# A simple, efficient and economical method for isolating and culturing human umbilical cord blood-derived mesenchymal stromal cells

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**Abstract.** Mesenchymal stromal cells (MSCs) hold broad therapeutic potential in various diseases, however, it is difficult to produce sufficient numbers of MSCs for clinical application, therefore, improved culture systems are required. The present study aimed to develop a novel method for isolating and culturing human umbilical cord blood-derived mesenchymal stromal cells (hUCB-MSCs). A sequential culture method was developed that uses two types of culture media to optimize the isolation and culture of hUCB-MSCs. First, DMEM supplemented with mesenchymal stem cell growth supplement was used to improve the colony formation and primary culture success rates of hUCB-MSCs. Then, after removing the heterogeneous cell population, ordinary DMEM was used from the fourth passage. This method obtained hUCB-MSCs with high culture efficiency and at a greatly reduced cost. The optimal culture conditions were determined and the hUCB-MSCs were phenotypically characterized after passaging. Taken together, this simple, efficient and economical method can produce a large number of high-quality hUCB-MSCs in <1 month, therefore facilitating the future clinical applications of hUCB-MSCs.

## Introduction

Mesenchymal stromal cells (MSCs) are a population of heterogeneous multipotent cells that differentiate into diverse cell types. Stromal cells contain various populations, including stem cells (1,2). MSCs have self-renewal ability, immunomodulation and multi-directional differentiation potential, and have become a practical source of cells in the field of gene therapy. MSCs have been extensively used for the treatment of several diseases including immune diseases and hematological diseases, based on their biological and therapeutic effects (3). MSCs can be isolated from different tissues, including bone marrow (4), adipose tissue (5), the umbilical cord (6), umbilical cord blood (UCB) (7), menstrual blood (8), the placenta (9), amniotic membrane (10), dental pulp (11) and tissue from the central nervous system (12). Compared with other sources of MSCs, human UCB-derived MSCs (hUCB-MSCs) have many advantages, such as extensive sources, convenient collection, strong proliferation and differentiation abilities, low immunogenicity and the lack of ethical issues surrounding their use (13-15). There are currently different methods for the *in vitro* isolation and culture of hUCB-MSCs, however, it is still difficult to obtain hUCB-MSCs in large numbers (16). Therefore, a simple, practical and low-cost method to obtain the largest proportion of MSCs from UCB is urgently needed.

Previously, hUCB-MSCs were cultured in media including DMEM/F12, DMEM, and α-MEM (17,18). Some methods added supplements including hydrocortisone, granulocyte-macrophage colony stimulating factor and insulin, while others used only MesenCult™ medium (19,20). However, these methods result in hUCB-MSCs that have a low success rate and are not suitable for scientific research, and clinical applications that require large numbers of hUCB-MSCs. Moreover, many commercially available special stem cell culture media are costly. Finally, the growth rate of osteoclast-like cells is also high during the primary culture process (21), which is not conducive to the subculture of hUCB-MSCs, and thus also reduces the culture success rate of hUCB-MSCs.

Due to the unique characteristics of hUCB-MSCs, there are several methods for their isolation and culture; however, there is no consensus on a standard method. In the present study, a simple, efficient and economical method for isolating and culturing high-quality hUCB-MSCs is described. Once this method becomes the accepted standard, it can be used in scientific research, clinical medicine and cell banking.

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## Materials and methods

*Isolation and culture of UCB mononuclear cells (MNCs).* All UCB samples (age, 24-38 years) were collected from the Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, China between February 2018 and December 2018. Typically, 30-60 ml of UCB samples were collected through the umbilical cord by way of gravity drainage. UCB was collected from the umbilical cord vein with written informed consent of the mother and in strict accordance with the ethical standards of the local ethics committee. All blood samples were processed 4-6 h after collection. To isolate and expand MSCs from the UCB, MNCs were isolated using a lymphocyte separation medium (ρ=1.077 g/l; TBDscience). D-Hanks balanced salt solution (Beijing Solarbio Science & Technology Co., Ltd.) was used to dilute 15-30 ml of fresh UCB at a 1:1 ratio, then the diluted blood was slowly added to the lymphocyte separation medium (taking care not to disturb the liquid surface). The diluted UCB was mixed with the lymphocyte separation media with a volume ratio of 2:1. After centrifugation at 1,317 x g for 20 min at room temperature, the white cloud-like MNCs in the interface layer were carefully aspirated, centrifuged as aforementioned, washed twice with PBS at 750 x g for 10 min at room temperature, and washed once with the DMEM (Gibco; Thermo Fisher Scientific, Inc.) at the same speed. After centrifugation, DMEM supplemented with 10% FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and 1% penicillin/streptomycin was added to the collected cells to prepare a single cell suspension. The cells were counted and inoculated into an FBS-coated T25 cell culture flask (area of 25 $cm^2$ required 1-1.5 ml FBS) at a density of $1x10^7$ cells/ml and incubated at 37˚C in a humidified atmosphere with 5% $CO_2$ (19). Upon reaching 70-80% confluency, the cells were digested with 0.1% trypsin and further subcultured. The growth and morphological characteristics of the primary cells were observed daily under an inverted light microscope (Leica Microsystems GmbH; magnification, x200).

