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Human Amniotic Mesenchymal Stem Cells Exhibit Similar Immunosuppressive Ability with Bone Marrow Mesenchymal Stem Cells and Possess a Higher Proliferation Activity and Clearer Stem Cell Properties *in vitro*

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Abstract

Objectives: Human bone marrow mesenchymal stem cells (hBMSCs) have been used for the prevention and treatment of acute graft-versus-host disease (aGVHD) in post-hematopoietic stem cell transplant patients. In this study, we compared the biological characteristics and immunosuppressive activity of human amniotic mesenchymal stem cells (hAMSCs) and hBMSCs to provide experimental evidence for the potential use of hAMSCs for treating aGVHD, thus solving the problem of insufficient hBMSCs sources.

Methods: HAMSCs were isolated by enzymatic digestion. hBMSCs were isolated using Ficoll-Hypaque density gradients. The biological characteristics of both stem cell types were compared by morphological analysis, analysis of cell growth, cell cycle profiling, immunophenotyping, and immunofluorescence assays. An *in vitro* co-culture of MSCs and peripheral blood mononuclear cells (PBMCs) was performed and lymphocyte proliferation measured using the Cell Counting Kit-8(CCK-8) assay. IFN-γ production was determined in the co-culture supernatant using enzyme linked immunosorbent assay (ELISA).

Results: Both hAMSCs and hBMSCs had fibroblast-like morphology. hAMSCs could be maintained for at least 15 culture passages, but hBMSCs started to show signs of aging and remarkably reduced proliferation at 6-7 passages. There was no significant difference in the proportion of cells in G2/M phase between hAMSCs and hBMSCs (P>0.05). Immunophenotyping revealed positive expression of CD105, CD90, and CD73 and negative expression of CD34, CD45, CD11b, CD19, and HLA-DR on the surface of both hAMSCs and hBMSCs. hAMSCs were positive for Oct-3/4, but hBMSCs were not. Both hAMSCs and hBMSCs expressed vimentin. PHA-stimulated PBMCs proliferation was inhibited by hAMSCs and hBMSCs. This inhibition was stronger, as the proportion of MSCs increased. There were not significant differences between the inhibitory effects of the two MSCs types on PBMCs proliferation (P>0.05). Interferon-γ (IFN-γ) production was lower when PBMCs were co-cultured with either hAMSCs or hBMSCs than when they were cultured alone (P<0.05). IFN-γ production was lower when PBMCs were co-cultured with hAMSCs than when they were co-cultured with hBMSCs (P>0.05).

Conclusion: The results of this study demonstrated that hAMSCs have higher proliferation activity and clearer stem cell properties than hBMSCs. Both hAMSCs and hBMSCs were able to suppress the proliferation of allogeneic peripheral blood lymphocytes and reduce IFN-γ secretion stimulated by PHA *in vitro*.

Introduction

Despite the use of extensive laboratory and clinical investigation into methods to prevent severe acute graft-versus-host disease (aGVHD), this complication remains a major cause for mortality and morbidity with allogeneic hematopoietic stem cell transplantations (allo-HSCT) [1,2]. Human bone marrow MSCs (hBMSCs), possess the capacity to differentiate into various types of cells, home to sites of inflammation, repair tissue, modulate immune or inflammation response, and support hematopoiesis, have been widely used for the prevention and treatment of aGVHD [3,4]. However, hBMSCs have character of can be recovered at extremely low cell counts. These cells are characterized by an age-related decline in proliferative and

differentiation potential, and by ahigh viral infection rates. The collection of hBMSCs from donors requires invasive bone marrow aspiration, which limits the potential sources of hBMSCs [4,5].

The human amniotic membrane (HAM), an extremely rich and easily accessible tissue with no ethical concerns, has been proposed as an attractive material in stem cell therapy. Amniotic mesenchymal stem cells (hAMSCs) derived from HAM can be easily acquired noninvasively without pain and risk of morbidity. HAMSCs also have a high proliferation rate, without the limitation that the proliferative potential and differentiation abilities decrease as the donor's age increases in the traditionally used hBMSCs [6].

