium [21], and placental amnion [22]. WJ is rich in hyaluronic acid, glycos-aminoglycans (GAGs), and collagen. These are also found in cartilage extracellular matrix; thus, WJ may be potentially used in cartilage tissue engineering [23]. In contrast, cells from UC vein are more inclined to differentiate into epithelial cells, and may be more suitable for tissue engineering of heart valves [24]. Fine structural, immunohistochemical [25–28], and *in vitro* functional studies [27,29] suggested that there are significant differences in the number and nature of cells among subamniotic, intervascular, and perivascular regions.

In this study, we isolated MSCs from whole UC as well as three different sections of UCs, and then investigated their immuno-modulatory activity and hematopoietic supporting roles. This study provides important evidence for the applications of MSCs derived from UCs in hematopoietic stem cell transplantation (HSCT).

Materials and Methods

Ethics statement

Written informed consents for a stem cell-oriented study were obtained from all of the donors prior to sample collection, and the study was approved by the independent ethics committee of Affiliated Hospital of Academy of Military Medical Sciences (Beijing, China).

Isolation and culture of MSCs derived from different tissues

UCs ($n = 38$; gestational ages, 37–40 weeks) were sterilely collected from the delivery room or the operating theater in Beijing Armed Police Hospital (Beijing, China). After repeated washing with phosphate buffered saline (PBS) to remove residual blood under sterile conditions, whole UCs were dissected to the amniotic, WJ, and vascular tissues, and then shredded into small pieces of $\sim 2\ mm^3$. The tissues were digested in 0.05% type II collagenase (Sigma, St Louis, USA) solution at 37°C for 2–3 h. The cell suspension was collected by filtering through a stainless steel mesh. After washing twice with PBS, cells were resuspended in α-MEM (Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS; Stemcell, Vancouver, Canada) and then seeded in 75-cm^2 culture bottles (Costar, Corning, USA). Cells were cultured at 37°C in 5% CO_2 atmosphere for 72 h with over 95% humidity, and then non-adherent cells were discarded. Media were changed every 3 to 4 days until cells were observed by an inverted microscope (Nikon, Tokyo, Japan) to grow to 80% to 90% confluence. After digestion with solution containing 0.05% Trypsin and 0.01% EDTA (Amresco, Solon, USA), cells were passaged at 4000–6000 cell/cm^2. Fifth-generation cells were collected for follow-up experiments. Cells were marked as UCMSCs (from whole UC), WJMSCs (from WJ), UVMSCs (from umbilical vessel, UV), and AMMSCs (from amniotic membrane, AM), respectively.

Isolation of peripheral blood $CD3^+$ cells and UC blood $CD34^+$ cells

Adult peripheral blood samples from healthy donors were collected from the blood center in Affiliated Hospital of Academy of Military Medical Sciences. $CD3^+$ T cells were separated from the peripheral blood by MidiMACS sorting according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). The purity of isolated cells was up to 97.87% ± 1.46% as detected by flow cytometry.

Healthy full-term fetal cord blood samples were sterilely collected from the delivery room or operating theatre in Beijing Armed Police Hospital. $CD34^+$ hematopoietic stem/progenitor cells were separated from these blood samples by MiniMACS sorting. The purity of isolated cells was up to 93.16% ± 2.74% as detected by flow cytometry.

Growth curve and cell cycle of cultured MSCs

MSCs derived from different tissues of UCs were cultured *in vitro* to the exponential growing phase and collected for further experiments. After trypsin digestion, cells were prepared as single cell suspensions at 1×10^4 cells/ml and seeded onto 24-well plates (Costar) with 1 ml per well. Cells were collected and counted in triplicates everyday for 8 days, and then a cell growth curve was plotted.

For cell cycle detection, 1×10^6 MSCs in the exponential growing phase were collected. After washing twice with PBS, cells were fixed in cold 70% ethanol at 4°C for 24 h. Then 50 μg/ml RNase A (Sigma) were added, and the cells were incubated at 37°C for 30 min, followed by the addition of 50 μg/ml propidium iodide (PI; Amresco) and a 30-min incubation at 4°C in the dark. Cell cycle was detected by flow cytometry. The results were analyzed by ModiFIT software (Verity Software House, Topsham, USA).

Osteogenic differentiation capacity

Cells in the exponential growth phase were seeded in 6-well plates at a density of 4×10^4 cells/well and then cultured at

37°C in 5% CO_2 for 24 h with over 95% humidity. Then, the cells were transferred to osteogenic HG-DMEM (Gibco) containing 10% FBS (Stemcell), 0.1 μM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerol phosphate (Sigma). The cells were incubated for 2 weeks. Media were changed every 3–4 days. Osteoblasts were identified by alkaline phosphatase staining (Sigma).

Adipogenic differentiation capacity

Cells in the exponential growth phase were seeded in 6-well plates at a density of 8×10^4 cells/well and then transferred to induction HG-DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX (Sigma) and 50 μM ascorbic acid. The cells were incubated for 2 weeks. Media were changed every 3–4 days. Fat droplets were identified by Oil red O staining (Amresco).

Chondrogenic differentiation capacity

Cells (4×10^5) in the exponential growth phase were transferred to chondroinductive HG-DMEM containing 0.1 μM dexamethasone, 50 μM ascorbic acid, 1 mM sodium pyruvate, 1 × ITS+1 (Sigma), and 10 ng/ml TGF-β3 (R&D, Minneapolis, USA). The cells were incubated for 4 weeks. Media were changed every 3–4 days. The extracellular matrix was detected by Alcian blue staining (Amresco).

