Co-culture of mesenchymal stem cell spheres with hematopoietic stem cells under hypoxia: a cost-effective method to maintain self-renewal and homing marker expression

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Abstract

Background  Hematopoietic stem cell (HSC) transplantation is considered a possible treatment option capable of curing various diseases. The aim of this study was the co-culturing of mesenchymal stem cell (MSC) spheres with HSCs under hypoxic condition to enhance the proliferation, self-renewal, stemness, and homing capacities of HSCs.

Methods and results  HSCs were expanded after being subjected to different conditions including cytokines without feeder (Cyto), co-culturing with adherent MSCs (MSC), co-culturing with adherent MSCs + hypoxia (MSC + Hyp), co-culturing with MSCs spheres (Sph-MSC), co-culturing with MSCs spheres + hypoxia (Sph-MSC + Hyp), co-culturing with MSC spheres + cytokines (Sph-MSC + Cyto). After 10 days, total nucleated cell (TNC) and CD34+/CD38− cell counts, colony-forming unit assay (CFU), long-term culture initiating cell (LTC-IC), the expression of endothelial protein C receptor (EPCR), nucleostemin (NS), nuclear factor I/X (Nfix) CXCR4, and VLA-4 were evaluated. The TNC, CD34+/CD38− cell count, CFU, and LTC-IC were higher in the Sph-MSC + Hyp and Sph-MSC + Cyto groups as compared with those of the MSC + Hyp group (P < 0.001). The expanded HSCs co-cultured with MSC spheres in combination with hypoxia expressed more EPCR, CXCR4, VLA-4, NS, and Nfix mRNA. The protein expression was also more up-regulated in the Sph-MSC + Cyto and Sph-MSC + Hyp groups.

Conclusion  Co-culturing HSCs with MSC spheres under hypoxic condition not only leads to higher cellular yield but also increases the expression of self-renewal and homing genes. Therefore, we suggest this approach as a simple and non-expensive strategy that might improve the transplantation efficiency of HSCs.

Keywords  Mesenchymal stem cell · Sphere · Hematopoietic stem cell · Co-culture · Hypoxia · Endothelial protein C receptor
Introduction

Hematopoietic stem cells (HSCs) are a heterogeneous population of stem cells that have stemness and self-renewal capability and can differentiate to all blood cell lineages [1, 2]. Based on their self-renewal potentialities, HSCs are mainly divided into two types, long-term HSCs and short-term HSCs [2, 3]. The transplantation of HSCs is considered a curative and/or supportive treatment for several hematologic and non-hematologic diseases [4, 5]. However, transplantation of short-term HSCs instead of long-term HSCs, and the poor homing rate are known as main obstacles of HSC transplantation [6–8].

So far, various studies have been conducted to address some of the mentioned obstacles [2, 9]. In vitro methods use different cytokine cocktails to expand HSCs, which might be an expensive approach. In recent decades, co-culturing mesenchymal stem cells (MSCs) with HSCs has been employed for harvesting more numbers of HSCs [10, 11].

MSCs are potent valuable cells that can be isolated from bone marrow, umbilical cord blood, umbilical cord tissue and, etc. [10–12]. MSCs and their derivatives are used in gene therapy and cell therapy for treating a variety of diseases [13–15]. On the other hand, MSCs have the potentiality to secrete stem cell factor (SCF), granulocyte monocyte colony-stimulating factor (GM-CSF), thrombopoietin (TPO), angiopoietins (Angs), Fms-related tyrosine kinase 3-Ligand (Flt3-L), and wingless-related integration site (Wnts) that are involved in HSCs proliferation, self-renewal, and stemness [3, 16–19]. MSC-secreted SCF has positive effects on the proliferation, survival, and self-renewality of HSCs [3]. Angs enhance the symmetric division of HSCs and preserve their stemness capacity [16, 20]. Wnts and their related signaling pathways play critical roles in the fate of stem cells including HSCs [21, 22]. Some of the applied methods for enhancing the expansion of HSCs are high-tech but they are costly as well.

