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# Simultaneous expansion and harvest of hematopoietic stem cells and mesenchymal stem cells derived from umbilical cord blood

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**Abstract** The simultaneous expansion and harvest of hematopoietic stem cells and mesenchymal stem cells derived from umbilical cord blood were carried out using bioreactors. The co-culture of umbilical cord blood (UCB)-derived hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) was performed within spinner flasks and a rotating wall vessel (RWV) bioreactor using glass-coated styrene copolymer (GCSC) microcarriers. The medium used was composed of serum-free IMDM containing a cocktail of SCF 15 ng·mL$^{-1}$, FL 5 ng·mL$^{-1}$, TPO 6 ng·mL$^{-1}$, IL-3 15 ng·mL$^{-1}$, G-CSF 1 ng·mL$^{-1}$ and GM-CSF 5 ng·mL$^{-1}$. Accessory stromal cells derived from normal allogeneic adipose tissue were encapsulated in alginate-chitosan (AC) beads and used as feeding cells. The quality of the harvested UCB-HSCs and MSCs was assessed by immunophenotype analysis, methylcellulose colony and multi-lineage differentiation assays. After 12 days of culture, the fold-expansion of total cell numbers, colony-forming units (CFU-C), CD34$^+$/CD45$^+$/CD105$^-$ (HSCs) cells and CD34$^-$/CD45$^-$/CD105$^+$ (MSCs) cells using the RWV bioreactor were (3.7 ± 0.3)- , (5.1 ± 1.2)- , (5.2 ± 0.4)- , and (13.9 ± 1.2)-fold respectively, significantly better than those obtained using spinner flasks. Moreover, UCB-HSCs and UCB-MSCs could be easily separated by gravity sedimentation after the co-culture period as only UCB-MSCs adhered on to the microcarriers. Simultaneously, we found that the fibroblast-like cells growing on the surface of the GCSC microcarriers could be induced and differentiated towards the osteoblastic, chondrocytic and adipocytic lineages. Phenotypically, these cells were very similarly to the MSCs derived from bone marrow positively expressing the MSCs-related markers CD13, CD44, CD73 and CD105, while negatively expressing the HSCs-related markers CD34, CD45 and HLA-DR. It was thus demonstrated that the simultaneous expansion and harvest of UCB-HSCs and UCB-MSCs is possible to be accomplished using a feasible bioreactor culture system such as the RWV bioreactor with the support of GCSC microcarriers.

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## 1 Introduction

It has been proven that the umbilical cord blood (UCB) collected from newborns is composed of at least two types of clinically valuable stem cells: the hematopoietic stem cells (HSCs) and the mesenchymal stem cells (MSCs). HSCs possess an enormous potential for clinical applications, including in gene therapy, tumor purification, bone

marrow transplantation, and especially in promoting hematopoiesis to treat cancer patients who have undergone chemo or radiotherapy [1, 2]. On the other hand, MSCs have been reported to possess the capacity to support and even improve the expansion potential of HSCs obtained from different sources—bone marrow (BM), peripheral blood (PB) and cord blood (CB)—in ex vivo conditions [3–5]. In addition, co-transplantation of both cell types has been demonstrated to accelerate the hematopoietic reconstruction in the murine system [6]. In the human body, the autologous co-engraftment of HSCs and MSCs would have similar therapeutic effects, reducing potential syndromes caused by engraftment synchronously [7]. Moreover, MSCs are often used as seed cells in cell therapies and functional carrier cells in gene therapy [8]. Thus, synchronal expansion and harvest of HSCs and MSCs from UCB would have a great significance for clinical applications.

The ex vivo co-culture of MSCs and HSCs from the same source is still at an early stage. Chen et al. [9] have successfully expanded BM-HSCs and -MSCs within a rotating wall vessel bioreactor, having harvested both stem cell types at the same time. Up to our knowledge, similar investigations for CB-derived HSCs and MSCs haven't yet been reported. The fact that the percentages of MSCs in the UCB are far lower than those in the BM is perhaps one of the real obstacles in accomplishing such co-culture process with the same rate of success.

Stromal cells, of which MSCs are an example, are known to play a pivotal role in supporting HSCs. They secrete and provide several cytokines and growth factors that promote expansion and regulate proliferation and differentiation of HSCs in vivo. Ex vivo, the co-culture of both cell types has also been proven through two different approaches: direct cell–cell contact system and indirect co-culture system. In the first case, stromal cells are exposed to an irradiation source to inactivate their expansion capacity, and are used as a layer of feeders to support HSCs, which are directly inoculated on top of the stromal feeder layer [10, 11]. Contrarily, the second pattern uses a physical separation of HSCs from the stromal cells by making use of bio-membranes, microencapsulation and microbeads, hence avoiding contamination of HSCs [12, 13]. While the use of a bio-membrane was found to be ineffective due to mass transfer limitations when used on three-dimensional (3D) dynamic systems, leading to poor co-culture outcomes, the use of micro-encapsulation and micro-beads provided an efficient means for improving the 3D dynamic cultures using bioreactors [14–16].

In order to simulate the in vivo hematopoietic microenvironment by taking into account the unique biological features of HSCs and MSCs, the co-culture system should provide both a suspension and an adhesion surface in ex vivo culture systems onto which both cell types, respectively, could proliferate. Due to their inherent characteristics, the combination of microcarrier and bioreactor technologies appears as a feasible solution to attain these properties in a single culture system. It has been demonstrated that glass coated styrene copolymer (GCSC) microcarriers present a great adhesiveness performance, and have been used in various 3D dynamic applications to support adherent cells during in vitro cultures [17]. Amongst various types of bioreactors, the spinner flask (SF) and the rotating wall vessel (RWV) bioreactor are known to be suitable for the in vitro culture of stem cells, providing a uniformly suspended culture environment with a very low shear stress [18]. In this work, we have tried to develop a novel system that, using encapsulated stromal cells, GCSC microcarriers and bioreactors (RWV bioreactor and SF), would simulate the in vivo microenvironment for the expansion and maintenance of UCB-HSCs and -MSCs simultaneously. Using this protocol, stromal cells could provide several types of stimulating and inhibitory factors that would support UCB-MNCs, partly replacing the need in the use of animal-derived serum. On the other hand, GCSC microcarriers would offer a growing and supporting surface for adherent cells while the bioreactor would provide enough space for the development of suspended cells in a uniform environment under low shear stress. Up to our knowledge, this study will be the first attempt to expand and harvest ex vivo UCB-HSCs and -MSCs synchronously by combining microcarriers and bioreactors.