This study aimed to compare the biological features and immunosuppressive properties of hAMSCs and hBMSCs in vitro, to provide experimental evidence for the potential use of hAMSCs for treating aGVHD, in order to solve the problem of insufficient hBMSCs sources.

Materials and Methods

Source of specimens

A fresh human amnion was donated by a healthy woman undergoing a caesarean section. A bone marrow sample (20 ml) and peripheral blood (15 ml) were donated by healthy volunteers from the Department of Hematology, Nanfang Hospital, Southern Medical University (Guangzhou, China). Human foreskin fibroblasts were a gift from the Gynecology and Obstetrics department of Nanfang Hospital, Southern Medical University (Guangzhou, China).

This study was consistent with the concerns of FDA Regulation of Tissues (FY 2012 Update) & (Homologous Use of Human Cells, Tissues, and Cellular and Tissue-Based Products Draft Guidance for Industry and FDA Staff). And it was approved by the Human Research Ethics Committees of Nanfang Hospital, Southern Medical University (Guangzhou, China). Signed informed consent was obtained from all participants after a detailed description of the purposes of our study.

Isolation, culture, and morphological observation of hAMSCs and hBMSCs

The amnion was isolated from fresh placental tissue under sterile conditions. The amnion was rinsed with physiological saline buffer, and cut into pieces measuring approximately 1 mm × 1 mm. Amnion pieces were digested with 0.25% trypsin (Sigma–Aldrich; St. Louis, MO, USA) for 30 min, and then filtered through a 200 mesh sieve. The filtered amnion specimens were digested with type II collagenase (Sigma–Aldrich) in a 37°C water bath (Taicang Experimental Instruments Factory; Taicang, China), and then filtered through a 200 mesh sieve. Cells were harvested, washed twice with PBS, and cultured in RPMI 1640 medium (Hyclone; Logan, UT, USA) for 48 h. When adherent cells reached 80% to 90% confluence cells were passaged at a ratio of 1:3 (first passage). Then, cells growing to approximately 90% confluence were passaged again (second passage). During the period of cell growth, the morphology of hAMSCs was observed under the microscope and photographed.

Bone marrow was diluted in PBS under sterile conditions. Mononuclear cells were isolated using lymphocyte separation medium (Tianjin Haoyang Biological Manufacture Co., Ltd.; Tianjin, China), and cultured in RPMI 1640 medium for 48 h. Non-adherent cells were removed and the medium was replaced completely. When the adherent

cells reached 80% to 90% confluence, cells were passaged at a ratio of 1:2 (first passage), then cells growing to approximately 90% confluence were passaged again (second passage). During the period of cell growth, the morphology of hBMSCs was observed under the microscope and photographed.

Growth analysis of hAMSCs and hBMSCs

Single-cell suspensions were prepared from passage 3 cultures of hAMSCs and hBMSCs. Cell density was adjusted to 1×10^4 cells/ml. Cells were seeded onto 96-well plates (100 μl per well) and triplicate wells were assigned for hAMSCs and hBMSCs. Cells were harvested daily, and incubated in 10 μl of CCK-8 reagents for 3 h, following medium replacement. The optical density (OD) was measured using a microplate reader (BioTek Instruments, Inc.; Winooski, VT, USA) at 450 nm. After successive measurement for 8 days, a cell growth curve plot was constructed.

Cell cycle analysis of hAMSCs and hBMSCs

Single-cell suspensions were prepared from passage 3 cultures of hAMSCs and hBMSCs. Cell density was adjusted to 1×10^6 cells/ml. Cells in approximately 1 ml of the single-cell suspensions were stained using a cell cycle detection kit (KeyGen Biotech Co., Ltd.; Nanjing, China), and then analyzed by fluorescence activated cell sorting (FACS), using a BD FACS Aria flow cytometer (Becton Dickinson; Franklin Lakes, NJ, USA).