According to our present study, reinforcement of MSC paracrine effects through the implementation of co-culturing MSCs with HSCs seems a feasible strategy to reach more numbers of potent engraftable cells. Culturing in the non-adherent phase that allows for the formation of spheres is one of the practical methods utilized for improving the differentiation and secretory capacities of MSCs [12, 17, 23, 24]. Suspension cultivation of MSCs somehow primes them to show more supportive effects on HSCs [25, 26]. Monolayer culturing of MSCs leads to diminished proliferation and secretory potentialities along with induced cellular senescence [27]. Moreover, according to in vitro studies, expanding HSCs on the MSC-feeder layer in combination with culturing under hypoxia conditions results in an increased number of CD34+ cells, enhanced clonogenicity, and improved homing and migration capacities [28, 29].

Based on our previous experience, this study aims to expand HSCs in a co-culturing system containing MSC spheres under hypoxia condition for improving their self-renewality and homing capacities. Our results demonstrated that co-culturing MSCs spheres with HSCs under hypoxic condition increases the number of HSCs, results in an increased colony-forming unit assay (CFU-assay) and long-term culture initiating cell (LTC-IC) capacities and expressed more EPCR, CXCR4, VLA-4, NS, and Nfix mRNA.

Materials and methods

Human samples

After filling consent form, umbilical cord samples were transferred from Milad Hospital to Iranian Blood Transfusion Organization (IBTO) cell culture laboratory immediately. Umbilical cord blood samples were obtained from public cord blood bank unit after defined processing and before freezing.

Isolation of Wharton jelly (WJ)-MSC spheres and evaluation of their growth factor secretion capability.

WJ-MSCs were subjected to suspension culturing conditions to form WJ-MSC spheres as described in our previous study. WJ is gelatinous mucous tissue of the umbilical cord containing different cellular and non-cellular components [12]. Briefly, the WJ-MSCs were separated from fresh umbilical cord samples after the necessary consent forms were filed by parents. The WJ-MSCs were seeded in poly-HEMA-coated plates and incubated under hypoxic condition containing 5% O₂ (New Brunswick, Germany) to support the forming of spheres. Next, the formed spheres were cultured in serum-free media for 48 h. The collected supernatant was concentrated using Vivaspin® 500 (Sartorius, Germany) with centrifugal force. Concentrations of growth factors including Angs, Wnts, SCF, and Flt3-L were measured using ELISA method (MyBioSource, USA) according to the manufacturer’s protocol.

Isolation and cultivation of HSCs

After the necessary consent forms were filed, HSCs were isolated from fresh umbilical cord blood using Ficoll gradient protocol as described previously [30]. CD34+ cells were positively selected using magnetic-activated cell sorting (MACS) according to the kit instructions (Miltenyi Biotec, Germany). The purity of CD34+ cells and their CD38, CD90, and CD45RA expression levels were analyzed using
flow cytometry (Life Technologies Attune NxTm, USA). The used antibodies and fluorochromes are as follows: CD34 APC/PE, CD38 FITC, CD45RA PE, and CD90 FITC (BD, Biosciences, Germany). The cells were mixed with serum-free stem span media (Stem Cell Technologies, USA) containing a cytokine cocktail (Flt3-L, TPO, and SCF) and then were seeded on culture plates. The culture medium was refreshed twice per week. This group of HSCs (expanded in cytokines without feeder layer condition) was named “Cyto”.

Co-culturing HSCs with adherent MSCs or MSC spheres under hypoxia or normoxia

MSC spheres were transferred into culture plates and the mixture of HSCs with stem span medium containing cytokines (named Sph-MSC + Cyto) or without cytokines (named Sph-MSC) were added to the plates. Co-culturing HSCs with adherent monolayer MSCs (named MSC) was considered as another experimental group. All of the mentioned culture plates were incubated at 37 °C for 10 days (20% O2 and 5% CO2). The plates containing MSC spheres were transferred into culture plates and incubated for 10 days. The plates containing MSC spheres with HSCs without cytokine addition and the plates containing adherent MSC with HSCs incubated under reduced O2 pressure (5% O2) to induce hypoxia (named Sph-MSC + Hyp and MSC + Hyp, respectively) were considered as other experimental groups. The culture medium was exchanged twice per week. After 10 days, MSC spheres were separated from HSCs using 30 μm nylon mesh. Then the number of CD34+/CD38− cells was also calculated based on the TNC counting and their defined percentage by flow cytometry. The fold change in TNC and CD34+/CD38− cell number was calculated by comparing with the initial number of the seeded cells.