## 2 Materials and methods

### 2.1 Isolation of UCB-MNCs

Nine UCB units were obtained from normal full-term deliveries after informed consent from parents in the First affiliated hospital of Dalian Medical University. MNCs were isolated from UCB by the Ficoll–Hypaque method as described before [12]. Briefly, 4 mL of Ficoll (1.077 g $mL^{-1}$) was added into a 15 mL sterile plastic centrifuge tube. Cord blood was diluted by same volume of $Ca^{+}$ and $Mg^{+}$ -free phosphate buffered saline (PBS) before gently dropped onto the surface of Ficolll within the tube (using a proportion of 1:1–2 between the Ficoll and UCB volumes). UCB-MNCs were isolated by density gradient centrifugation over Ficoll and centrifuged horizontally at 2500 rpm for 25 min at room temperature. Then MNCs at the interface were collected and washed twice with PBS, centrifuged twice at 1000 rpm for 5 min and finally resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, USA).

### 2.2 Isolation and culture of stromal cells

The stromal cells in this study were adipose-derived stem cells (ADSCs). Adipose tissue was collected from surgical patients after informed consent. Adipose tissue was digested using 0.25% trypsin solution and 0.1% collagenase solution in alternate turns, and the mononuclear cells (MNCs) were suspended in the lower layer. This procedure was repeated 2–3 times. After that, the collected cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% FBS to stop digestion, and transferred to T-flasks. Following to this, cells were put into a humidified atmosphere of 5% $CO_2$ and 20% $O_2$ at 37°C with regular medium change for later use. Cultured cells were harvested at Passage 3 and analyzed for their immunophenotype and multilineage differentiation potential in order to confirm that the cells after in vitro culture were ADSCs [19].

### 2.3 Aginate-chitosan (AC) encapsulation of ADSCs

A suspension of ADSCs was added to 1% sodium alginate solution, and mixed uniformly to attain a final concentration of $2 \times 10^5$ cells.$mL^{-1}$ ADSCs-Alginate solution was injected into 3% calcium chloride solution using a 10 mL syringe with a 5# needle that was fixed on an electrostatic generator at 3 kV. After 8 min of magnetic stirring, the diameters of the formed beads were between 1 and 1.5 mm. After that, the encapsulated beads were washed twice with PBS and then polymerized using a 1% chitosan solution for 8 min. Following to this, the beads were washed twice with PBS for later use.

### 2.4 Pretreatment of GCSC microcarrier

The desiccated GCSC microcarriers were immersed in $Ca^+$ and $Mg^+$-free PBS (50–100 mL $g^{-1}$) for at least 3 h in a siliconizing glass container, and the mixture was stirred once in a while. After this, the microcarriers were washed twice with $Ca^+$ and $Mg^+$-free PBS, and steam-sterilized at 115°C for 15 min for further experiments.

### 2.5 Static co-culture of UCB-MNCs and -MSCs

Freshly isolated UCB-MNCs were seeded into 6-well plates that were covered with GCSC microcarriers (5 mg $mL^{-1}$) at a density of $5 \times 10^5$ cells $mL^{-1}$ (4 mL per well). Afterwards, the encapsulated ADSCs-AC beads were co-cultured with UCB-MNCs (proportion of 1:5 between ADSCs and MNCs) for 9 days in a 100% humidified atmosphere of 5% $CO_2$ and 20% $O_2$ at 37°C. Each well was supplemented with IMDM containing the following purified recombinant human cytokines cocktail (Peprotech, USA): stem cell factor (SCF) 15 ng $mL^{-1}$, flt3 ligand (FL) 5 ng $mL^{-1}$, thrombopoietin (TPO) 6 ng $mL^{-1}$, interleukin 3 (IL-3) 15 ng $mL^{-1}$, granulocyte-macrophage-colony stimulating factor (GM-SCF) 5 ng $mL^{-1}$, granulocyte-colony stimulating factor (G-SCF) 1 ng $mL^{-1}$). The suspended MNCs in each well were counted every day. Around 0.2 mL of medium containing GCSC microcarriers was aspirated from the co-cultured 6-well plates at the end of 72 and 216 h, respectively, and the expanded cells were collected after treatment for 10 min with 0.125% trypsin containing 0.02% EDTA. The percentages of $CD34^+CD45^+CD105^-$ cells (HSCs) and $CD34^-CD45^-CD105^+$ cells (MSCs) within the expanded cellular population were evaluated by flow cytometry analysis. At each sample point, adherent MSCs were obtained by digesting GCSC microcarriers with 0.125% trypsin solution containing 0.02% EDTA. These were then evaluated by immunophenotypic analysis and multiple differentiation potentials. At the end of the co-culture process, UCB-HSCs and -MSCs were separated and harvested respectively by a free settling method.

### 2.6 Dynamic co-culture of UCB-MNCs and -MSCs in bioreactors

Freshly isolated UCB-MNCs were seeded into 6-well plates that were covered with GCSC microcarriers (5 mg $mL^{-1}$) at a density of $1 \times 10^7$ cells $mL^{-1}$, and the mixture was incubated for 24 h. Afterwards, the encapsulated ADSCs-AC beads were co-cultured with UCB-MNCs (proportion of 1:5 between ADSCs and MNCs), and the density of MNCs was adjusted to $5 \times 10^5$ cells $mL^{-1}$ by supplementing IMDM. The cellular suspensions were then transferred to spinner flasks (100 mL 20–30 rpm) and the RWVB (40 mL, 6 rpm) and cultured in a humidified atmosphere of 5% $CO_2$ at 37°C for 12 days. Assays of the expanded cells were performed as described above.

### 2.7 Flow cytometry analysis of UCB-HSCs and -MSCs

Flow cytometry analysis consisted of two parts for the 3 culture systems. The first one intended to analyze the immunophenotypes of the adherent cells onto the GCSC microcarriers for certifying UCB-MSCs; the second to study the percentages of HSCs and MSCs in freshly isolated UCB MNCs and expanded cells after co-culture, respectively. This study was performed using a Becton–Dickinson Flow Cytometer, using the following protocol. Cells on GCSC microcarriers were digested by 0.125% trypsin solution containing 0.02% EDTA, washed once with PBS and finally resuspended at a density of ca. $5 \times 10^5$ cells per 200μL in PBS. A sample was kept unstained as control and 7 other samples were single-stained in sequence using 5μL of each of the following

antibodies: anti-CD13-PE, anti-CD34-FITC, anti-CD44-FITC, anti-CD45-PE, anti-CD73-PE, anti-CD105-FITC and anti-HLA-DR-PE. Samples were incubated at 4°C in the dark for 30 min, washed once in PBS and finally resuspended in PBS containing 0.1% sodium azide for flow cytometry analysis.