Immunophenotypic analyses

Fifth-generation cells in the exponential growth phase were collected, digested conventionally, and suspended in PBS containing 20 g/l BSA (ExCell Biology, Shanghai, China) to a concentration of 1×10^6 cells/ml. Cells (0.5 ml) were added into individual tubes and incubated with phycoerythrin (PE)-conjugated CD31, CD34, CD40, CD73, CD80, CD105, CD106, or CD166, fluorescein isothiocyanate (FITC)-conjugated CD14, CD44, CD86, CD90, CD154 (CD40L), HLA-ABC, HLA-DR, or TRA-1-60, and peridinin chlorophyll protein (PerCP)-conjugated CD45 (BD; Franklin Lakes, USA) at 4°C for 30 min in the dark. Isotype controls were also included. After washing twice with PBS containing 20 g/l BSA, the cells were fixed in 1% paraformaldehyde at 4°C and then analyzed by flow cytometry.

Lymphocytic transformation test and mixed lymphocyte reaction

^{3}H-TdR incorporation assay was performed to investigate whether MSC inhibition of T-cell proliferation was induced by mitogen or allogeneic lymphocytes. $CD3^+$ T cells were purified by the MidiMACS cell immunomagnetic beads sorting system. Purified $CD3^+$ T cells were seeded as effector cells at 2×10^5 cells/well on top of the UCMSC, WJMSC, UVMSC, or AMMSC feeder cells, which had been seeded in 96-well plates at 2×10^4 (1 : 10), 1×10^4 (1 : 20), 5×10^3 (1 : 40), or 2.5×10^3 (1 : 80) cells per well overnight, respectively. Four duplicates were included in each group and each cell gradient. For the lymphocytic transformation assay, 10 μg/ml phytohemagglutinin (Sigma) was added to stimulate the proliferation of effector cells. For the mixed lymphocyte reaction test, allogeneic peripheral blood lymphocytes were irradiated by 15 Gy ^{60}Co and suspended in RPMI 1640 containing 20% FBS. These cells were plated at 1×10^5 cells/well to stimulate the proliferation of effector cells. Cells were incubated at 37°C in 5% CO_2 with 100% humidity for 48–72 h. ^{3}H-TdR (Atomic Energy Research Institute of Chinese Academy of Sciences, Beijing, China) was added at a dose of 0.2 μCi/well. After 12 h, cells were collected on cellulose acetate filter paper. The paper was dried and placed in a counting vial. Scintillation fluid was then added, followed by the detection of count per minute with a liquid scintillation counter.

Co-culture experiment

Cells in the exponential growth phase were seeded in 24-well plates at 4×10^4 cells/well as trophoblasts. After 24 h, the original culture media was disposed, and then fresh sorting $CD34^+$ cells were added at 1×10^4 cells/well in StemSpan™ H5100 media (Stemcell) containing 50 ng/ml stem cell factor (SCF), 50 ng/ml flt3 ligand (FL), and 10 ng/ml IL3 (PeproTech, Rocky Hill, USA). Cells were incubated at 37°C in 5% CO_2 with 100% humidity for 7 days. Half of the media was replaced after 3 days. Cells were collected before the co-culture and after 7 days of co-culture. The number of mononuclear cells (MNCs) was stained using trypan blue and counted under the inverted microscope, and the purity and number of $CD34^+$ cells were analysed by flow cytometry. Cells were cultured for 12–14 days in a standard hematopoietic colony culture system with semi-solid media containing 1% methyl cellulose (Sigma), 30% FBS (Stemcell), 1% BSA, 2 mM L-glutamine (Amresco), 0.1 μM 2-Mercaptoethanol (Sigma), 50 ng/ml SCF, 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 20 ng/ml IL3, 20 ng/ml IL6 (PeproTech), and 3 U/ml erythropoietin (EPO) (Kirin, Tokyo, Japan). Hematopoietic stem and progenitor cell colony-forming units culture (CFU-C), burst-forming units erythrocyte (BFU-E), colony-forming units granulocyte-macrophage (CFU-GM), and colony-forming units-mixed (CFU-Mix) were counted under the inverted microscope. Amplification efficiencies were calculated with the following formulas:

$$\begin{aligned}&\text{CD34}^+\text{ cells amplification efficiency}\\&=\frac{\begin{array}{c}\text{Number of proliferating cells after one week}\\\times\text{ Purity of CD34}^+\text{ cells after one week}\end{array}}{\begin{array}{c}\text{Number of cells before proliferation}\\\times\text{Purity of CD34}^+\text{ cells before proliferation}\end{array}}\times 100\%\end{aligned}$$

$$\text{CFU amplification efficiency} = \frac{\begin{array}{c}\text{CFU production rate after one week} \\ \times \text{Number of proliferation cells per} \\ \text{well after one week/seeded cells} \\ \text{per well after one week}\end{array}}{\begin{array}{c}\text{CFU production rate before proliferation} \\ \times \text{Number of proliferation cells per} \\ \text{well before proliferation/seeded cells} \\ \text{per well before proliferation}\end{array}} \times 100\%$$

RNA extraction and real-time polymerase chain reaction to determine the mRNA expression of cytokines

