Evaluation of Expansion and Maintenance of Umbilical Cord Blood CD34+ Cells in The Co-Culture with Umbilical Cord Blood-Derived Mesenchymal Stem Cells in The Presence of Microcarrier Beads

Sepideh Naseri Mobaraki, Ph.D.[1], Saeid Abroun, Ph.D.[1*], Amir Atashi, Ph.D.[1, 2*], Saeid Kaviani, Ph.D.[1]

1. Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Stem Cell and Tissue Engineering Research Center, Shahroud University of Medical Sciences, Shahroud, Iran

Abstract

Objective: Umbilical cord blood (UCB) is an accessible and effective alternative source for hematopoietic stem cell (HSC) transplantation. Although the clinical application of UCB transplantation has been increased recently, quantitative limitation of HSCs within a single cord blood unit still remains a major hurdle for UCB transplantation. In this study we used microcarrier beads to evaluate the *ex vivo* expansion of UCB-derived HSCs in co-cultured with UCB-derived mesenchymal stem cells (MSC).

Materials and Methods: In this experimental study, we used microcarrier beads to expand UCB-derived MSCs. We investigated the simultaneous co-culture of UCB-derived CD34+ cells and MSCs with microcarrier beads to expand CD34+ cells. The colony forming capacity and stemness-related gene expression on the expanded CD34+ cells were assessed to determine the multipotency and self-renewal of expanded cells.

Results: Our results indicated that the microcarrier-based culture significantly increased the total number and viability of UCB-derived MSCs in comparison with the monolayer cultures during seven days. There was a significant increase in the UCB-derived CD34+ cells expanded in the presence of microcarrier beads in this co-culture system. The expanded UCB-derived CD34+ cells had improved clonogenic capacity, as evidenced by higher numbers of total colony counts, granulocyte, erythrocyte, monocyte, megakaryocyte colony forming units (CFU-GEMM), and granulocyte–monocyte colony forming units (CFU-GM). There were significantly increased expression levels of key regulatory genes (*CXCR4, HOXB4, BMI1*) during CD34+ cells self-renewal and quiescence in the microcarrier-based co-culture.

Conclusion: Our results showed that the increase in the expansion and multipotency of CD34+ cells in the microcarrier-based co-culture can be attributed to the enhanced hematopoietic support of UCB-derived MSCs and improved cell-cell interactions. It seems that this co-culture system could have the potential to expand primitive CD34+ cells.

Keywords: Co-Culture, Hematopoietic Stem Cell, Mesenchymal Stem Cells, Microcarrier

Introduction

Hematopoietic stem cell (HSC) transplantation has become the standard treatment for many patients with hematologic malignancies, immunodeficiency diseases, and metabolism-based disorders (1). An umbilical cord blood (UCB) is an accessible and effective alternative source of bone marrow (BM) and peripheral blood stem cell (PBSC). High proliferative capacity and the ability to tolerate some degree of human leukocyte antigens (HLA) disparity with the recipient, make UCB an attractive supply of donor cells (2). Additionally, studies have indicated that in compression with BM, UCB is composed of a significantly higher proportion of immature and committed hematopoietic progenitor cells (HPCs), including colony-forming units- granulocytes, erythrocytes, macrophages and megakaryocytes (CFU-GEMMs) (3, 4). However, the clinical UCB transplantation application encounters some limitations. The transplantation of one UCB unit may not provide adequate cells for hematopoietic reconstitution, particularly in adult recipients, because of a fixed number of HPCs in a single UCB unit. Additionally, suboptimal numbers of HPCs may also result in slow hematopoietic recovery, prolonged immune reconstitution, and high risk of graft failure (5). These barriers have encouraged researchers to develop *ex vivo* expansion of HPCs to improve CB transplantation outcomes.

In an attempt to develop an effective HPC expansion

Royan Institute
Cell Journal (Yakhteh)

strategy, many researchers explored methods that cultured cells in media supplemented with exogenous cytokine cocktails and *ex vivo* co-cultures of hematopoietic cells with the components of the hematopoietic microenvironment (6). The combined use of recombinant cytokines and a stromal cell feeder layer was also utilized to increase the expansion of CD34+ cells (7). However, unfavorable differentiation of HPC into less potent long-term engrafting cells using the recombinant cytokines administration for expansion raised a question about these methods for *ex vivo* expansion (8, 9). A report by Jang et al. (3) demonstrated that committed HPCs and CD34+ cells could be expanded in culture conditions supplemented only with a cell feeder layer composed of UCB-derived mesenchymal stem cells (UCB-MSCs) in the absence of recombinant cytokines.

It has been demonstrated that microcarrier-based culture systems could simulate a three-dimensional (3D) environment to support anchorage-dependent cells. The attachment of adherent cells to microcarrier beads forms cell-microcarrier complexes suspended in the growth medium and increases the culture density. The biopharmaceutical industry frequently uses the microcarrier-based expansion of culture adherent cells for vaccine production (10). Adherent cells expand on the microcarrier beads and can be retrieved at the end of the expansion time period (11). Additionally, the larger surface area to volume ratio and provided by the addition of microcarriers in comparison with a monolayer culture enables cellular scale-up.

Furthermore, microcarrier-based expansion processes that use different kinds of commercially available microcarriers in rotary reactors or spinner flasks have been developed to expand MSCs from different cell tissue sources. Researchers have successfully utilized different types of microcarriers for robust and scalable MSCs manufacturing from various sources (12-16). Interestingly, after expansion, the MSCs maintained their capability to differentiate into osteoblast, adipocyte, and chondroblast lineages and expressed the human BM-MSC immunophenotype (16).

Co-culture systems that use cell feeder layers to support HPCs simulate the *in vivo* hematopoietic microenvironment where direct cell-cell interactions, as well as cytokines and growth factor secretion, promote expansion and regulate the proliferation and differentiation of HPCs (17). On the other hand, developing a system where stromal cells could also proliferate would mimic the *in vivo* hematopoietic microenvironment for the simultaneous expansion and maintenance of UCB-HPCs and UCB-MSCs.

In this study, we exploited the superior expandability of UCB-MSCs on microcarrier beads in an attempt to maximize UCB-CD34+ cells support through elevated growth factor secretion and cell-cell interactions. We demonstrated that, in the absence of externally added recombinant cytokines, the support that UCB-CD34+ cells received from UCB-MSCs in the 3D system resulted in significantly higher numbers of CD34+ cells that retained their pluripotent potential. The expanded UCB-CD34+ cells also expressed higher levels of the stemness-related genes and showed a higher colony-forming ability than conventional two-dimensional (2D) co-culture systems.

Materials and Methods

The Ethical Committee of the Tarbiat Modares University, Tehran, Iran, approved this study protocol and informed consent form (IR.MODARES.REC.1398.193). In this experimental study, UCB samples were obtained from the Iran National Cord Blood Bank, Iran Blood Transfusion Organization, Tehran, Iran. The UCB samples were collected after receipt of written informed consent from women who delivered normal full-term infants.

Mononuclear cells isolation from human umbilical cord blood

The UCB samples of normal full-term infants were collected into standard bags (123456789, JMS, Singapore) containing citrate phosphate dextrose adenine (CPDA) solution. Mononuclear cells (MNCs) were isolated within 24 hours by density gradient centrifugation using Ficoll-Paque PLUS (17144002, GE Healthcare, UK, density 1.077 g/ml). Briefly, UCB was diluted by the same volume of phosphate-buffered saline (PBS), gently overlaid onto the Ficoll surface in a 15-mL sterile plastic centrifuge tube, and centrifuged at 400 g for 30 minutes at room temperature. After centrifugation, the MNCs were collected and diluted with PBS and then centrifuged at 300 g for 10 minutes at room temperature.