*Effects of different media on the culture of hUCB-MSCs.* In the present study, the culture efficiency of hUCB-MSCs was compared in two types of media. DMEM supplemented with 10% FBS (n=16; DMEM group) and DMEM supplemented with mesenchymal stem cell growth supplement (ScienCell Research Laboratories, Inc.) and 10% FBS [n=22; the mesenchymal stem cell medium (MSCM) group]. To reduce costs, a sequential culture method consisting of two media was used in the MSCM group. hUCB-MSCs in the MSCM group were cultured in DMEM supplemented with 10% mesenchymal stem cell growth supplement (ScienCell Research Laboratories, Inc.) and 10% FBS for three passages, and on the fourth passage the culture media was replaced with DMEM supplemented with 10% FBS. The isolated hUCB-MSCs were inoculated into an FBS-coated T25 cell culture flask at a density of $1x10^7$/ml (5 ml/flask) and cultured at 37˚C in a humidified atmosphere with 5% $CO_2$. The growth and morphological characteristics of the primary cells were observed daily under an inverted microscope and images were captured. The numbers of hUCB-MSCs in the two groups were counted on the 5, 7 and 14th day under the same magnification using a light microscope. After uniform spindle fibroblast-like cells, which grew in a whirled manner, were cultured successfully, the culture success rates and effects of the different types of media on the culture of hUCB-MSCs were compared.

*Effects of different inoculation densities on the culture of hUCB-MSCs.* MNCs from nine blood samples were divided equally into three subgroups and were inoculated in T25 cell culture flasks at cell densities of $1x10^6$, $1x10^7$ and $1x10^8$/ml (with each density representing one subgroup). The cells were cultured in DMEM supplemented with mesenchymal stem cell growth supplement and 10% FBS. The growth of hUCB-MSCs was observed daily under an inverted microscope, and the extension time and primary culture time were recorded.

*Effects of the first medium changes on the culture of hUCB-MSCs.* MNCs from nine blood samples were divided equally and inoculated in a T25 cell culture flask at a cell density of $1x10^7$/ml; the cells were cultured in DMEM supplemented with mesenchymal stem cell growth supplement and 10% FBS. The medium was changed on the 3, 4, 5 and 7th day after inoculation. The media was transferred to a new culture flask for >5 days and further observed to determine whether MSCs could grow, and to determine the time of first medium change.

*Growth curves.* Passage 3 and 10 of hUCB-MSCs were prepared into single cell suspensions, the cell concentrations were adjusted and the cells were inoculated into a 24-well plate. The number of cells added to each well was $1x10^3$. Cells were cultured in DMEM supplemented with 10% FBS in a 37˚C humidified atmosphere with 5% $CO_2$. Cells were counted twice per day and the mean was used as the final cell number. Any increase in the number of cells was calculated for 12 consecutive days. Growth curves of hUCB-MSCs were plotted according to the number of cells, and the population doubling time was calculated from the growth curves. All experiments were performed in triplicate.

*Detection of cell surface markers using flow cytometry.* hUCB-MSCs from passage 3 and 10 were digested with 0.1% trypsin. After centrifugation at 750 x g for 10 min at room temperature, the supernatant was removed and washed with PBS. Aliquots of ~$1x10^6$ cells for each antibody were obtained. The harvested cells were stained with phycoerythrin-mouse anti-human CD29 (cat. no. 561795; 20 $\mu$l/$10^6$ cells; BD Biosciences), FITC mouse anti-human CD44 (cat. no. 560977; 20 $\mu$l/$10^6$ cells; BD Biosciences) and FITC mouse anti-human CD45 (cat. no. 560976; 20 $\mu$l/$10^6$ cells; BD Biosciences). After 30 min of incubation at room temperature in the dark, the cells were centrifuged at 750 x g for 5 min at room temperature and the unconjugated antibody was removed. The stained cells were resuspended in 500 $\mu$l PBS and analyzed using a flow cytometer (Beckman-Coulter, Inc.), and CytExpert software 2.0 (Beckman-Coulter, Inc.) was used for data analysis. All experiments were performed in triplicate.