CFU-assay

On the 10th day of the experiment, the expanded cells of Cyto, MSC, MSC + Hyp, Sph-MSC, Sph-MSC + Hyp, and Sph-MSC + Cyto groups were cultivated in Iscove’s MDM medium (Sigma, Germany) containing 1% methylcellulose, and they were incubated for 14 days. Clusters containing 50 or more cells were counted to calculate CFU capacity. Granulocyte macrophage-colony forming units (CFU-GM), erythroid burst/colony-forming units (BFU-E/CFU-E), and granulocyte erythrocyte monocyte megakaryocyte-colony forming units (CFU-GEMM) were scored and counted in triplicate by laboratory experts. The CFU fold change of expanded cells was estimated by comparing it with the CFU of fresh unexpanded cells.

LTC-IC-assay

LTC-IC assay was conducted on an irradiated feeder. The expanded CD34+ cells were seeded in MyelocultMyeloM5300 (Stem Cell Technologies, USA) and the medium was changed twice a week. After 6 weeks, the viability of harvested cells was determined using Trypan blue exclusion dye, and two thousand cells were subjected to CFU-assay according to the previously mentioned procedure. The enumerated colonies were considered as LTC-IC. The LTC-IC fold change of the expanded cells was estimated in comparison to the LTC-IC of fresh unexpanded cells.

Real-time reverse transcription polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to measure the expression levels of NS and Nfix as self-renewal genes as well as CXCR4 and VLA-4 as homing genes using Syber green dye. Briefly, 6 μl of Syber green master mix (Takara, Japan), 1 μl of synthesized cDNA (BIONEER, South Korea), and 10 μmol/l of specific primers were mixed and the total volume was adjusted to 12 μl. The primer sequences were as follows: CXCR4 F: 5′-CTATTGAAACCCCATCCTGCT-3′, R: 5′-TCCACGATGAACTGCTTCTTT-3′, VLA-4 F: 5′-ATGTTGCGATGTTCTACTG-3′, R: 5′-AGGCTTCCACTAAATATGAG-3′, NS F: 5′-CTGTATGCTTGAGTTGGATGC-3′, R: 5′-CATGTACAGTTGAAGGC-3′, Nfix F: 5′-CATTTCTGTCCCGTTCCTG-3′, R: 5′-TCCCCTGAAAGCATCAC-3′ and B-actin F: 5′-GATACC GCAAATGTGACAGC-3′, R: 5′-GGGCTCAAGGAACA GTCTT-3′. RT-qPCR was performed using the LightCycler® 96 System (Roche, Bavaria, Germany) in triplicate. PCR conditions were as follows: initial denaturation at 95 °C for 15 min followed by 40 cycles of 20 s at 95 °C, 60 s at 60 °C for VLA-4, NS, Nfix, and β-actin, and 63 °C for CXCR4. The specificity of PCR amplification was checked by identity of all samples peaks in the PCR melting curve. The relative expression of the mentioned genes in comparison with the expression of β-actin was determined using the 2^(−ΔΔCT) method [31–34].

Immunophenotyping for EPCR, CXCR4, and VLA-4 expression

After 10 days of HSCs expansion, the expression of EPCR, CXCR4, and VLA-4 was assayed using flow cytometer (Life Technologies Attune NxTm, USA). The cells were incubated and stained with an optimized amount of related antibodies at room temperature for 30 min in the dark. Then, the cells were checked by the flow cytometer instrument.
Western blot analysis

Total protein of expanded HSCs was extracted using lysis buffer (Roche, Germany). After electrophoresis of the protein samples on 12% SDS-polyacrylamide gel, they were transblotted onto polyvinylidene fluoride membrane (Roche, Germany) using semi-dry blotter (Bio-Rad, USA) in an optimized voltage for an appropriate duration. Then, the membranes were incubated with anti-NS (Abcam, UK), anti-Nfix (Merck, Germany) and anti-β-actin (Sigma, USA) antibodies at optimized conditions (for anti-NS antibody: 1:3000 dilution, 3 h at room temperature with shaking, for anti-Nfix antibody: 1:5000 dilution, 3 h at room temperature with shaking, and for anti-β-actin antibody: 1:2000, 2 h at room temperature with shaking). After immersing the membranes in the diluted (1: 1000) secondary antibody (Abcam, UK), ECL substrate solution was poured on the membranes. Furthermore, images were captured using a gel doc imager (Bio-Rad, USA) and the protein expression levels were semi-quantified using Image Lab software.

