Original Article

Isolation and Characterization of Mesenchymal Stem Cells Derived from Human Umbilical Cord Blood Mononuclear Cells

Chih-Yang Chang[1], Po-Han Chen[2], Chia-Jung Li[2], Shang-Chieh Lu[2], Yu-Chun Lin[3], Po-Huang Lee[3], Ying-Hsien Kao[2]

Objectives: Human umbilical cord blood-derived mononuclear cells (UCB-MNCs) have been widely used for the isolation and development of hematopoietic and mesenchymal stem cells (MSCs). This study aimed to establish a research-grade human UCB-MNC bank at E-Da Hospital and to characterize the multipotent differentiation capacity of the isolated cells after ex vivo expansion.

Methods: Human UCB-MNCs were isolated from donor mothers using gradient density centrifugation. After ex vivo expansion, adherent cells were tested for MSC surface markers by flow cytometry and their ability to undergo adipogenesis, chondrogenesis, osteogenesis, and hepatogenesis.

Results: Despite a low percentage of MNCs from UCB being adherent, the adherent cells grown in MSC expansion medium showed typical mesenchymal morphology and a rapid growth rate. After cryopreservation and thawing, the cells still displayed high viability. No difference was noted between cells stored using either cryopreservation technique. Flow cytometry demonstrated that all isolated adherent UCB-MNCs expressed MSC-characteristic surface antigens, including CD45, CD73, CD90, and CD105, without hematopoietic markers. Moreover, gene expression and phenotypic analyses showed that isolated adherent UCB-MNCs were capable of being induced for adipogenesis, chondrogenesis, osteogenesis, and hepatogenesis. These results support the conclusion that the adherent UCB-MNCs are multipotent MSCs suitable for cell transplantation.

Conclusions: We have established a research-grade UCB cell bank with standardized protocols for the isolation and cryopreservation of UCB-MNCs. The multipotency of isolated UCB-MSCs suggests their applicability in further pre-clinical studies and in the development of clinical therapeutic strategies at E-Da Hospital.

Key words: adipogenesis, chondrogenesis, osteogenesis, hepatogenesis, umbilical cord blood-derived mononuclear cells

From the [1]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, and [2]Laboratory of Regenerative Medicine, Department of Medical Research, and [3]Department of Surgery, E-Da Hospital, I-Shou University, Kaohsiung, Taiwan.

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Address reprint request and correspondence to: [1]Ying-Hsien Kao, Laboratory of Regenerative Medicine, Department of Medical Research, and [2]Po-Huang Lee, Department of Surgery, E-Da Hospital, No. 1, Yida Road, Jiaosu Village, Yanchao District, Kaohsiung City 82445, Taiwan.

[1]Tel: +886-7-6151100 ext. 5059, Fax: +886-7-6150945, E-mail: danyhkao@gmail.com

[2]Tel: +886-2-23123456 ext. 65104, Fax: +886-2-23568810, E-mail: pohuang1115@ntu.edu.tw

Introduction

There have been global ethical concerns about the use of embryonic stem cells.[1] Nevertheless, recent research raises the possibility of deriving pluripotent stem cells from somatic cells using epigenetic reprogramming approaches.[2,3] Somatic stem cells have been shown to reside in various tissues such as bone marrow, brain, liver, skeletal muscle, and the dermis.[3] In addition, human umbilical cord blood (UCB) has long been widely used for the isolation of mononuclear cells (MNCs) and further development of hematopoietic stem cells.[4] The UCB-derived MNCs (UCB-MNCs) contain adequate progenitor cells with characteristics different from those of adult bone marrow or peripheral blood stem cells. During the past two decades, human UCB units have been donated worldwide, and stored for use by unrelated patients with hematologic malignancies and/or bone marrow disorders, who have no matched donors.[5] Cord blood units have also been stored privately for personal use as well as for the purpose of autologous transplants. Recent advances in stem cell research support the notion that UCB cells are a more primitive population than adult marrow cells, with several distinct advantages over other adult stem cell sources. UCB cells have longer telomeres, higher proliferation potential, a reduced risk of viral contamination, a reduced risk of graft-versus-host disease during allogeneic grafts, and a better tolerance for human leukocyte antigen mismatches compared to bone marrow.[6] The main advantages of UCB cells are that they can be collected non-invasively, easily characterized, and easily banked.[7] Thus, UCB is a very powerful and readily accessible cell source for allogeneic or autologous cell transplantation. Furthermore, ex vivo expansion and processing of UCB-derived MNCs can give rise to adherent mesenchymal stem cells (MSCs) that exhibit the capacity for multilineage differentiation.[8] They have been demonstrated to be pluripotent and able to differentiate into specific functional cell types present in adult organisms, including chondrocytes, osteocytes, adipocytes, myocytes, and neurons.[9] These capabilities strongly suggest that UCB-MNCs are potentially useful for the treatment of non-hematopoietic diseases. In light of the need for developing both basic research and personalized cell-based therapeutics at E-Da Hospital, this study set forth to establish standard protocols for the isolation and cryopreservation of human UCB-MNCs. To guarantee the clinical applicability of UCB-MNCs, the adherent cells were isolated, expanded ex vivo, and validated as multipotent MSCs.

Materials and Methods

Human subjects

Human UCB samples were obtained from twenty mother donors (less than 40 years of age) in the Department of Obstetrics and Gynecology, E-Da Hospital, with informed consent (IRB approval no. EMRP-099-058 and EMRP16101N). Donor sera were screened for HBsAg, HIV, VDRL before enrollment and all test results were negative.

Isolation and ex vivo expansion of UCB-MNCs

UCB-MNCs were obtained from 40 mL of UCB with citrate phosphate dextrose (Sigma-Aldrich, St. Louis, MO) as anticoagulant and underwent density gradient centrifugation through Ficoll-Paque (1.077 g/cm^3; Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. MNC fractions were washed with phosphate-buffered saline (PBS), counted using trypan blue exclusion staining, and plated onto fibronectin-coated tissue culture flasks (Becton Dickinson) in MSC expansion medium. The expansion medium consists of Iscove modi-

fied Dulbecco medium (IMDM; Life Technologies/Gibco, Grand Island, NY) and 20% fetal bovine serum (FBS; Hyclone, Logan, UT) supplemented with 10 ng/mL recombinant human bFGF (Peprotech, Rocky Hill, NJ), 100 U penicillin, 100 U streptomycin, and 2 mM L-glutamine (Life Technologies/Gibco). Cells were allowed to adhere overnight and nonadherent cells were washed out with medium changes. For long-term maintenance and cellular expansion, adherent cells reaching approximately 60-70% confluence were passaged with 1X TrypLE solution (Life Technologies/Gibco) and replated at 1:4 under the same culture conditions.