Real-time polymerase chain reaction (PCR) was performed to detect the mRNA expression of cytokines associated with immuno-modulatory and hematopoietic function in MSCs, including SCF, FL, leukemia inhibition factor (LIF), hepatocyte growth factor (HGF), macrophage colony-stimulating factor (M-CSF), GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, thrombopoietin (TPO), vascular endothelial growth factor (VEGF), IL3, IL6, IL10, IL11, and transforming growth factor beta 1 (TGFβ1). RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, USA). cDNA was synthesized using the Rever Aid TM First Strand cDNA Synthesis Kit (Fermentas, Waltham, USA) according to the manufacturer's protocol. Real-time PCR was performed using the SYBR® Green Real-time PCR Master Mix-Plus kit (Toyobo, Osaka, Japan). *β-actin* served as a reference gene. UC samples were used as the positive control for calibration. The gene expression profiles of WJMSC, UVMSC, and AMMSC samples were compared with the calibration sample and analyzed using the method of $2^{-\Delta\Delta Ct}$. All of the primers were synthesized by Life Technologies. Primer sequences and the sizes of the fragments are shown in **Table 1**.

Statistical analyses

Statistical analyses were performed with the statistical software SPSS 13.0 (SPSS Inc., Chicago, USA). Data were presented as mean ± SD. Comparisons of continuous variables between more than two groups were performed by one-way analysis of variance. $P < 0.05$ was considered of statistically significant difference. All of the experiments were performed at least in triplicate.

Results

Morphology and growth characteristics of MSCs derived from UCs

After UC tissues were digested by collagenase, different sized and shaped cells were visible under the inverted microscope. Most of the cells began to adhere within 24 h. Cell proliferation was very rapid, and 80% confluence occurred after 7 days of culture. The cells had a tight arrangement, stretched bodies, predominantly swirling growth, and relatively homogeneous long-spindle morphology. In addition, cells had vortex-like growth and maintained their morphology and proliferative capability up to passage 14 (**Fig. 1A**, a–h). The proliferation of MSCs in each group was detected using the cell counting method. The results showed that fifth-generation MSCs derived from UCs underwent exponential

Table 1. The primers for immune and hematopoietic related genes used in this study

Gene	Forward	Reverse	Size (bp)	GenBank No.
β-actin	TGACGTGGACATCCGCAAAGA	CTGGAAGGTGGACAGCGAGG	205	NM_001101.3
SCF	CATTGTTGGATAAGCGAGAT	CACGCACTCCACAAGGTC	147	NM_000899.4
FLT3	AGTGTCAGCCCGACTCCTCA	GCCAGGTCAGTGCTCCACAA	245	NM_001459.2
LIF	CCCTGCTGTTGGTTCTGC	CAAGGTACACGACTATGCGGTA	309	NM_002309.3
HGF	CAAGGGAACAGTATCTATCAG	GTCACAGACTTCGTAGCG	191	NM_000601.4
M-CSF	CCTGCGTCCGAACTTTCTAT	TCACTGCTAGGGATGGCTTT	205	NM_000757.4
GM-CSF	TGCTGCTGAGATGAATGAAAC	GGCCCTTGAGCTTGGTGA	131	NM_000758.2
G-CSF	AGATGGAAGAACTGGGAATGGC	GGGCAAGGTGGCGTAGAA	159	NM_000759.3
EPO	TATGCCTGGAAGAGGATGGAG	GAGCCCGAAGCAGAGTGGT	190	NM_000799.2
TPO	AAGGTGCGTTTCCTGATGC	GCTGAGGCAGTGAAGTTTGTCTCCA	158	NM_000460.2
VEGF	AAGAAATCCCGGTATAAGTCCTG	TTCGTTTAACTCAAGCTGCCTC	201	NM_001025366.2
IL3	AGTTTACAGAACGCATCAGC	TTCCAGTCACCGTCCTTG	116	NM_000588.3
IL6	GTGAGGAACAAGCCAGAGC	TACATTTGCCGAAGAGCC	232	NM_000600.3
IL10	GGAGAACCTGAAGACCCTC	CTCACTCATGGCTTTGTAGAT	142	NM_000572.2
IL11	GCAGCGGACAGGGAAGGGTT	CCACAGGCTCAGCACGACCA	120	NM_000641.2
TGFβ1	CAGCAACAATTCCTGGCGATAC	GCTAAGGCGAAAGCCCTCAAT	139	NM_000660.4