Statistical analysis

Parametric ANOVA test was used for data analysis and the P-value of P < 0.05 was considered significant. The experiments were carried out in duplicate in three independent experiments.

Results

WJ-MSCs form spheres with higher SCF, Wnts, Angs, and Flt3-L secretion ability under hypoxic condition

WJ-MSCs were isolated successfully in both adherent (Fig. 1Sa) and non-adherent spheroid form (Fig. 1Sb). As shown in Fig. 1c, cultivation of WJ-MSCs in poly HEMA-coated plates for suspension culturing under hypoxic condition enhanced their paracrine effect especially in the case of Flt-3 (P < 0.001). The concentrations of SCF, Wnts, and Angs were more in the conditioned medium of MSC spheres in comparison to those of the conditioned medium of adherent MSCs (P < 0.001) (Fig. 1Sc).

HSCs showed higher proliferation rate while co-cultured with MSC spheres under hypoxic condition.

MACS-separated cells expressed CD34/CD90, however, did not express CD38/CD45RA.

In other words, MACS-separated CD34 cells expressed CD90 (Fig. 1aII) while these cells did not express CD38 and CD45RA (Fig. 1aIII).

These cells expressed very a low level of EPCR (Fig. 1aIV). HSCs were expanded in different conditions including cytokines without feeder (Cyto), co-culturing with adherent MSCs (MSC), co-culturing with adherent MSCs + hypoxia (MSC + Hyp), co-culturing with MSCs spheres (Sph-MSC), co-culturing with MSCs spheres + hypoxia (Sph-MSC + Hyp), and co-culturing with MSC spheres + cytokines (Sph-MSC + Cyto). Figure 1b and c represent MSC + Hyp and Sph-MSC + Hyp groups after 10 days, respectively.

According to the cell enumeration results, the count and fold increase of TNC along with CD34+/CD38− cells were significantly higher (P < 0.001) in Sph-MSC + Hyp and Sphe-MSC + Cyto than those of the MSC and MSC + Hyp groups co-cultured with adherent MSCs (Table 1S). The fold expansion average of TNC for Cyto, MSC, MSC + Hyp, Sph-MSC, Sph-MSC + Hyp, and Sph-MSC + Cyto groups were 32.8 ± 2.9, 27.9 ± 1.1, 32.95 ± 2.7, 47.9 ± 2.7, 54.3 ± 3.3, and 59.1 ± 2.9, respectively. We observed a 48.9 ± 2.5-fold increase in CD34+/CD38− cells in the Sph-MSC + Hyp group. The mentioned parameters did not significantly differ in the Sph-MSC + Cyto group as compared to the Sph-MSC + Hyp group (Table 1S). The fold change of TNC and CD34+/CD38− cells was calculated in comparison with the initial number of seeded cells.

HSCs co-cultured with MSC spheres showed more enhanced CFU and LTC-IC capacities than those of HSCs co-cultured with adherent MSCs

CFU assay and LTC-IC were performed to evaluate the clonogenicity of HSCs. According to the results presented in Fig. 2aI–III, the expanded cells could form CFU-GM, BFU-E/CFU-E, and CFU-GEMM. We observed more clonogenic capacity in Sph-MSC + Hyp and Sph-MSC + Cyto in comparison to that of the MSC + Hyp group (P < 0.001) (Fig. 2b). The CFU fold increase numbers in Sph-MSC + Hyp vs. MSC + Hyp were determined as follows: a 1.9-fold increase in total CFU, a 1.7-fold increase of CFU-GM, a 1.6-fold increase of BFU-E/CFU-E, and a 2.1-fold increase of CFU-GEMM. The fold change of different colonies in Sph-MSC + Cyto was not meaningfully different from that of Sph-MSC + Hyp except for CFU-GEMM (P < 0.05) (Fig. 2b).

The results of the LTC-IC assay indicated a higher fold increase in long-term HSCs co-cultured with MSC spheres both with or without the addition of cytokines (P < 0.01). On the other hand, Sph-MSC + Cyto and Sph-MSC + Hyp had more improvement in LTC-IC capacity in comparison to that of MSC + Hyp (P < 0.01) (Fig. 2c). The fold change
of CFU and LTC-IC was calculated in comparison with fresh unexpanded cells.