Cryopreservation of human UCB-MNCs

All isolated UCB-MNCs were suspended in two commercially available cold preservation solutions, including Synth-a-Freeze® (Life Technologies/Gibco) and CellBanker II® (Zenoaq, Nippon Zynyaku Kogyo Co., Japan) cell cryopreservation media, respectively. The cells were then cryopreserved for at least 3 months using either uncontrolled-rate isopropylalcohol freezing (IPA) or controlled-rate freezing (CRF) method.[10] For IPA cryopreservation, isolated UCB-MNCs were suspended at 1.0×10^7 cells/mL in cryo-media and frozen in a Cryo -1˚C/min freezing container (Nalgene, USA). After overnight in a -70˚C freezer, cells were stored in vapor phase of liquid nitrogen tank for at least 3 months. For CRF method, cells were frozen using a rate-controlled freezer (Kryo 360-1.7, Planer PLC, Middlesex, UK) using program as follows: 1˚C/min to -4˚C, 20˚C/min to -45˚C, 10˚C/min to -10˚C, 1˚C/min to -40˚C and 10˚C/min to -100˚C. The frozen cells were immediately transferred into vapor phase of a liquid nitrogen tank and cryopreserved for at least 3 months. For cell thawing, the cryopreserved cells were rapidly thawed in a 37˚C water bath and diluted with 10 volumes of IMDM expansion medium. After resuspension in fresh medium, cell number and viability were measured by the trypan blue exclusion method or replated for ex vivo expansion.

Flow cytometry analysis

The MSC phenotype of isolated adherent UCB-MNCs were characterized by using a commercially available kit for human MSC analysis (Stemflow™, BD Biosciences, San Jose, CA). In brief, the adherent cells at second passage were trypsinized and stained with fluorochome-conjugated antibodies according to manufacturer's instruction. The antibodies used for MSC-expressing markers included CD44 PE, CD73 APC, CD90 FITC, and CD105 PerCP-Cy5.5, while an antibody cocktail against CD45/CD34/CD11b/CD19/HLA-DR PE was used for MSC negative expression markers. Isotype-identical monoclonal antibodies served as controls. The percentages of CD markers were measured in a flow cytometer (Accuri™, BD Biosciences) with initial gating to rule out dead cells and debris.

In vitro MSC characterization

To test the robustness and in vitro differentiating capacities of expanded UCB-MNCs, the adherent cells were treated with induction medium and subjected to measurements for differentiation markers as previously described.[11,12] To induce adipogenic differentiation, adherent UCB-MNCs at third passage (plated at a density of 3×10^3 cells/cm^2) were treated with adipogenic medium for 3 consecutive weeks. The adipogenic induction medium consisted of IMDM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 0.1 mM indomethacin (Sigma-Aldrich), and 1 μM hydrocortisone (Sigma-Aldrich). Medium changes were carried out twice weekly and specimens for differentiation induction were assessed at weekly intervals.

To induce osteogenic differentiation, the expanded cells at third- to fifth-passage were

treated with osteogenic medium for 3 consecutive weeks and medium was changed twice weekly. The osteogenic induction medium consisted of IMDM supplemented with 10 mM β-glycerol phosphate (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), and 0.2 mM ascorbic acid (Sigma-Aldrich). The osteoblastic phenotype was measured by expression of osteoblastic marker genes, including Runx2, osteopontin and osteocalcin, while the mineralized matrix was visualized by Alizarin red staining.

To induce chondrogenic differentiation, the cells at third- to fifth-passage were transferred into 15-mL polypropylene eppendorf tubes and centrifuged at 1000 rpm for 5 min. The pelleted micromass containing 1 × 10^6 cells at the tube bottom was then treated with serum-free chondrogenic medium for 3 consecutive weeks. The chondrogenic induction medium consisted of high-glucose DMEM (Gibco) supplemented with 0.1 μM dexamethasone, 100 μg/mL sodium pyruvate (Gibco), 40 μg/mL proline (Sigma-Aldrich), 50 μg/mL ascorbic acid, 10 ng/mL TGF-β1 (Peprotech), and 50 mg/mL ITS+ premix (BD Biosciences). At weekly intervals, mRNA levels of syndecan and perlecan, two marker genes for chondrocytes, were quantified by real-time PCR.

Hepatogenic differentiation

To induce hepatogenic differentiation in vitro, the adherent cells at third passage were grown at 50% confluency and treated with two-stage induction of hepatogenesis as previously described.[11,12] The differentiation induction medium consisted of IMDM supplemented with 20 ng/mL HGF (Peprotech), 0.5 μM dexamethasone, and 50 mg/mL ITS+ premix. After 2 weeks, cells were changed to maturation medium consisting of IMDM supplemented with 20 ng/mL oncostatin M (Peprotech), 0.5 μM dexamethasone, and 50 mg/mL ITS+ premix. Medium changes were carried out twice weekly and samples were collected at weekly intervals for hepatogenesis assessment. Morphological alteration was digitally documented under inverted microscope (Axio-

Table 1. Primers used for PCR detection

Target genes	Genbank accession no.	Primer sequence (5’→3’)	Amplicon size (bp)
PPAR-γ	NM_005037.5	S: CAGTGATATCGACCAGCTGAA A: CCCATCATTAAGGAATTCATGTC	180
Runx2	NM_001015051.3	S: ACCTATCACAGAGCAATTAAAG A: GTGCAGAGTTCAGGGAG	187
Osteocalcin	NM_199173.4	S: CTATCGGCGCTTCTACG A: CCTCTTCTGGAGTTTATTTGG	181
Osteopontin	NM_000582.2	S: GGCAGACACAGCATCGTCGGG A: ACTTGGAAGGGTCTGTGGGGCT	273
Syndecan	NM_001006946.1	S: GAAGGACGAAGGCAGCTA A: GTTCTTCAAGGAAGAGGCAA	162
Perlecan	NM_001291860.1	S: AGCTATGTGAATGCAATGG A: ATGTTCTCTGGGTTGGT	187
α-Fetoprotein	NM_001134.2	S: TGCAGCCAAAGTGAAGAGGGAAGA A: CATAGCGAGCAGCCCAAAGAAGAA	217
TAT	NM_000353.2	S: TGAGCAGTCTGTCCACTGCCT A: ATGTGAATGAGGAGGATCTGAG	359
Albumin	NM_000477.5	S: TGCTTGAATGTGCTGATGACAGGG A: AAGGCAAGTCAGCAGGCATCTCATC	162

TAT: Tyrosine aminotransferase; S: Sense primer; A: Anti-sense primer

vert 200, Carl Zeiss, Germany) at weekly intervals. Morphometrical analysis on percentage of transdifferentiated cells was calculated by using ImageJ software (NIH, USA).