Immunophenotyping of hAMSCs and hBMSCs

Single-cell suspensions were prepared from passage four cultures of hAMSCs and hBMSCs. Cell density was adjusted to 1×10^5 cells/ml. Cells in 100 μl of the single-cell suspension were transferred to FACS tubes. Cells were incubated with mouse anti-human CD11b, CD19, CD34, CD45, CD73, CD90, CD105 and HLA-DR monoclonal antibodies (Becton Dickinson; Franklin Lakes, NJ, USA) at room temperature for 30 min (20 μl of each antibody was added to each sample). Mouse anti-mouse IgG monoclonal antibody (Becton Dickinson; Franklin Lakes, NJ, USA) was used as a control. Cells were washed once with PBS, mixed evenly with 200 μl PBS, and subjected to FACS analysis.

Single-cell suspensions were prepared from passage 4 cultures of hAMSCs and hBMSCs. Cell density was adjusted to 1×10^5 cells/ml. Cells were then fixed in 200 μl of 1% paraformaldehyde at 37°C for 10 min, washed once with PBS, mixed with 200 μl of 90% methanol and incubated on ice for 30 min. Subsequently, cells were washed twice with PBS, and incubated in 20 μl of mouse anti-human Oct-3/4 monoclonal antibody (Becton Dickinson; Franklin Lakes, NJ, USA) at room temperature for 30 min. Mouse anti-mouse IgG monoclonal antibody served as a control. Cells were then washed once in PBS, mixed evenly with 200 μl PBS in each tube, and subjected to FACS analysis.

Immunofluorescence assay

Passage four cultures of hAMSCs and hBMSCs of 80% to 90% confluence were washed twice with DPBS, fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, and blocked in DPBS supplemented with 0.2% Triton X-100, 5% goat serum, and 1% BSA for 45 min. Both hAMSCs and hBMSCs were incubated with anti-vimentin primary antibody (D21H3; Cell

Signaling Technology, Inc.; Danvers, MA, USA) overnight at 4°C in a humidifier (Thermo Fisher Scientific; Cleveland, OH, USA). Cells were washed three times with PBS, and incubated with a fluorescent secondary antibody at room temperature for 45 min. Cells were then washed twice in PBS, subjected to DAPI nuclear staining (Invitrogen; Carlsbad, CA, USA) at room temperature for 2 min, washed twice with PBS, and stored protected from light. Cells were visualized under an inverted fluorescence microscope (Olympus; Tokyo, Japan).

Assessment of immunosuppressive activity of hAMSCs and hBMSCs

Peripheral blood was diluted with PBS under sterile conditions. Mononuclear cells were isolated from peripheral blood using lymphocyte separation medium. After washing twice with PBS, cells were cultured for 24 h. The cells in suspension were counted and harvested for subsequent experiments.

Single-cell suspensions were prepared from passage five cultures of hAMSCs and hBMSCs. Cell density was adjusted to 1×10^5 cells/ml. Cells were seeded onto 24-well plates at densities of 1×10^4, 2×10^4, and 1×10^5 cells/ml. After an overnight incubation, cells were treated with 10 μg/ml mitomycin C (Roche, Switzerland). MSCs in each well were mixed with peripheral blood mononuclear cells (PBMCs; at a density of 1×10^5 cells/ml) that were stimulated, or not (unstimulated control), with 10 μg/ml PHA (SigmaAldrich). Triplicate wells were assigned to each group. After 72 h of culture, the supernatant was harvested, and stored at 20°C for further analysis.

PBMCs were harvested and transferred to 96-well plates containing RPMI 1640 medium (100 μl in each well). Cells were incubated with 10 μl CCK-8 reagents for 3 h, and then OD values were measured at 450 nm using a microplate reader.