**Sph-MSC + Cyto and Sph-MSC + Hyp showed more up-regulated expression of self-renewal and homing genes**

NS and Nfix along with CXCR4 and VLA-4 expression were quantified using RT-qPCR. The up-regulation of NS, Nfix, CXCR4, and VLA-4 expression was detected (Fig. 4a). HSCs expansion in presence of MSC spheres and hypoxia led to enhanced expression of self-renewal and homing genes especially in terms of Nfix and CXCR4 (P < 0.001) (Fig. 3a). The expression of NS, Nfix, CXCR4, and VLA-4 in Sph-MSC + Hyp in comparison to MSC + Hyp was significantly up-regulated (P < 0.001). The different expression levels of NS, CXCR4, and VLA-4 were not significant in Sph-MSC + Cyto vs Sph-MSC + Hyp. However, a more up-regulated expression of Nfix was detected in Sph-MSC + Cyto (P < 0.05) (Fig. 3a).

**The expression of NS and Nfix proteins are elevated in HSCs co-cultured with MSC spheres**

Western blot was performed to confirm the expression of NS and Nfix proteins. The sample image of protein bands is presented in Fig. 3b. The protein density was semi-quantified. The increased amount of protein levels was detected in Sph-MSC + Cyto and Sph-MSC + Hyp, not only in terms of NS but also in terms of Nfix as compared with those of MSC + Hyp (P < 0.001, P < 0.01) (Fig. 3c).
MSC spheres induce a higher level of EPCR, CXCR4, and VLA-4 in the expanded HSCs

Co-culturing HSCs with MSC spheres under hypoxia conditions resulted in a higher level of EPCR expression in comparison with that of HSCs co-cultured with adherent MSCs under hypoxia. Figure 4aI and aII represent flow cytometry graphs related to the EPCR expression in CD34 positive cells in the MSC + Hyp and Sph-MSC + Hyp groups, respectively. The percentage of EPCR expression was quantified in all of the experimental groups. As shown in Fig. 4b, Sph-MSC + Hyp had the most EPCR expression level (P < 0.001).

Furthermore, the CXCR4 surface marker was expressed about 50% in Sph-MSC + Hyp that was higher than its expression in MSC + Hyp (21.08%) (Fig. 5aI, aII). Quantification of CXCR4 expression revealed that Sph-MSC + Hyp and Sphe-MSC + Cyto expressed more CXCR4 (Fig. 5b) (P < 0.001). Sph-MSC + Hyp also expressed VLA-4 on their surface even higher than Sphe-MSC + Cyto (Fig. 5cI, cII, d).

As demonstrated in Fig. 5d, hypoxia and co-culturing with MSC spheres induced a higher level of VLA-4 expression (P < 0.01).

These results proved that MSC spheres harbor superior hematoprotective potentialities in comparison with those of adherent MSCs. The results of co-culturing with MSC spheres without cytokine addition along with hypoxia induction are comparable to those of culturing under cytokine addition. MSC sphere-based co-culturing with or without cytokine addition showed similar supportive effects on proliferation and self-renewal/homing marker expression of HSCs.

Discussion

According of our results, MSC spheres co-cultured under hypoxia condition showed supportive effects on proliferation, self-renewal, and homing capability in the majority
of HSCs which was similar to those of using as cytokine addition condition. Improving the surrounding microenvironment of HSCs is critical for their expansion and obtaining an adequate number of cells ex vivo. Co-culturing with MSCs is one of the applicable methods for the expansion of HSCs [10, 11]. Following our previous experience in increasing the supportive effects of MSCs on HSCs expansion [30], in this study, we examined the impacts of different co-culturing systems on the expansion of HSCs. WJ-MSCs were used as a feeder layer. Advantages of birth-associated tissue-derived MSCs have been described previously [11, 12]. The spheroid form of MSCs was used for co-culturing with HSCs along with hypoxia. We previously reported the improvement of plasticity and differentiation potentialities of MSCs under suspension cultivation [12, 35]. Moreover, the paracrine effects of MSCs improve under spheroid conditions [17, 23, 24, 36]. Prior reports had already shown the positive effects of hypoxia on the therapeutic applications of MSCs [28, 29, 37].