On the other hand, the percentages of HSCs ($CD34^+CD45^+CD105^-$) and MSCs ($CD34^-CD45^-CD105^+$) pre- and post- culture were examined as follows. Cellular suspensions with GCSC microcarriers were digested according to the method previously described and washed once with PBS. After centrifugation, the resuspended cells were adjusted to a density of ca. $1 \times 10^6$ cells per 200 μL in PBS. Samples were single-stained using 5 μL of each of the following antibodies: anti-CD34-PE-cy5, anti-CD45-PE and anti-CD105-FITC. These were then incubated at 4°C in the dark for 30 min. The cells were then washed again with PBS, resuspended in 1 mL of PBS containing 0.1% sodium azide and analyzed on a FACSCalibur. At least 20,000 events were acquired per sample and analyzed using CellQuest Pro.

### 2.8 CFU-Cs assay of UCB-MNCs

Cultured MNCs within the 6-well plates, spinner flask and RWV bioreactor were harvested after 0, 72 and 216 h of culture respectively. After this, $2 \times 10^5$ harvested MNCs were resuspended in 1 mL methylcellulose medium, added to a 35 mm culture dish, and then putted in a humidified atmosphere of 5% $CO_2$ at 37°C and cultured for 12 days. The colonies consisting of 50 or more cells were counted under an inverted microscope. Culture medium was IMDM containing 0.9% methylcellulose (Sigma), 30% FBS, 1% BSA (Sigma), 50 U $mL^{-1}$ penicillin, 50 mg $mL^{-1}$ streptomycin, 2 mM L-glutamine, $10^{-4}$ M 2-mercaptoethanol (Sigma), 10 ng $mL^{-1}$ recombinant human GM-CSF (Peprotech), 50 ng $mL^{-1}$ recombinant human SCF (Peprotech), 10 ng $mL^{-1}$ recombinant human IL-3 (Peprotech) and 3U $mL^{-1}$ recombinant human EPO (Peprotech).

### 2.9 Multilineage differentiation potential for assessing UCB-MSCs

Cultured MSCs found on the GCSC microcarriers cultured in the 6-well, spinner flask and RWV bioreactor systems were harvested for multi-differentiation examination after 12 days of culture. The harvested stromal cells were plated in 24-well plates at a density of $5 \times 10^4$ cells $mL^{-1}$ (1 mL per well), and cultured in IMDM containing 10% FBS for 3 days. After this, inducing medium for the osteogenic, chondrogenic, and adipogenic lineages were added to each well according to the experimental design, and the media was changed every three days for a period of 3 weeks of culture. The expression of alkaline phosphatase (ALP) was observed in the early osteogenic induction after one week while calcified nodules were observed by staining with von-Kossa stain after 2 weeks of culture. Moreover, intracellular lipid droplets could also be observed via Oil Red staining after 2 weeks. Differentiated chondrocytes could be observed after 3 weeks of culture by staining with the fluorescent dye connexin43 [20–22].

Osteogenic-inducing medium consisted of IMDM containing 10% FBS, 50 μM ascorbate (Sigma), 10 mM $\beta$-glycerophosphate (Sigma) and 0.1 μM dexamethasone (Sigma). Medium for adipogenesis was composed of IMDM containing 10% FBS, 1 μM dexamethasone (Sigma), 0.1 mM 3-isobutyl-1- methylxanthine (IBMX) (Wako), 10 μM insulin and 0.2 μM indomethacin. And finally the chondrogenic inducer included IMDM with 10% FBS, 50 μg $mL^{-1}$ ascorbate (Sigma), 0.1 μM dexamethasone (Sigma), 10 ng $mL^{-1}$ TGF-$\beta$3, 500 ng $mL^{-1}$ bone morphogenetic protein (BMP-6), 100 μg $mL^{-1}$ sodium pyruvate and 50 mg $mL^{-1}$ ITS+.

Differentiation rates of UCB-MSCs towards adipocytes, osteoblasts, and chondrocytes were analyzed by a semi-quantitative method as follows. Cells that expressed ALP and calcified nodules were used for calculating the osteogenic differentiation rate, i.e., the percentage of ALP-expressing cells in the total population and the area of calcified nodules in respect to the total area in 10 photographs taken. In order to evaluate the percentage of the differentiation rate of chondrogenesis and adipogenesis, it was determined the percentage of cells that stained positively for toluidine blue or oil red, respectively, in respect to the total number of cells in 10 photographs taken.

### 2.10 Scanning electron microscope (SEM) observation for UCB-MSCs

MSCs-microcarrier constructs from 6-well plates, spinner flask and RWV bioreactor were fixed with 2.5% glutaraldehyde (pH 7.2–7.4) in centrifuge tubes for 3 h at room temperature, and washed 2 times with PBS. The supernatant was then removed and 1 mL of 30% ethanol was added into the tube. After 5 min, 0.4 mL of 50% ethanol was used to replace the same volume of previous ethanol. Then the gradient-elution was performed as proportion above until 100% ethanol (50/75/85/95/100/100% in water). Afterwards, tertiary butyl alcohol gradient-elution was performed the same way as previously described for ethanol gradient-elution, and finally the constructs were vacuum dried, ion sputtered and observed under a scanning electron microscope (SEM).

### 2.11 Statistical analysis

All experiments were performed in triplicate. Results are expressed as the mean ± SD. The statistical significance of differences within each group was evaluated by one-way analysis of variance (1-Way ANOVA) using the software Origin7.0 (OriginLab Corporation, USA).

