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Mesenchymal stem/progenitor cells in human umbilical cord blood as support for e*x vivo* expansion of CD34+ hematopoietic stem cells and for chondrogenic differentiation

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A B S T R A C T

Background and Objectives. Human mesenchymal stem/progenitor cells (MSPC) ar pluripotent, being the precursors for marrow stroma, bone, cartilage, muscle and connective tissues. Although the presence of hematopoietic stem/progenitor cells (HSPC) in umbilical cord blood (UCB) is well known, that of MSPC has been not fully evaluated.

Design and Methods. In this study, we examined the immunophenotype, the supporting function in relation to *ex vivo* expansion of hematopoietic stem progenitor cells and the chondrogenic differentiation of cultured cells with characteristics of MSPC from UCB. When UCB nucleated cells were isolated and 10^7 cells cultured in IMDM with 20% fetal bovine serum, the mean number of adherent fibroblastlike colonies was $3.5\pm0.7/10^6$ monuclear cells.

Results. UCB-derived MSPC could be expanded for at least 15 passages. In their undifferentiated state, UCB-derived MSPC were $CD13^+$, $CD29^+$, $CD90^+$, $CD105^+$, $CD166^+$, $SH2^+$, $SH3^+$, $SH4^+$, $CD45^-$, $CD34^-$, and $CD14^-$; they produced stem cell factor, interleukin 6 and tumor necrosis factor α. UCB-derived MSPC cultured in chondrogenic media differentiated into chondrogenic cells. UCB-derived MSPC supported the proliferation and differentiation of $CD34^+$ cells from UCB *in vitro*.

Interpretation and Conclusions. UCB-derived MSPC have the potential to support *ex vivo* expansion of HSPC and chondrogenic differentiation. UCB should not be regarded as medical waste. It can serve as an alternative source of mesenchymal stem cells and may provide a unique source of fetal cells for cellular and gene therapy.

Key words: UCB-derived mesenchymal stem/progenitor cells, support to expansion of hematopoietic stem/progenitor cells, chondrogenic differentiation.

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It is now clear that adult human bone marrow contains a rare population of mesenchymal stem/progenitor cells (MSPC). These cell can be extensively expanded *ex vivo* and, when cultured under specific permissive conditions, retain their ability to differentiate into multiple lineages including bone, cartilage, tendon, muscle, nerve, and stromal cells.[1-6] MSPC are of great therapeutic potential because of their ability to self-renew and differentiate into multiple tissues.[7] Adult bone marrow-derived MSPC engraft in numerous organs and differentiate along tissue-specific lineages when transplanted into fetal sheep.[8] They enhance engraftment of donor hematopoietic cells after co-transplantation in animal models,[9-11] and they migrate into areas of muscle degeneration to undergo myogenic differentiation in immunodeficient mice.[12] In humans, MSPC have been used to regenerate the marrow microenvironment after myeloablative therapy.[13] Recent work has shown that mesenchymal progenitors are also present at low frequencies in adult peripheral blood[14] and in umbilical cord blood (UCB).[15-18] Jäger *et al.* showed the culture condition difference of bone marrow and cord blood derived mesenchymal stem cells. The term *unrestringated somatic stem cell* (USSC) is recommended for cord blood-derived mesenchymal stem cells.[19] The aim of this study was to isolate and characterize MSPC in UCB, to study their potential for chondrogenic differentiation and to evaluate their capacity to support *ex vivo* expansion of $CD34^+$ hematopoietic stem/ progenitor cells (HSPC). We show that MSPC can be isolated from human UCB, readily expanded, and induced to differentiate into chrondogenic cells *in vitro*.

Design and Methods

Umbilical cord blood (UCB)

UCB, collected for research purposes, was kindly provided by the Obstetrics Department of the Zhejiang Gynecological and Obstetric Hospital, Hangzhou. Twenty-four parturient women with a median age of 27 years (range 25 to 30 years) and a median weight of 60 kg (range 52 to 68 kg), gave written consent to the use of UCB for research purposes in accordance with procedures approved by the Human Experimentation Committee at Zhejiang Public Health Bureau, Hangzhou, P. R. China. The collected UCB was heparinized.