Reverse transcription and qPCR analysis

Total RNA was extracted from differentiated cells using Trizol solution (Invitrogen, Gaithersburg, MD). Two micrograms of total RNA was used for reverse transcription reaction, followed by conventional and qPCR analyses as previously described.[13] The gene-specific primers listed in Table 1 were synthesized according to a previous study[11] with partial modification.

Western blotting

Total protein extracts from differentiated cells were obtained by lysing the cells in ice-cold RIPA buffer in the presence of a cocktail of protease inhibitors (Roche, Molecular Biochemicals, Mannheim, Germany). Protein content was quantified using a bicinchonic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and an equal amount of total protein for each lane was subjected to SDS-PAGE, followed by immunodetection as previously described.[13]

Statistical analysis

All data are presented as mean ± standard deviation (s.d.). Differences between groups of independent samples were assessed by Student's *t*-test for paired and unpaired data when appropriate. Significance is declared when p value is less than 0.05.

Results

Isolation and ex vivo expansion of human UCB-MNCs

Human UCB-MNCs were isolated from 20 donor mothers, cryopreserved, and stored for at least one month. The UCB-MNCs were then thawed and tested in a cell viability assay. In this study, only three lines of isolated UCB-MNCs showed adherent cell colonies and typical MSC-like morphology (Fig. 1). The rapid growth rate of the adherent cells remained for at least five passages in vitro. Unfortunately, the isolation rate of adherent and proliferative UCB-MNCs was lower than anticipated. Although we isolated adherent cells from 45% of donor samples (9 out of 20) on fibronectin-coated dishes, only 15% of donor samples (3 out of 20) continued to expand ex vivo for further analyses.

Cyropreservation efficiency of UCB-MNCs

We sought to evaluate and compare the cryopreservation efficiency of UCB-MNCs

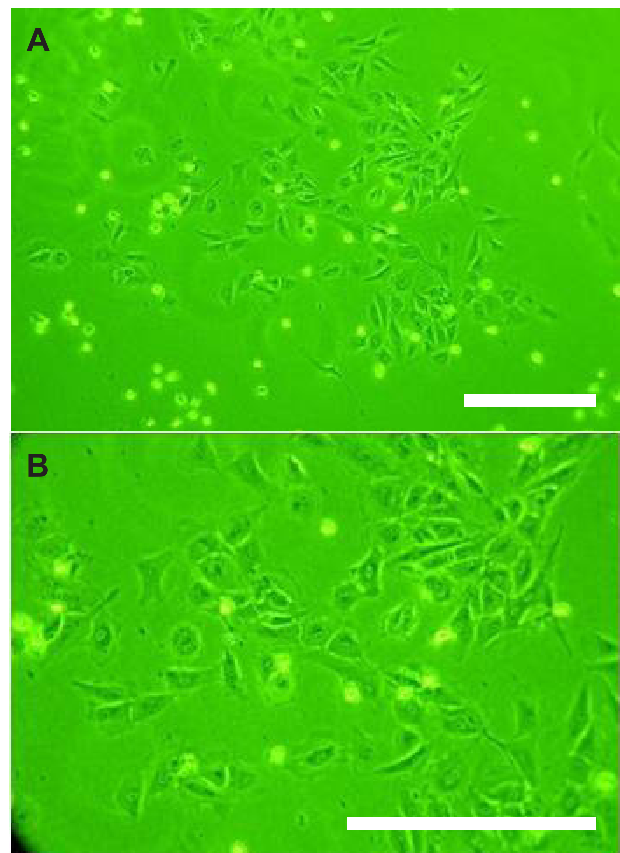

Fig. 1 Morphology of adherent mononuclear cells derived from human umbilical cord blood (UCB-MNCs). After 7 days of cultivation on fibronectin-coated culture dishes, human UCB-MNCs adhered onto dishes and continued to form colonies. Sample images of colonies are shown at (A) low magnification and (B) high magnification. Scale bars are 100 μm.

Table 2. Evaluation on cell viability of isolated human umbilical cord blood mononuclear cells (UCB-MNCs) before and after cryopreservation (CP).

Cell viability (before CP)	Cell viability (after CP)			
	IPA method		CRF method	
	Solution A	Solution B	Solution A	Solution B
94.3 ± 2.6	80.9 ± 2.5[a]	82.0 ± 3.6[a]	87.3 ± 2.3[a,b]	88.1 ± 1.7[a,b]

Isolated human UCB-MNCs were immediately cryopreserved in two commercially available cryopreservation solutions (anonymously shown as solution A and solution B) using both uncontrolled-rate isopropyl alcohol (IPA) and controlled-rate freezing (CRF) methods. Cell viability of freshly isolated cells and those stored in liquid nitrogen for at least 3 months was determined by using trypan blue dye exclusion assay. Data are expressed as mean ± s.d.

[a] $p < 0.05$ compared before CP; [b] $p < 0.05$ compared with IPA methods in the same CP solution.

when different preservation solutions and freezing procedures were used. Samples from the primary isolated UCB-MNCs were preserved in one of two commercially available solutions (anonymously presented as products A and B) at the same cell density, frozen using one of two procedures (i.e., IPA and CRF), respectively, and subsequently stored in liquid nitrogen. After at least one month of storage, the cells were thawed and immediately tested for viability. As shown in Table 2, the averaged viability of freshly isolated UCB-MNCs was 94.3 ± 2.6% before cold preservation. The comparative analysis revealed that after thawing there was no difference in viability between the cells preserved with the two solutions. Although significant loss of viability was