Isolation of umbilical cord blood CD34+ cells

CD34+ progenitors were isolated by immunomagnetic bead selection on an affinity column using magnetic-activated cell-sorting (MACS) CD34 Isolation Kit (130046702, Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, the MNCs were suspended in PBS (up to 108 total cells). Then, 100 μl human Fc receptor (FcR) blocking reagent and 100 μl anti-CD34 microbeads were added, mixed and incubated at 4°C for 30 minutes. Subsequently, the MNCs were washed with PBS and centrifuged at 300 g for 10 minutes. The labeled MNCs were then re-suspended in 3 mL PBS and passed through an LS separation column (130042401, Miltenyi Biotec, Bergisch-Gladbach, Germany) placed in a magnetic field to capture the CD34+ cells. In order to collect the CD34+ cells, the column was removed from the magnetic field and flushed with a PBS and 2 mM EDTA washing solution. The purity of CD34+ cells was assessed by flow cytometry using anti-human phycoerythrin (PE) -conjugated CD34 antibody (550761, Pharmingen, San Diego, CA, USA).

Isolation and characterization of UCB- MSC

For UCB-MSCs isolation, a total of 1×10^6 MNCs/cm²

were resuspended in the Iscove's Modified Dulbecco's Medium (IMDM, 12440053, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (10270106, FBS, Gibco, USA), penicillin (100 units/mL)/streptomycin (100 µg/mL) (15140, Gibco, USA) in 75 cm² tissue-culture flasks. The cells were maintained at 37°C in a humidified, 5% CO_2 incubator, and the culture medium was changed twice per week. Non-adherent cells were removed after 48 hours and the fibroblas-like cell colonies remained in the flasks. MSCs from 3rd passages were used for further experiments. The isolated MSCs were characterized by flow cytometry (Applied Biosystems, USA) analysis to evaluate their ability to express specific surface markers (CD105, CD73, CD90) and inability to express CD34, CD45, and HLA-DR. In addition, osteogenic and adipogenic differentiation capability of UCB-MSCs was investigated by using standard commercial differentiation media. Briefly, adipogenic induction was performed by feeding MSC cells for two weeks with a medium that consisted of 1 mM (100 nM) dexamethasone (D4902, Sigma Chemical Co., St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylxanthine (I5879, IBMX; Sigma, USA), 10 µg/mL recombinant human insulin, 100 mM indomethacin (I7378, Sigma, USA), and 10% FBS. Differentiation to osteogenic lineage was induced when UCB-MSCs were placed on a six-well plate of the osteogenic induction medium that consisted of 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.2 mM ascorbate, 0.05 mM L-ascorbic acid 2-phosphate (A8960, Sigma, USA), and 10% FBS for two weeks. Differentiation of these cells toward adipogenic and osteogenic phenotypes was assessed by oil red O and Alizarin red staining, respectively (18) (Details are available upon request).

Pretreatment of microcarrier beads

The microcarriers beads (Z378682, Sigma-Solohill, UK) were pretreated for the cell culture in PBS and sterilized in an autoclave at 121°C for 20 minutes according to the manufacturer's protocol.

Expansion of UCB-MSCs on microcarrier beads

The conditioning medium was carefully removed from the pretreated microcarrier beads in order to avoid aspirating the microcarriers and we added fresh, prewarmed IMDM medium supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 units/mL penicillin. The UCB-MSCs were seeded at a density of 6000 cells/cm² at 37°C in humidified air that contained 5% CO_2. As described by Nienow et al. (19), the optimum microcarrier and cell densities were approximately 6000 microcarriers/mL and 5 cells/microcarriers, respectively. Briefly, the culture medium was carefully removed, and the cells on the microcarriers were washed twice with 2 mL of prewarmed PBS. The UCB-MSCs were then detached with trypsin (0.25%, w/v)/EDTA (Lonza, UK). After 5 minutes, 4 mL of complete media containing FBS was added to stop the enzymatic activity, and the

cell plus microcarrier suspension was filtered through a 100 mm Cell Strainer (BD Biosciences, USA). The cell suspension was then centrifuged and re-suspended in culture medium to evaluate the cell number and viability. We harvested the UCB-MSCs according to a detachment protocol described by Nienow et al. (19).

***Ex vivo* expansion of CD34+ cells**

The UCB-CD34+ cells were cultured in a co-culture with the feeder cell UCB-MSCs in the presence and absence of microcarrier beads for 7 days. In the co-culture experiments, 1×10^5 UCB-MSCs and UCB-CD34+ cells were cultured on the microcarrier beads for seven days at 37°C and 5% CO_2. To address the importance of cell-cell interactions between the UCB-CD34+ cells and UCB-MSCs, we used 0.4 µm pore size transwell inserts (6.5 mm diameter, 0.4 µm pore size, SPL Life Sciences, Seoul, Korea) in our co-culture groups. The culture groups used in this study were classified into five groups: A. Freshly isolated CD34+ cells, B. CD34+ cells co-cultured with a monolayer of UCB-MSCs, C. CD34+ cells co-cultured with UCB-MSCs expanded on the microcarrier beads, D. CD34+ cells and UCB-MSC as a non-contact co-culture using transwell and E. CD34+ cells and UCB-MSCs expanded on microcarrier beads as a non-contact co-culture using 0.4 µm pore size transwell inserts. Figure 1 illustrates the schematic diagrams of the culture conditions. In further functional studies, UCB-MSCs and CD34+ cells were easily separated by sedimentation due to the distinct weight differences between microcarriers containing adherent MSCs and the suspended CD34+ cells.
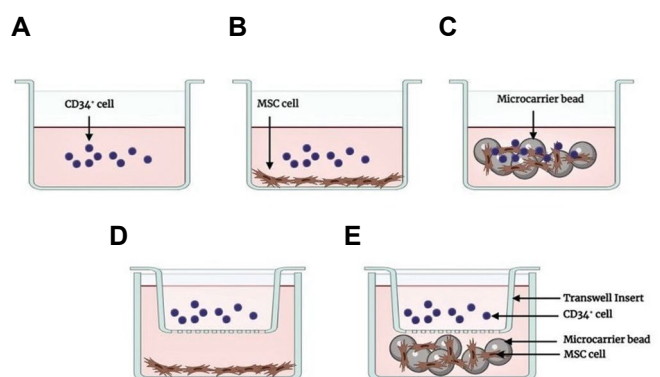

Fig.1: Schematic diagrams of the culture groups studied. **A.** Freshly isolated CD34+ cells, **B.** CD34+ cells co-cultured with a monolayer of UCB-MSCs, **C.** CD34+ cells co-cultured with UCB-MSCs expanded on microcarrier beads, **D.** CD34+ cells and UCB-MSC as a non-contact co-culture using transwell inserts, and **E.** CD34+ cells and UCB-MSCs expanded on microcarrier beads as a non-contact co-culture using transwell inserts. UCB-MSCs; Umbilical cord blood-mesenchymal stem cells.

Scanning electron microscopy

The surface attachment of UCB-MSCs on the microcarrier beads and homing of UCB-CD34+ cells onto the 3D constructs of the microcarriers beads were

observed by scanning electron microscopy (SEM). The UCB-MSC-microcarrier constructs from six-well plates were fixed in 2.5% glutaraldehyde (Shandong Minglang Chemical, china) in PBS (pH=7.2) for 1 hour at room temperature. The samples were dehydrated with serial dilutions of ethanol (50, 75, 85, 95, and 100%). The constructs were gold sputtered and observed by SEM (FEI ESEM Quanta 200, Hillsboro, OR, USA).

