Effects of Culture Conditions on the Biological Characteristics and MiRNA Expressions of Human Adipose Mesenchymal Stem/Stromal Cells

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Abstract. Mesenchymal stem/stromal cells (MSCs) have been studied intensively due to their great potential in regenerative medicine. Before therapeutic application, *in vitro* expansion of MSCs is normally required to achieve a sufficient number of cells. However, previous studies have indicated that different expansion procedures may result in an alteration of MSC growth kinetics and their biological properties, such as microRNA (miRNA) expression. This study aimed to assess the effects of culture conditions on adipose-derived MSCs (ASCs) behaviours during long-term *in vitro* expansion. Three different mediums, including the traditional DMEM supplemented with 10% fetal bovine serum (FBS), a commercially reduced serum medium, and a xeno-free medium were used for ASC culture. The changes in cell morphology, population doubling time, cell proliferation, intracellular reactive oxygen species (ROS) level, and expression levels of miR-21, miR-223, and miR-423 of cultured ASCs were analyzed at passage (P) 7, P9, and P11. A gradual decrease in proliferation rate and prolonged population doubling time was observed in ASCs cultured in reduced serum and xeno-free mediums over time, whereas the cells rapidly underwent cellular senescence and stopped proliferating at P9 in DMEM supplemented with 10% FBS. The levels of ROS varied greatly depending on particular culture conditions. A reduction of miR-21 and miR-223 expression was detected at the later passages, whereas the miR-423 level remained unaffected under xeno-free condition. Our study indicated that an appropriate selection of culture condition is important to retain the desired biological properties of ASCs in order to utilize their potential in therapeutic applications.

Keywords: Adipose MSCs · miR-21 · miR-223 · miR-423

1 Introduction

Mesenchymal stem/stromal cells (MSCs) are an attractive candidate in regenerative medicine and cell therapy due to their great potential in multilineage differentiation and immunomodulatory activity [1, 2]. Among the MSCs derived from various tissues,

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adipose-derived MSCs (ASCs) are widely accessible, considering the abundance of their origin and less invasive collecting procedures compared to bone marrow or umbilical cord MSCs [3–6]. Even though, the clinical application of ASCs still requires large-scale expansion to obtain sufficient cell numbers and reduce the heterogeneity of freshly extracted ASCs, posing significant challenges to maintaining their characteristics during culture processes. Many studies have demonstrated that the biological characteristics and functionality of ASCs are impacted by in vitro expansion, thereby affecting their therapeutic efficacy [7, 8]. Hence, it is essential to assess the influence of culture procedure on cellular behaviour prior to the development of ASC-based applications.

The choice of culture medium is an important aspect of the large-scale culture of ASCs. Traditionally, fetal bovine serum (FBS) is the most widely used, xenogenic additive for culturing ASCs despite significant concerns about xenogeneic immune reactions and zoonotic viral infection [9, 10]. To overcome these limitations, several chemically defined formulations or xeno-free, serum-free mediums have been developed as alternatives which are also in use in clinical approaches [11]. Although they have been well optimized and characterized, their effectiveness on cell morphology, growth kinetics, and expression profile of ASCs varied greatly in different research [12, 13].

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs that play crucial roles in the regulation of gene expression, impacting various cellular processes including proliferation, differentiation, and apoptosis [14]. Numerous studies have underlined the importance of miRNA functionality in the biogenesis of MSC derived from different tissues. Several miRNAs were reported to mediate the self-renewal and differentiation potentials of MSCs [15, 16]. In particular, miR-21 has been involved in replicative senescence of MSCs by targeting ensin homolog on chromosome ten (PTEN) or programmed cell death 4 (PDCD4) factor [16, 17]. MiR-223 participated in cell cycle regulation through its interaction with Forkhead Box O3 (FOXO3), while it promoted cartilage formation of MSCs by down-regulating NLR family pyrin domain containing 3 (NLRP-3) expression [18, 19]. During MSC differentiation, miR-423 was identified as a negative regulator of osteoblastogenesis and cell viability by modulating SMAD Family Member 3 (SMAD3) level thereby impacting its downstream factors such as cyclin D2 (CCND2) and the cyclin-dependent kinase inhibitor 1B (p27) [20, 21]. In addition, it has been mentioned that the therapeutic effects of MSCs and their secretomes are influenced by miRNA expression profiles [22]. Nevertheless, little is known about these miRNA levels in ASCs and even less about how culture condition may impact miRNA expressions in order to exploit their therapeutic potential.