Isolation and culture of UCB-derived MSPC

A median of 72 mL of UCB (range 60 to 83 mL) was centrifuged at 450×g for 10 min within 12 hours after collection. The pellet was diluted with Iscove's modified Dulbecco's medium (IMDM; HyClone, Logan, UT) and then layered onto Ficoll-Hypaque (1.077±0.001 g/mL; Sigma, St. Louis, MO, USA), and centrifuged at 300×g for 20 min. Low-density mononuclear cells (MNC) from the gradient interface were collected and washed three times with IMDM, and were then diluted with the complete medium (20% fetal bovine serum (FBS; Sigma) in IMDM with 50 μM 2-mercaptoethanol and 2 mM L-glutamine (Gibco BRL, Life Technologies, Paisley, UK). The resuspended cells were cultured in 25-cm^2 flasks at a density of 4.2×10^5 cells/cm^2, and incubated in 100% humidified 5% CO_2 in air at 37°C. After 4 days, when cells had adhered to the flask, the supernatant and non-adherent cells were removed, and the complete medium was replaced. At 90% confluence, cells were harvested with 0.25% trypsin and 1 mM EDTA (Stem Cells Technology, Vancouver, BC, Canada) for 5 minutes at 37°C. Harvested cells were washed twice with PBS/1%FBS and then CD34 negative cells were isolated using a MACS laboratory separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The $CD34^-$ cells were diluted with complete media and cultured in 25-cm^2 flasks at a density of 5×10^3 cells/cm^2, in 100% humidified 5% CO_2 in air at 37°C as the F_1 passage. At near confluence, cells were harvested and cultured by a passage as described above. In order to measure the growth kinetics, cells from passages F_1, F_5, F_{10} and F_{15} were plated into a 6-well plate at 10^4 cells/cm^2. Growth curves were assessed once every four days, by counting the number of adherent cells, for 24 days.

Flow cytometry analysis of cultured UCB- derived MSPC

UCB-derived adherent cells (at the end of passages F_1, F_5, F_{10} and F_{15}) were trypsinized and stained with anti-CD34-FITC, CD45-FITC, CD14-FITC, CD105-FITC, CD90-PE, CD166-PE, CD13-PE, CD29-PE (Becton Dickinson, UK), SH2, SH3, and SH4 monoclonal antibodies (Osiris Therapeutics, Baltimore, MD, USA) and were analyzed by FACScalibur flow cytometry (Becton Dickinson).

Cytokine production in the conditioned medium of cultured UCB-derived MSPC

When adherent cells in passages F_1, F_5 and F_{10} were confluent, the conditioned media were collected and used to measure any cytokines produced by the UCB-derived adherent cells. The presence of stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) in the media was quantitatively determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The limit of detection of each ELISA kit was 4.0 pg/mL for SCF, 7.8 pg/mL for IL-3, 20 pg/mL for GM-CSF, 0.09 pg/mL for IL-6, and 0.18 pg/mL for TNF-α.

Co-culture of UCB-derived MSPC and $CD34^+$ hematopoietic cells

The UCB-derived adherent cell layer from the F_5 passage was selected for the experiments investigating the support offered to *ex vivo* expansion of HSPC. This passage was chosen in consideration of the homogeneous state of cells, their growth potential and cytokine production.

MNC from UCB were isolated as above. $CD34^+$ cells, selected from MNC preparations with anti-CD34 antibodies (Miltenyi Biotec) conjugated with microbeads and eluted through MiniMACS columns according to the manufacturer's instructions, were resuspended in IMDM (20% FBS) with or without 100 ng/mL each of recombinant human stem cell factor (rhSCF), recombinant human granulocyte colony-stimulating factor (rhG-CSF) and recombinant human megakaryocyte growth and development factor (rhMGDF) (Amgen Inc., Thousand Oaks, CA, USA), and then seeded at the density of 1.6×10^4 cells/cm^2 in 25-cm^2 flasks with the UCB-derived adherent MSPC. As a control system, $CD34^+$ cells were cultured in IMDM (20% FBS) with the same concentration of three exogenous cytokines but without the UCB-derived adherent MSPC. The UCB-derived $CD34^+$ cells were cultured in 100% humidified 5% CO_2 in air at 37°C using a two-step cul-