MTT assay

Cell Growth Determination Kit (Sigma, USA) was used for this assay in accordance with the manufacturer's protocol. The cells were seeded at a density of 4×10^3 cells per well and incubated at 37°C in 5% CO_2. For seven consecutive days, we added 50 µl MTT solution [5 mg/ml in DMEM (Invitrogen, USA)] to the cells seeded in each well. The plates were incubated at 37°C for 4 hours to enable conversion of MTT to formazan crystals by mitochondrial dehydrogenases in the viable cells. The supernatant was removed for dissolution of dark blue intracellular Formazan, and a constant amount of dimethyl sulfoxide solvent was added. Optical density was read in a microplate reader at 570 nm (BioTek Instruments, Winooski, VT, USA). Finally, the cell numbers were calculated using a standard curve.

Colony-forming cell assay

Clonogenic capability of the CD34+ cells was assessed by a semi-solid culture using MethoCult GF H4434 medium (Stemcell Technologies, Inc., Vancouver, Canada) according to the manufacturer's instructions. Briefly, 1×10^3 CD34+ cells from freshly isolated (day 0) and expanded CD34+ cells in were seeded into 3 ml semi-solid culture media and cultured for 14 days in a humidified incubator at 37°C and 5% CO_2. Subsequently, hematopoietic colonies that included erythroid burst-forming unit (BFU-E), granulocyte–monocyte colony forming unit (CFU-GM), and CFU-GEMM were identified and counted by an inverted microscope (Nikon, Japan). Each cluster of 50 cells was counted as one colony.

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

The relative expression of genes that are important in self-renewal ability and maintenance of HSCs such as CXCR4, HOXB4, and BMI1 were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) from isolated CD34+ cells and expanded CD34+ cells after 3 and 7 days of co-culture, according to the manufacturer's protocol. RNA integrity was determined using electrophoresis with RNA loading dye on a 1% agarose gel. Subsequently, the extracted RNA was reverse transcribed to total cDNA with a SuperScript First Strand cDNA System (Thermo Fisher Scientific, Waltham, MA, USA). The primers were designed and checked by AlleleID 6 and Primer-BLAST (Table S1, See Supplementary Online Information at www.celljournal.org). Quantitative RT-PCR was carried out by using SYBR Green Master Mix (Ampliqon, Odense, Denmark) and a StepOne Real-Time PCR System (Applied Biosystems, USA) (20). GAPDH gene was chosen as the internal control for mRNA quantification.

Statistical analysis

All experiments were performed in triplicate. Results are presented as mean ± SD. The data were analyzed using one-way analysis of variance with the GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA, USA). The results were considered statistically significant when P<0.05.

Results

Isolation and characterization of umbilical cord blood mesenchymal stem cells

Flow cytometry analysis results indicated that the UCB-MSCs were >95% positive for the MSC-associated markers (CD73, CD90 CD105) and <5% positive for hematopoietic lineage markers (CD34, CD45, and HLA-DR) (Fig.2A). We stimulated the UCB-MSCs to verify their ability to differentiate into adipocytes and osteoblasts. After 21 days in lineage-specific differentiation media, UCB-MSCs were stained with oil red O to evaluate for adipogenic differentiation, which was shown by the apparent formation of lipid vacuoles, and they were also assessed for alkaline phosphatase, which demonstrated calcium deposit-producing osteoblasts by Alizarin red staining (Fig.2B).

Umbilical cord blood mesenchymal stem cell attachment and proliferation on microcarrier beads