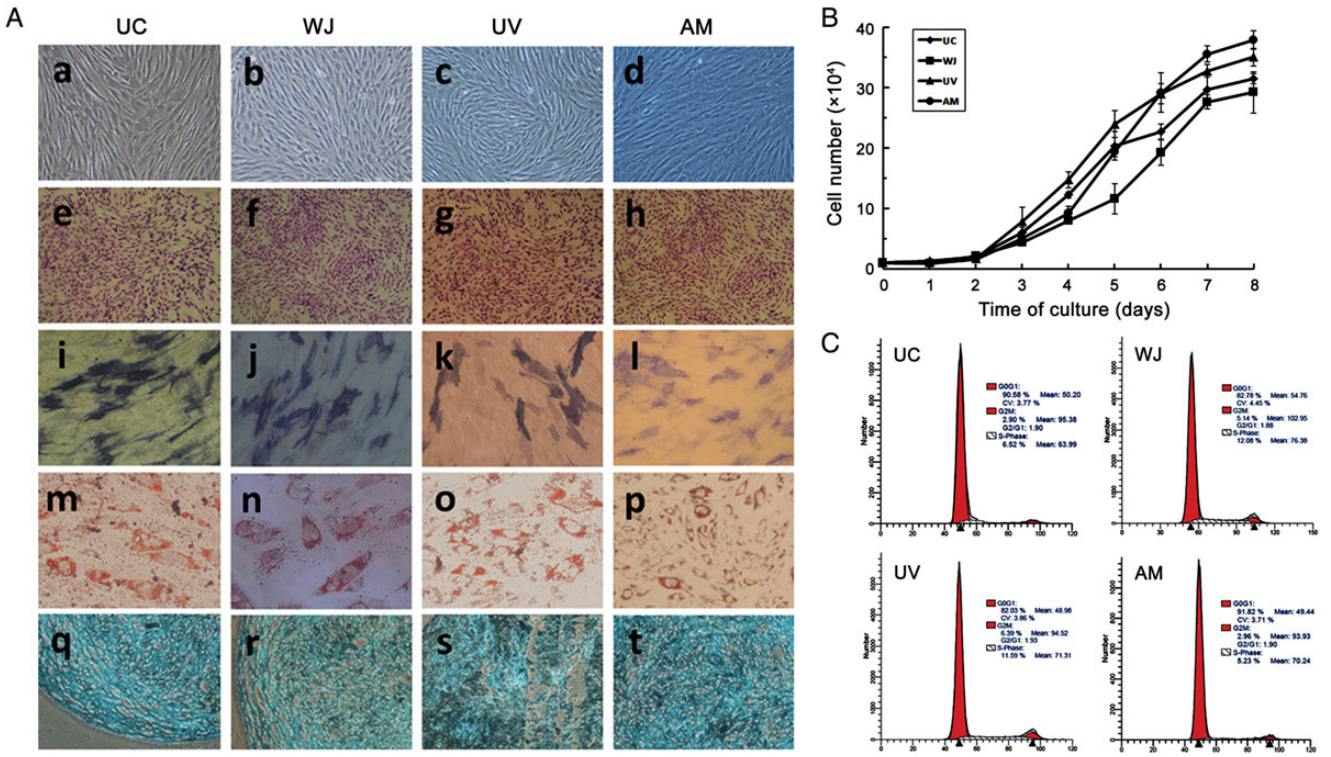

Figure 1. The growth and pluripotency characteristics of MSCs derived from UCs (A) MSCs derived from UC, WJ, UV, and AM were digested by collagenase and amplified to the fifth-generation *in vitro*. Cell morphology and cell differentiation are shown. a–d, typical MSCs (×100); e–h, Wrights-Gimsa staining of MSCs (×40); i–l, alkaline phosphatase staining of osteoblasts differentiated from MSCs (×100); m–p, Oil red O staining of adipocytes differentiated from MSCs (×200); q–t, Alcian blue staining of chondrocytes differentiated from MSCs (×200). (B) The representative cell growth curves of fifth-generation MSCs derived from UC, WJ, UV, and AM. (C) The representative cell-cycle plots of fifth-generation MSCs derived from UC, WJ, UV, and AM.

growth from 2 to 7 days with similar population doubling times, 24.76 ± 5.47 h (UCMSC), 24.36 ± 4.18 h (WJMSC), 25.12 ± 4.94 h (UVMSC), and 23.24 ± 3.65 h (AMMSC) (**Fig. 1B**). Cell-cycle analysis performed by flow cytometry demonstrated that >80% of fifth-generation MSCs were in the G_0/G_1 phase (UCMSC: 89.51% ± 3.23%, WJMSC: 86.79% ± 6.29%, UVMSC: 86.81% ± 4.22%, AMMSC: 90.58% ± 2.80%), whereas a small population of cells was engaged in proliferation (G_2/M phase: 2.71% ± 1.49%, 3.60% ± 1.90%, 3.81% ± 2.35%, 2.47% ± 1.80%; S phase: 7.78% ± 3.72%, 9.61% ± 4.44%, 9.39% ± 1.91%, 6.95% ± 1.49%, respectively). Cell-cycles across four groups showed no statistical differences. Thus, all of the cultured MSCs derived from UCs showed stem cell cell-cycle characteristics. Generally, most of cells were in a quiescent state, i.e., in the G_0/G_1 phase. **Figure 1C** shows the representative cell-cycle plots of MSCs derived from UC, WJ, UV, and AM.

Multi-lineage differentiation potential

To examine the multi-lineage differentiation potential of fifth-generation MSCs, we added differentiation inducers for osteoblasts, adipocytes, or chondrocytes directly into the media. After one week of culture in the osteoblast induction system, MSCs began to be polygon shaped. After 2 weeks, ALP staining was positive (**Fig. 1A**, i–l). After 3 days of culture in the adipocyte induction system, MSCs showed obvious morphology changes, including larger and round cell bodies and intracellular tiny fat droplets, which gradually increased as the induction time increased and turned bright red when stained with Oil red O after 10–14 days of induction (**Fig. 1A**, m–p). After 4 weeks of culture in the chondrocyte induction system, cells were fixed with 10% neutral formalin, embedded with paraffin, and sliced. Alcian blue staining revealed collagen fibers and GAGs in cartilage matrix (**Fig. 1A**, q–t). These results demonstrated that MSCs derived from different sections of UCs had multi-lineage differentiation potential.