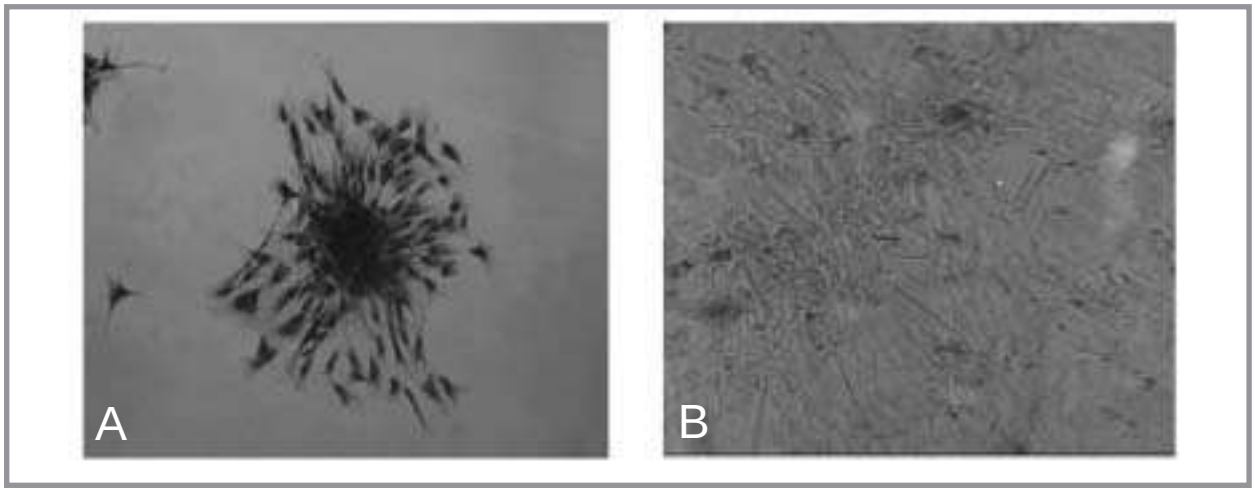

Figure 1. UCB-derived adherent cells (×400) after one week (A) and four weeks (B) under culture conditions.

ture system as previously described.[20] At the beginning (the first day) and at the end (the 14th day) of culture, non-adherent cells in the culture medium were assayed for colony-forming cells in complete methylcellulose without erythropoietin (Gencyte, Amherst, NY, USA) as the progenitor cell assay.[21]

Chondrogenic differentiation and analysis of collagen gene expression

The differentiating ability of MSPC was assessed at passages F_1, F_5 and F_{10} of UCB-derived MSPC (n=5). Chondrogenic medium containing DMEM supplemented with 10% FBS, 10 ng/mL transforming growth factor-β1 (TGF-β1) (R&D Systems), 50 μg/mL ascorbic acid, 100 U/mL penicillin and 100 μg/mL streptomycin was used to induce chondrogenic differentiation as previously described.[22]

After 3 weeks of induction, the induced cells were fixed with 10% formalin solution for 1 h, washed once with distilled water, and then stained in 1% toluidine blue solution for 2-3 h as described elsewhere.[23]

Total RNA was prepared by the guanidinium thiocyanate/phenol method.[24] Reverse transcription was performed by denaturing RNA and dT_{18} primers in the presence of 0.1 mol/L methylmercuric hydroxide, followed by quenching with 20 mmol/L β-mercaptoethanol and extension in a total of 20 μL with Superscript II reverse transcriptase as recommended (GIBCO, Carlsbad, CA, USA). Polymerase chain reactions (PCR) were performed using 2.0 μL RNase-treated cDNA with Taq polymerase (Perkin Elmer, Foster City, CA, USA) in a total of 50 μL.

The PCR reactions were performed with an initial denaturation of 94°C for 2.0 minutes, and then at 94°C for 0.5 minutes, 58°C for 0.75 minutes, and 68°C for 0.75 minutes for 10 cycles, followed by 94°C for 0.5 minutes, 58°C for 0.75 minutes, and 73°C for 0.75 minutes for another 25 cycles. The primer set used for PCR of collagen cDNA was as follows: 5' TTC AGC TAT GGA GAT GAC AAT C 3' and 5' AGA GTC CTA GAG TGA CTG AG 3'. Fifteen microliters of PCR reaction were fractionated by agarose gel electrophoresis.