The surface attachment of UCB-MSCs on microcarriers beads was investigated by electron microscopy imaging on the 4th day (Fig.3A). Additionally, SEM imaging indicated that the microcarriers could provide a 3D environment created by microcarriers covered with UCB-MSCs and CD34+ cells. As shown in Figure 3B, the number of UCB-MSCs increased slightly in the microcarrier-based culture within the first four days, and also the number of UCB-MSCs increased significantly in the microcarrier group ($1.57 \pm 0.07 \times 10^5$) in comparison with the 2D group ($1.24 \pm 0.08 \times 105$, P<0.001) at 5th day. The same elevation was continued for 6th and 7th days, and also, the number of MSCs substantially increased in the microcarrier group was compared to the monolayer culture. The monolayer culture also showed a gradual increase in the number of cells over seven days of culture. The UCB-MSCs culture showed a significantly higher number of cells in microcarrier bead culture compared with monolayer culture [(3.27 $\pm 0.13 \times 10^5$ vs. $2.19 \pm 0.14 \times 10^5$), Fig.3B, P<0.0001]. Cell viability assay showed the same scenario where the number of viable MSC cells was significantly higher in the microcarrier-based culture between 4th and 7th days (Fig.3C, P<0.0001).
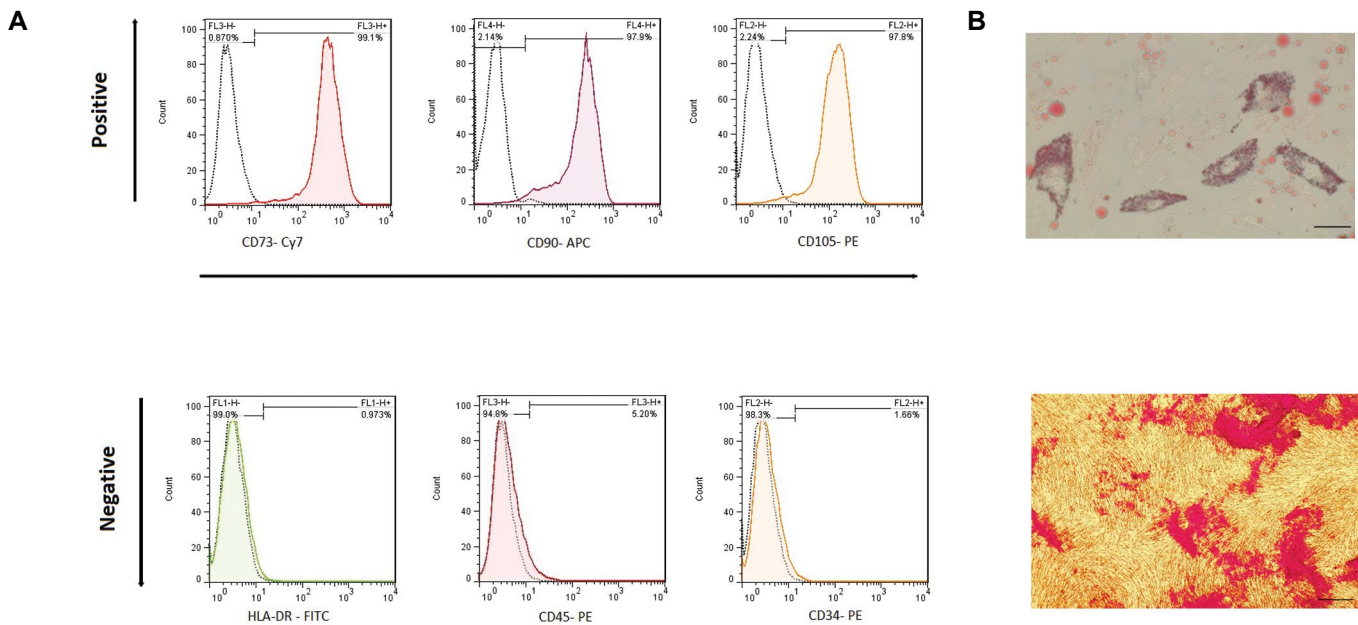
Fig.2: Characterization and differentiation potential of the UCB-MSCs. **A.** Flow cytometry results for the MSC-associated markers (CD73, CD90, CD105) and hematopoietic lineage markers (CD34, CD45, HLA-DR). Cell surface expression profile antigens (—), isotype control (….). **B.** Differentiation of UCB-MSCs into adipocytes and osteoblasts. **B1.** Adipocytes stained with the oil red O after 21 days (scale bar: 50 μm), and **B2.** Osteoblasts stained with the Alizarin red after 21 days (scale bar: 100 μm). UCB-MSCs; Umbilical cord blood-mesenchymal stem cells.
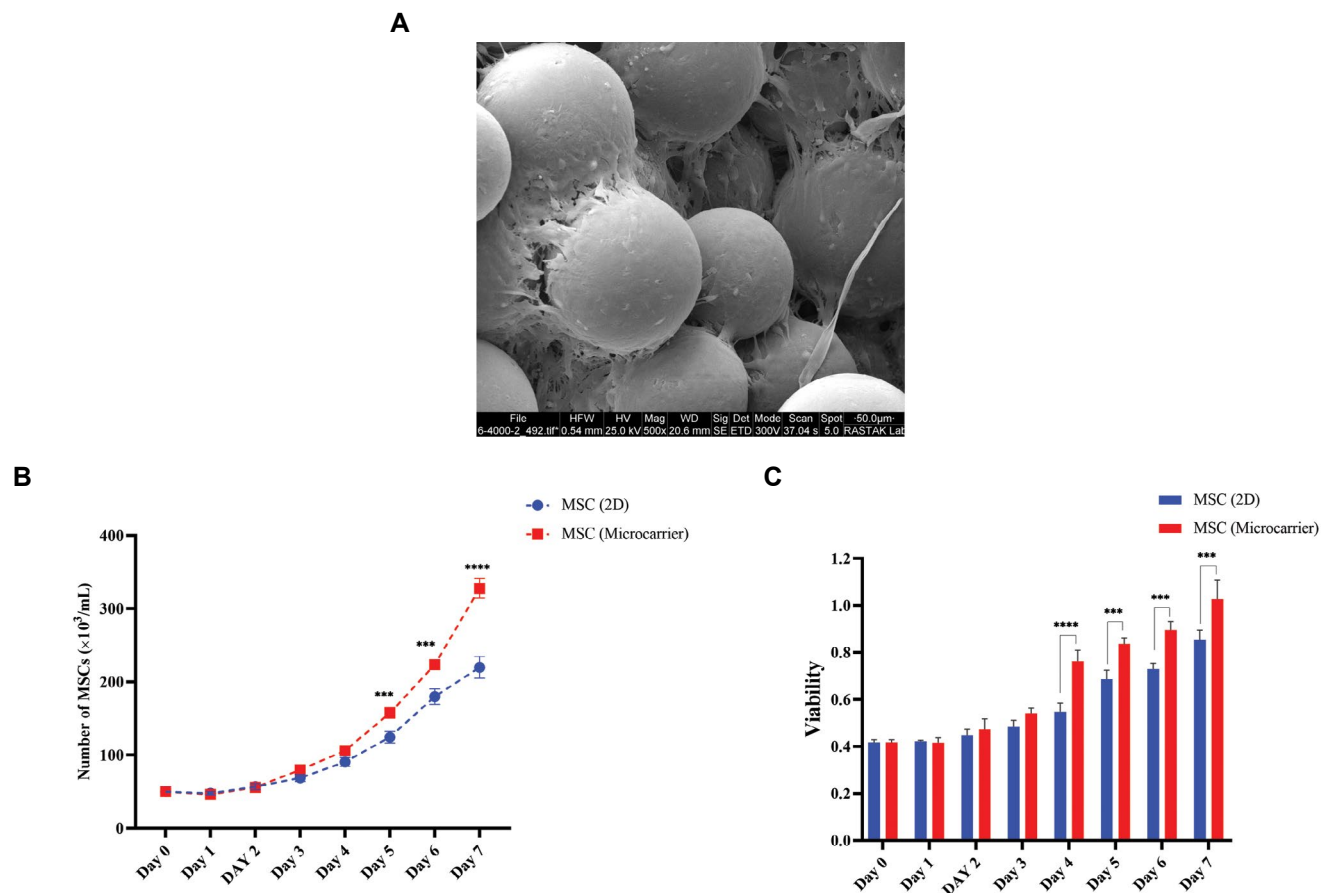

Fig.3: Attachment and expansion of UCB-MSCs on the microcarrier beads. **A.** At the 4th day of culture, Surface attachment of UCB-MSCs in a three-dimensional (3D) network, which created by microcarrier beads was explored by scanning electron microscopy (SEM). The image shows the presence of UCB-HPCs in the network created by the UCB-MSCs and microcarriers. **B.** *Ex vivo* expansion of the UCB-MSCs in a microcarrier-based culture system (red) in comparison with a monolayer culture (blue) over seven days of culture. Cell expansion was plotted as cell number/ml. **C.** Cell viability of the UCB-MSCs was examined in a microcarrier-based culture system (red) in comparison with a monolayer culture (blue) over seven days of culture. The results are presented as mean ± standard deviation (n=3, ***; P<0.001, ****; P<0.0001). UCB-MSCs; Umbilical cord blood-mesenchymal stem cells.

188

Ex vivo expansion of umbilical cord blood CD34+ cells co-cultured with UCB mesenchymal stem cells

Flow cytometry results confirmed that the purity of MACS-isolated CD34+ HSCs was at least 86% of the population of isolated cells (Fig.4A). The isolated CD34+ cells were co-cultured UCB-MSCs in the presence (B group) or absence (C group) of microcarrier beads for seven days. We also used 0.4 μm pore size transwell inserts in the D group (UCB-MSCs) and the E group (UCB-MSCs+microcarrier beads) to determine the importance of physical contact between the MSCs and CD34+ cells.

As mentioned previously, SEM imaging results showed expansion of the UCB-MSCs and UCB-CD34+ cells in a 3D environment created by the microcarriers (Fig.3A). Figure 4B shows the number of CD34+ cells that was determined by a hemocytometer-mediated cell counting. Expansion of CD34+ cells in the microcarrier-based 3D systems was significantly higher than the other co-culture groups at 7th day and reached $10.5 \pm 0.8 \times 10^5$ cells/ml. Overall, we observed a 5.5- fold increase in the number of CD34+ cells expanded in the B group, a 9.5-fold increase for the C group, a 4.3-fold increase for the D group, and a 7.6-fold increase in the number of CD34+ cells expanded in the E group at 7th day of culture (Fig.4B). The percentage of CD34+ cells in the C group was significantly higher in comparison with the other co-culture groups on 3rd and 7th days (Fig.4C). More than 65% of the cells had the CD34+ marker on the 7th day, which manifested the potential of microcarrier beads to provide a superior microenvironment for the UCB-CD34+ cell proliferation. The percentage of CD34+ cells in the D group was the lowest among the other co-culture groups. In the D category CD34+ cell rate significantly reduced on both 3rd day ($51.3 \pm 6.6\%$, $P<0.001$) and 7th day ($28 \pm 3.7\%$, $P<0.001$) in comparison with the C group. In the E group, the effect of prevention of direct cell-cell contacts on proliferation and the purity of the CD34+ cells were partly alleviated by the presence of the microcarrier beads. However, there was a significant reduction in the CD34+ cells on 3rd day ($64 \pm 7.0\%$, $P<0.01$) and 7th day ($42.6 \pm 4.1\%$, $P<0.01$).