## 3 Results

### 3.1 Expansion of MNCs

UCB-MNCs were co-cultured with stromal cells in spinner flasks and a RWV bioreactor both for 12 days and in 6-well plates for 9 days, respectively, with the culture medium being partly changed throughout the culture period by using a diluting method. In 6 W plate, the medium was diluted to one-third of primary density at 72 h. In spinner flask, the media were diluted to half of primary densities at 72 h and 216 h, respectively. In RWV bioreactor, the media were diluted to half of primary densities at 72 and 196 h, respectively. Figure 1a–b shows the change of cellular density and fold-expansion of UCB-MNCs with time, respectively. As shown in Fig. 1a, UCB-MNCs cultured in the static 6-well plates represented the shortest growth cycle and reached the peak of cellular density at the end of 168 h of culture. On the other hand, the growth cycles for UCB-MNCs cultured in the dynamic spinner flasks and RWV bioreactor were similar and very long, reaching the highest cellular density at the end of 240 and 216 h of culture, respectively. Figure 1b shows that UCB-MNCs cultured in 6-well plates, spinner flasks and the RWV bioreactor had a fold-expansion of (4.0 ± 0.3)- , (4.8 ± 0.4)- , and (3.7 ± 0.3)-fold, respectively.

### 3.2 Expansion of UCB-HSCs and UCB-MSCs

The percentage of $CD34^{+}CD45^{+}CD105^{-}$ cells (HSCs) and $CD34^{-}CD45^{-}CD105^{+}$ cells (MSCs) in the total MNCs population that was cultured and collected from each of the three systems described was assessed by flow cytometry and is shown in Fig. 3. Moreover, the partial fold-expansion of each targeted cell type (HSCs and MSCs) is presented in Fig. 2. As shown in Fig. 3, the percentage of HSCs within the 6-well plates initially increased and then decreased, whilst that within the spinner flasks remained unchanged and within the RWV bioreactor showed a continuous increase. On the other hand, the percentage of MSCs in the total population cultured and harvested from both the 6-well plates and the spinner flasks increased initially and then diminished, whilst that of the cells collected from the RWV bioreactor showed a continuous increase. Figure 2 shows that the highest fold-expansions obtained for the cultured UCB-HSCs in the 6-well, spinner flask and RWV bioreactor systems were (2.6 ± 0.1)-fold (after 72 h), (3.2 ± 0.2)-fold

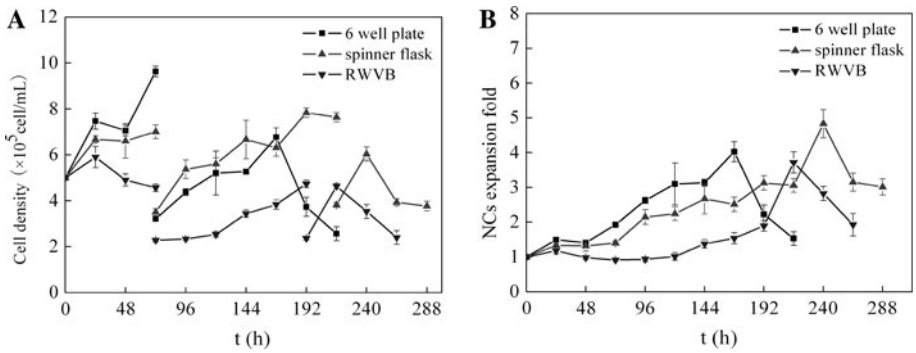


**Fig. 1** Change of cell density (**a**) and fold-expansion (**b**) of MNCs

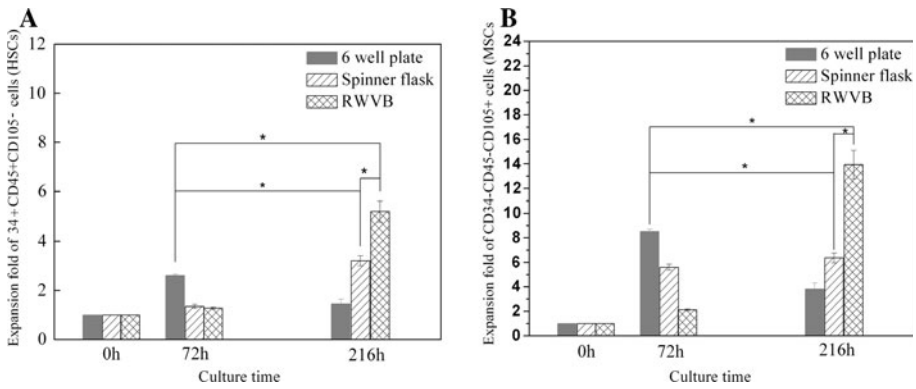


**Fig. 2** Fold-expansions of UCB derived HSCs and MSCs. (**a**) Expansion fold of HSCs ($CD34^{+}CD45^{+}CD105^{-}$); (**b**) Expansion fold of MSCs ($CD34^{-}CD45^{-}CD105^{+}$). (*$P < 0.05$)