The supernatants of hAMSCs-PBMCs and hBMSCs-PBMCs co-cultures (1:1), PHA-stimulated PBMCs (positive control), and unstimulated PBMCs (negative controls) were collected, and used to measure IFN-γ by ELISA (Neobioscience Technology Company; Shenzhen, China). OD values were measured at 450 nm using a microplate reader.

Statistics

Data are expressed as mean ± standard deviation (SD). Student t tests were used to compare the means of two independent samples, while differences among groups were tested for statistical significance using one-way analysis of variance (ANOVA). All statistical analyses were performed using the statistical software SPSS version 19.0 (SPSS, Inc.; Chicago, IL, USA), and a P value<0.05 was considered statistically significant.

Results

Analysis of morphology and growth of hAMSCs and hBMSCs

After 48 h of culture, 40% to 50% of cells in the primary culture of hAMSCs were adhered to the wall of the wells. Microscopy analysis showed that the majority of hAMSCs were fusiform or polygonal, and that the minority of the cells were round or oval. After approximately a week of culture, the morphology of hAMSCs gradually became uniform; the cell body became thin and long, with acceleration seen in cell proliferation (Figure 1A). The flask bottom was completely covered

with hAMSCs after 10 to 14 days. After three or more passages, hAMSCs exhibited a fibroblast-like morphology, and the growth of the cells accelerated. In addition, hAMSCs retained high proliferative activity at passage 6 (Figure 1B).

After 48 h of culture, approximately 10% of the cells in the primary culture of hBMSCs were adhered to the wall of the wells, and a cluster of growing cells could be observed. After 10 days in culture, the proliferation of hBMSCs accelerated, and cells appeared fusiform or polygonal (Figure 1C). The flask bottom was completely covered with hBMSCs after approximately 2 weeks. Like hAMSCs, hBMSCs had fibroblast-like morphology that did not change after several passages (Figure 1D). At five or six passages, hBMSCs started to age. Aging was characterized by intra-nuclear vacuolation, increased cytoplasm, and slow proliferation.
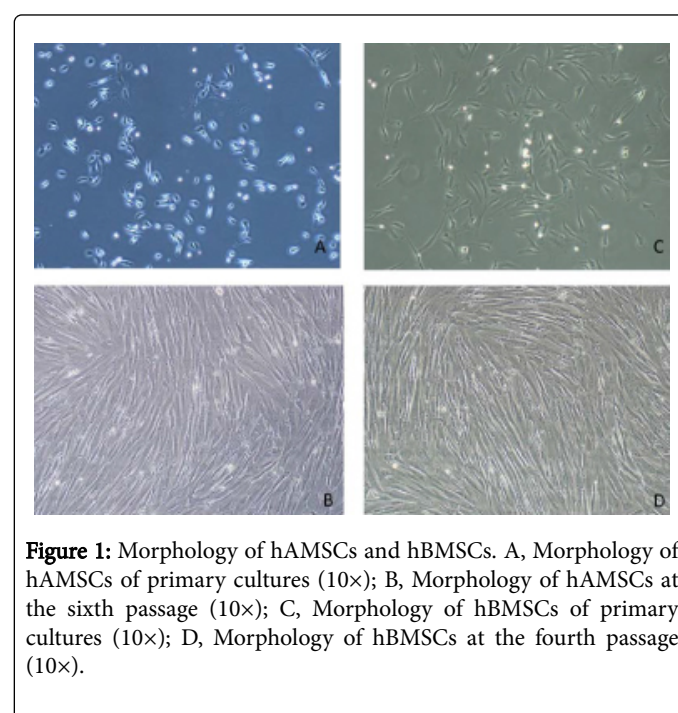