*Reverse transcription (RT)-PCR.* hUCB-MSCs ($1x10^6$) from passage 3 and 10 were harvested and the total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher

Scientific, Inc.), according to the manufacturer's instructions, and the RNA concentrations were determined. RNA sample (500 ng) was reverse transcribed into cDNA using a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd.). The temperature protocol was as follows: 37˚C for 30 min and then 85˚C for 5 sec. Primers were designed according to the sequences of octamer-binding transcription factor 4 (Oct4), Sex determining region Y-box 2 (Sox2) and the homeobox protein Nanog (Table I). The PCR protocol was as follows: 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 40 sec. The reaction was completed with a final extension at 72˚C for 5 min. GAPDH was used as a positive control. PCR products were visualized with ethidium bromide on a 1% agarose gel.

*RT-quantitative (q)PCR*. The cDNA from passage 3 and 10 of the hUCB-MSCs was used for RT-qPCR using the SYBR Premix Ex Taq (Vazyme) using the ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixture (20 $\mu$l) consisted of 2 $\mu$l template (50 ng/$\mu$l), 10 $\mu$l 2X SYBR mix (Vazyme), 0.5 $\mu$l each primer (10 $\mu$mol/l) and 7 $\mu$l deionized water. The primer sequences were the same and are listed in Table I. The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 9˚C for 10 sec and 60˚C for 30 sec. The relative gene expression level was determined using the $2^{-\Delta\Delta Cq}$ method (22). GAPDH was used for normalization.

*Adipogenic differentiation*. hUCB-MSCs from passage 3 and 10 were used for adipogenic differentiation. Briefly, for the differentiation assay, passage 3 and 10 of hUCB-MSCs were seeded in a 6-well plate at a density of $2x10^4$ cells/well and grown to 80% confluence. Subsequently, the growth medium was substituted for adipogenic differentiation medium (Cyagen Biosciences, Inc.) containing 1 $\mu$mol/l dexamethasone (Sigma-Aldrich; Merch KGaA), 10 $\mu$g/ml insulin (Sigma-Aldrich; Merck KGaA), 200 $\mu$mol/l indomethacin (Sigma-Aldrich; Merck KGaA) and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck KGaA), the medium was replaced every 3 days. After 14 days, the cells were washed with PBS and fixed using 4% formaldehyde for 30 min at room temperature. Adipogenic differentiation was determined using 0.3% Oil Red O staining for 30 min at room temperature. All experiments were performed in triplicate.

*Statistical analysis*. All experiments were performed in triplicate. All data were analysed using SPSS 22.0 software (IBM Corp.). Data were expressed as the mean ± SD. Student's t-tests were performed to compare differences between groups, whereas the differences among multiple groups were determined using one-way ANOVA, followed by post hoc Duncan's tests. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Comparison of the effects of different media on the culture efficiency of hUCB-MSCs*. Using the same isolation procedure, 5 of 16 samples in the DMEM group obtained a uniform culture of hUCB-MSCs, indicating a culture success rate of 31.25%, whereas 18 of 22 samples in the MSCM group obtained a uniform culture of hUCB-MSCs, indicating a culture success rate of 81.81%, which was significantly higher than the DMEM group ($P<0.05$). The numbers of hUCB-MSCs in the MSCM group on the 5, 7 and 14th days were significantly higher than the DMEM group on the same days ($P<0.05$), and the hUCB-MSCs grew rapidly in a whirled manner in the MSCM group (Table II). In the DMEM group, the MNCs were round on the first day (Fig. 1A), after 7 days, the hUCB-MSCs began to expand (Fig. 1B); the primary cultured cells reached a confluency of 10-20% on day 14 (Fig. 1C) and 80-90% on day 40. In the MSCM group, the MNCs were round on the first day (Fig. 1D), then after five days, the hUCB-MSCs began to expand; the primary cultured cells reached a confluency of 10-15% on day 7 (Fig. 1E), 30-40% on day 14 (Fig. 1F) and 80-90% on day 22. The cultured cells were passaged twice to obtain a more uniform culture of hUCB-MSCs at passage 3. The UCB samples that were not cultured successfully contained a small numbers of MSCs. In addition, the volume of MSCs in the unsuccessful cultures was not as large as the volume of osteoclast-like cells, and a small number of MSCs were mixed with the osteoclast-like cells and could not expand normally. Osteoclast-like cells occupied the bottom of the culture flask and reached 80% confluence after 3-4 weeks; the cells could not be trypsinized from the base of the flask. After passaging, MSCs in the cultures initially determined to be unsuccessful could no longer be cultured, ultimately leading to cell death.