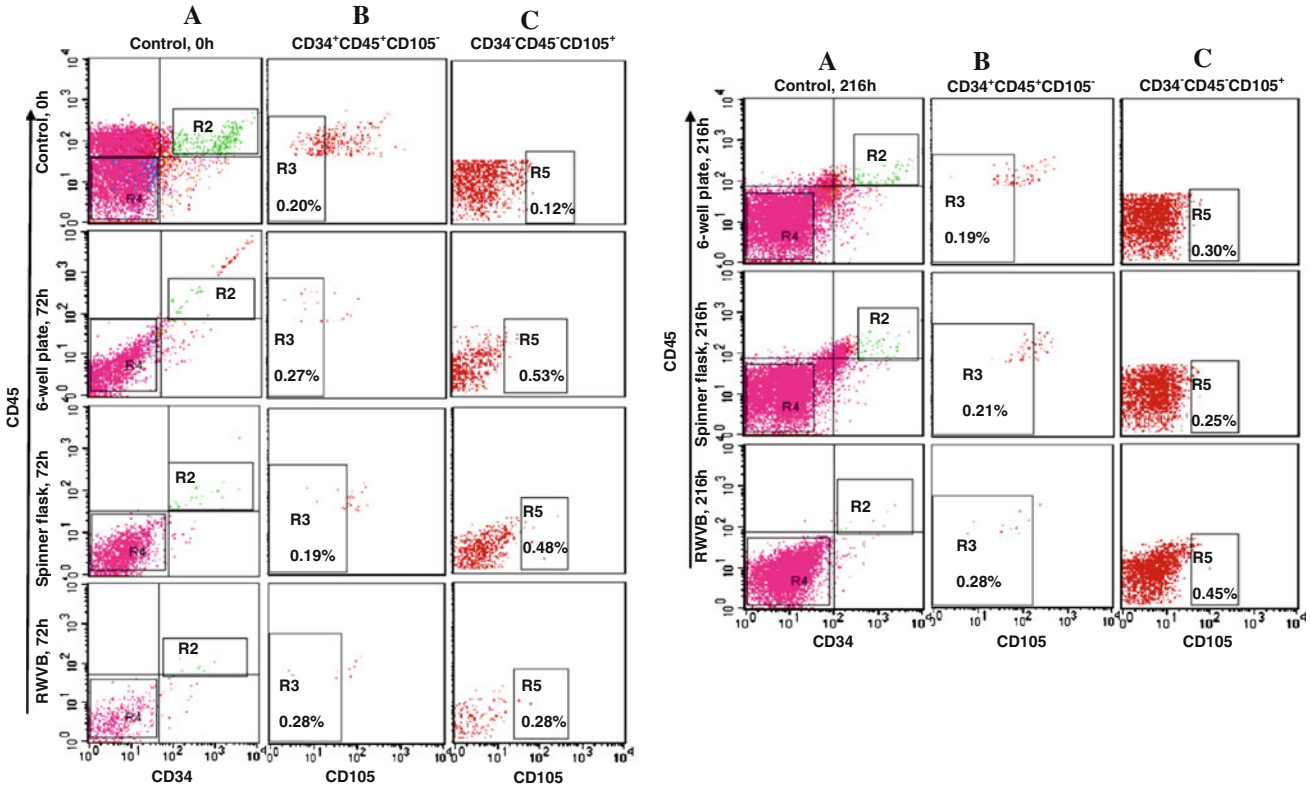


**Fig. 3** Flow cytometry analysis of UCB-derived HSCs and MSCs. **a** Negative control; **b** percentage of the sub-population $CD34^+CD45^+CD105^-$ (HSCs); **c** percentage of the sub-population $CD34^-CD45^-CD105^+$ (MSCs)

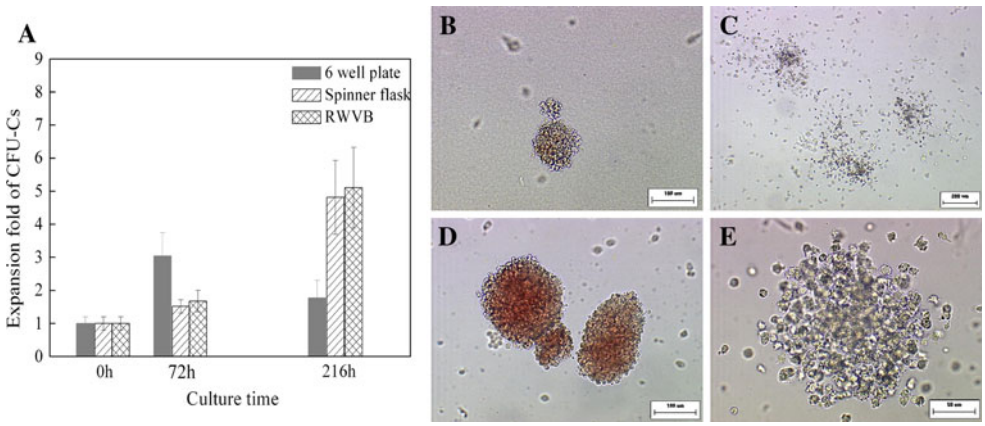


**Fig. 4** Fold-expansion and morphology of CFU-Cs. **a** Fold-expansion of CFU-Cs; **b**-**e** morphology of the observed CFU-Cs: **b** CFU-Mix's × 100, **c** CFU-GMs × 40, **d** BFU-Es × 100 and **e** CFU-GMs × 200 ($P < 0.05$)

(after 216 h), (5.2 ± 0.4)-fold (after 216 h), respectively, with statistical significant differences amongst them ($P < 0.05$). In respect to the UCB-MSCs, these were expanded in the 6-well, spinner flask and RWV bioreactor systems by (8.5 ± 0.2)-fold (after 72 h), (6.4 ± 0.4)-fold (after 216 h) and (13.9 ± 1.2)-fold (after 216 h), respectively, also with statistical significant differences amongst the three groups ($P < 0.05$). It is clear that the maximum fold-expansion obtained for both cell types, UCB-HSCs and UCB-MSCs, was using the RWV bioreactor.

### 3.3 Expansion of CFU-Cs

Figure 4 summarizes the results obtained for the CFU-Cs assays performed on the 3 culture systems after 0, 72, and 216 h of culture, respectively. The colonies of CFU-Cs those were possible to be observed included BFU-Es, CFU-GMs and CFU-Mix's as presented in Fig. 4b–d. In addition, Fig. 4a shows that the fold-expansions obtained using the 3 culture systems used were (3.0 ± 0.7)-fold (after 72 h), (4.8 ± 1.1)-fold (after 216 h) and (5.1 ± 1.2)-fold

(216 h), respectively, but there wasn't any statistically significant difference between the three groups ($P > 0.05$).

### 3.4 Cellular immunophenotypes of UCB-MSCs

The immunophenotypes of the adherent cells growing on the GCSC microcarriers collected from the three systems being investigated in this experimental work were characterized. The results obtained for the cultures in 6-well plates are presented in Fig. 5, while the expression of MSCs surface markers from cells cultured in each culture system is shown in Table 1. Cells harvested from 6-well, spinner flask and RWV bioreactor systems all strongly expressed CD13 and SH3 (CD73), while the expression of both CD44 and SH2 (CD105) was relatively weak and the absence of expression of CD34, CD45 and HLA-DR antibodies was also observed. These results are coincident with those found in literature [23, 24]. Table 2.

### 3.5 Multipotential differentiation of MSCs

The multipotential differentiation of MSCs cultured in the 6-well, spinner flask and RWV bioreactor systems is shown in Fig. 6, having been shown that the percentage of these cells that undergone differentiation after induction wasn't significantly different amongst the 3 culture systems. Approximately 90% of the cells showed ALP positive expression after being induced for 1 week, while in just 30% of them appeared mineralized nodules when induced for two weeks. In addition, extracellular matrix that stained for toluidine blue was around 90% positive after induction for 3 weeks. It was also observed that around 10% of the cellular population showed lipid droplet. It was clear that almost all cultured cells that attached onto the GCSC microcarriers showed a differentiation potential towards the osteogenesis and chondrogenesis lineages, while a minority of the cells possessed adipogenic potential. Surface marker analysis and multi-differentiation potential assays both indicated that the expanded cells found on the GCSC microcarriers collected from the 3 culture systems are in fact UCB-MSCs.