Statistics

Results are expressed as mean±SEM, and statistical comparisons were performed using the Student's t test.

Results

Adhesion, fibroblastic and growth characteristics of UCB-derived MSPC

Cells adhered to flasks and formed individual colonies consisting of several dozen to a few hundred spindle-shaped fibroblastic cells by day 7-9 after UCB MNC had been inoculated at a density of 4.2×10^5 cells/cm^2 in IMDM containing only 20% FBS with no additional growth factors (Figure 1A). The frequency of adherent colonies was $3.5\pm0.7/10^6$ MNC. After 15-19 days of culture, numerous fibroblast-like cells could be observed. By the end of four weeks, a homogeneous layer of fibroblastoid cells occupied the whole surface of the flask and had become confluent (Figure 1B).

The growth kinetics of UCB-derived adherent cells (n=5) was measured at passages F_1, F_5, F_{10} and F_{15} (Figure 2). Cells were allowed to divide for 24 days, duplicate cultures were harvested once every four days, and cell counts were performed. Growth curves depicted an initial lag phase of 4 days, followed by a log phase in which cells divided at an exponential rate for 8 to 12 days. The log phase was followed by a

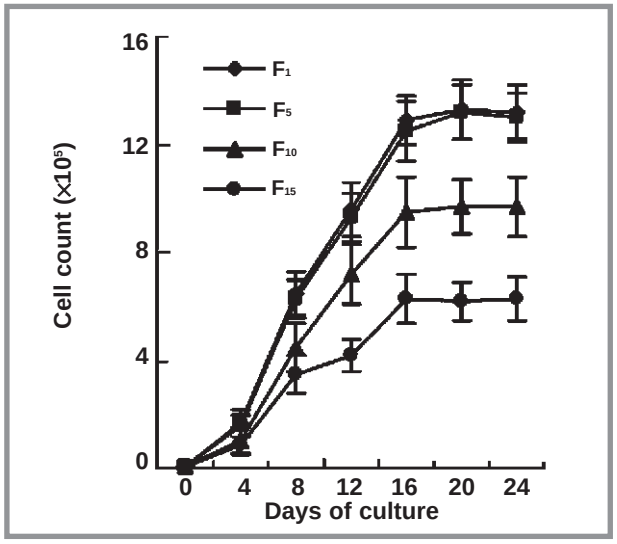

Figure 2. Growth curves of MSPC from UCB. UCB-derived MSPC were plated at a density of 10^4 cells/cm²; duplicate cultures were harvested every four days for 24 days, and the number of adherent cells was determined. Results are expressed as mean ± SEM (n=5).

Table 1. Cytokine production in the conditioned media of cultured UCB-derived mesenchymal stem/progenitor cells (pg/mL).

	SCF	*IL-3*	*IL-6*	*GM-CSF*	*TNF-α*
F_1	129.6 ±11.2*	–	75.2±9.8*	–	57.4±7.7*
F_5	34.2 ±9.7	–	78.2±11.2	–	61.2±9.7
F_{10}	32.1±12.4	–	77.4±13.1	–	62.2±7.8

*F_1, F_5 and F_{10} are F_1 passage, F_5 passage and F_{10} passage, respectively. SCF: stem cell factor; IL-3: interleukin 3; IL-6: interleukin 6; GM-CSF: granulocyte-macrophage colony-stimulating facto; TNF-α: tumor necrosis factor α. Comparison with F_5 passage: *p <0.05. –: undetected.*[23]

plateau phase. UCB-derived adherent cells could be readily expanded *in vitro* by successive cycles of trypsinization, seeding, and culture every 17 to 20 days for 15 passages. Cells that had undergone up to 15 passages displayed no visible change in their morphology, their forward and side scatter properties, or their growth patterns, but fold expansion decreased from F_{10} to F_{15}.

In order to assess the capacity of UCB-derived adherent cells to secrete hematopoietic cytokines, conditioned medium was sampled from the F_1 confluent passage. SCF , IL-6 , and TNF-α levels were detected in conditioned media while IL-3 and GM-CSF were not detected (Table 1).