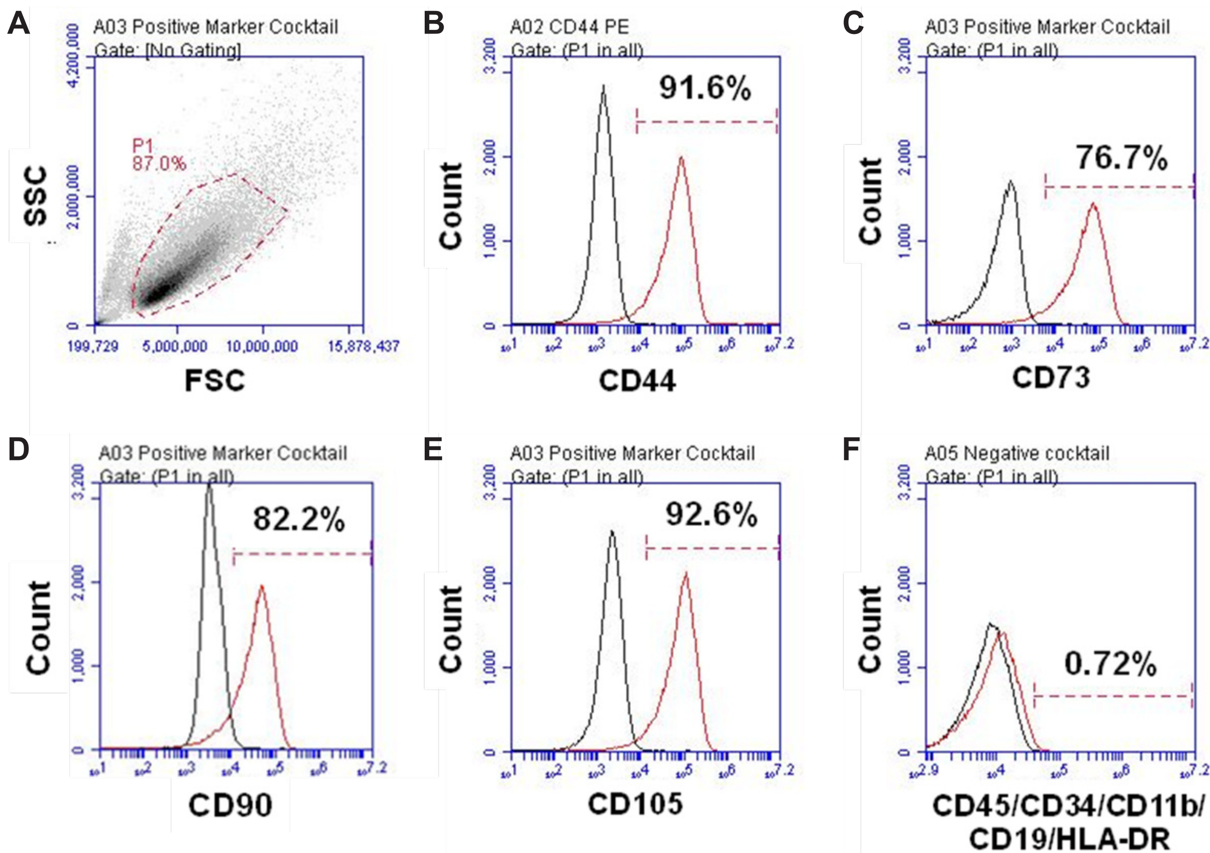

Fig. 2 Expression of mesenchymal stem cell surface markers on adherent mononuclear cells derived from human umbilical cord blood (UCB-MNCs). Primary adherent UCB-MNCs at their third passage were stained with monoclonal antibodies and subjected to flow cytometry for counting based on their surface antigens. Representative data of UCB-MNCs derived from Donor 2 are shown in a dot plot (A). Histogram profiles from Donor 2 for the positive expression of CD44 (B), CD73 (C), CD90 (D), CD105 (E). or the negative expression of the hematopoietic lineage markers CD45/CD34/CD11b/CD19/HLA-DR (F) are shown.

noted for cells under every condition, greater preservation efficiency was achieved using the CRF method than the IPA method (Table 2).

Detection of MSC markers by flow cytometry

We next examined whether the isolated adherent UCB-MNCs express surface antigens characteristic of MSC. Cells at their third passage of growth were subjected to immunostaining for cluster of differentiation (CD) markers and analyzed by flow cytometry. Figure 2 shows representative CD marker histograms of adherent UCB-MNCs isolated from Donor 2. The histograms clearly show that UCB-MNCs express all MSC-characteristic markers, including CD44, CD73, CD90, and CD105. The percentage expression of MSC-characteristic markers for three lines of isolated adherent UCB-MNCs is shown in Table 3. It is notable that although less than 90% of isolated UCB-MNCs were CD90 positive, only 0.72% of cells displayed the CD45/CD34/CD11b/CD19/HLA-DR antigenicity characteristic of hematopoietic cell types, implying little to no contamination with hematopoietic progenitor cells. This result strongly suggests that the isolated adherent UCB-MNCs are MSCs.

In vitro induction of adipogenesis

To characterize the adipogenic differentiation capacity of isolated UCB-MNCs, the cells were cultured in induction medium for three weeks. Morphological changes and the intracellular formation of neutral lipid vacuoles were noticeable after one week of induction. Neutral lipid droplets were seen under phase-contrast microscope after 4 days of induction (Fig. 3A), while the lipid droplets in the cells after 7 days of induction were prominently stained by Oil-Red O staining (Fig. 3B). Moreover, analysis by qPCR (Fig. 3C) and western blot (Fig. 3D) revealed that expression of peroxisome proliferator-activator receptor-γ (PPAR-γ), an adipocyte-specific marker, was significantly increased in cells following 7 days of induction. These results demonstrate that the adherent UCB-MNCs can be induced to differetiate into adipocytes.

In vitro induction of osteogenesis

The isolated UCB-MNCs were treated with an osteogenic induction medium to test their ability to differentiate into osteoblasts. Following incubation under serum-free osteogenic conditions, cell morphology gradually became flattened and broadened. The induced cells showed matrix mineralization and calcium deposition, as revealed by Alizarin red staining (Fig. 3E). Analysis of transcript levels in these cells by qPCR showed significantly elevated expression of three osteoblast specific genes, runx2, osteopontin and osteocalcin (Fig. 3F). Therefore, these results demonstrate that isolated UCB-MNCs can be induced to differentiate into osteoblasts.

Table 3. Flow cytometry characterization by detecting mesenchymal stem cell(MSC) CD marker expression in isolated adherent human umbilical cord blood mononuclear cells (UCB-MNCs).