### 3.6 Morphology of UCB-MSCs-microcarrier constructs

The morphology of the UCB-MSCs that were found on the GCSC microcarriers was observed under an inverted and a scanning electron microscopes. As shown in Fig. 7, the

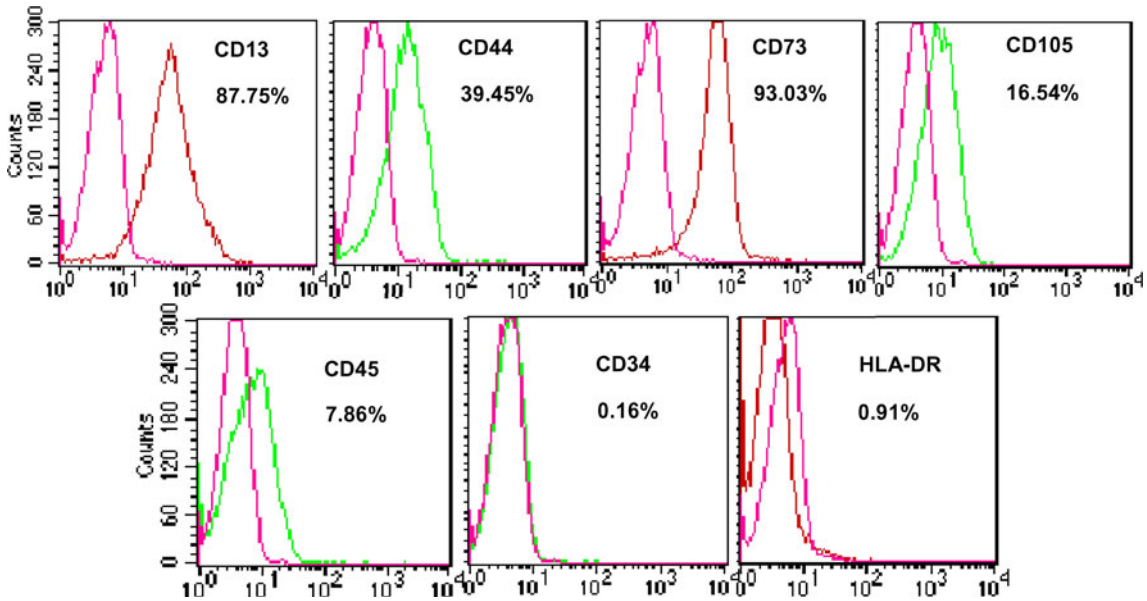


**Fig. 5** Immunophenotype analysis of the UCB-MSCs that adhered onto the GCSC microcarriers in the 6-well plates. These cells stained positively to the antigens CD13, CD73, CD44 and CD105, and negatively to HLA-DR, CD34 and CD45

**Table 1** Immunophenotyp analysis of cells adherent onto the GCSC microcarriers in the different culture systems used in the experiment

| Culture system | CD13(%) | CD34 (%) | CD44 (%) | CD45 (%) | CD73 (%) | CD105 (%) | HLA-DR(%) |
|---|---|---|---|---|---|---|---|
| 6-well plate | 87.75 | 0.16 | 39.45 | 7.86 | 93.03 | 16.54 | 0.91 |
| Spinner flask | 78.63 | 0.22 | 36.74 | 5.79 | 91.25 | 20.54 | 1.34 |
| RWV bioreactor | 86.58 | 0.12 | 45.69 | 4.55 | 88.86 | 16.52 | 2.47 |

**Table 2** Comparison of experimental results and literature results

| Source of cells | Combination of cell factors and volume($ng \cdot mL^{-1}$) | Culture medium | Stroma cells | Types of bioreactors | MNCs expansion fold | CFU-Cs expansion fold | HSCs expansion fold | MSCs expansion fold | References |
|---|---|---|---|---|---|---|---|---|---|
| UCB-MNCs | SCF 15,FL 5,TPO 6,IL-3 15,G-CSF 1,GM-CSF 5 | IMDM | AT-MSCs | Well plates | 4.0 | 3.0 | 2.6 | 8.5 | This study |
| UCB-MNCs | SCF 15,FL 5,TPO 6,IL-3 15,G-CSF 1,GM-CSF 5 | IMDM | AT-MSCs | Spinner flasks | 4.8 | 4.8 | 3.2 | 6.4 | This study |
| UCB-MNCs | SCF 15,FL 5,TPO 6,IL-3 15,G-CSF 1,GM-CSF 5 | IMDM | AT-MSCs | RWVB | 3.7 | 5.1 | 5.2 | 13.9 | This study |
| BM-MNCs | SCF 50, IL-3 10, IL-6 10,$10^{-6}$M hydrocortisone | Myelo-cult™ | None | RWVB | 9 | | 8 | 29 | [9]Chen |
| UCB-MNCs | SCF 50,FL 50,TPO 50,IL-3 25 | IMDM | Xenogenic-MSCs | RWVB | 107 | 19 | 26 | – | [26]Liu Y |
| BM-CD34$^+$ | SCF 100,FL 100,LIF 10U·$mL^{-1}$,bFGF 5 | QBSF-60 | Autogeneic-MSCs | T-flask | – | – | 4.7 | – | [27] Goncalves |
| BM-CD34$^+$ | SCF 100,FL 100,LIF 10U·$mL^{-1}$,bFGF 5 | QBSF-60 | Xenogenic -MSCs | T-flask | – | – | 0.8 | – | [27] Goncalves |
| UCB-CD34$^+$ | SCF 50,FL 50,IL-6 10 | X-VIVO-20 | None | Well plates | 3 | 2 | 1 | – | [28]Yildirim |
| UCB-CD34$^+$ | SCF 50,FL 50,IL-6 10 | X-VIVO-20 | Endotheliocyte | Well plates | 55 | 19 | 15 | – | [28] Yildirim |
| UCB-CD34$^+$ | SCF 50,FL 50,IL-6 10,VEGF 50 ,IL-1 10 | X-VIVO-20 | None | Well plates | 10 | 4 | 3 | – | [28] Yildirim |
| UCB-CD34$^+$ | SCF 50,FL 50,IL-6 10,VEGF 50 ,IL-1 10 | X-VIVO-20 | Endotheliocyte | Well plates | 77 | 32 | 23 | – | [28] Yildirim |

*BM* bone marrow, *AT* adipose tissue

shapes of the UCB-MSCs were short fusiform or round. Moreover, MSCs adhered more tightly to the surface of the microcarriers in the 6-well plates than in the spinner flasks or the RWV bioreactor. Due to shearing forces and cellular disruption caused by the collision of microcarriers within the spinner flask and the RWV bioreactor, cells were separated from the microcarriers and have grown in suspension.