Immunophenotype of UCB-derived MSPC

The immunophenotype of UCB-derived adherent cells from passages F_1, F_5, F_{10} and F_{15} was determined by flow cytometry. As shown in Table 2, UCB-derived monolayer adherent cells stained positively for CD13, CD29, CD90, CD105, CD166, SH2, SH3 and SH4. $CD34^+$ cells were not detected in UCB adherent cells from any passage because this cell population had been depleted at the beginning of passage F_1. Low levels of CD14 and CD45 were detected in F_1 probably due to contaminating cells, but no significant staining was seen at F_5 and F_{10}. This profile is consistent with a non-hematopoietic cell and confirmed that hematopoietic cells had been depleted from the cultures. The immunophenotypic profile of UCB adherent cells did not change significantly after 10 passages in culture (p >0.05).

Ability of UCB-derived MSPC to support ex vivo expansion of HSPC

To determine whether MSPC from UCB were capable of supporting the *ex vivo* proliferation and differentiation of HSPC, confluent monolayer MSPC of passage F_5 (n=5) were used to co-culture human $CD34^+$ cord blood cells. $CD34^+$ cells were overlaid and maintained in culture for up to 14 days. As controls, UCB $CD34^+$ cells were seeded into the 25-cm^2 flask with the same medium but without UCB-derived MSPC as a stromal cell feeder layer. Figure 3 shows the growth kinetics of the non-adherent cells in the three culture systems. The expansion of total nucleated cells (TNC) in the co-culture system with exogenous cytokines was higher than that in the co-culture system without exogenous cytokines and in the control system (p<0.01). The

Table 2. Percentage of UCB-derived monolayer adherent cells expressing specific phenotypes (%).

	CD13	*CD14*	*CD29*	*CD45*	*CD90*	*CD105*	*CD166*	*SH2*	*SH3*	*SH4*
F_5	1.8±8.7°	3.6±2.3*	48.8±7.8°	8.4±2.1°	62.4±10.5°	45.2±15.2°	68.8±9.3°	45.2±4.9°	53.0±9.5°	39.8±10.4°
F_5	76.4±5.2*	1.8±1.7	81.2±14.2*	3.2±1.0*	87.2±12.7	58.0±11.3*	91.6±18.1	61.8±7.2*	77.6±5.4	63.8±11.2*
F_{10}	83.2±12.4	0.5±0.1	87.4±9.5	1.2±0.8	82.8±8.8	64.2±6.9	93.2±12.7	77.4±12.5	82.8±7.6	74.4±8.3
F_{15}	82.7±9.2	0.6±0.2	88.6±11.3	1.0±0.9	83.1±10.7	66.0±8.8	93.1±11.2	78.4±11.3	83.1±9.2	75.8±9.9

*F_1, F_5, F_{10} and F_{15} are the F_1 passage, F_5 passage, F_{10} passage and F_{15} passage, respectively. Comparison with F_{10} passage: *p<0.05, °p<0.01.*

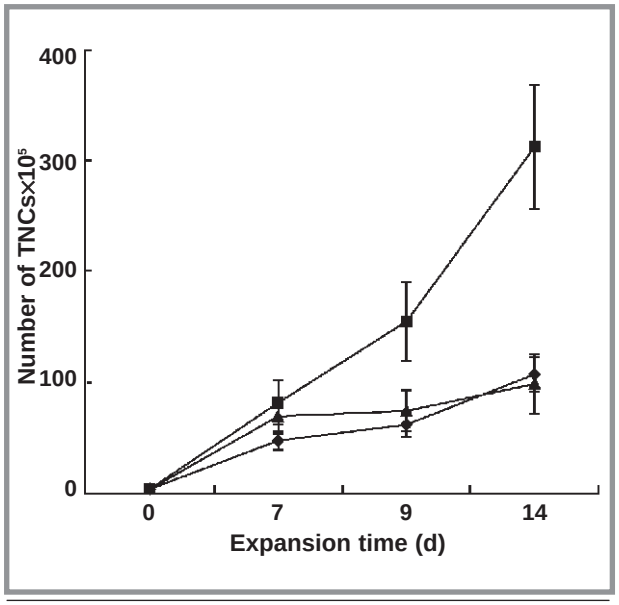

Figure 3. Expansion trends of UCB HSPC. —■—: co-culture system with exogenous cytokines; —◆— : co-culture system without exogenous cytokines; —▲—: control system.