Our results indicated elevated levels of Angs, Wnts, SCF, and Flt3-L in the conditioned medium of MSC spheres cultivated under hypoxic condition. MSCs secrete Angs which is involved in the process of regeneration [38]. Not only it influences the niche and fate of HSCs in vivo, but it is
also involved in their survival and stemness ex vivo [16, 20, 38]. Flt3-L and SCF are well-known crucial growth factors for the proliferation, survival, and self-renewal of HSCs [3, 18]. Activation of Wnt and its related signaling pathways, especially the β-catenin-dependent pathway, improves the proliferation of HSCs and consequently increases the number of long-term HSCs [21, 22].

Co-culturing MSC spheres and HSCs led to an increased number of expanded CD34+CD38− cells. The percentage of expanded CD34+ cells and the duration of expansion are the variable parameters in the reported studies. Different expansion conditions, various defined times for CD34 evaluation, and interchangeability of the CD34+ and CD34− populations [2, 39] might lead to different results.

In 2017, Ahmadinejad et al. expanded HSCs under different conditions including co-culturing with MSCs, co-culturing with MSCs + cytokines, and cytokines only. In the mentioned study, a maximum increase in the number of CD34+ cells was observed when HSCs were co-cultured with MSCs + cytokines after 7 days. They concluded that the high-level expression of DNA methyltransferase 1 was the mechanism underlying this event [40]. Our results showed the increased fold change of CFU and LTC-IC. Co-culturing of MSC spheres with HSCs under hypoxia led to an enhanced amount of Angs, Wnts, SCF, and Flt3-L which resulted in an improved level of clonogenicity along with long-term cells in the expanded cells. In 2014, Oubari et al. assayed CFU and LTC-IC capacity of HSCs in expansion conditions, cytokine cocktail containing SCF, TPO, and Flt3-L, placenta-derived MSC feeder layer, and placenta-derived MSC layer with Flt3-L. Their results indicated that using placenta-derived MSCs only in the presence of Flt3-L led to an increased LTC-IC along with an enhanced CFU capacity which was similar to the effects of using cytokine cocktail without the presence of any feeder layer. They emphasized the crucial role of Flt3-L in HSCs expansion in vitro [19].

In our study, an increased expression level of endothelial protein C receptor (EPCR) was detected by flow cytometry in co-culturing of HSCs with MSC spheres. EPCR is a potential long-term engraftment marker of HSCs [41, 42]. The subgroup of CD34+CD90+CD45− cord blood HSCs express EPCR [42]. EPCR and its related signaling pathways control the recruitment of long-term HSCs by decreasing nitric oxide production and increasing the affinity of the VLA-4 homing marker [43]. The paracrine effects of MSC spheres particularly by Wnt5 maintain the stemness and long-term capability of EPCR positive HSCs [22] and cause a slow cell cycling state [44]. In 2017, Fares et al. studied the effects of UM171, as an agonist of HSCs, on the proliferation rate of HSCs ex vivo and their reconstitution ability in vivo. Their study reported that UM171 caused an increased expression of EPCR among long-term HSCs [43]. Furthermore, various other chemical components have been used to induce EPCR expression and to harvest more potent primitive HSCs [45].

Expansion of HSCs in co-culture with MSC spheres up-regulated genes involved in the survival, self-renewality, and homing of HSCs. NS influences different physiologic and pathologic processes since it can coordinate cell self-renewal [46]. It has effects on cell proliferation without any interference with the differentiation behavior of the cells [47]. Nfix plays an essential role in the survival of primitive HSCs by elevating the expression of c-Mpl, reducing apoptosis, and protecting the cells against stressful conditions [48, 49]. In 2013, Holmfeldt and colleagues knocked down the Nfix gene in hematopoietic stem progenitor cells (HSPCs). The Nfix-depleted HSPCs lost their clonogenicity due to the down-regulation of survival genes such as Erg, Mecom, and Mpl. The mice receiving the Nfix-depleted HSPCs showed poor hematopoietic repopulation because of the increased number of apoptotic cells in the bone marrow.
In 2018, Hall et al. over-expressed \( \text{Nfix} \) in HSCs and expanded them in different conditions. They extended the period of expansion from 20 to 40 days [48]. In a study, expression of NS, as a stemness monitoring marker, was measured in different sub-fractions of primitive HSCs such as \( \text{CD150 hap} / \text{CD48 neg} / \text{CD34 neg} \) and \( \text{CD150 hap} / \text{CD48 neg} / \text{CD34 pos} \) LT-HSCs. This marker was expressed in both of the abovementioned sub-fractions [1].