Table I. Primers used in the present study.

| Gene | Sequence (5'-3') |
|---|---|
| Oct4 | Forward: AGTGAGAGGCAACCTGGAGA |
| | Reverse: GTGAAGTGAGGGCTCCCATA |
| Nanog | Forward: CAGAAGGCCTCAGCACCTAC |
| | Reverse: GAATTTGGCTGGAAGTGCAT |
| Sox2 | Forward: ACCAGCTCGCAGACCTACAT |
| | Reverse: GGTAGTGCTGGGACATGTGA |
| GAPDH | Forward: TGGTGAAGACGCCAGTGGA |
| | Reverse: GCACCGTCAAGGCTGAGAAC |

Table II. Comparison of cell numbers in the two types of media during the culture of human umbilical cord blood-derived mesenchymal stromal cells.

| | Culturing time (day) | | |
|---|---|---|---|
| Group | 5 | 7 | 14 |
| MSCM | 6.3±1.5[a] | 20.4±3.1[a] | 42.6±5.7[a] |
| DMEM | 2.1±1.3 | 10.8±2.7 | 19.3±3.4 |

Cell numbers were observed under the same field of view. [a]$P<0.05$ vs. DMEM. MSCM, mesenchymal stem cell medium.

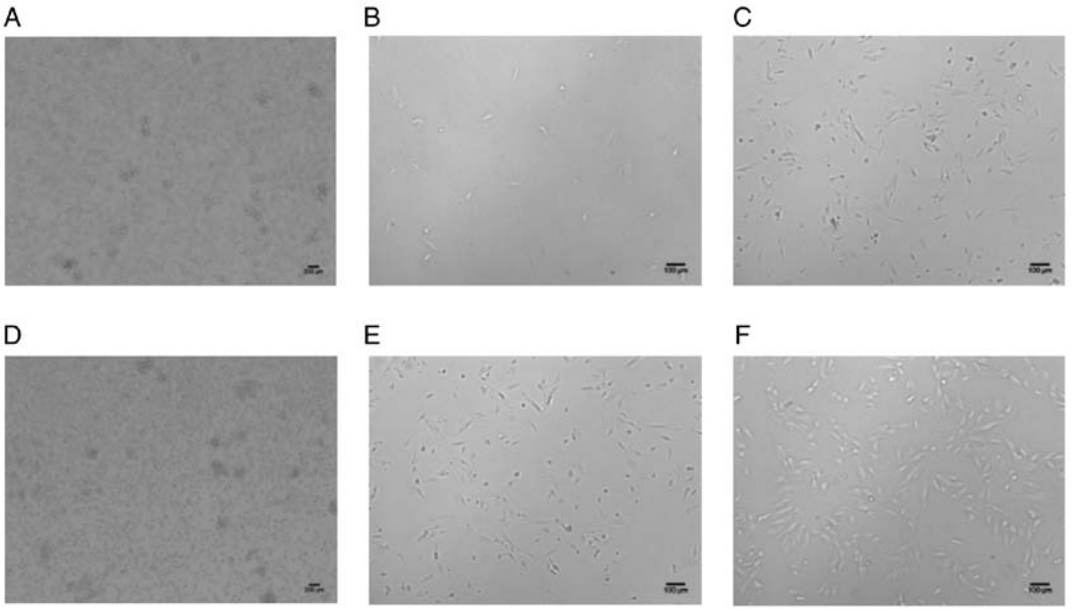


Figure 1. Primary culture of hUCB-MSCs. Primary cultures of hUCB-MSCs using DMEM. In the DMEM group, (A) MNCs were round on the first day, (B) a few spindle fibroblast-like MSCs can be seen on day 7 and (C) hUCB-MSCs reached 10-20% confluence on day 14. Primary cultures of hUCB-MSCs using DMEM supplemented with mesenchymal stem cell growth supplement. In the MSCM group, (D) MNCs were round on the first day, (E) hUCB-MSCs reached 10-15% confluence on day 7 and (F) 30-40% confluence on day 14. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells; MNCs, mononuclear cells; MSCM, mesenchymal stem cell medium.