## 4 Discussion

In this study, we have investigated the feasibility of co-culturing UCB-HSCs and -MSCs in static 6-well plates and dynamic spinner flasks and a RWV bioreactor in the presence of low doses of growth factors and stromal cells in a serum-free environment. The results showed that there was no significant differences in the fold-expansion of MNCs using any of the three culture systems, whilst the fold-expansion of HSCs ($CD34^+CD45^+CD105^-$) and MSCs ($CD34^-CD45^-CD105^+$) was significantly different ($P < 0.05$). The numbers of HSCs cultured in the 6-well plates initially increased and then decreased during the culture period, while the growth behavior of these cells within the dynamic environment of the spinner flask and the RWV bioreactor remained unchanged and always increased, respectively. When HSCs and MSCs were co-cultured and expanded together in the RWV bioreactor the resulting fold-expansion at the end of the culture period was ($5.21 \pm 0.43$)- and ($13.9 \pm 1.2$)-fold, for each cell type respectively, with both values statistically higher than those obtained for when using the spinner flasks or the 6-well plates. Detection by cellular surface protein markers and induced potential for multi-differentiation has demonstrated the cells adhering onto the GCSC microcarriers were in fact the UCB-MSC.

Animal-derived serum is a commonly established practice in conventional cell cultures [25]. However, even though it does supply indispensable nutrients and important proteins to the cells, it is unsuitable for cell cultures intended for clinic use due to its unknown and non-standardized composition and potential viral contamination. Thus, research towards the in vitro expansion of HSCs has been targeting methods to overcome the use of animal-derived serum. In order to examine the potential of the method described in this study for the expansion of HSCs and MSCs under serum-free conditions, a comparison with the currently established protocols has also been performed. As shown in tab. 2, HSCs and MSCs harvested from the bone marrow and cultured in a RWV bioreactor have been shown to have a greater expansion capacity than those used in this study, which have been harvested from cord-blood, a source known to possess a greater expansion

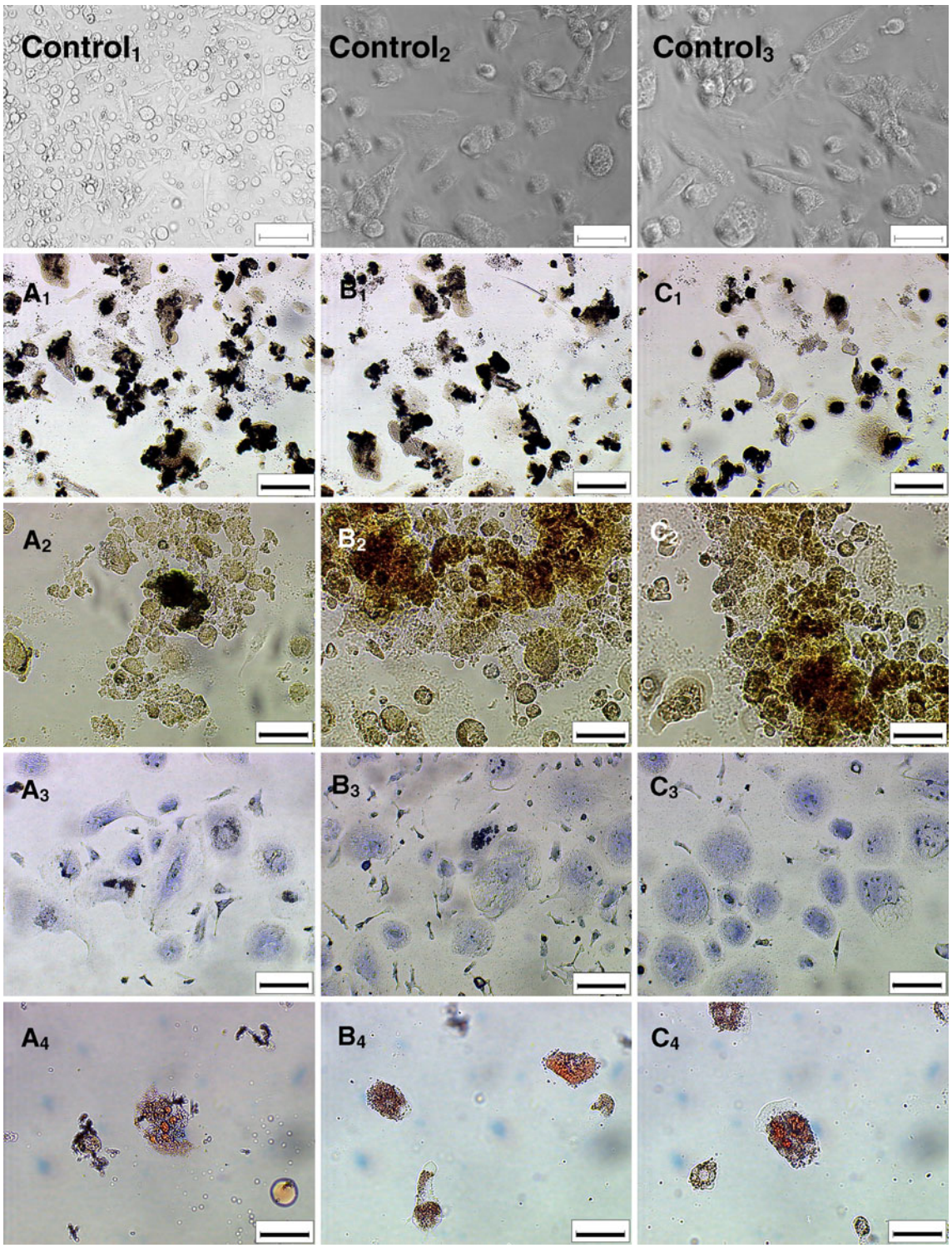


**Fig. 6** Multi-lineage differentiation potential of the UCB-MSCs that adhered onto the GCSC microcarriers. $Control_1$: primary culture of UCB-MSCs, $Control_{2\&3}$: Subculture of UCB-MSCs; $Control_{1–3}$: × 100. 6-well plates (**a**), spinner flask (**b**) and RWVB (**c**), respectively. 1: ALP staining for osteogenic differentiation (1st week); 2: von-Kossa staining for osteogenic differentiation (2nd week); 3: toluidine blue staining for chondrogenic differentiation (3rd week); 4: oil red staining for adipogenic differentiation (2nd week). $\mathbf{a_1}$–$\mathbf{c_4}$: × 100

potential. This observation can be partly attributed to the higher doses of growth factors [9] and different cell sources used. Content of MSCs in the harvested marrow during the referred study was about (2–5) MSCs/$10^6$ MNCs, while that in UCB used was only (0.05–2.8) MSCs/$10^6$ MNCs. Taking into account the great influence of the cell seeding