Immunophenotypic characterization

Flow cytometry was used to detect surface markers of UCMSCs, WJMSCs, UVMSCs, and AMMSCs. As shown in **Table 2**, there were high levels of CD44, CD73 (SH-3,4), CD90 (Thy-1), CD105 (SH-2/endoglin), CD166 (ALCAM), and HLA-ABC (MHC-I molecule), low levels of CD106 and TRA-1-60, and no expression of CD14, CD31, CD34, CD45, CD80 (B7-1), CD86 (B7-2), CD40, CD40L (CD154), or HLA-DR (MHC-II molecule) on MSCs derived from different sections of the UC. We compared the

Table 2. Surface markers of MSCs derived from UC, AM, WJ, and UV

Surface marker	UC (%)	WJ (%)	UV (%)	AM (%)	*P*
CD14	0.9 ± 1.1	0.7 ± 1.2	0.1 ± 0.2	0.4 ± 0.5	0.188
CD31	1.2 ± 1.3	0.6 ± 0.8	0.4 ± 0.2	1.0 ± 0.8	0.165
CD34	0.4 ± 1.1	1.2 ± 1.0	0.7 ± 0.7	0.8 ± 0.8	0.321
CD40	1.4 ± 1.6	0.8 ± 0.9	0.3 ± 0.3	0.6 ± 0.7	0.253
CD40L	1.4 ± 1.8	1.1 ± 1.8	0.2 ± 0.1	1.2 ± 1.4	0.535
CD44	52.5 ± 23.1	42.4 ± 29.3	70.2 ± 23.5	59.5 ± 26.7	0.381
CD45	1.1 ± 1.9	1.0 ± 1.5	0.2 ± 0.3	0.4 ± 0.5	0.333
CD73	98.9 ± 1.6	98.4 ± 1.6	97.4 ± 2.9	95.5 ± 6.9	0.301
CD80	1.7 ± 1.7	0.8 ± 1.0	0.2 ± 0.2	1.1 ± 1.4	0.107
CD86	1.9 ± 2.1	1.2 ± 1.7	1.7 ± 0.9	1.9 ± 3.2	0.658
CD90	99.1 ± 1.1	99.6 ± 0.3	97.9 ± 2.1	98.3 ± 2.6	0.172
CD105	99.0 ± 1.6	98.7 ± 1.7	96.5 ± 3.5	97.2 ± 3.4	0.240
CD106	33.0 ± 26.4**	17.9 ± 21.4	4.0 ± 4.4	19.6 ± 18.2	0.019
CD166	89.1 ± 17.2**	61.4 ± 33.6	79.9 ± 16.3	84.0 ± 13.8*	0.040
HLA-ABC	94.5 ± 4.1	77.0 ± 19.6	77.9 ± 24.0	89.3 ± 12.1	0.134
HLA-DR	1.2 ± 1.7	1.1 ± 1.5	0.2 ± 0.1	1.2 ± 2.1	0.442
TRA-1-60	21.3 ± 27.8	8.0 ± 17.1	1.7 ± 2.2	19.2 ± 24.3	0.127

*$P < 0.05$, **$P < 0.01$.

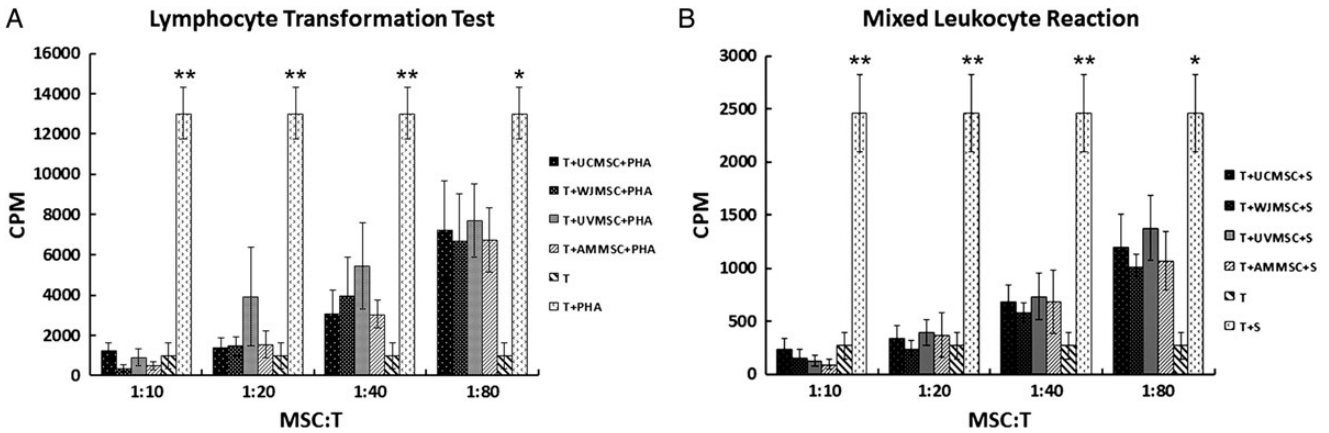

Figure 2. Immuno-modulatory activity of MSCs derived from UCs (A) UCMSCs, WJMSCs, UVMSCs, and AMMSCs were incubated with ^{3}H-TdR labeled CD3^{+} T lymphocytes and mitogen phytohaemagglutinin (PHA). (B) UCMSCs, WJMSCs, UVMSCs, and AMMSCs were incubated with ^{3}H-TdR labeled CD3^{+} T lymphocytes and allogeneic lymphocytes. T-cell counts are shown as radioactivity (count per minute). *$P < 0.05$, **$P < 0.01$.

phenotype profiles of each subtype of MSCs and found that there were no differences in the levels of CD14, CD31, CD34, CD40, CD40L, CD44, CD45, CD73, CD80, CD86, CD90, CD105, HLA-ABC, HLA-DR, or TRA-1-60. However, the surface expression of CD106 on UCMSCs was increased as compared with UVMSCs ($P < 0.01$). In addition, the expression of CD166 on UCMSCs ($P < 0.05$) and AMMSCs was increased as compared with WJMSCs ($P < 0.05$).