Specimens	CD marker positivity (%)				
	CD44	CD73	CD90	CD105	CD45/CD34/CD11b/CD19/HLA-DR
Donor 1	88.5	70.6	85.6	87.4	0.66
Donor 2	91.6	76.7	82.2	92.6	0.72
Donor 3	82.8	84.5	74.8	82.3	1.53
Average	87.6 ± 4.5	77.3 ± 6.9	80.9 ± 5.5	87.4 ± 5.2	0.97 ± 0.48

Primary human adherent UCB-MNCs at third passage were stained with monoclonal detecting antibodies and subjected to flow cytometrical detection for MSC-specific surface antigens, including CD44, CD73, CD90, and CD105. CD45/CD34/CD11b/CD19/HLA-DR are markers for hematopoietic stem cells. Averaged positivity data are expressed as mean ± s.d.

In vitro induction of chondrogenesis

To test their chondrogenic potential, the adherent UCB-MNCs were subjected to pelleted culture in a serum-free chondrogenic medium for three weeks. Intriguingly, a round-shape micromass was observed after 7 days of

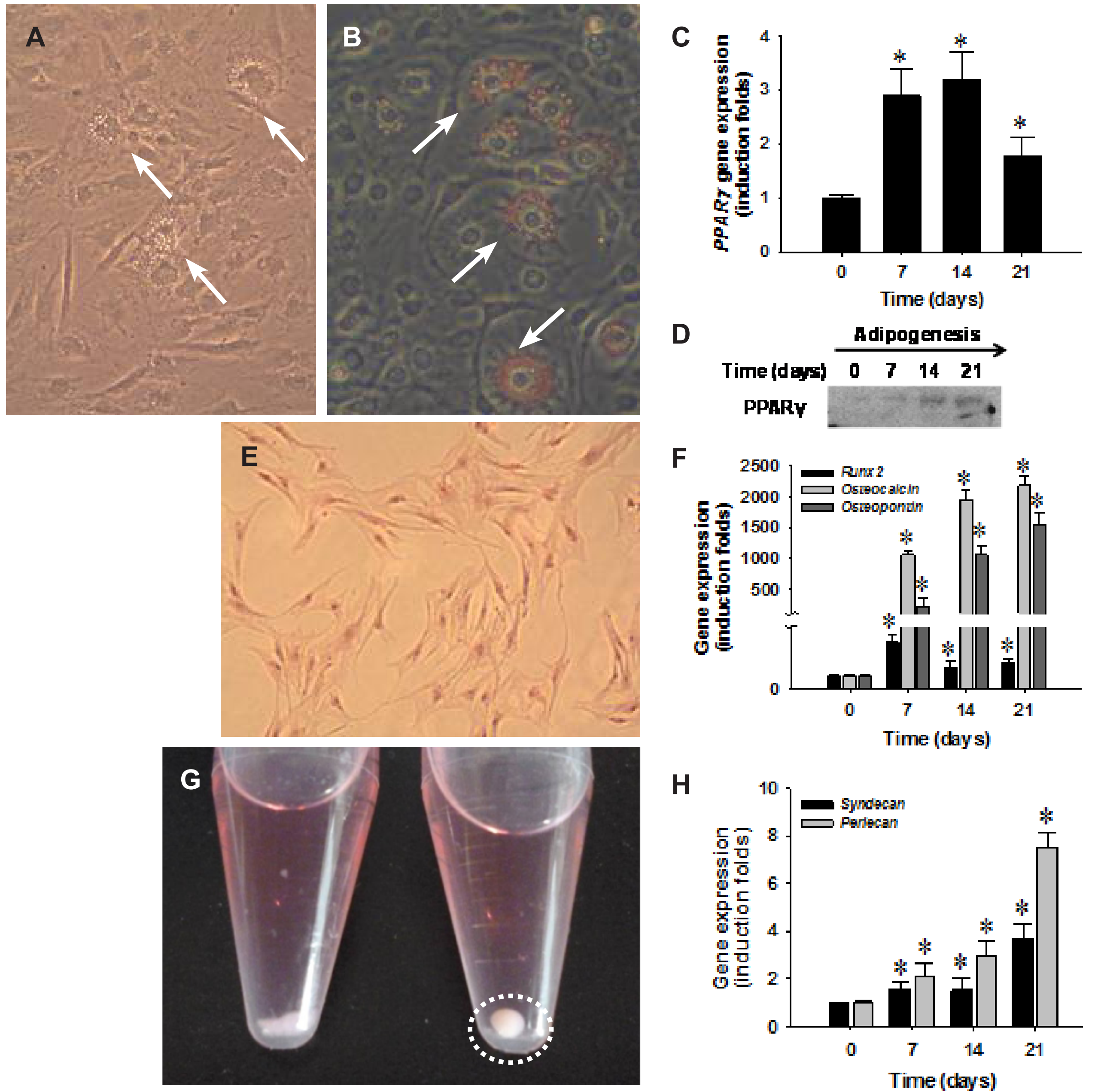

Fig. 3 In vitro differentiation induction of adherent mononuclear cells derived from human umbilical cord blood (UCB-MNCs). (A-D) Primary adherent UCB-MNCs were grown in adipogenic induction medium for 2 weeks. After 4 days of culture, abundant vacuole-like structures (indicated by arrows) were seen in the cytoplasm under phase contrast microscope (A). Perinuclear localization of neutral lipid droplets (indicated by arrows) was visualized by Oil-Red O staining in cells after 7 days of adipogenic induction (B). PPAR-γ expression was evlauated by qPCR (C) and PPAR-γ protein level was evaluated by western blot (D). (E-F) UCB-MNCs were grown in osteogenic induction medium for 3 weeks, subjected to Alizarin red staining for matrix mineralization (E), and subjected to qPCR analysis of osteoblast-specific gene expression (runx2, osteocalcin and osteopontin) (F). (G-H) Chondrogenesis was induced for 7 days. An image shows formation of a round micromass characteristic of chondrogenesis seen after 7 days of induction (right tube, dashed circle), but absent at day sero (left tube) (G). qPCR analysis of syndecan and perlecan mRNA levels in the micromass are shown (H). All quantitative data are expressed as mean ± s.d. * indicates $p < 0.05$ compared with the zero time-point control.

induction (Fig. 3G). Moreover, mRNA levels of both syndecan and perlecan, two chondrocyte-specific genes, were significantly increased following 7 days of induction and thereafter (Fig. 3H). These observations indicate that adherent UCB-MNCs can be induced to differentiate into chondrocytes.