In this work, we investigated the effects of different culture conditions on growth kinetics, oxidative stress, and miRNA expression of ASCs. Our data presents the disparity of ASCs cultured in reduced serum or xeno-free conditions, compared to those grown and expanded under conventional DMEM supplemented with 10% FBS. These results indicated that the in vitro expansion of ASC should be carefully considered for its therapeutic utility, depending on the pertained characteristics of the selected cell source and their biological properties.

2 Materials and Methods

2.1 Cell Culture

Human ASCs were obtained from Thermo Fisher. Upon receipt, the ASCs were thawed and subcultured in MesenPRO RS Medium (MesenPRO) (Thermo Fisher, USA) at 37 °C and 5% CO2 until passage 5, following the manufacturer's guidelines. In passage 6, the ASCs were gradually adapted in three different culture conditions, including the Mesen, a mixture of Mesen and StemMACS MSC Expansion Medium (StemMACS) (Miltenyi Biotec, Germany) at a 50%:50% volume ratio, and a mixture of Mesen and Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at a 50%:50% volume ratio. From passage 7 onward, the ASCs were cultured in three distinct media: MesenPRO, StemMACS, and DMEM+10% FBS. The detailed compositions of tested culture media are listed in Table 1. The ASCs were collected at P7, P9, and P11 for subsequent analysis.

Table 1. The composition of three culture media.

	MesenPRO RS	StemMACS	DMEM
Medium	MesenPRO basal medium	Serum-free, xeno-free medium	Dulbecco's Modified Eagle Medium
Growth supplement	provided growth supplement with 2% FBS	provided growth supplement	10% FBS
Other components	- 1% L-glutamine - 1% Pen/Strep	- 1% Pen/Strep - Phenol red	- 1% L-glutamine - 1% Pen/Strep - Phenol red

2.2 Cell Counting

Total cell number and cell viability were assessed using a hemocytometer and the trypan blue exclusion method. A 0.4% trypan blue solution (15250061, Gibco, USA) was prepared in phosphate-buffered saline (PBS), pH 7.2–7.3. A 1:1 mixture of cell suspension and trypan blue solution was prepared and stained for 30 s, and then 10 μL of the mixture was loaded onto a hemocytometer. Total number of cells and the number of stained cells were counted.

2.3 Cell Growth Rate and Doubling Time

ASCs were cultured for 72 h in a 12-well plate. Then, ASCs were collected and counted to calculate the growth rate and cell doubling time using the following equations as previously described in Eq. 1 and Eq. 2 [23]:

$$\text{Growth rate} = \frac{\ln\left(\frac{\text{Final concentration}}{\text{Initial concentration}}\right)}{\text{Duration}} \quad (1)$$

$$\text{Doubling rate} = \frac{\text{Duration} \times \ln(2)}{\ln\left(\frac{\text{Final concentration}}{\text{Initial concentration}}\right)} \quad (2)$$

2.4 Cell Viability

Cell viability was determined using cell counting kit 8 (CCK8) colourimetric assay (ab228554, Abcam, UK) following the manufacturer's protocol. In brief, ASCs were seeded into a 96-well plate at a density of 5000 cells/well. After an incubation of 24 h, 10 μL of CCK8 solution was added to each well. The plates were incubated at 37 °C for 3 h in a 5% CO2 incubator. Absorbance was measured by Varioskan LUX multimode microplate reader (Thermo Fisher) at 460 nm wavelength.

2.5 Reactive Oxygen Species (ROS) Assay

Intracellular ROS levels were measured using 2′,7′-dichlorofluorescein diacetate (DCFDA) (ab113851, Abcam, UK). Briefly, ASCs were seeded at a density of 5000 cells/well in black-walled, clear-bottom 96-well plates. The cells were then incubated for 24 h at 37 °C and 5% CO2. For ROS measurement, the ASCs were washed with PBS and incubated with 25 μM DCFDA for 45 min in the dark at 37 °C and 5% CO2. After the incubation, cells were washed with PBS, and fluorescence intensity was measured at excitation/emission wavelengths of 485/535 nm using Varioskan LUX multimode microplate reader (Thermo Fisher).