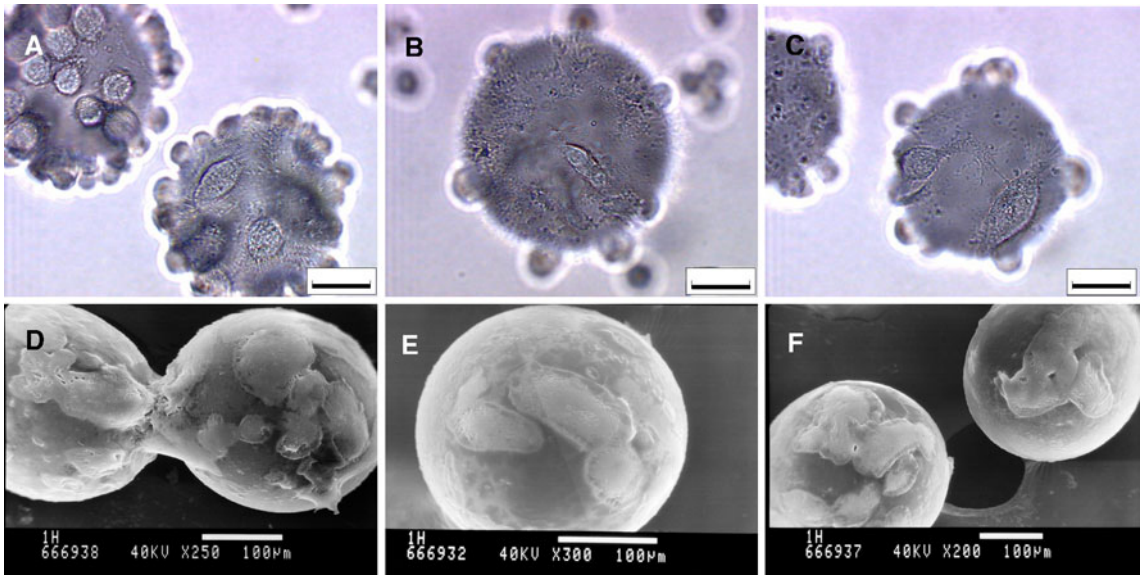


**Fig. 7** Cellular morphology of MSCs-microcarrier constructs. **a**–**c**: under inverted microscope (*bright field*) at day 12; **d**–**f**: under SEM at day 12; **a** and **d** are from cultures in the 6-well plates, **b** and **e** in the spinner flasks and **c** and **f** in the RWV bioreactor

density towards the expansion potential of the cellular population itself, the fewer MSCs used in our experiments have definitely influenced the outcome, increasing the difficulty for a successful in vitro culture that is inherent to the limited cell numbers obtained from the source. In addition, research from Liu et al. [26] has shown that UCB-HSCs cultured in a RWV bioreactor could be successfully expanded using a cocktail composed of high doses of cell factors and stromal cells. Similarly, Yildirim et al. have also achieved a great expansion of HSCs under similar conditions but under the static environment of culture plates [27]. Hence, the low doses of cell factors used in our study are probably the cause for the lower expansion results found in comparison with others reported in literature.

Furthermore, we used encapsulated ADSCs in AC microbeads as feeder cells to secrete growth factors and cell factors into the culture, replacing the conventional protocols that use serum and/or exogenously added cell factors. Comparing to the study of Liu et al. [26], in which the total nucleated cells, $CD34^{+}$ cells, and CFU-GM expanded more than 430- , 30- , and 20-fold, respectively, in a RWV bioreactor using serum and high doses of cell factors, which has been demonstrated that stromal cells couldn't completely replace serum or cell factors.

The fold-expansion results obtained in our study using the RWV bioreactor were clearly better than those obtained using either the static 6-well plates or the stirred spinner flasks. This is mainly due to the capacity of the RWV bioreactor to provide a 3-dimensional dynamic and suspension culture environment under low shear stress. It has also not only allowed reducing concentration gradients of nutrients, oxygen and metabolic waste, but also mimicked well the in vivo hematopoietic microenvironment. The spinner flask could also provide this 3-dimensional culture environment, but the shear stress produced by the impeller was larger than that observed in the RWV bioreactor, leading to a higher disruption of the sensible microenvironment and thus promoting lower expansion capabilities to the cultured cells.

Up to now, HSCs can be used in hematopoietic reconstruction, gene therapy, tumor purification and immunological therapy [2, 29]. Importantly, hematopoietic stem cells transplantation (HSCT) has been used as a method for hematopoietic reconstruction in post-radiotherapy cancer patients. Moreover, due to their high self-renewal and multipotential capacities, MSCs can be used as seed cells for cellular therapy and cell carriers for gene therapy [8]. In addition to the applications mentioned, the combination of HSCs and MSCs has also been gradually used for clinical therapies. During the process of HSCT, combined transplantation of HSCs and MSCs could not only promote the hematopoietic reconstruction, but also effectively reduced the disease incidence usually accompanying HSCT [7]. Based on extensive and in fact brilliant applications, we have thus investigated the feasibility of simultaneously expanding and harvesting UCB-HSCs and UCB-MSCs in this study. Obviously, our work is quite fundamental and there is still the need for further research to make the proposed protocol suitable for clinical trials, such as the types or dosages of microcarriers and cell factors that are optimally required.

## 5 Conclusion

In short, UCB-HSCs and UCB-MSCs can be successfully expanded and harvested simultaneously in a rotating wall vessel bioreactor in the presence of low doses of cell factors (SCF 15 ng $mL^{-1}$, FL 5 ng $mL^{-1}$, TPO 6 ng $mL^{-1}$, IL-3 15 ng $mL^{-1}$, G-CSF 1 ng $mL^{-1}$, GM-CSF 5 ng $mL^{-1}$) when using microencapsulated feeder cells and GCSC microcarriers.

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