Assessment of the multipotency and stemness of expanded CD34+ hematopoietic stem cells

Hematopoietic colony morphology that included CFU granulocytes (CFU-Gs), CFU monocytes (CFU-Ms), CFU-GMs, BFU-Es, CFU erythrocytes (CFU-E), and CFU-GEMMs after 14 days of incubation in semi-solid media are presented in Figure 5A.
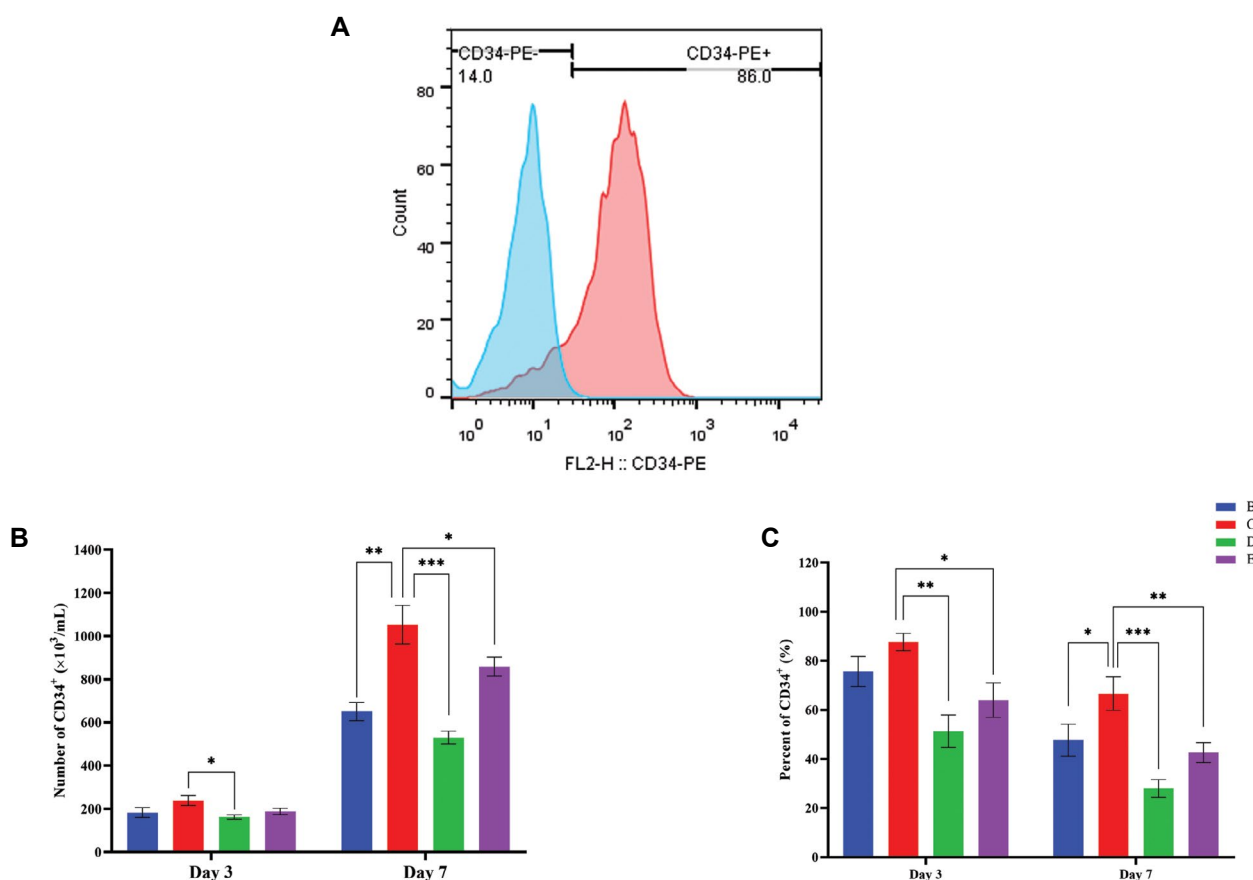

Fig.4: Evaluation of purity and expansion of the UCB-CD34+ cells. **A.** Flow cytometry data show that 86% of magnetic-activated cell-sorting (MACS) isolated cells express CD34. **B.** Hemocytometer analysis of the number of UCB hematopoietic stem cells (UCB-HPCs) cells co-cultured with UCB-MSCs on 3rd and 7th days in a different culture group. **C.** The purity of CD34+ cells in the UCB-HPCs cells co-cultured with the UCB-MSCs at 3rd and 7th days in a different culture group. We used a PE-conjugated antibody against CD34 for all sample staining procedures. The results are presented as mean ± standard deviation (n=3, ***; $P<0.001$, **; $P<0.01$, *; $P<0.05$). UCB; Umbilical cord blood, MSCs; Mesenchymal stem cells, B; CD34+ cells co-cultured with monolayer UCB-MSCs, C; CD34+ cells co-cultured with UCB-MSCs expanded on microcarrier beads, D; CD34+ cells and UCB-MSCs in non-contact co-culture using transwell inserts, and E; CD34+ cells and UCB-MSCs expanded on microcarrier beads in non-contact co-culture using transwell inserts.

There was a significant increase in the total colony number in the C category, and also the colony formation capacity of CD34+ cells reduced profoundly in the D category (Fig.5B). The number of colonies increased significantly in the C group by 85 ± 1.5 (P<0.01) on the 3rd day and 95 ± 5.6 (P<0.01) on the 7th day in comparison with the freshly isolated CD34+ cells (A group). We also separately evaluated the number of each lineage in different conditions and assessed the statistical differences (Fig.5C). We observed significantly more CFU-GEMM colonies (12.5 ± 0.07) in the C category on the 7th day in comparison with the freshly isolated UCB-CD34+ cells (5.55 ± 2, P<0.05). Interestingly, there was a decrease in the CFU-GEMM colony count in the absence of microcarrier beads in the physically separated UCB-CD34+ cells and MSCs, which was time-dependent. The C group also exhibited a significant elevation of CFU-GM colonies at 3rd day in comparison with the other co-culture groups. There were more CFU-G colony numbers (12.5 ± 2.1) in the C group in comparison with the other groups on the 7th day; however, there was no significant difference in the CFU-G colony count among our different cultural groups on the 3rd day. Similarly, the CFU-M colony count was higher in the C group in comparison with the freshly isolated CD34+ on the 7th day (8 ± 1.4 vs. 3 ± 1.4, P<0.05) and the D group (2.5 ± 0.7, P<0.05). Additionally, despite the sharp increase in the BFU-E colonies in expanded CD34+ cells, the CFU-E colonies significantly decreased over time. As shown in Figure 5C, at 7th day, the number of CFU-E colonies were significantly higher in the freshly isolated CD34+ cells in comparison with the other co-culture groups, except for the C group.