2.6 MicroRNA Isolation

Total miRNAs were isolated from 10^5 cultured cells using the Hybrid-RTM miRNA extraction kit (#325-150, GeneAll Biotechnology, Korea) according to the manufacturer's instructions. In brief, pellet cells were mixed with 500 μL RiboEx solution and incubated for 5 min at room temperature (RT). Following the addition of 200 μL chloroform, the mixture was shaken vigorously for 15 s, incubated for 2 min at RT, and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was transferred to a Type B column and centrifuged at ≥10,000 g for 30 s at RT. The pass-through solution was mixed with an equal volume of 100% ethanol, transferred to a Type W column, and centrifuged at ≥10,000 g for 30 s at RT. The column was washed twice with 500 μL RBW and once with 500 μL RNW; each washing was centrifuged at ≥10,000 g for 30 s at RT. A volume of 25 μL RNase-free water was added to the column in a new RNase/DNase-free tube, then centrifuged at ≥10,000 g for 1 min at RT. Isolated miRNAs were stored at –70 °C for further analysis. MiRNA concentration was determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA).

2.7 MiRNA Quantification

Reverse transcription of isolated miRNAs was performed using the miRCURY LNA RT Kit (#339340, Qiagen, Germany) according to the manufacturer's protocol. The cDNA

was diluted with nuclease-free water at a 1:5 ratio. MiRNA expression was quantified using the miRCURY LNA SYBR Green PCR Kit (#339345, Qiagen, Germany) with pre-designed miRCURY LNA miRNA PCR Assays for miR-21, miR-223, and miR-423. MiR-103a was used as internal control as previously described [24]. The PCR reaction was initiated with a denaturation step at 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. Relative miRNA expression levels were calculated using the comparative cycle threshold (ΔCt) method. A Ct value ≥40 was considered undetermined. All samples were performed in triplicate.

2.8 Statistical Analysis

Data are presented as mean±standard deviation (SD) unless otherwise stated. Student's t-test was used for the two-group comparison. For comparison between more than two groups, one-way ANOVA with Tukey's post-hoc test was used, unless otherwise specified. All statistical tests were performed using GraphPad Prism 8; $p < 0.05$ was considered significant.

3 Results

3.1 Changes in Growth Kinetics of ASCs Under Different Culture Conditions

To investigate the effect of culture conditions on the growth and population doubling time, ASCs were either maintained in DMEM+10% FBS, reduced serum medium (MesenPRO) as recommended by the cell supplier, or commercial xeno-free medium (StemMACS) through several passages (Fig. 1).

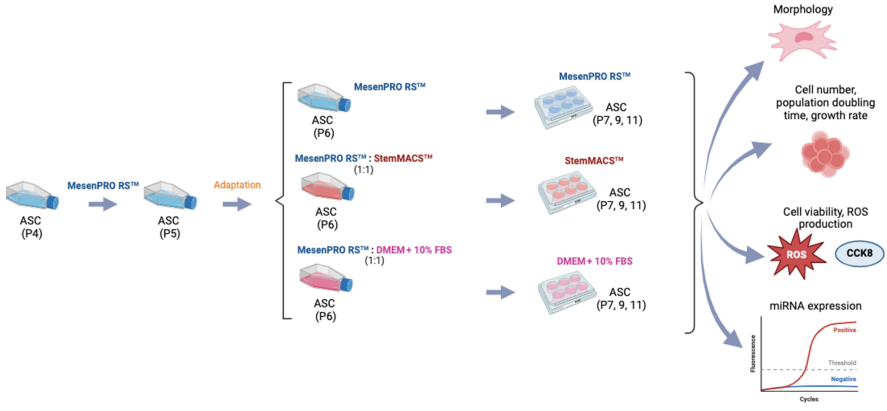

Fig. 1. Experimental scheme.

In all culture conditions, cultured ASCs presented normal fibroblastic and spindle-shaped morphology (Fig. 2A). Generally, the total number of ASCs significantly decreased at later passages, in which the highest yield was observed for the cells cultured

in StemMACS, compared to those in MesenPRO and DMEM+10% FBS (Fig. 2B). In DMEM+10% FBS, cultured ASCs did not propagate beyond passage 9, indicating this medium was insufficient to support cell growth long-term. Therefore, no further analysis was conducted at passage 11 for DMEM+10% FBS condition. The cultured ASCs presented a significant prolong of cell doubling time from 27–35 h at P7 to 53–70 h at P11 (Fig. 2C), while a two-fold decrease in growth rate was also documented (Fig. 2D). Taken together, these results indicated a massive reduction in the proliferative capability of ASCs during in vitro expansion that may be decelerated by the selection of appropriate culture conditions.