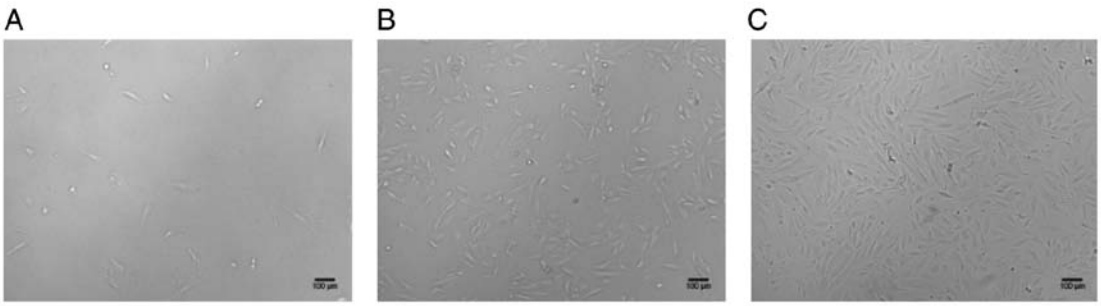


Figure 2. Morphological observation of hUCB-MSCs following the change of media. Primary cultures of hUCB-MSCs in the changed media on (A) day 5 after inoculation and (B) day 4 after inoculation. (C) Subculture of hUCB-MSCs in the changed media on day 4 after inoculation. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells.

*Comparison of different inoculation densities on the culture efficiency of hUCB-MSCs*. DMEM supplemented with mesenchymal stem cell growth supplement was used to compare the culture efficiency using three inoculation densities. As shown in Table III, an inoculation density of $1x10^7$ cells/ml ($2x10^6$ cells/$cm^2$) resulted in a shorter extension time and primary culture time than the other densities tested; the difference was statistically significant ($P<0.05$), indicating that this density was good for the culture of hUCB-MSCs.

*Comparison of the first medium changes on the culture efficiency of hUCB-MSCs*. The medium was changed for the first time on the 3, 4, 5 and 7th day after inoculation. Changing the media on the 7th day did not result in the growth of MSCs in the media removed; only a small number of MSCs grew in the new culture flask on the 5th day, and achieving a high cell density was difficult (Fig. 2A). When media was changed on the 3rd or 4th day MSCs could be obtained following long-term culture, therefore, the first medium change was set at four days after inoculation (Fig. 2B). Compared with changing the media on day 5 after inoculation, the subculturing efficiency was significantly higher at day 4 after inoculation, with a more uniform MSC population obtained when subcultured at passage 3 (Fig. 2C).

*Constructing the growth curve of hUCB-MSCs*. The cell expansion rate of hUCB-MSCs before passage 3 was slower than that of passage 4 or of later passages. As hUCB-MSCs were gradually purified, the osteoclast-like cells were removed, and the doubling rate of each passage became more stable. Furthermore, to reduce costs, starting with passage 4, conventional DMEM was also used to subculture hUCB-MSCs in the MSCM group. After several rounds of passaging, the growth rate of cells was almost equivalent as in the stem cell media and there were no significant differences in the morphological characteristics of the cells (Fig. 3). The growth curves of hUCB-MSCs showed that the growth latency was 1-3 days. Starting on the 4th day, cells entered the logarithmic phase of growth and began to proliferate, the cell density increased rapidly. The proliferation rate reached

Table III. Effect of the inoculation densities of the isolated cells from human umbilical cord blood on the growth of human umbilical cord blood-derived mesenchymal stromal cells.

| Inoculation density (/ml) | Extension time (h) | Primary culture time (day) |
|---|---|---|
| $1x10^6$ | 126.3±6.1 | 31.3±3.5 |
| $1x10^7$ | 93.5±5.4[a,b] | 22.5±2.1[a,b] |
| $1x10^8$ | 128.6±6.3 | 28.1±3.7[a] |

Extension time indicates the duration of time required for the cells to change from round cells to fibroblast-like cells after inoculation. Primary culture time indicates the time required to reach confluency. [a]P<0.05 vs. $1x10^6$, [b]P<0.05 vs. $1x10^8$ groups.

a peak on the 10th day and began to slow, and plateaued. The population doubling time for the entire hUCB-MSCs population was 68 h. There were no significant differences in the proliferation rate and growth cycle of hUCB-MSCs between passages 3 and 10 (Fig. 4).

*Analysis of cell surface markers of hUCB-MSCs*. Flow cytometry was used to detect the cell surface markers of hUCB-MSCs. hUCB-MSCs from passage 3 showed little to no expression of the hematopoietic marker CD45 (0.06%), however, the cells did stably express the stem cell markers CD29 (98.81%) and CD44 (98.41%). hUCB-MSCs stably expressed CD29 (99.29%) and CD44 (98.59%) until passage 10, and did not express CD45 (0.10%; Fig. 5). The expression levels of the cell surface markers of the passage 10 cells was almost equivalent to the passage 3 cells, which is consistent with a previous study investigating bone marrow derived mesenchymal stem cells (BM-MSCs) by Nagamura-Inoue and He (15).