Our qRT-PCR results showed a significant increase in the RNA expression levels of CXCR4, HOXB4, and BMI1 in the C group in comparison with the other groups (Fig.6). This finding was time-dependent. Interestingly, on the 3rd day, the E group had significantly more transcript-level expression of CXCR4 than the B group. Additionally, there was a 3.6-fold (P<0.01) and 5-fold (P<0.01) increase in the HOXB4 expression level in the C group in comparison with the B group at 3rd and 7th day, respectively. The BMI1 expression increased significantly after co-culture of CD34+ cells with UCB-MSCs cultured on the microcarrier bead on the 3rd day (6.3-fold, P<0.001) and 7th day (8.9-fold, P<0.001) in comparison with the B group.
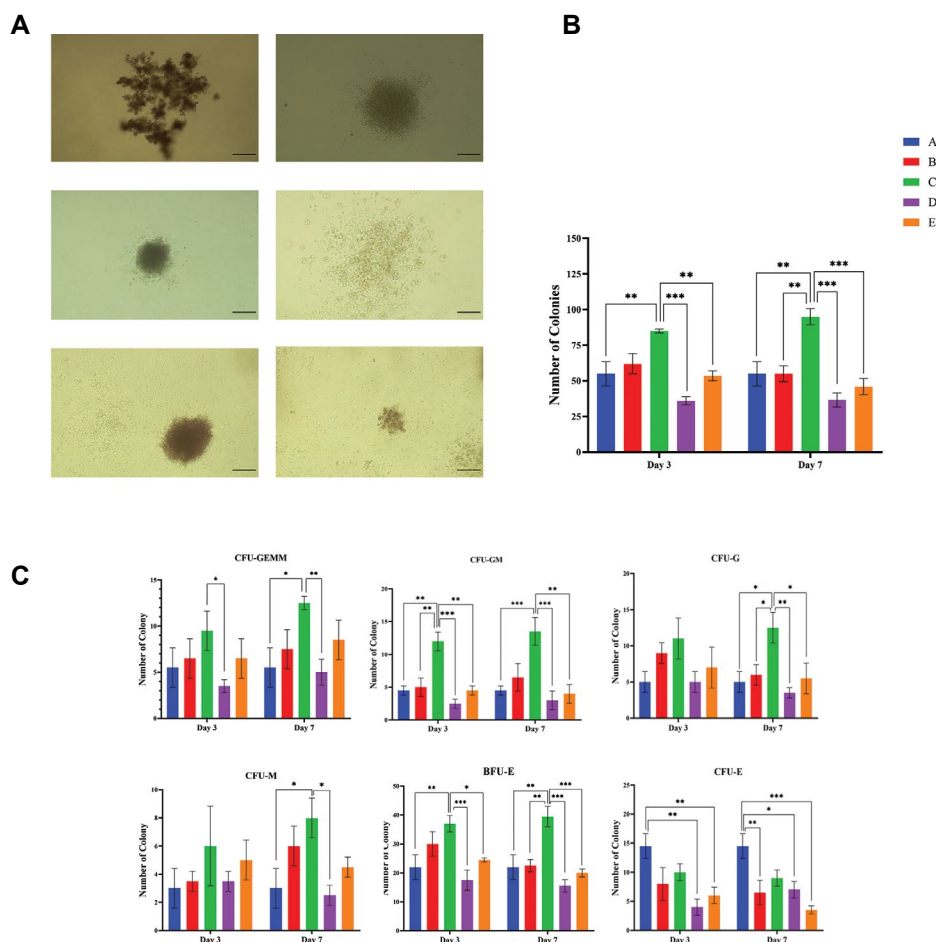

Fig.5: Colony-forming cell (CFC) assay results of UCB-CD34+ HSCs at 3rd and 7th days in different co-culture groups. **A.** Hematopoietic colony morphology after 14 days of incubation in semi-solid media. CFU-GEMM (up left); Granulocyte, erythrocyte, monocyte, megakaryocyte CFU (scale bar: 500 µm), CFU-GM (middle right); Granulocyte-monocyte CFU (scale bar: 500 µm), CFU-G (up left); CFU granulocyte (scale bar: 200 µm), CFU-M (middle right); CFU monocyte (scale bar: 500 µm), BFU-E (down left); Erythrocyte burst-forming unit (scale bar: 200 µm), CFU-E (down right); CFU erythrocyte (scale bar: 200 µm) **B.** The total colony numbers of UCB-CD34+ HSCs in different co-culture groups. **C.** A comparison of clonogenic capacities of CD34+ cells expanded under different culture conditions over 7 days. CFC assay results are presented as mean ± SD (n=3, ***; P<0.001, **; P<0.01, *; P<0.05). A; Freshly isolated UCB-CD34+ cells, B; CD34+ cells co-cultured with monolayer UCB-MSCs, C; CD34+ cells co-cultured with UCB-MSCs expanded on microcarrier beads, D; CD34+ cells and UCB-MSCs in non-contact co-culture using transwell inserts, and E; CD34+ cells and UCB-MSCs expanded on microcarrier beads in non-contact co-culture using transwell inserts.
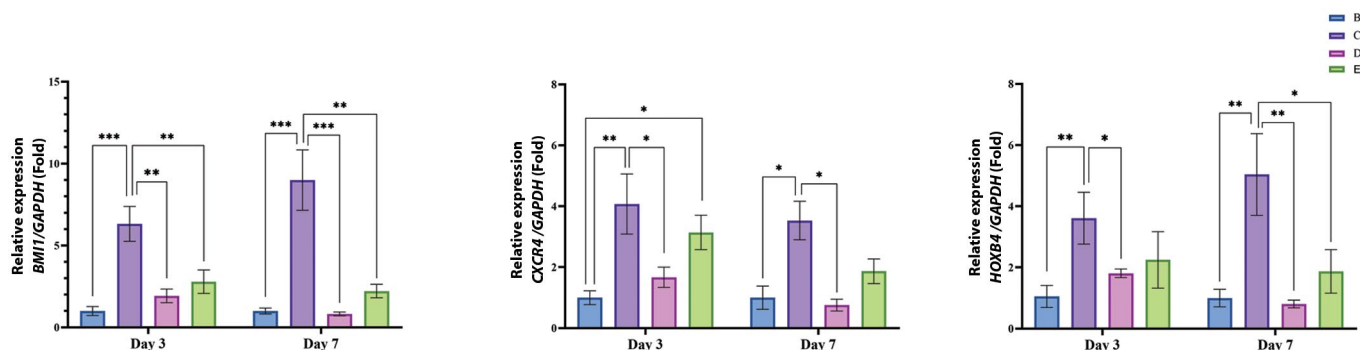
Fig.6: Mean fold changes in RNA expression levels of *CXCR4, HOXB4,* and *BMI1* genes in UCB-CD34^{+} HSCs at 3rd and 7th day in the different co-culture group by qRT-PCR. *GAPDH* transcription level was used for data normalization. Data are presented as mean ± SD (n=3, ***; P<0.001; **; P<0.01, *; P<0.05). B; CD34^{+} cells co-cultured with monolayer UCB mesenchymal stem cells (UCB-MSCs), C; CD34^{+} cells co-cultured with UCB-MSCs expanded on microcarrier beads, D; CD34^{+} cells and UCB-MSCs in non-contact co-culture using transwell inserts, E; CD34^{+} cells and UCB-MSCs expanded on microcarrier beads in non-contact co-culture using transwell inserts, UCB; Umbilical cord blood, HSCs; Hematopoietic stem cell, and qRT-PCR; Quantitative real-time polymerase chain reaction.