In vitro induction of hepatogenesis

UCB-MNCs have been previously reported to differentiate into endodermal cell types such as hepatocytes.[11] Therefore, we next tested the hepatogenic differentiation potential of our isolated adherent UCB-MNCs by repeating the published protocol. Briefly, the induction protocol includes 2 weeks of induction and 2 weeks of maturation.[11] After 1 week of hepatogenic induction, changes in cell morphology, such as cell flattening and enlargement, were discernible (Fig. 4). The differentiating cells assumed a round shape after 2 weeks, and were heavily vacuolated after 3 weeks. Morphometric analysis indicated that 16.8 ± 1.6% and 27.5 ± 5.4% of initially seeded cells had a matured hepatocyte-like phenotype after 3 and 4 weeks of induced differentiation, respectively. More intriguingly, a minor portion of the differentiated cells displayed binuclear morphology, which is one characteristic of adult hepatocytes. Molecu-

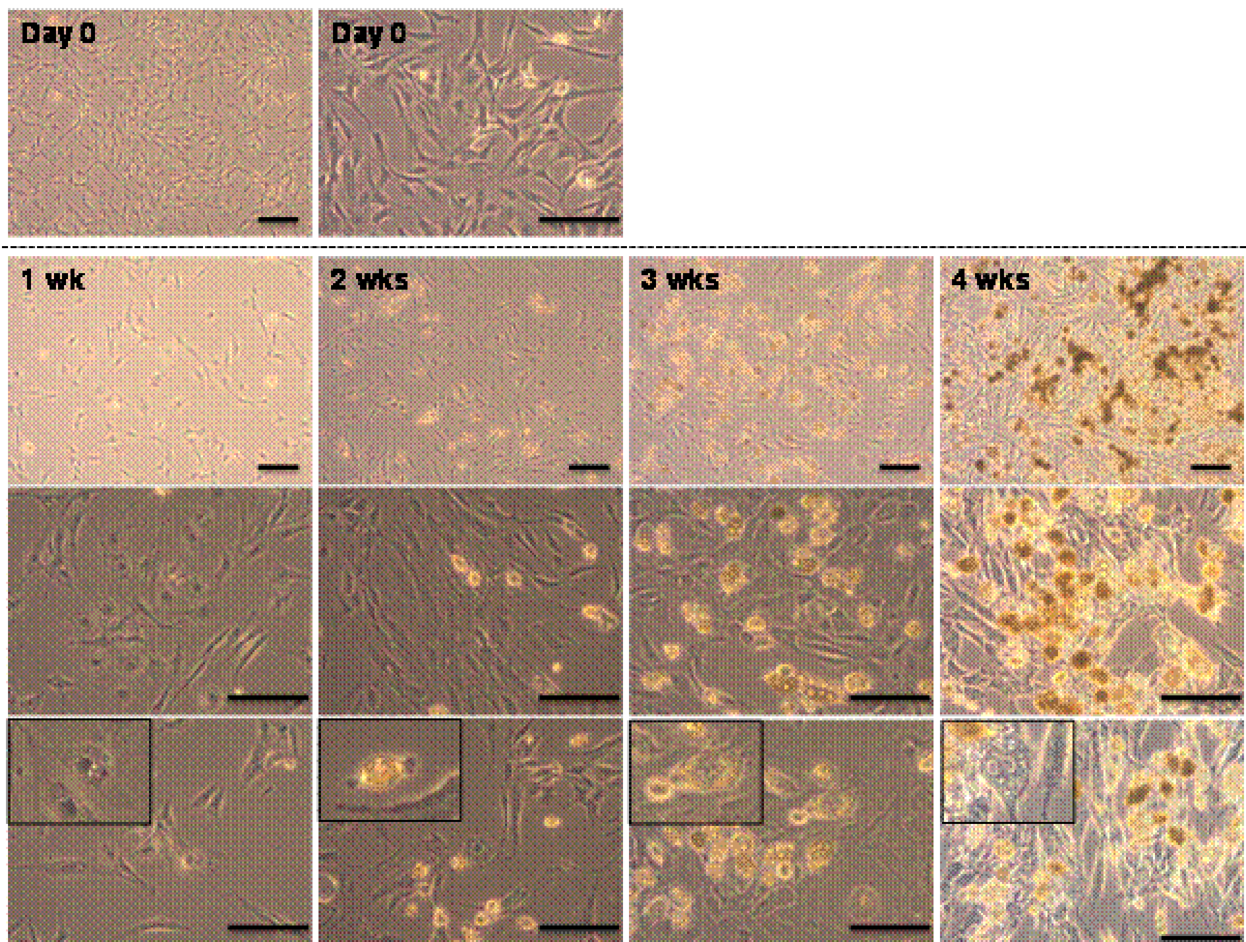

Fig. 4 Hepatocyte-like morphology of the adherent mononuclear cells derived from human umbilical cord blood (UCB-MNCs) following hepatogenic induction. Primary adherent UCB-MNCs at their fifth passage were grown in two-stage hepatogenic media for 4 weeks. After 1 week, cell flattening and enlargement were discernible by phase-contrast microscopy. After 2 weeks, differentiating cells became rounded. After 3 weeks, the dilated cytoplasm was filled with vacuolated ultrastructures. After 4 weeks of induction, differentiated cells displayed binuclear morphology, which is characteristic of adult hepatocytes. Insets show typical morphology of differentiated cells. Scale bars represent 50 μm.

lar detection using RT-PCR (Fig. 5A) and qPCR (Fig. 5B) confirmed that expression of hepatocyte-specific functional genes including α-fetoprotein (*AFP*), albumin (*ALB*), and tyrosine aminotransferase (*TAT*), was significantly increased. Furthermore, western blot analysis demonstrated that ALB, a maturation marker for functional hepatocytes, was significantly induced by hepatogenic treatment (Fig. 5C). Both the morphologic and molecular findings support the conclusion that isolated human UCB-MNCs have the ability to differentiate into hepatocyte-like cells.