Figure 1: Morphology of hAMSCs and hBMSCs. A, Morphology of hAMSCs of primary cultures (10×); B, Morphology of hAMSCs at the sixth passage (10×); C, Morphology of hBMSCs of primary cultures (10×); D, Morphology of hBMSCs at the fourth passage (10×).
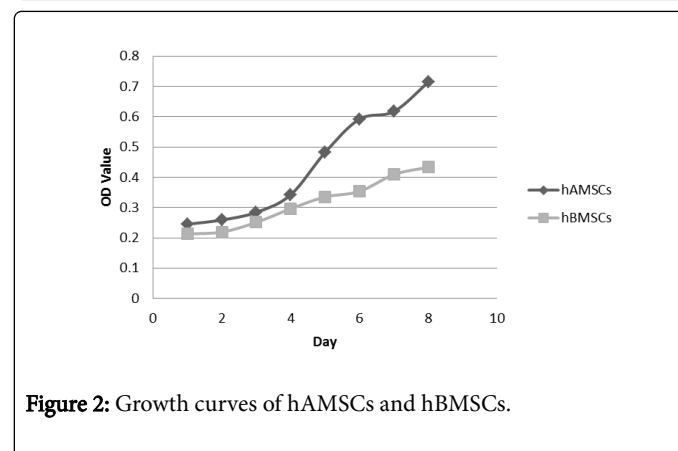

Figure 2: Growth curves of hAMSCs and hBMSCs.

The latency period of hAMSCs and hBMSCs was 1 to 3 days post-inoculation. hAMSCs exhibited accelerated growth on days 3 to 8, and then entered into the log growth phase. hBMSCs exhibited accelerated growth on days 3 to 7, but reduced their proliferation after 7 to 8 days

of culture. hAMSCs exhibited higher proliferation rates than hBMSCs (Figure 2).

Analysis of the cell cycle in hAMSCs and hBMSCs

FACS analysis of passage 3 cells revealed no significant differences in the proportion of cells in G2/M phase between hAMSCs and hBMSCs (P=0.645).
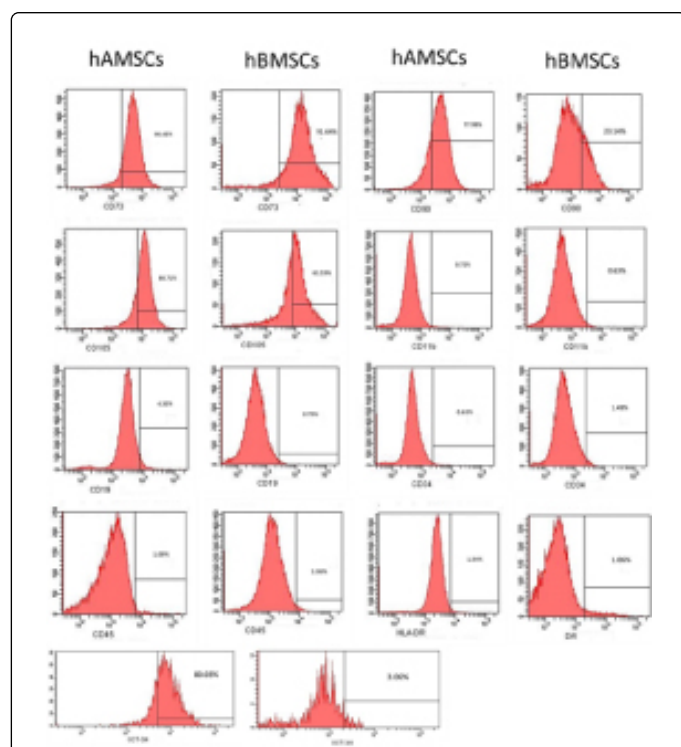