*Gene expression of Oct4, Sox2 and Nanog*. RT-PCR was used to detect the gene expression levels of the embryonic stem cell-specific genes Oct4, Sox2 and Nanog in hUCB-MSCs. It was found that Oct4, Sox2 and Nanog mRNAs were present in the cells from passage 3 (Fig. 6A) and 10 (Fig. 6B). Moreover, RT-qPCR was used to analyze the gene expression levels of Oct4, Sox2 and Nanog in hUCB-MSCs. As shown in Fig. 6C, the gene expression levels of Oct4, Sox2 and Nanog showed no significant difference between passage 3 and 10 (P>0.05), indicating that the hUCB-MSCs had stem cell characteristics.

*Adipogenic differentiation*. An adipogenic differentiation assay confirmed that hUCB-MSCs from passage 3 and 10 could undergo adipogenic differentiation after being exposed to specific induction media. The formation of lipid droplets was observed under an inverted microscope following Oil Red O staining (Fig. 7), further indicating that the hUCB-MSCs had multi-directional differentiation potential.

## Discussion

The cellular components of UCB are complex, and the primary cultures present as heterogeneous cell populations.

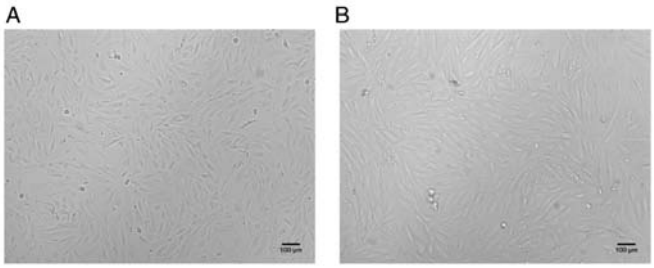


Figure 3. Morphological observation of a primary culture and subculture of hUCB-MSCs. (A) Primary culture of hUCB-MSCs and (B) at passage 10. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells.

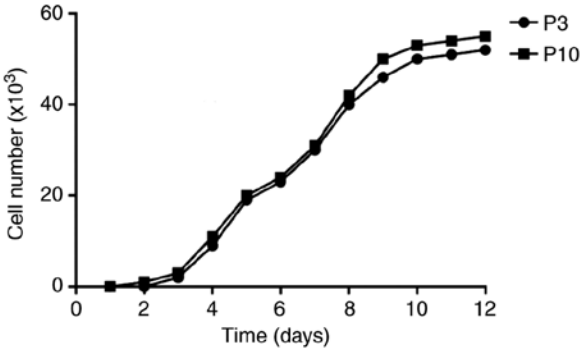


Figure 4. Growth curve of hUCB-MSCs. hUCB-MSCs (1,000 cells/well) were plated in 24-well plates. Cells were counted twice per day and the mean number of cells was calculated. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells; P3, passage 3; P10, passage 10.

There are two main types of adherent cells in UCB: Spindle fibroblast-like MSCs and round osteoclast-like cells (23). Osteoclast-like cells predominate in many UCB samples, with only a small number of MSCs found with the osteoclast-like cells; these MSCs cannot expand normally and achieve higher confluence, and, therefore, cannot be passaged (24). Moreover, other factors may affect the adherence of MSCs. For example, a large number of red blood cells will occupy the bottom of the culture flask, and as the cells grow in a mass, this makes it difficult for MSCs to come into contact with the culture flask (25). Therefore, some interfering factors during initial cell adherence should be eliminated in order to promote adherence and facilitate the growth of MSCs.

The medium is an important factor in cultivating hUCB-MSCs. Most researchers have adopted DMEM (low or high glucose) supplemented with 5-20% FBS, though the success rate is low (17,18,26). Bieback *et al* (19) used MesenCult™ medium to improve culture results. MesenCult™ medium is an acidic medium that includes basal medium and supplements, such as pre-separated serum and glutamine, and does not require the addition of other cytokines. As MesenCult™ can promote the proliferation of hUCB-MSCs and inhibit the growth of other adherent cells, it is a suitable medium for growing hUCB-MSCs (19). However, this medium is expensive and MSCs have a long culture period, which creates high costs for the average laboratory.

In the present study, a sequential culture method was introduced that uses two types of culture media. First, DMEM supplemented with mesenchymal stem cell growth supplement