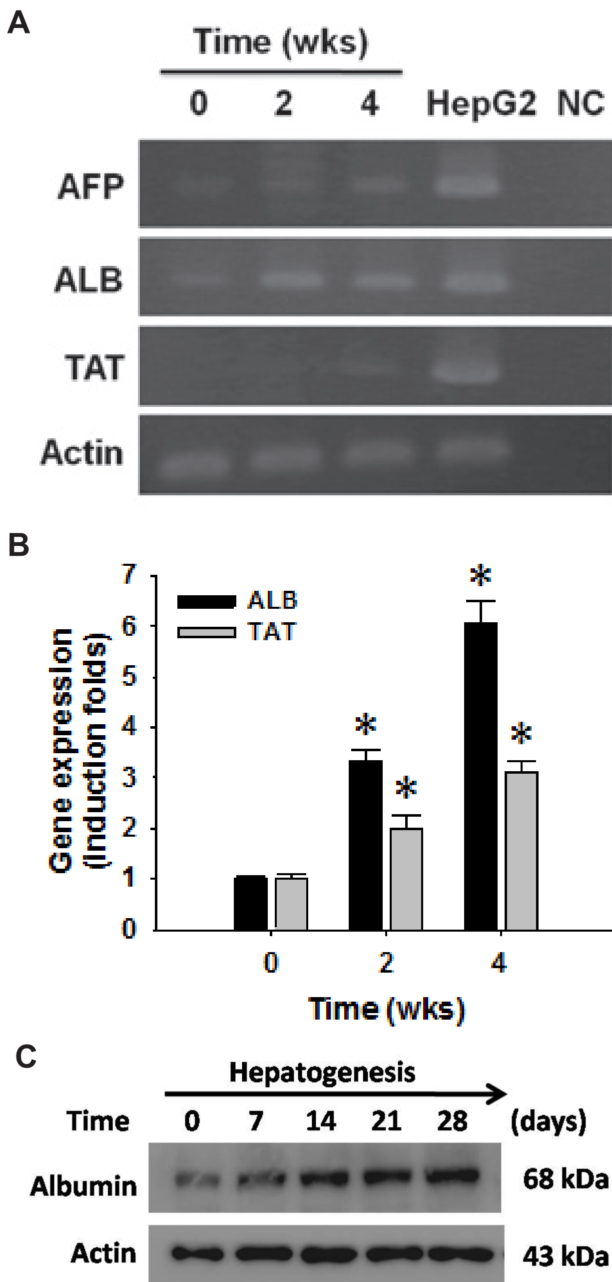

*Fig. 5 Induction of hepatogenic maker genes in adherent mononuclear cells derived from human umbilical cord blood (UCB-MNCs). Primary adherent UCB-MNCs at their third passage were grown in hepatogenic media for 2 or 4 weeks. Total RNA was extracted and subjected to RT-PCR (A) and qPCR (B) for detection of α-fetoprotein (AFP), albumin (ALB), and tyrosine aminotransferase (TAT) gene expression. ALB protein content in induced hepatocyte-like cells was measured by western blot (C). Data are expressed as mean ± s.d. * indicates p <0.05 compared with the zero time-point control.*

Discussion

This study was conducted to establish protocols for isolation, cryopreservation, ex vivo expansion, and characterization of human UCB-MNCs. We reported a limited isolation efficiency of adherent UCB-MNCs from 20 mother donors. Only 45% of UCB-MNC samples were adherent and around 15% of samples could be expanded ex vivo for further characterization as MSCs. Consistent with our results, Barilani et al. recently reported their 9-year experience that nearly one out of two UCB units retains the potential to give rise to MSC colonies, while only 46% of clones can be cultured up to passage eight, but one-fourth of those can reach higher passages.[14] Using magnetically activated cell sorting with CD271 as the enrichment factor, Attar et al. observed that CD271-positive UCB cells did not show outgrowth ex vivo, but a 54.5% isolation rate of MSCs was still obtained from non-enriched UCB cells.[15] Similarly, Vasaghi and co-workers did not observe outgrowth of MSC-like cells using the same bFGF-supplemented medium, but achieved a 40% isolation rate of MSCs using a commercial Mesen-Cult proliferation kit.[16] Besides, Pievani et al. reported a low isolation rate of adherent UCB-MNCs using expansion medium with 20% FBS supplementation.[17] In addition to using the bFGF-supplemented media for the isolation of adherent MSCs from UCB-MNCs, there are other opportunities to modify and improve

our protocol for ex vivo MSC expansion. For instance, hypoxic culture conditions (under 1-5% oxygen tension) were recently shown to facilitate proliferation and maintain the undifferentiated state of UCB-MSCs.[18] This modified protocol is being tested in our laboratory to verify the improved efficiency of ex vivo MSC expansion.

In the context of cryopreservation efficiency, two commercially available cryopreservation solutions and two freezing methods were tested on UCB-MNCs in the current study. We did not note any difference in preservative ability between the two cryopreservation solutions, but found that the CRF method was clearly superior to the IPA freezing procedure. Undoubtedly, the programmable freezing procedures in the CRF method provided maximal protection of cellular structure and function, mainly due to lower crystallization of buffer salts under well-controlled temperature conditions. However, it is noteworthy that the instrumentation and the 15-20 L for each run of procedures make CRF more expensive than IPA. Our data provide a useful technical reference for clinical cost-benefit evaluation.

The International Society for Cellular Therapy (ISCT) has proposed a minimal set of three standard criteria to be used universally for defining a multipotent MSC.[19] MSCs must have plastic adherence, express a set of specific surface antigens, and be capable of adipogenesis, osteogenesis, and chondrogenesis. In terms of surface antigen expression, the phenotype of multipotent MSCs is defined as, at a minimum, the co-expression of the surface antigens CD105, CD73, and CD90 (> 95% positive) in the absence of hematopoietic lineage markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR (< 2% positive).[20] In the present study, were characterized the isolated adherent UCB-MNCs by analyzing their expression of surface CD markers using flow cytometry. We used a screening kit for MSC identification, and demonstrated that the isolated adherent UCB-MNCs expressed all the CD markers characteristic of MSCs. Conversely, the expression rate for a set of negative markers was lower than 1%, strongly supporting the conclusion that these isolated cells are MSCs and are potentially useful for cell therapies. UCB contains a heterogeneous population of stem/progenitor cells expressing a range of surface antigens not limited to the above-mentioned CD markers, but including other relevant markers such as CD29, CD34, CD45, and CD133.[21] Previous attempts have been made to sort UCB cells in order to purify a pluripotent cell population based on surface markers. CD34 is the marker of choice in progenitor selection strategies. It is limited to early hematopoietic progenitors and can be used as a surrogate marker for engraftment potential of both peripheral and cord blood stem cell collections.[22,23] In addition, co-expression of CD34 and CD45 is widely used to identify hematopoietic progenitor cells in UCB.[24] Better understanding of the expression profiles of CD34, CD45, and CD133 surface antigens in isolated UCB-MNCs is crucial for the development of UCB transplantation.