Figure 3: Immunophenotyping of hAMSCs and hBMSCs. Both MSCs types were positive for CD73, CD90, and CD105 and negative for CD11b, CD19, CD34, CD45, and HLA-DR.
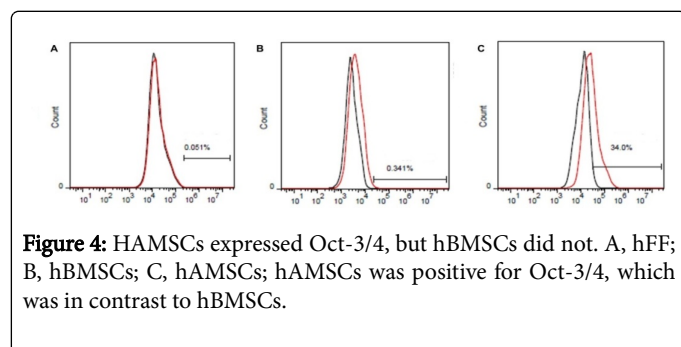

Figure 4: HAMSCs expressed Oct-3/4, but hBMSCs did not. A, hFF; B, hBMSCs; C, hAMSCs; hAMSCs was positive for Oct-3/4, which was in contrast to hBMSCs.

Immunophenotyping of hAMSCs and hBMSCs

Both hAMSCs and hBMSCs expressed CD73, CD90, and CD105, but did not express CD34, CD45, HLA-DR, CD19 or CD11b (Figure 3). hAMSCs, but not hBMSCs, were positive for Oct-3/4 (Figure 4).

Detection of vimentin in hAMSCs and hBMSCs

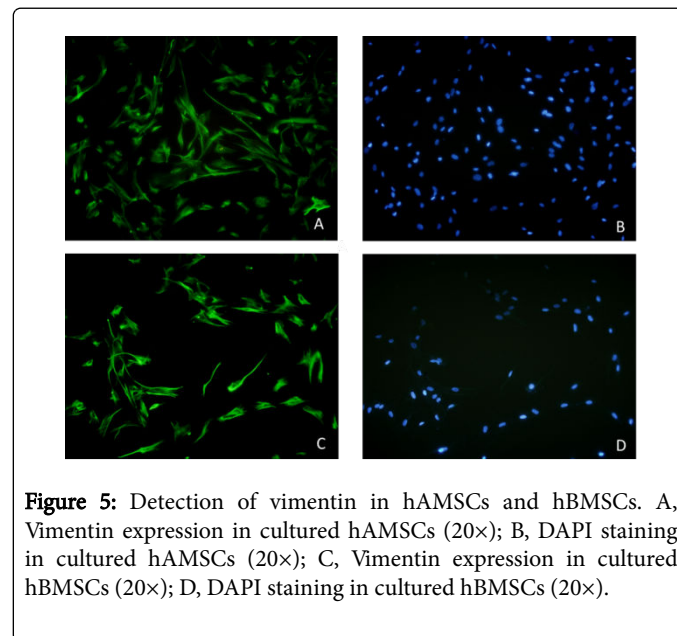Immunofluorescence microscopy showed that vimentin was expressed in both hAMSCs and hBMSCs (Figure 5).

Figure 5: Detection of vimentin in hAMSCs and hBMSCs. A, Vimentin expression in cultured hAMSCs (20×); B, DAPI staining in cultured hAMSCs (20×); C, Vimentin expression in cultured hBMSCs (20×); D, DAPI staining in cultured hBMSCs (20×).

Inhibition of PBMC proliferation by hAMSCs and hBMSCs

Both hAMSCs and hBMSCs suppressed the proliferation of PHA-stimulated PBMCs (P<0.05), as revealed by the CCK-8 assays. Increasing the proportion of hAMSCs or hBMSCs further diminished PBMCs proliferation (Table 1). However, there were not significant differences between the inhibition of PBMC proliferation by hAMSCs or hBMSCs (P>0.05).