Immuno-modulatory activity

The immuno-modulatory activity of MSCs derived from UCs was investigated using a lymphocyte transformation experiment (**Fig. 2A**) and mixed lymphocyte culture test (**Fig. 2B**). The results showed that the immuno-modulatory activity was similar among UCMSCs, WJMSCs, UVMSCs, and AMMSCs. All of the groups inhibited T-lymphocyte proliferation stimulated by mitogen and allogeneic antigens in a dose-dependent manner. In both assays, the T-lymphocyte count gradually decreased with increasing concentrations of MSCs.

Functional analysis of CD34^{+} cells co-cultured with MSCs

In a serum-free culture system, CD34^{+} cells separated from fresh UC blood were cultured separately for 7 days or

co-cultured with UCMSCs, WJMSCs, UVMSCs, and AMMSCs, respectively, for 7 days. The proliferation of hematopoietic cells was examined under the inverted microscope. The results showed that the number of cells in the suspension culture group (no MSCs) was significantly lower than that in the co-culture group (**Fig. 3A**). In a standard hemopoietic colony culture system, $CD34^+$ cells from all of the groups were cultured for 12–14 days and then observed for hematopoietic differentiation under the microscope. The results showed that cells from the control group and four experimental groups were able to differentiate into colonies of erythroid cells, granulocytes, macrophages, and multipotential progenitors (**Fig. 3B**). We detected the number of MNCs, $CD34^+$ cells, and hematopoietic colonies in different groups after 7 days. As shown in **Table 3**, we found that the numbers of MNCs, $CD34^+$ cells, CFU-C, BFU-E, CFU-GM, and CFU-Mix in all of the co-culture groups were significantly higher than those in the suspension culture group ($P < 0.01$), except in the AMMSC group ($P = 0.063$). In addition, the numbers of MNCs, $CD34^+$ cells, and CFU-C on the feeding layer of UCMSCs were significantly higher than those on the feeding layer of WJMSCs, UVMSCs, or AMMSCs ($P < 0.01$), and the efficacy of CFU-C was similar. There were no significant differences in the efficacy of CFU-Mix among all of the groups; however, the amplification efficiencies of CFU-GM and BFU-E of WJMSCs were significantly higher than that of AMMSCs ($P < 0.05$) (**Fig. 3C,D**).

mRNA expression of cytokines

Real-time PCR was performed to detect the mRNA expression of cytokines associated with immuno-modulatory and hematopoietic functions of MSCs. The relative mRNA expression of cytokines on MSCs from UC, WJ, UV, and AM was compared. The results showed that there was positive expression of immune-related cytokines, including IL6, IL10, IL11, and TGFβ1 (**Fig. 4A**), and hematopoietic-related cytokines, including SCF, FLT3, LIF, IL3, IL6, IL11, HGF, EPO, TPO, VEGF, M-CSF, GM-CSF, and G-CSF (**Fig. 4B**), in UCMSCs, WJMSCs, UVMSCs, and AMMSCs. For the expression of IL3, IL10, LIF, TGFβ1, HGF, TPO, EPO, VEGF, M-CSF, and GM-CSF, no statistical differences among the subtypes of MSCs were found. However, the mRNA expression of IL6 in UCMSCs was significantly higher than that in WJMSCs, UVMSCs, and AMMSCs ($P < 0.0001$). There was also significance between the WJMSC and AMMSC groups ($P < 0.01$). The expression of IL11 in UCMSCs was statistically higher than that in AMMSCs ($P < 0.01$). SCF expression was significantly elevated in UCMSCs as compared with AMMSCs ($P < 0.05$). Finally, the expression of FLT3 in UCMSCs and UVMSCs was higher than that in WJMSCs ($P < 0.01$).