To address the third ISCT-proposed criteria, we demonstrated the multi-lineage differentiation capacity of the UCB-MNCs isolated in this study. We showed that our UCB-MNCs were capable of adipogenesis, osteogenesis, and chondrogenesis. In addition to three mesenchymal cell types, UCB-MSCs can reportedly differentiate into endodermal lineage cells, including functional hepatocyte-like cells[11,12] and neuronal cells from the ectodermal lineage.[25] The present study reproducibly demonstrated the hepatogenic differentiation capacity of the isolated adherent UCB-MSCs, using morphologic and molecular evidence. The potential functionality of the differentiated hepatocyte-like cells generated here was supported by a remarkable increase in albumin expression after two weeks of hepatogenic induction. These data reflect

the possible use of the multipotent UCB-MSCs in treating chronic liver diseases. In fact, the existence of hepatic progenitor cells in human UCB has long been discussed,[26] although their therapeutic potential remains debatable. The in vitro hepatogenic potential of multipotent UCB-MSCs suggests they have potential clinical use in promoting liver regeneration. In this regard, further pre-clinical study is warranted to address their future therapeutic application in liver diseases.

The establishment of a UCB banking technique is important to provide the cells necessary for the development and application of regenerative cell therapies. The banked UCB cells can be applied to at least two current cell-based therapies, namely UCB transplantation and MSC therapy. UCB transplantation was once limited to children due to low cell dose. The majority of UCB transplants is now also performed in adults, thanks to improved techniques of cell banking, reduced intensity transplants, and double transplants. Not surprisingly, UCB transplantation has been suggested to be applicable in a wide variety of diseases. Numerous preclinical studies have shown that UCB cell administration improves neurological recovery in rats with strokes,[27] preserves cardiac function and reduces myocardial infarct size by decreasing apoptosis.[28,29] For MSC therapy, the multipotency of MSCs has greatly increased the clinical interests in these cells. However, their diversity presents a challenge to both clinicians and researchers who want to work with them. For this reason, the research-grade UCB cell bank established in our lab may help open new avenues for the development of regenerative medicine at E-Da Hospital.

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References

1. Wils JP: Umbilical cord blood stem cell transplantation - ethical problems. Biomed Ethics 1999;4:92-101.
2. Leeb C, Jurga M, McGuckin C, et al.: Promising new sources for pluripotent stem cells. Stem Cell Rev 2010;6:15-26.
3. Okita K, Yamanaka S: Induction of pluripotency by defined factors. Exp Cell Res 2010;316:2565-70.
4. Knudtzon S: In vitro growth of granulocytic colonies from circulating cells in human cord blood. Blood 1974;43:357-61.
5. Shapira MY, Hai AA, Tsirigotis P, et al.: Hematopoietic stem cell therapy for malignant diseases. Ann Med 2007;39:465-73.
6. Rocha V, Wagner JE, Jr., Sobocinski KA, et al.: Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. N Engl J Med 2000;342:1846-54.
7. McGuckin CP, Forraz N: Umbilical cord blood stem cells--an ethical source for regenerative medicine. Med Law 2008;27:147-65.
8. Goodwin HS, Bicknese AR, Chien SN, et al.: Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant 2001;7:581-8.
9. Karahuseyinoglu S, Cinar O, Kilic E, et al.: Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem Cells 2007;25:319-31.
10. Woods EJ, Liu J, Pollok K, et al.: A theoretically optimized method for cord blood stem cell cryopreservation. J Hematother Stem Cell Res 2003;12:341-50.
11. Lee OK, Kuo TK, Chen WM, et al.: Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669-75.
12. Lee KD, Kuo TK, Whang-Peng J, et al.: In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40:1275-84.
13. Kao YH, Lin YC, Tsai MS, et al.: Involvement of the nuclear high mobility group B1 peptides released from injured hepatocytes in murine hepatic fibrogenesis. Biochim Biophys Acta 2014;1842:1720-32.
14. Barilani M, Lavazza C, Vigano M, et al.: Dissection of the cord blood stromal component reveals predictive parameters for culture outcome. Stem Cells Dev 2015;24:104-14.
15. Attar A, Ghalyanchi Langeroudi A, Vassaghi A, et al.: Role of CD271 enrichment in the isolation of mesenchymal stromal cells from umbilical cord blood. Cell Biol Int 2013;37:1010-5.

16. Vasaghi A, Dehghani A, Khademalhosseini Z, et al.: Parameters that influence the isolation of multipotent mesenchymal stromal cells from human umbilical cord blood. Hematol Oncol Stem Cell Ther 2013;6:1-8.
17. Pievani A, Scagliotti V, Russo FM, et al.: Comparative analysis of multilineage properties of mesenchymal stromal cells derived from fetal sources shows an advantage of mesenchymal stromal cells isolated from cord blood in chondrogenic differentiation potential. Cytotherapy 2014;16:893-905.
18. Drela K, Sarnowska A, Siedlecka P, et al.: Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner. Cytotherapy 2014;16:881-92.
19. Dominici M, Le Blanc K, Mueller I, et al.: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.
20. Wang HS, Hung SC, Peng ST, et al.: Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 2004;22:1330-7.
21. McGuckin CP, Pearce D, Forraz N, et al.: Multiparametric analysis of immature cell populations in umbilical cord blood and bone marrow. Eur J Haematol 2003;71:341-50.
22. Van haute I, Lootens N, De Smet S, et al.: Viable CD34+ stem cell content of a cord blood graft: which measurement performed before transplantation is most representative? Transfusion 2004;44:547-54.
23. Delalat B, Pourfathollah AA, Soleimani M, et al.: Isolation and ex vivo expansion of human umbilical cord blood-derived CD34+ stem cells and their cotransplantation with or without mesenchymal stem cells. Hematology 2009;14:125-32.
24. Hassanein SM, Amer HA, Shehab AA, et al.: Umbilical cord blood CD45(+)/CD34(+) cells coexpression in preterm and full-term neonates: a pilot study. J Matern Fetal Neonatal Med 2010;
25. Hou L, Cao H, Wang D, et al.: Induction of umbilical cord blood mesenchymal stem cells into neuron-like cells in vitro. Int J Hematol 2003;78:256-61.
26. Kakinuma S, Tanaka Y, Chinzei R, et al.: Human umbilical cord blood as a source of transplantable hepatic progenitor cells. Stem Cells 2003;21:217-27.
27. Vendrame M, Cassady J, Newcomb J, et al.: Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. Stroke 2004;35:2390-5.
28. Ma N, Stamm C, Kaminski A, et al.: Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. Cardiovasc Res 2005;66:45-54.
29. Wu KH, Zhou B, Yu CT, et al.: Therapeutic potential of human umbilical cord derived stem cells in a rat myocardial infarction model. Ann Thorac Surg 2007;83:1491-8.