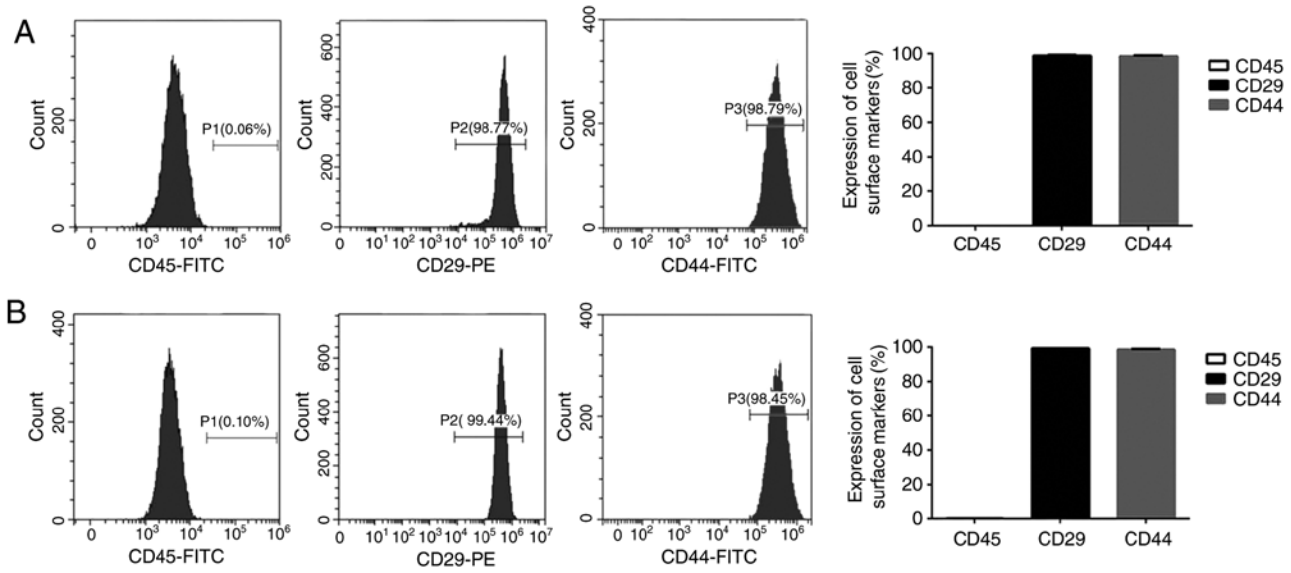


Figure 5. Flow cytometry analysis of hUCB-MSC cell surface markers. CD29, CD44 and CD45 expression in hUCB-MSCs at (A) passage 3 and (B) 10. Cells were prominently positive for CD29 and CD44, and negative for CD45. Error bars represent the mean ± SD of three independent experiments. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells; PE, phycoerythrin.

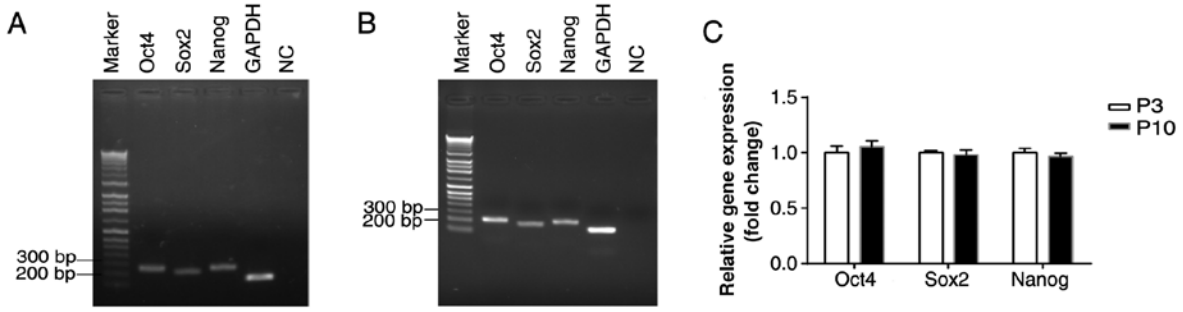


Figure 6. Gene expression analysis of Oct4, Sox2 and Nanog in hUCB-MSCs. Embryonic stem cell-specific gene expression in hUCB-MSCs at (A) P3 and (B) P10. hUCB-MSCs expressed markers of the embryonic stem cell transcription factors Oct4 (273 bp), Sox2 (265 bp) and Nanog (275 bp). GAPDH (138 bp) was used as a positive control. (C) The expression levels of Oct4, Sox2 and Nanog as determined by reverse transcription-quantitative PCR. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells; NC, negative control; Oct4, octamer-binding transcription factor 4; Sox2, Sex determining region Y-box 2; P3, passage 3; P10, passage 10.