CXCR4/SDF-1 axis is fundamental for homing and trafficking of any type of stem cells [39, 50]. On the other hand, the expression of VLA-4 is critical for HSCs mobilization and homing [19, 39]. In 2018, Mousavi et al. expanded HSCs in three-dimensional nanofiber scaffolds. The expression of homing genes such as \( \text{VLA-4} \) and \( \text{CXCR4} \) was evaluated. Consistent with our results, they reported an increased fold change for the expression of \( \text{CXCR4} \) and \( \text{VLA-4} \) [39]. In another study, the impact of culturing HSCs on the expression of CD49d, a VLA-4 subunit, with or without feeder layer with using different cytokine cocktails was reported and it was concluded that the combination of MSC feeder layer and Flt-3 addition induced high levels of VLA-4 surface expression in HSCs [19].

In 2016, Huang et al. designed a three-dimensional coculturing system using bone-made scaffolds. These researchers mixed HSCs with MSCs or/and osteoblasts and seeded them on the scaffolds. CFU and LTC-IC capacities were reinforced in HSCs co-cultured with MSCs and osteoblasts. A high engraftment rate was confirmed after transplantation of these HSCs to immunodeficient mice [25]. In 2015, Kadekar et al. compared the supportive effects of placenta-derived and umbilical cord-derived MSC feeder on HSCs. They reported superior supportive effects of placenta-derived MSC feeder on HSCs especially in terms of proliferation and CFU ability [11]. Culturing MSCs on polydimethylsiloxane polymer led to enhanced secretion of Ang-1 and TPO. Co-culturing these MSCs with HSCs increases the myeloid differentiation capacity of HSCs and induces CD123 expression [23].

**Fig. 5** Flow cytometry analysis for CXCR4 and VLA-4 on the 10th day of expansion. a) Flow cytometry graph for the expression of CD34 and CXCR4 in MSC + Hyp group. aII Flow cytometry graph for the expression of CD34 and CXCR4 in the Sph-MSC + Hyp group. b) Quantification of CXCR4 expression in different groups. Sph-MSC + Hyp and Sph-MSC + Cyto expressed the CXCR4 homing marker more than MSC + Hyp. c) Flow cytometry graph for the expression of CD34 and VLA-4 in the MSC + Hyp group. cII Flow cytometry graph for the expression of CD34 and VLA-4 in Sph-MSC + Hyp group. d) Quantification of VLA-4 expression in different groups. The expression of the VLA-4 homing marker was enhanced when co-cultured with MSC spheres in the Sph-MSC + Hyp, and Sph-MSC + Cyto groups. Data is shown as mean ± SD. ***P < 0.001, **P < 0.01 Sph-MSC + Cyto and Sph-MSC vs. MSC + Hyp. CXCR4 C-X-C chemokine receptor type 4, VLA-4 very late antigen-4, Cyto cytokines without a feeder, MSC co-culturing with adherent MSCs, MSC + Hyp co-culturing with adherent MSCs under hypoxic condition, Sph-MSC co-culturing with MSC spheres, Sph-MSC + Hyp co-culturing with MSC spheres under hypoxic condition, Sph-MSC + Cyto co-culturing with MSC spheres with cytokine addition.
Conclusion

The expanded HSCs demonstrated more rates of EPCR, NS, Nfix expression indicating that these cells exhibit superior self-renewal capability and stemness ability than those of HSCs co-cultured with monolayer adherent MSCs under the same oxygen pressure. The up-regulated expression of C-X-C chemokine receptor type 4 (CXCR4) and very late antigen (VLA-4) in HSCs co-cultured with MSCs spheres proved their homing potentiality that might result in enhanced engraftment rate after transplantation. Using an MSC sphere-based co-culture system under hypoxia condition might be a potent and non-expensive alternative for cytokine cocktails that are usually used for the expansion of HSCs.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were approved by Hamadan University of Medical Sciences Ethic Committee (No. IR.UMSHA.REC.1399.035). These procedures were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Human umbilical cord tissue/blood was taken after filling of consent form.

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