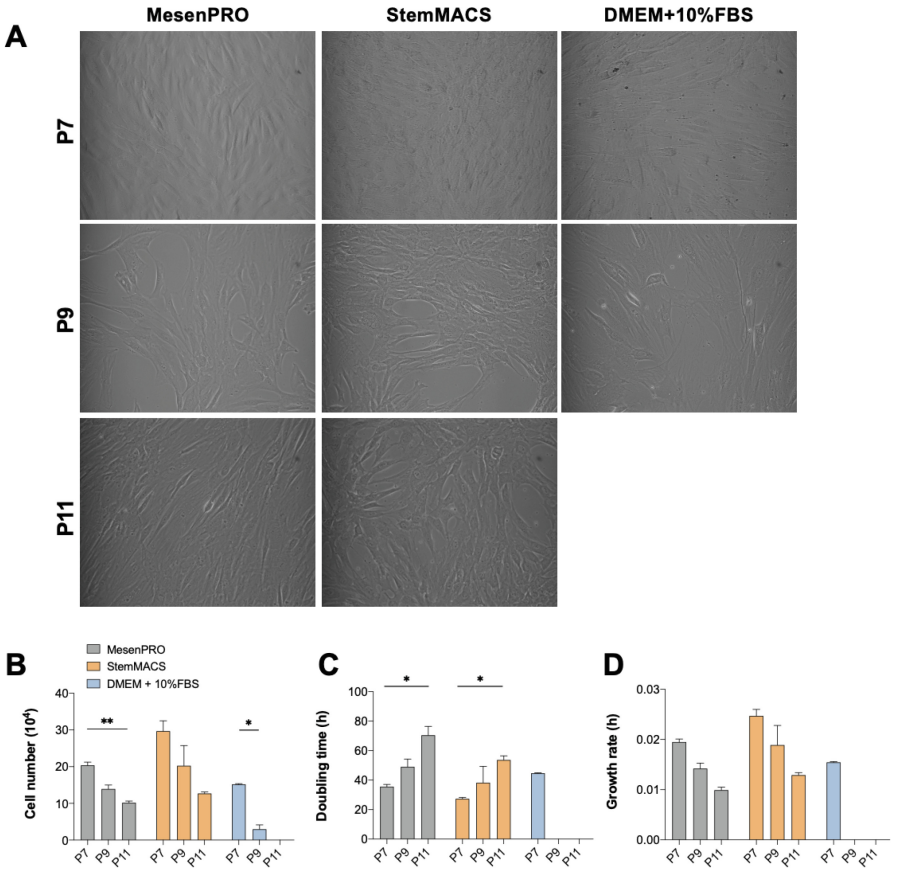

Fig. 2. ASC morphology and growth kinetics in different culture conditions. (A) Representative images of ASCs cultured in MesenPRO, StemMACS, and DMEM+10% FBS at P7, P9, and P11; (B) Total cell number, cell counts were performed using a hemocytometer, data are mean±SEM; (C) Population doubling time calculated for 72-h culture intervals; (D) The growth rate of ASCs P7, P9, and P11. Statistical significance was determined using ANOVA, with $p < 0.05$ considered significant.

3.2 The Effects of Culture Conditions on Cell Viability and ROS Production of ASCs

Previous studies have demonstrated that extended passages can influence the metabolic activity of ASCs and their ROS levels. To assess ASC viability, the CCK-8 assay was performed. At passage 7, the number of viable ASCs cultured in StemMACS was twice as high as those in MesenPRO and DMEM+10% FBS, with no difference seen between MesenPRO and DMEM+10% FBS (Fig. 3A). This pattern persisted at passage 9, in which the viable cells in StemMACS remained significantly higher number. In contrast, ASCs cultured in DMEM+10% FBS exhibited a marked reduction in cell viability (Fig. 3A). By passage 11, there were no differences in ASC viability among tested conditions.