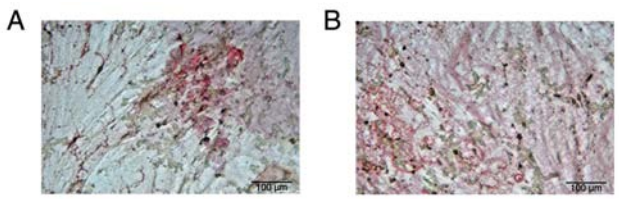


Figure 7. Adipogenic differentiation potential of hUCB-MSCs. Oil Red O staining results showed that hUCB-MSCs from (A) passage 3 and (B) 10 could undergo adipogenic differentiation after being exposed to specific induction media. hUCB-MSCs, human umbilical cord blood-derived mesenchymal stromal cells.

was used to improve the primary culture success rate of hUCB-MSCs. After removing the heterogeneous cell population, standard DMEM was adopted from the 4th passage. hUCB-MSCs can be passaged multiple times and the cell population expands rapidly; these features allow the biological characteristics of MSCs to be maintained and greatly reduces the cost of culture. In addition, FBS-coated culture flasks were used in the present study, which may also have contributed to cell adherence and growth (27). The reason for this enhanced adherence and growth may be a result of FBS covering the surface of heterogeneous antigens in the culture flasks, thus facilitating the adherence of MSCs and allowing them to adapt to the environment quicker. The FBS coating may also provide additional growth and adherence factors, favoring the growth of MSC-like cells (27).

To provide an efficient and practical method for isolating hUCB-MSCs, the effects of different inoculation densities and different timings for first media change on culture efficiency were investigated. The proportion of MSCs in UCB is small and the inoculation density is an important factor affecting cell culture. During the process of primary culture and subculture, hUCB-MSCs show density dependence (28). If the cell density is very low, the few cells present are unable to form the microenvironment required for cell growth, therefore, osteoclast-like cells become the dominant cell type, and MSCs gradually undergo cell death due to aging. Moreover, if the cell density is very high, this may affect the expansion of adherent

cells (29). The present study showed that the optimal cell inoculation density of the primary culture was $1x10^7$ cells/ml ($2x10^6$ cells/$cm^2$); this was the density at which the expansion ability of hUCB-MSCs was significantly increased.

The adherence time of hUCB-MSCs is longer than that of BM-MSCs (30). Therefore, the appropriate time when the medium is first changed is an important factor for ensuring a high yield of hUCB-MSCs. If the medium is changed too early, this may cause unnecessary cell loss. If the medium is changed too late, the nutrient deficiency in the culture medium will hinder normal growth. According to the results of the present study, the optimal time for the first medium change was 4 days after inoculation. This time-point preserved the maximum number of active cells without affecting cell growth, and allowed a high yield of hUCB-MSCs to be obtained.

MSCs are a mixed cell population and their expression of cell surface markers is not uniform (31). The integrin family member CD29 is considered as an important cell surface marker on MSCs (7). In the present study, flow cytometry analysis showed that hUCB-MSCs did not express the hematopoietic precursor cell surface marker CD45 (0.06%), however, they did stably express CD29 (98.81%) and CD44 (98.41%). Compared with BM-MSCs, hUCB-MSCs stably expressed CD29 (99.29%) and CD44 (98.59%) until passage 10, and their morphological characteristics and proliferative activity did not change significantly, which was supported by the growth curves of hUCB-MSCs. In addition, mRNA for Oct4, Sox2 and Nanog was present in the hUCB-MSCs from passage 3 and 10, and the expression levels showed no significant differences. Oct4, Sox2 and Nanog are embryonic stem cell-specific genes (32). An adipogenic differentiation assay showed that hUCB-MSCs from passage 3 and 10 could undergo adipogenic differentiation. The aforementioned results indicated that compared with BM-MSCs, hUCB-MSCs are more naïve, have stronger proliferative ability and differentiation potential, which is consistent with the findings previously reported by Baksh *et al* (33). Several MSC populations, for example BM-MSCs, are being tested in the field of immunotherapy, however, donor variance, *ex vivo* expansion, senescence and immunogenicity are among the main factors influencing the effectiveness of hUCB-MSCs (2,34), in order to develop more standardized culture methods and procedures further studies are required.

In summary, the present study described a sequential culture method that uses two types of culture media to optimize the isolation and culture of hUCB-MSCs. This method has a short culture period and produces a high cell purity with a low economic cost. It also provides an important experimental basis for the large-scale cultivation and clinical applications of hUCB-MSCs in the future.

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**Availability of data and materials**

All data generated or analyzed during the present study are included in this published article.

**Authors' contributions**

HX and JHZ designed, analyzed the experiments and wrote the manuscript. JLZ performed the experiment for cultured cells. QM analyzed some experimental data and co-wrote the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was conducted in accordance with the declaration of Helsinki. The present study was conducted with approval from the Ethics Committee of Shaanxi Normal University. Written informed consent was obtained from all participants.

**Patient consent for publication**

All participants within this study provided consent for the publication of their data.

**Competing interests**

The authors declare that they have no competing interests.

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