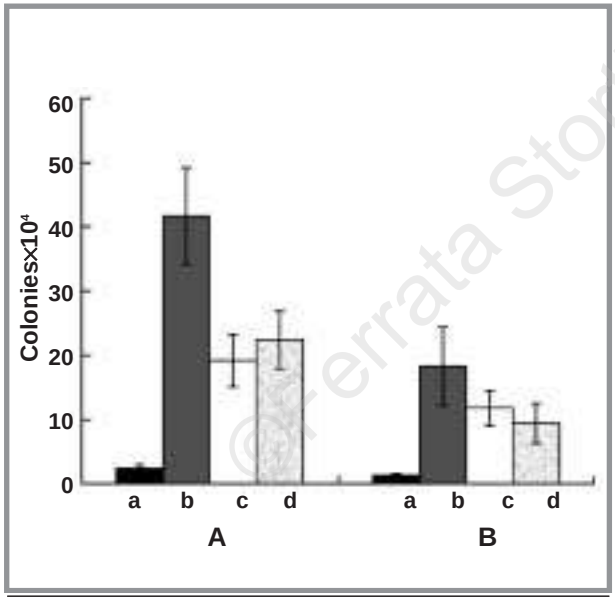

Figure 4. Expansion of granulocyte-macrophage colony-forming cells (GM-CFC) and high proliferative potential colony-forming cells (HPP-CFC). A: GM-CFC, B: HHP-CFC; a: the of colonies from the starting CD34+ cell fraction; b: the number of colonies from expanded cells in a co-culture system with exogenous cytokines; c: the number of colonies from expanded cells in a co-culture system without exogenous cytokines; and d: the number of colonies from expanded cells in the control system.

number of expanded TNC in the co-culture system with exogenous cytokines was 3.16 (±0.3)-fold higher than that in the control system. The level of progenitor cells was assayed from non-adherent cells in the culture medium at the beginning (the first day) and at the end (the 14th day) of culture. As shown in Figure 4, MSPC from UCB increased the expansion of GM-CFC and HPP-CFC ($p<0.05$). The mean number of GM-CFC and HPP-CFC in the starting CD34+ cell fraction was 2.3 (±0.6)×10⁴ and 1.4 (±0.3)×10⁴, respectively. After expansion, there were 41.8 (±7.5)×10⁴ GM-CFC and 18.4 (±6.1)×10⁴ HPP-CFC for the co-culture system with exogenous cytokines, and 22.5 (±4.5)×10⁴ GM-CFC and 9.5 (±3.1)×10⁴ HPP-CFC for the control system.

The percentage of CD34+ cells decreased after 14 days of expansion (5.7%, 4.8% and 2.9%, respectively, for the co-culture system with exogenous cytokines, the co-culture system without exogenous cytokines and the control system) compared to the starting percentage (97.1% for CD34+ cell fraction). However, the co-culture conditions resulted in 6.2-fold higher numbers of CD34+ cells than those grown in the control culture.

Differentiation of UCB-derived MSCP into chondrocytes

The chondrocytic differentiation potential of UCB-derived MSPC in passages F_1, F_5 and F_{10} was studied by culturing cells under conditions suitable for inducing chondrogenic differentiation. After 3 weeks of culture in chondrogenic medium, induced cells formed chondrocyte-like lacunae which were visualized by light microscopy; furthermore, the induced cells secreted a metachromatic matrix, which was positive with toluidine blue staining (Figure 5). To confirm this differentiation, we used RT-PCR to analyze collagen gene