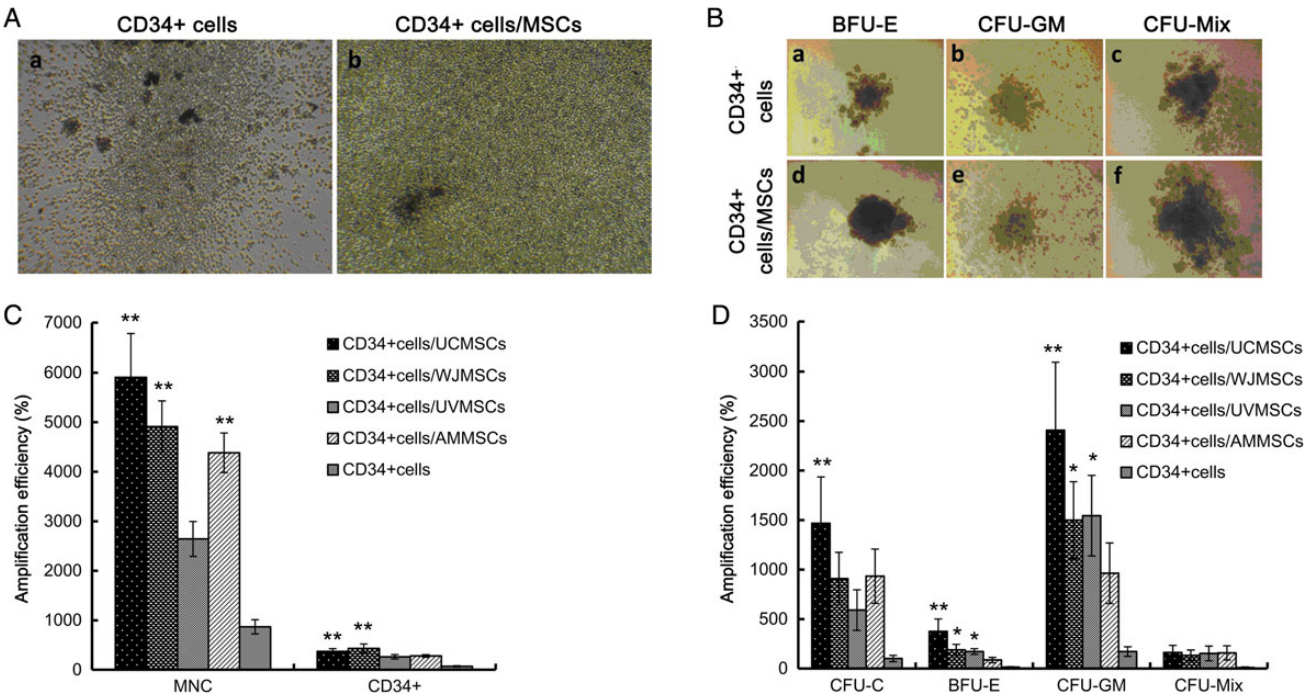

Figure 3. MSCs derived from UCs were co-cultured with $CD34^+$ cells from cord blood (A) MSCs derived from UCs were co-cultured with $CD34^+$ cells sorted from cord blood for 7 days. The cell number in individually cultured groups (a, ×40) was significantly lower than that in MSC co-cultured groups (b, ×40) on day 7. (B) The colony formation of cells from individually cultured groups (a, b, and c, ×50) and co-cultured groups (d, e, and f, ×50) after 7 days of culture. (C) The propagation efficiencies of the MSC feeder layer for MNCs and $CD34^+$ cells. (D) The propagation efficiencies of the MSC feeder layer for CFU-C. *$P < 0.05$, **$P < 0.01$.

Table 3. The propagation efficiencies of MNC/CD34$^+$ on day 7 and of colony-forming units on day 12–14 were superior in co-cultured groups

	MNC (%)	CD34$^+$ (%)	CFU-C (%)	BFU-E (%)	CFU-GM (%)	CFU-Mix (%)
CD34$^+$cells	869.2 ± 144.0	70.3 ± 11.8	101.7 ± 32.0	14.8 ± 4.8	172.2 ± 48.2	10.5 ± 6.2
CD34$^+$cells/UCMSCs	5908.3 ± 874.3**	372.6 ± 54.9**	1467.8 ± 1468.7**	376.3 ± 124.8**	2408.0 ± 687.4**	162.8 ± 72.4**
CD34$^+$cells/WJMSCs	4912.5 ± 519.1**	432.2 ± 89.3**	908.8 ± 265.7**	188.7 ± 56.5**	1499.2 ± 388.8**	133.5 ± 54.5**
CD34$^+$cells/UVMSCs	2644.2 ± 352.8**	263.5 ± 45.2**	591.2 ± 204.4**	172.7 ± 28.6**	1545.0 ± 406.7**	153.5 ± 74.3**
CD34$^+$cells/AMMSCs	4384.2 ± 399.4**	280.2 ± 25.8**	933.8 ± 274.2**	86.5 ± 26.8	963.8 ± 305.4**	158.7 ± 71.9**