Discussion

Over the last 30 years ,there is a considerable increase in the UCB application for HSC transplantation. Quantitative limitation of HSCs within a single CB unit is a major obstacle for UCB transplantation, particularly in adult recipients. Different approaches have been explored to address this limitation, including use of recombinant cytokine cocktails and small molecules, as well as a co-culture with feeder stromal cells (21-23). The use of pre-expanded UCB-CD34^{+} is reported to be a safe, efficient procedure without the increased chances of graft-versus-host disease (GVHD) (23). However, studies commonly used extremely high concentrations of recombinant cytokines compared to concentrations detected *in vivo* in the BM microenvironment (24). As a result, this could impact the quality of transplanted HSCs, as the primitive progenitors responsible for the long-term BM reconstitution. In recent years, it has been shown that co-culture of HSCs with stromal cells increases their ability to expand and maintain their primitive phenotype (6, 25). It has been hypothesized that either cell-cell contact or soluble factors provided by the MSCs in *ex vivo* co-culture systems closely resemble the BM microenvironment for HSCs proliferation and maintenance (26).

Our results showed that the total number of UCB-MSCs was higher in the microcarrier-based cultures than monolayer cultures after seven days. Microcarrier beads provide a higher surface area to volume ratio compared to conventional 2D culture systems and facilitate the scalability of adherent cells, including MSCs. Different types of microcarriers have been used for fast, reliable ex vivo expansion of MSCs for further clinical applications. Eibes et al. (12) used gelatin microcarriers (Cultispher S) for *ex vivo* expansion of BM-MSCs in stirred culture systems and achieved an 8.4-fold increase in the number of expanding cells within eight days. Expansion of BM-MSCs with fibronectin-coated, plastic microcarriers in stirred spinner flasks resulted in a 2.86-fold increase in cells after six days (11). Rafiq et al. (27) determined that among a panel of 13 microcarriers for the culture of human

BM-MSCs, the Solohill microcarrier performed best with respect to cell growth in either the static or agitated conditions. They found that the collagen-coated Solohill microcarrier yielded more viable MSCs in either static and stirred culture systems. This finding was consistent with our results, where the number of viable UCB-MSCs in the microcarrier-based culture group showed a significant increase after 5 days of culture in comparison with the monolayer culture.

Our data demonstrated that co-culture with UCB-MSCs maintained the proliferation capacity of UCB-CD34^{+} cells and did not significantly change their primitive immunophenotype over higher numbers of cell divisions. UCB-CD34^{+} cells increased significantly at 7th day and reached 10.5×10^5 cells/ml (9.5-fold increase) with a purity of over 65% of the CD34^{+} marker. In a Kedong et al. (28) study, co-culture of UCB-HPCs and MSCs within a rotating wall vessel bioreactor using glass-coated styrene copolymer (GCSC) microcarriers in serum-free containing a cocktail of cytokines resulted in 3.7-fold expansion of total cell numbers in the bioreactor. Jang et al. (3) studied the ex vivo expansion capacity of UCB-MSCs as a cell feeder layer to support UCB-derived committed HPCs in the presence or absence of recombinant cytokines. Although, they found that the expansion kinetics of committed HPCs reached a plateau at seven culture days in the co-culture, there was a significant increase in the CFU numbers.

Previous studies showed that the CD34^{+} cell numbers could increase up to several-fold in some cytokine-driven cultures. However, their expansion was accompanied by concomitant differentiation and gradual loss of stemness. Kögler et al. (29) reported that the expansion and multilineage differentiation of CD34^{+} cells was significantly higher in the cells that were cultured under the support of UCB-derived unrestricted somatic stem cells (USSC) in comparison with the BM-derived MSC-supported cultures. They found that UCB-derived USSC produced soluble hematopoietic active cytokines, which appeared to be involved in the lineage-specific

differentiation and proliferation of progenitor cells.

MSCs play a crucial role in the adult BM microenvironment and hematopoiesis (30). Here, we studied the impact of cell-cell contact between UCB-CD34$^+$ cells and MSCs on the expansion of CD34$^+$ cells. Although, the physical contacts between CD34$^+$ cells co-cultured with UCB-MSCs was avoided by using 0.4 µm pore size transwell inserts, the culture media could float between the two cell types for transmission of secreted cytokines. Prevention of physical contact between CD34$^+$ cells and MSCs caused a significant decrease in the number of CD34$^+$ cells in the absence of microcarrier beads. We assumed that elevated production of soluble growth factors, by soaring numbers of UCB-MSCs in the 3D network of microcarrier beads improved CD34$^+$ cell proliferation.

As the *ex vivo* expansion may result in a change in the HSCs multipotency and self-renewal, we evaluated the in vitro differentiation capacity of the expanded UCB-CD34$^+$ cells in the microcarrier-based culture and compared them with other cell culture conditions. The colony assay results revealed an elevation in the total number of colonies in the microcarrier-based co-culture, which was reflected in the greater number of total CFUs, CFU-GEMM, and CFU-GM. This data agreed with a previous investigation of the higher ratio of CFU-GEMM/CFU-GM in the MSC and HSC co-culture in the 3D nanofiber structures, which indicated that the combination of the 3D-structure and MSCs provided a better environment for stemness preservation of HSCs (31). Similarly, UCB-HPCs maintained their multipotency in the protein-coated nano-scaffold culture system, as lineage amplification was enhanced following the co-culture with MSCs (32).

Overexpression of genes that are crucial for HSC homing, self-renewal, and maintenance such as CXCR4, HOXB4, and BMI1 in expanded UCB-CD34$^+$ cells in the microcarrier-based culture suggested a more primitive phenotype of HSCs. CXCR4 gene is a well-known receptor for stromal-derived factor-1 (SDF-1), a strong chemo-attractant for HSCs, which is crucial for homing of HSCs in the adult BM niche (33). HOXB4 gene is a member of the homeobox (Hox) family gene that promotes HSC self-renewal (34). Intracellular regulatory protein BMI1 is a key component of the polycomb repressive complex 1, that regulates the proliferation and maintenance of HSCs (20, 35). Additionally, the elevated RNA expression level of CXCR4 in the CD34$^+$ cells co-cultured with UCB-MSCs have been reported in synthetic 3D structures, and offers more efficient homing capabilities for HSCs (31, 32). Interestingly, downregulation of CXCR4, HOXB4, and *BMI1* genes in the physically separated UCB-CD34$^+$ cells and MSCs suggested that secreted growth factors alone, failed to compensate for the lack of cell-cell contact between the expanding cells.

Conclusion

The results of this study showed that UCB-MSCs could successfully scale up in the microcarrier-based culture system. Furthermore, we found that the expanded UCB-MSCs on microcarriers created a microenvironment that could mimic the niche by providing the necessary growth factors and cell-cell interactions for CD34$^+$ cells proliferation and maintenance in comparison with the 2D culture systems, which was reflected in the increased number of CD34$^+$ cells. Our data also demonstrated that the 3D microcarrier-based culture system established by the co-culture of MSCs and CD34$^+$ cells on microcarrier beads, could effectively promote HPC self-renewal and multipotency. This study suggests that a microcarrier-based culture is a possible option for a scalable culture of UCB-MSCs and CD34$^+$ cell expansion.

Acknowledgments

Authors' Contributions

S.A., A.A.; Conceived of the presented idea. S.N.M.; Carried out the experiments and wrote the manuscript under supervision of S.A. and A.A. S.K.; Advised on the project. All authors contributed to the interpretation of the results and also approved the final version of this manuscript for submission.