It is known that ROS can cause cellular damage and impact ASC viability, differentiation, and function. To determine the effects of culture condition on oxidative stress; hydroxyl, peroxyl, and other ROS activity were measured by DCFDA staining. A higher level of ROS was detected in ASCs maintained under StemMACS, than that of MesenPRO and DMEM+10% FBS (Fig. 3B). Notably, a gradual increase of ROS has been noticed in ASCs cultured in DMEM+10% FBS (Fig. 3B). An increase in cell metabolism or stress response can result in accelerating ROS level, a normalization of ROS over CCK8 values showed comparable ratios for all tested conditions (Fig. 3C), except for P9 ASCs in DMEM+10% FBS, probably due to the progression of ASC senescence as they stopped proliferating afterwards.

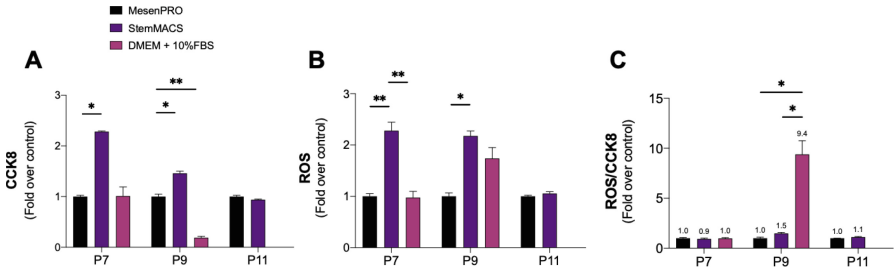

Fig. 3. Cell viability and ROS production in ASCs cultured in different media. (A) Cell viability of ASCs was assessed using CCK8 assay; (B) ROS production of cultured ASCs; (C) Normalized ratio of ROS/CCK8. Statistical significance was determined using ANOVA with uncorrected Fisher's LSD, with $p < 0.05$ considered significant.

3.3 MiRNA Expression Under Different Culture Conditions

Several miRNAs have been described to play crucial roles in regulating various cellular processes, including proliferation, differentiation, and apoptosis of MSCs. As changes in miR-21, miR-223, and miR-423 levels were previously detected in bone marrow-derived MSCs during in vitro ageing and differentiating processes; thus, we also examined their expressions in this study. The ASCs cultured in MesenPRO medium were used as a normalized control. Our results show that miR-21 expression was comparable in

three testing conditions at P7 (Fig. 4A). However, its level elevated significantly in ASCs maintained in StemMACS, whereas decreased in those in DMEM+10% FBS at passage 9 (Fig. 4A). In passage 11, no significant difference was observed among the three conditions (Fig. 4A). A different expressive pattern was observed for miR-223. Compared to the MesenPRO, the ASCs cultured in StemMACS and DMEM+10% FBS presented significantly higher levels of miR-233 at early passage (Fig. 4B). A progressive decrease of miR-223 was detected in ASCs from all culture conditions at later stages (Fig. 4B). For miR-423, ASCs cultured in StemMACS exhibited significantly higher expression than those in MesenPRO and DMEM+10% FBS at P7 (Fig. 4C). A similar pattern was observed at passage 11, despite a transient reduction of miR-423 level at P9 (Fig. 4C).

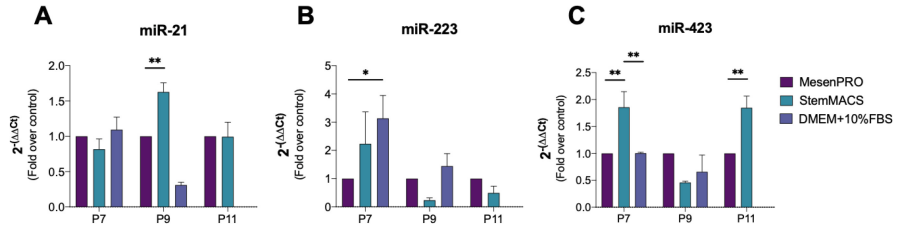

Fig. 4. Expression levels of miR-21, miR-223, and miR-423 in ASCs cultured in different media. MiRNA expression levels are normalized and presented as fold change over ASCs culture in MesenPRO (control). (A) MiR-21 expression of cultured ASCs at P7, P9, and P11; (B) MiR-223 expression of cultured ASCs at P7, P9, and P11; (C) MiR-423 expression of cultured ASCs at P7, P9, and P11. Statistical significance was determined using ANOVA, with $p < 0.05$ considered significant.