Group	OD value
hAMSC/PBMC + PHA (1:1)	0.136 ± 0.016[*]
hBMSC/PBMC + PHA (1:1)	0.149 ± 0.006[*]
hAMSC/PBMC + PHA (1:5)	0.153 ± 0.027[*]
hBMSC/PBMC + PHA (1:5)	0.161 ± 0.007[*]
hAMSC/PBMC + PHA (1:10)	0.182 ± 0.014[*]
hBMSC/PBMC + PHA (1:10)	0.167 ± 0.008[*]
PBMC + PHA	0.297 ± 0.033
PBMC	0.215 ± 0.019
[*]P<0.05 vs. the PBMC + PHA group.	

Table 1: Lymphocyte proliferation in MSCs co-cultured with PMBCs measured using the CCK-8 assay (mean ± SD)

IFN-γ production in the supernatant of PBMCs co-cultured with MSCs

The IFN-γ level in the supernatant of PHA-stimulated PBMCs was lower when these cells were co-cultured with either hAMSCs or hBMSCs (P<0.05) (Table 2 and Figure 6). However, the IFN-γ level in

the supernatant of PHA-stimulated PBMCs was lower when these cells were co-cultured with hAMSCs than when they were co-cultured with hBMSCs (P=0.056).
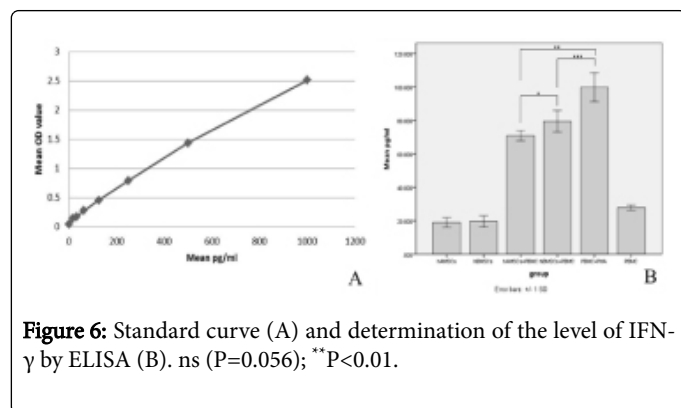

Figure 6: Standard curve (A) and determination of the level of IFN-γ by ELISA (B). ns (P=0.056); **P<0.01.

Group	IFN-γ
hAMSC	18.945 ± 2.938**
hBMMSC	19.582 ± 3.427##
hAMSC+PBMC+PHA (1:1)	70.918 ± 3.073
hBMSC+PBMC+PHA (1:1)	79.546 ± 6.497△△
PBMC+PHA	99.874 ± 8.629▲▲
PBMC	27.873 ± 1.611

**P<0.01 vs. the hAMSC + PBMC + PHA group;

##P<0.01 vs. the hBMMSC + PBMC + PHA group; ▲▲P<0.01 vs. the hAMSC + PBMC + PHA group; △△P<0.01 vs. the PBMC + PHA group.

Table 2: Supernatant level of IFN-γ in each group measured by ELISA (mean ± SD, pg/ml, n=3).

Discussion

Allogeneic hBMSCs were used for the treatment of a patient with aGVHD in 2004 with satisfactory outcomes [5]. Since then, there have been an increasing number of experimental and clinical studies pertaining to MSCs. Most studies have focused on bone marrow-derived stem cells. A recent systematic review and meta-analysis of clinical trials examined the safety of MSCs. Lalu et al. demonstrated that there was not an association between MSCs and acute infusional toxicity, organ system complications, infection, death, or malignancy [7]. The combination of hBMSCs and immunosuppressants was effective against steroid-refractory aGVHD [8,9]. With the continuation of hBMSCs research and of its application, there is a huge need for MSCs. However, it is difficult to meet clinical needs using hBMSCs because these cells have limited proliferation activity *in vitro*.