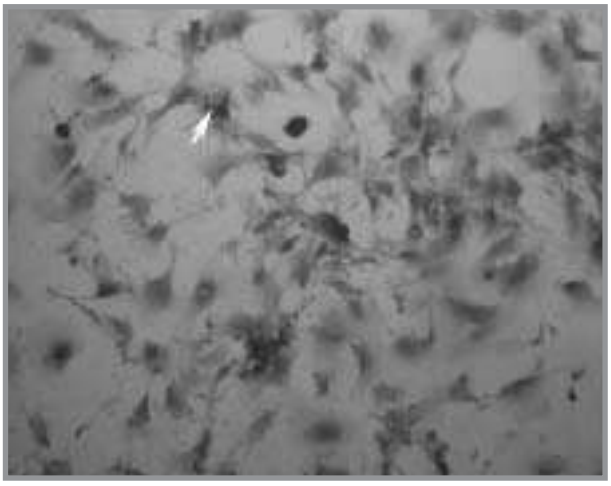

Figure 5. Induced cells stained with toluidine blue (×400). Induced cells secreted a metachromatic matrix that showed positive staining with toluidine blue.

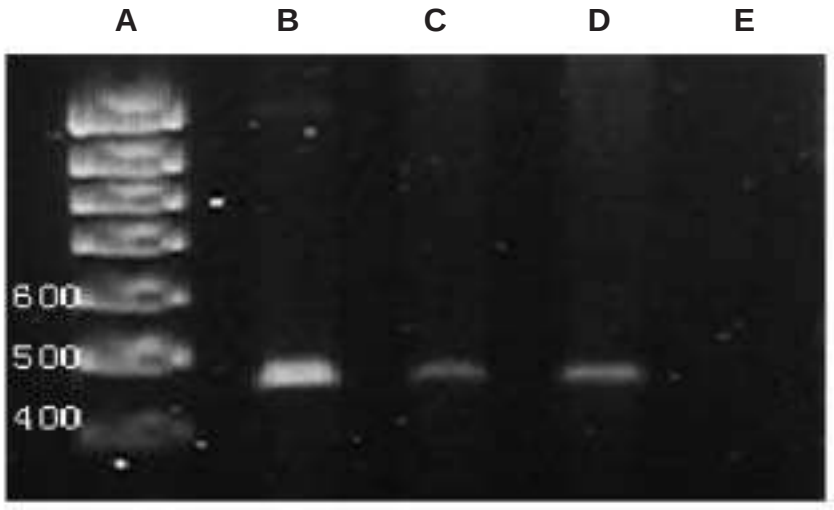

Figure 6. Expression of procollagen II mRNA in chondrocyte-like cells induced from UCB MSC with TGF-β1 (RT-PCR). A: marker; B: induced cells from MSC in passage F_1; C: induced cells from MSC in passage F_5; D: induced cells from MSC in passage F_{10}; E: undifferentiated MSC in passage F_1.

expression. Total RNA from differentiated chondrocyte-like cells and undifferentiated UCB MSC was prepared and cDNA of total mRNA was synthesized by reverse transcription. A fragment of approximately 500bp was amplified from differentiated chondrocyte-like cells by RT-PCR with a pair of primers specific for the collagen gene (Figure 6). By contrast, no amplified products were found from undifferentiated MSPC. This chondrocytic differentiation capacity was retained for passages F_5 and F_{10}.

Discussion

It has been demonstrated that there is a higher $CD34^+CD38^-$ cell fraction in UCB than in normal adult bone marrow, suggesting that very primitive progenitor cells may be more abundant in UCB.[25] Transplantation studies with UCB products have been shown to provide long-term durable engraftment *in vivo*, but the time to achieve neutrophil and platelet engraftment is longer in UCB recipients than in recipients of bone marrow and peripheral blood products.[25] These and many other studies are consistent with UCB containing higher levels of primitive stem cells.[26] Other studies have demonstrated that human UCB can be routinely cultured to form a confluent adherent feeder layer.[15-18] It is well known that adult bone marrow-derived MSC can be rapidly expanded, differentiate into multiple terminal cells, and support expansion of hematopoietic stem cells *in vitro*. MSPC from UCB also have these characteristics and functional properties such as supporting proliferation of hematopoietic stem cells,[15] and differentiating into osteogenic, adipogenic, or neurogenic cells.[17] In this study we demonstrated that UCB-derived MSPC can also be differentiated into chondrogenic cells. The MSPC from UCB that we identified are similar to those present in adult bone marrow. Their morphology and immunophenotype are similar to those of adult marrow MSC, and they are clearly non-hematopoietic and non-endothelial, showing $CD45^-$, $CD34^-$, and $CD14^-$, as reported for adult MSC.[3,27] The MSPC that we established were $CD14^-$, unlike the $CD14^+$ UCB-derived monolayer adherent cells established by Ye *et al.*[15] and Gutierrez-Rodriguez *et al.*,[28] but similar to the MSPC in human first-trimester fetal blood, liver, and bone marrow established by Campagnoli *et al.*[29] Like adult MSC, UCB MSPC are uniformly positive for some mesenchymal markers, such as SH-2, SH-3, and SH-4.