References

1. Li HW, Sykes M. Emerging concepts in haematopoietic cell transplantation. Nat Rev Immunol. 2012; 12(6): 403-416.
2. Danby R, Rocha V. Improving engraftment and immune reconstitution in umbilical cord blood transplantation. Front Immunol. 2014; 5: 68.
3. Jang YK, Jung DH, Jung MH, Kim DH, Yoo KH, Sung KW, et al. Mesenchymal stem cells feeder layer from human umbilical cord blood for ex vivo expanded growth and proliferation of hematopoietic progenitor cells. Ann Hematol. 2006; 85(4): 212-225.
4. Mayani H, Gutiérrez-Rodríguez M, Espinoza L, López-Chalini E, Huerta-Zepeda A, Flores E, et al. Kinetics of hematopoiesis in Dexter-type long-term cultures established from human umbilical cord blood cells. Stem Cells. 1998; 16(2): 127-135.
5. Rocha V, Gluckman E; Eurocord-Netcord registry and European Blood and Marrow Transplant group. Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. Br J Haematol. 2009; 147(2): 262-274.
6. Baron F, Ruggeri A, Nagler A. Methods of ex vivo expansion of human cord blood cells: challenges, successes and clinical implications. Expert Rev Hematol. 2016; 9(3): 297-314.
7. Köhler T, Plettig R, Wetzstein W, Schaffer B, Ordemann R, Nagels HO, et al. Defining optimum conditions for the ex vivo expansion of human umbilical cord blood cells. Influences of progenitor enrichment, interference with feeder layers, early-acting cytokines and agitation of culture vessels. Stem Cells. 1999; 17(1): 19-24.
8. Chivu M, Diaconu CC, Bleotu C, Alexiu I, Brasoveanu L, Cernescu C. The comparison of different protocols for expansion of umbilical-cord blood hematopoietic stem cells. J Cell Mol Med. 2004; 8(2): 223-231.
9. McNiece IK, Almeida-Porada G, Shpall EJ, Zanjani E. Ex vivo expanded cord blood cells provide rapid engraftment in fetal sheep but lack long-term engrafting potential. Exp Hematol. 2002; 30(6): 612-616.
10. Koh B, Sulaiman N, Fauzi MB, Law JX, Ng MH, Idrus RBH, et al. Three dimensional microcarrier system in mesenchymal stem cell culture: a systematic review. Cell Biosci. 2020; 10: 75.

11. Heathman TR, Glyn VA, Picken A, Rafiq QA, Coopman K, Nienow AW, et al. Expansion, harvest and cryopreservation of human mesenchymal stem cells in a serum-free microcarrier process. Biotechnol Bioeng. 2015; 112(8): 1696-1707.

12. Eibes G, dos Santos F, Andrade PZ, Boura JS, Abecasis MM, da Silva CL, et al. Maximizing the ex vivo expansion of human mesenchymal stem cells using a microcarrier-based stirred culture system. J Biotechnol. 2010; 146(4): 194-197.

13. Elseberg CL, Leber J, Salzig D, Wallrapp C, Kassem M, Kraume M, et al. Microcarrier-based expansion process for hMSCs with high vitality and undifferentiated characteristics. Int J Artif Organs. 2012; 35(2): 93-107.

14. Petry F, Smith JR, Leber J, Salzig D, Czermak P, Weiss ML. Manufacturing of human umbilical cord mesenchymal stromal cells on microcarriers in a dynamic system for clinical use. Stem Cells Int. 2016; 2016: 4834616.

15. Rafiq QA, Brosnan KM, Coopman K, Nienow AW, Hewitt CJ. Culture of human mesenchymal stem cells on microcarriers in a 5 l stirred-tank bioreactor. Biotechnol Lett. 2013; 35(8): 1233-1245.

16. Santos Fd, Andrade PZ, Abecasis MM, Gimble JM, Chase LG, Campbell AM, et al. Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions. Tissue Eng Part C Methods. 2011; 17(12): 1201-1210.

17. Lv D, Hu Z, Lu L, Lu H, Xu X. Three-dimensional cell culture: a powerful tool in tumor research and drug discovery. Oncol Lett. 2017; 14(6): 6999-7010.

18. Zhang Y, Khan D, Delling J, Tobiasch E. Mechanisms underlying the osteo- and adipo-differentiation of human mesenchymal stem cells. ScientificWorldJournal. 2012; 2012: 793823.

19. Nienow AW, Hewitt CJ, Heathman TRJ, Glyn VAM, Fonte GN, Hanga MP, et al. Agitation conditions for the culture and detachment of hMSCs from microcarriers in multiple bioreactor platforms. Biochem Eng J. 2016; 108: 24-29.

20. Ajami M, Soleimani M, Abroun S, Atashi A. Comparison of cord blood CD34 + stem cell expansion in coculture with mesenchymal stem cells overexpressing SDF-1 and soluble /membrane isoforms of SCF. J Cell Biochem. 2019; 120(9): 15297-15309.

21. de Lima M, McNiece I, Robinson SN, Munsell M, Eapen M, Horowitz M, et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. N Engl J Med. 2012; 367(24): 2305-2315.

22. Maung KK, Horwitz ME. Current and future perspectives on allogeneic transplantation using ex vivo expansion or manipulation of umbilical cord blood cells. Int J Hematol. 2019; 110(1): 50-58.

23. McNiece I, Kubegov D, Kerzic P, Shpall EJ, Gross S. Increased expansion and differentiation of cord blood products using a two-step expansion culture. Exp Hematol. 2000; 28(10): 1181-1186.

24. Sica RA, Terzioglu MK, Mahmud D, Mahmud N. Mechanistic basis of ex vivo umbilical cord blood stem progenitor cell expansion. Stem Cell Rev Rep. 2020; 16(4): 628-638.

25. Walenda T, Bork S, Horn P, Wein F, Saffrich R, Diehlmann A, et al. Co-culture with mesenchymal stromal cells increases proliferation and maintenance of haematopoietic progenitor cells. J Cell Mol Med. 2010; 14(1-2): 337-350.

26. Vaidya A, Kale V. Hematopoietic stem cells, their niche, and the concept of co-culture systems: a critical review. J Stem Cells. 2015; 10(1): 13-31.

27. Rafiq QA, Coopman K, Nienow AW, Hewitt CJ. Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors. Biotechnol J. 2016; 11(4): 473-486.

28. Kedong S, Xiubo F, Tianqing L, Macedo HM, LiLi J, Meiyun F, et al. Simultaneous expansion and harvest of hematopoietic stem cells and mesenchymal stem cells derived from umbilical cord blood. J Mater Sci Mater Med. 2010; 21(12): 3183-3193.

29. Kögler G, Radke TF, Lefort A, Sensken S, Fischer J, Sorg RV, et al. Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. Exp Hematol. 2005; 33(5): 573-583.

30. Fajardo-Orduña GR, Mayani H, Montesinos JJ. Hematopoietic Support capacity of mesenchymal stem cells: biology and clinical potential. Arch Med Res. 2015; 46(8): 589-596.

31. Darvish M, Payandeh Z, Soleimanifar F, Taheri B, Soleimani M, Islami M. Umbilical cord blood mesenchymal stem cells application in hematopoietic stem cells expansion on nanofiber three-dimensional scaffold. J Cell Biochem. 2019 (ahead of print).

32. Atashi A, Islami M, Mortazavi Y, Soleimani M. Homing genes expression in fucosyltransferase VI-treated umbilical cord blood CD133+ cells which expanded on protein-coated nanoscaffolds. Mol Biotechnol. 2018; 60(7): 455-467.

33. Walenkamp AME, Lapa C, Herrmann K, Wester HJ. CXCR4 ligands: the next big hit? J Nucl Med. 2017; 58 Suppl 2: 77S-82S.

34. Teichweyde N, Kasperidus L, Carotta S, Kouskoff V, Lacaud G, Horn PA, et al. HOXB4 promotes hemogenic endothelium formation without perturbing endothelial cell development. Stem Cell Reports. 2018; 10(3): 875-889.

35. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature. 2003; 423(6937): 302-305.