**$P < 0.01$.

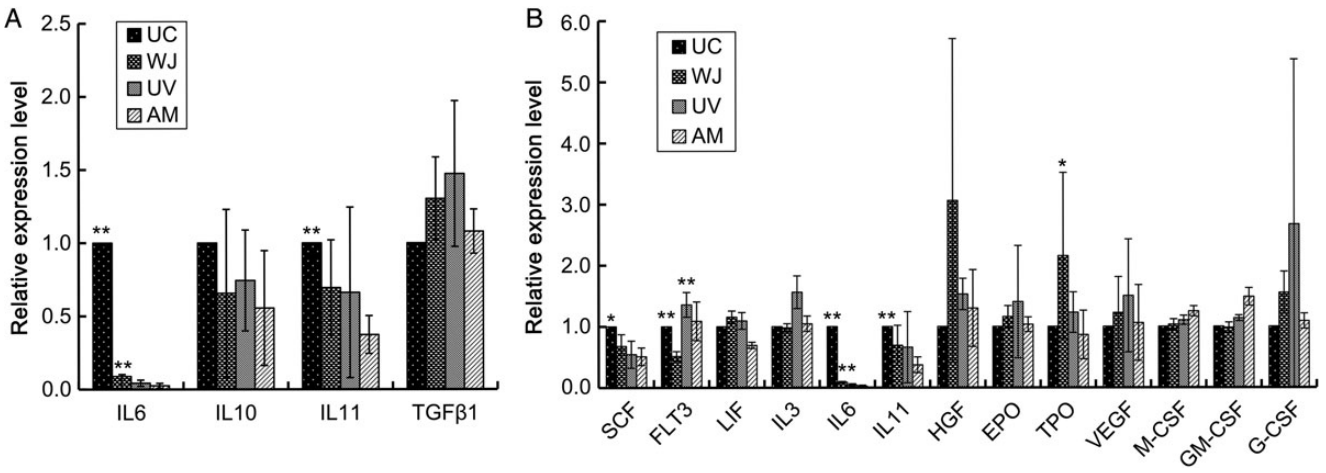

Figure 4. The mRNA of immune and hematopoietic factors expressed in MSCs derived from UCs (A) Real-time PCR was performed to detect the relative mRNA expression of immune-related cytokines in UCMSCs, WJMSCs, UVMSCs, and AMMSCs. (B) Real-time PCR was performed to detect the relative mRNA expression of hematopoietic cytokines in UCMSCs, WJMSCs, UVMSCs, and AMMSCs. *$P < 0.05$, **$P < 0.01$.

Discussion

We successfully isolated MSCs from human UC, WJ, UV, and AM and compared their biological immunomodulation and hematopoietic support features. Our UCMSC, WJMSC, UVMSC, and AMMSC results are similar to previous reports [30]. MSCs separated from different sources had uniform shape as typical long spindles and were arranged in parallel or spiral-shaped, and had strong proliferative capabilities. The growth curve for 8 days indicated that cells were in the exponential growth phase, with rapid proliferation from 2 to 7 days. The cell-cycle results showed that >80% of the cells in each group were in the G_0/G_1 phase, indicating that MSCs derived from UCs had typical stem cell cell-cycle characteristics.

Currently, the identification of MSCs relies on the analysis of multi-lineage differentiation potential and cell phenotype. Our research showed that MSCs derived from different sections of the UC had similarly high differentiation abilities. MSCs can be differentiated into osteoblasts, adipocytes, and chondrocytes under different induction systems. Thus, UCMSCs are excellent candidates for stem cell transplantation.

The MSC phenotypes in this study were similar and in line with previous studies, except that higher expressions of CD106 and CD166 were found in UCMSCs. The adhesion molecules CD106 and CD166 are associated with hematopoietic function and cell migration [31–34]. The difference between UCMSCs in our study and other subtypes of MSCs suggests that there is heterogeneity of MSCs in UC. Thus, MSCs derived from different sections of UC may exert different immune-modulatory and hematopoietic functions.

HSCT is an effective treatment for malignant hematological diseases. However, the biggest problem in allogeneic HSCT (Allo-HSCT) is graft-versus-host disease (GVHD), which has become a common complication and one of the leading causes of non-recurrent death after transplantation. In Allo-HSCT, the incidence of acute GVHD in HLA haploidentical transplantation is about 30%–50%. However, in HLA-mismatched transplantation the incidence is up to 60%–80% [35]. During GVHD, T lymphocytes from the donor recognize cell surface antigens of the recipient and induce an immune response, resulting in multiple organ injury [36]. In this process, T cells are a key factor for the development and progression of GVHD. The mechanism of the inhibitory effect of MSCs on T-cell function is not clear.

Some researchers have shown that MSCs inhibit T-cell proliferation through contacting T cells or secreting regulatory factors such as IL6, IL10, and TGFβ [37]. MSCs have immunosuppressive effects only in the presence of IFN-γ or other proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), IL1α, or IL1β [38]. These cytokines interact with MSCs and promote the expression of chemokines and indoleamine-2,3-dioxygenase, which induces apoptosis of T cells by degrading tryptophan and inhibits the proliferation of T cells [39]. Our study confirmed that MSCs inhibit T-lymphocyte transformation and respond to antigens *in vitro*, indicating strong immune-modulatory activity.

The hematopoietic microenvironment, also known as the hematopoietic stem cell niche, supports and regulates the proliferation, maturation, and differentiation of hematopoietic cells within the environment [40]. It mainly consists of stromal cells, extracellular matrix, and hematopoietic growth factors. As an important component of the microenvironment, MSCs are precursors of fibroblasts, osteoblasts, adipocytes, and other bone marrow stromal cells. These cells support and regulate the proliferation and differentiation of hematopoietic stem cells through secreting a variety of hematopoietic growth factors and chemokines, such as SCF, FLT3 ligands, IL3, M-CSF, GM-CSF, VEGF, PDGF-1, and others [41]. Although there are more $CD34^+$ cells in UC blood of full-term neonates than in normal bone marrow and peripheral blood [42], the low content of MSCs and limited volume of cord blood limit the application of UC blood in transplantation. This problem may be solved by adding MSCs derived from UC to propagate $CD34^+$ hematopoietic stem cells.

In summary, we demonstrated that MSCs derived from UCs, including whole UC, amnion, WJ, and vasculature, had a high proliferative rate, multi-lineage differentiation potential, and low immunogenicity. Thus, MSCs derived from UCs could be used as stem cells in various fields of cell therapy in the future.

Funding

This study was supported by the grants from Eleventh Five-Year Drug Discovery of Major Projects (No. 2009 ZX09503023-003), National High Technology Research and Development Program ('863' Program) of China (No. 2013AA020103), and National Natural Science Foundation of China (No. 31070996).

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