It is clear that in adult bone marrow, mesenchymal cells provide signals for differentiation and proliferation of hematopoietic stem cells and their progeny through direct cell-cell interactions[30] and secretion of hematopoietic growth factors and cytokines.[31-33] It has also been demonstrated that proliferation and differentiation of HSPC occurs in a number of histologically distinct microenvironments (yolk sac,[34] ventral aorta,[35] fetal liver, thymus, spleen, and bone marrow)[36] during human ontogeny. Our results here show that MSPC from UCB support the proliferation and differentiation of HSPC from the same tissue, but the relationship between MSPC and HSPC in human UCB during ontogeny remains to be determined. Hematopoietic growth factors and cytokines play a critical role in the proliferation and differentiation of HSPC. ELISA assays were used to screen for SCF, IL-3, IL-6 GM-CSF and TNF-α production. Only SCF, IL-6 and TNF-α were detected in the conditioned medium of UCB adherent cell layer cultures. There is some controversy about GM-CSF secretion by human bone marrow derived-adherent cells.[37-38] Ye *et al.* also failed to detect endogenous IL-3 and GM-CSF in the conditioned medium of UCB-derived adherent cell layer cul-

tures.[15] We should, however, note the TNF-α detected in the conditioned media. This may induce cells to express mRNA of many hematopoietic factors, such as IL-1, IL-6, IL-8 and GM-CSF.[39] Co-ordinated regulation of various growth factors produced or induced by human UCB-derived adherent cells in co-culture may support *ex vivo* proliferation and differentiation of human UCB-derived HSPC. Although other cytokines in the non-co-culture system (such as IL-6 and TNF-α) were not examined in the co-culture systems, *ex vivo* expansion of HSPC in co-culture with exogenous cytokines was, indeed, greater than that in the non-co-culture. A co-culture experiment without exogenous cytokines also showed that UCB-derived adherent cells supported the expansion of HSPC.

Perhaps other hematopoietic growth factors and cytokines that were undetected by us also play an important role in *ex vivo* expansion of HSPC. The dynamics of cytokine production in co-culture and non-co-culture systems during *ex vivo* expansion of HSPC and the cytokines' effects on the *ex vivo* expansion remaun to be determined.

Co-transplantation of adult MSC has been shown to enhance engraftment of hematopoietic stem cells in a fetal sheep model,[9-11] suggesting that co-transplantation of MSPC and HSPC from human UCB may also result in accelerated rapid, medium-term and long-term engraftment of hematopoietic stem/progenitor cells.

In conclusion, a population of MSPC was isolated from UCB. These cells possess morphologic, immunophenotypic, and functional characteristics similar to those of adult bone marrow-derived MSC. MSPC from UCB could provide an alternative approach for establishment and manipulation of *ex vivo* expansion of hematopoietic stem cells and for differentiation of pluripotent cells. This may facilitate studies on the mechanisms of development, maintenance, and differentiation of hematopoietic stem/progenitor cells.

J-FW: designing the research project and writing the paper; L-JW: isolation, culture and flow cytometry analysis of UCB-derived MSPCs; Y-FW: co-culture of UCB-derived mesenchymal stem/progenitor cells and CD34+ hematopoietic cells; YX: ELISA detection of cytokine production; C-GX: chondrogenic differentiation and RT-PCR analysis of collagen gene expression; B-BJ: statistical analysis; JH: assisted J-FW in designing the project; IKMcN: sent some cytokines for this research, reviewed and modified the original manuscript.

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