4 Discussions

In this study, we investigated the environmental influences on growth kinetics, ROS level, and miRNA expressions of cultured ASCs in commonly used DMEM+10% FBS medium, a commercially reduced serum medium (MesenPRO), and xeno-free formulated medium (StemMACS). Similar morphology was observed for ASCs cultured in the three conditions. However, the ASCs in DMEM+10%FBS rapidly underwent cellular senescence with increased ROS levels at later passages, compared to other culture media. These cells also presented massive reductions of miR-21, miR-223, and miR-423 levels during their in vitro ageing process. Our data indicated that the expansion of ASCs in reduced serum or xeno-free mediums was more convenient compared to DMEM+10%FBS as the cells could be maintained in longer intervals with a slower rate of cellular senescence.

In recent years, MSCs have become an attractive candidate for cell-based therapies because of their great potential in secreting a variety of paracrine factors and modulating immune responses [25, 26]. Among the MSCs obtained from different tissue origins, adipose-derived MSCs (ASCs) are representative sources for therapeutic application, considering their abundance and ease of isolation and expansion in vitro [27].

Nevertheless, large-scale production of ASCs strictly requires the ensure of cell growth without altering its phenotype and biological characteristics [28]. Appropriate selection of culture medium is thus a critical aspect of obtaining a sufficient amount of qualified ASCs.

Numerous culture media have been reported for the in vitro expansion of ASC with inconsistent results. For instance, morphological dissimilarity was observed for Wharton's jelly derived-MSCs cultured in xeno-free and FBS-containing mediums [12]. In an attempt to replace FBS by human activated pure platelet-rich plasma (P-PRP) during trans-differentiation of ASCs, Elgamal's group showed a similar cell shape between the 10% FBS, 10% PRP, and 20% PRP culture [29]. In line with the report of Elgamal's group, we did not obtain significant alteration of ASC morphology in all our testing conditions. The variation of tissue origin and donor may also contribute to the heterogeneity of MSCs.

Previous measurement of population doubling time has identified a prolonged of ASCs cultured in DMEM+10% FBS compared to the serum-free medium during the first five passages after its primary isolation [30]. In our study, a longer turnover time was also observed for ASCs in DMEM+10% FBS even at extended passages. Collectively, this information suggests that DMEM+10% FBS may not be an optimal condition for ASC expansion.

The increased age of ASC donors was found to be associated with cellular senescence and elevated ROS production [31]. Our results suggested that in vitro ageing of ASCs also manifested by a significant accumulation of ROS at the cellular level that was confirmed in cells losing their proliferative capability at P9 in DMEM+10% FBS. Hence, culture condition needs to be carefully considered to diminish its negative impacts on ASC functionality.

The effects of different expansion protocols on miRNA expression in cultured ASC is not well explored. MiR-21 has been suggested as a major molecule determining MSC proliferation and differentiation in which a comprehensive miRNA profiling of MSCs derived from different tissue sources revealed a higher expression of miR-21 in bone marrow MSCs compared to amniotic fluid MSCs [32]. In addition, miR-21 overexpression was shown to enhance significantly bone marrow MSC proliferation [33]. An increased level of miR-21 was also detected in ASCs with higher proliferative capability in our study. Upregulation of miR-223 has been documented in MSCs during an active proliferating phase of the wound healing process [34]. Following *in vitro* ageing, we have observed downregulation of miR-223 in cultured ASCs. Little is known about miR-423 expression in ASCs, though it has been described to negatively regulate MSC differentiation [21]. In human umbilical vein endothelial cells, miR-423 secreted by ASCs promoted cell proliferation and migration [35]. Since a stably high level of miR-423 was found in ASCs cultured in StemMACS, this feature could be exploited further to utilize the therapeutic effects of miR-423.

The main limitation of this study is that we only assess the impacts of culture conditions on ASCs collected from a single donor. Therefore, future investigation is required to evaluate the environmental impacts on ASCs isolated from different donors. Besides, extended studies of ASCs cultured in other commercialized or in-house developed media

will assist in a proper selection of culture condition to maintain ASC properties during *in vitro* expansion.

5 Conclusion

In summary, ASC expansion is accompanied by a series of biological changes which are influenced by cell culture conditions. Therefore, the selection of appropriate culture media and expansion protocol deserves more concern to maximize ASC expansion without compromising their biological characteristics and therapeutic efficacy.

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Conflicts of Interests. The authors declared that they have no conflicts of interest.

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