Both hAMSCs and hBMSCs are derived from the embryonic mesoderm, and share similar biological characteristics. We showed that both hAMSCs and hBMSCs have fibroblast-like morphology; however hAMSCs exhibited higher proliferation activity than hBMSCs. On days 1 to 3 (the latency period), both types of cells grew slowly. FACS analysis of the 3 day cell cultures revealed no significant difference in the proportion of cells in G2/M phase (P>0.05) between hAMSCs and hBMSCs. On days 3 to 8 of culture, hAMSCs entered into the log phase, and showed rapid proliferation. hBMSCs showed lower proliferation than hAMSCs on day 3, and entered into the plateau phase of proliferation gradually on days 7 and 8. In addition, hBMSCs showed slow proliferation and aging at passages 5 to 6, while hAMSCs were still growing well at passage 6 and could be used for further passages. Such a finding is consistent with a previous report indicating that hAMSCs could be maintained for 15 or more passages [10].

In the current study, both hAMSCs and hBMSCs were found to express vimentin, CD105, CD73, and CD90, but not CD11b, CD19, CD34, CD45, or HLA-DR. This immunophenotyping pattern is in agreement with the criteria for identifying MSCs defined by the International Society for Cellular Therapy [11]. HAMSCs expressed Oct-3/4, but hBMSCs did not. Oct-3/4 plays a critical role in the proliferation and multipotent differentiation potential of embryonic stem cells, and is a surface marker of embryonic stem cells. Our findings indicate that hAMSCs exhibit clearer stem cell characteristics and may have greater multipotent differentiation potential than hBMSCs [12].

MSCs have immunoregulatory functions [13]. These cells may modulate the immune response through the direct contact with immune cells, or through the secretion of multiple cytokines [14,15]. MSCs have been reported to suppress allogeneic T lymphocyte proliferation in a dose dependent manner [16-18]. In our experiments both hAMSCs and hBMSCs suppressed PBMCs proliferation in a dose-dependent manner, which is consistent with previous studies. However, there was no significant difference between hAMSCs and hBMSCs in the suppression of PBMCs proliferation.

In the present study, co-culturing PHA-stimulated PBMCs with MSCs significantly suppressed IFN-γ secretion (P<0.01). There were lower IFN-γ levels in the supernatants of the PHA-stimulated PBMCs co-cultured with hAMSCs than in those of PHA-stimulated PBMCs co-cultured with hBMSCs, but the difference was not statistically significant (P=0.056). IFN-γ is an inflammatory cytokine secreted by CD4+ Th1 cells. It plays a critical role in the activation of CD4+ T cells, NK cells, NKT cells, B cells, and antigen-presenting cells. Our findings showed that both hAMSCs and hBMSCs inhibited IFN-γ secretion by lymphocytes, which may result in negative feedback regulation of Th1-mediated inflammation. It has been reported that MSCs may express MHC class II molecules on the surface for antigen presentation, and that they secrete small amounts of IFN-γ to positively regulate the expression of MHC class II molecules. A reduction in the level of MHC class II molecules on MSCs has been reported after the increase of IFN-γ levels, resulting in immunosuppression [19]. Multiple soluble factors are involved in MSC-mediated immunosuppression, including hepatocyte growth factor, transforming growth factor-beta 1, indoleamine 2,3, dioxygenase, and prostaglandin E2. IFN-γ may directly or indirectly promote the secretion of these soluble factors to positively regulate MSC-induced immunosuppression [18].

In conclusion, the results of this study demonstrate that the hAMSCs and hBMMSCs have similar biological characteristics and immunosuppressive effects. However, hAMSCs exhibit clearer stem cell characteristics and higher proliferation activity than hBMSCs. Our results suggest that hAMSCs may be a better choice than hBMSCs for the treatment of aGVHD.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University.

Ethics, Consent and Permissions

Signed informed consent with permissions to report individual patient data was obtained from all participants after a detailed description of the purposes of our study.

Availability of Data and Materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Funding

Authors' Contributions

B-h P and H-t S designed the research. Y G and J-q H analyzed the data and prepared the typescript. The other authors provided the subject data. All authors read and approved the final manuscript.

Acknowledgements

Competing Interests

The authors declare that they have no competing interests.

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