Sertoli cells promote proliferation of bone marrow-derived mesenchymal stem cells in co-culture

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Bone marrow-derived mesenchymal stem cells (BMSCs) are a major source for cell transplantation. The proliferative ability of BMSCs is an important determinant of the efficiency of transplant therapy. Sertoli cells are “nurse” cells for development of sperm cells. Our recent study showed that Sertoli cells promoted proliferation of human umbilical cord mesenchymal stem cells (hUCMSCs) in co-culture. Studies by other groups also showed that Sertoli cells promoted growth of endothelial cells and neural stem cells. In this study, we investigated the effect of Sertoli cells on proliferation of BMSCs. Our results showed that Sertoli cells in co-culture significantly enhanced proliferation of BMSCs ($P < 0.01$). Moreover, co-culture with Sertoli cells also markedly increased mRNA and/or protein expressions of Mdm2, p-Akt and Cyclin D1, and decreased p53 expression in BMSCs ($P < 0.01$ or $< 0.05$). These findings indicate that Sertoli cells have the potential to enhance proliferation of BMSCs.

Keywords: BMSCs, Cell proliferation, Cyclin D1, hUCMSCs, p53 expression, Transplant therapy

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stromal cells that can differentiate into cells of different lineages, such as osteocytes, adipocytes, endothelial cells, cardiomyocytes and neurons. Currently, they are widely used in regenerative medicine. The proliferative ability of BMSCs is the most important determinant for the efficiency of the transplant therapy. High proliferative ability ensures more BMSCs survival in the receipts after the transplantation.

Studies on techniques to stimulate the proliferation of MSCs are not uncommon. Few tried by regulating the gene expression or application of some chemical stimulators. For instance, a recent study tried to enhance the proliferation and differentiation of human synovium-derived MSCs through overexpression of TGF-β1 in these cells. However, transplantation of these gene operated cells would cause many biological uncertainties in the later life of the receipts. In addition, most chemicals have potential to cause cell apoptosis or have the teratogenicity to the cells. Recently, application of co-culture with other cell types that able secret growth factors or use of conditioned medium from the co-culture system to stimulate the proliferation of MSCs had gradually attracted attention of many groups. Use of the co-culture or the conditioned medium to enhance MSC proliferation could successfully avoid the above-mentioned disadvantages. In most cases, we can utilize the co-culture with the autologous cells or the homologous cells to stimulate the proliferation of MSCs.

Sertoli cells are a kind of “nurse” cells in the testes involved in the formation of testis and spermatogenesis. Previous studies have shown that Sertoli cells promote proliferation of endothelial cells and neural stem cells in the in vitro co-culture states through excreting growth factors and cytokines such as epithelial growth factor, nerve growth factor and IL-6. Our recent study also showed that co-culture with Sertoli cells increased proliferation of human umbilical cord mesenchymal stem cells. Whether Sertoli cells affect proliferation of BMSCs has not been examined until now. Here, we investigated the effect of Sertoli cells on proliferation of BMSCs and the related mechanisms.

Materials and Methods

Materials
ProlongH Gold Antifade Regent was purchased from Invitrogen™/Life Technologies (Grand Island, NY, USA); RNeasy Mini-Kit, DNase I Kit and SuperScript II 1st Strand DNA Synthesis Kit from

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Invitrogen (Carlsbad, CA, USA); FITC-conjugated CD90, PE-conjugated CD44, Sox9, CyclinD1 and \( \beta \)-actin antibodies from Abcam (Cambridge, MA, USA); P53, Mdm2 and phospho-Akt antibodies from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA); and protein extraction kit, BCA protein assay kit, PVDF membrane, DAB detection IHC kit and PCR reaction mix were purchased from Henan Tianchi Biological Technology Co., Ltd. (Zhengzhou, Henan, China). Hematoxylin, eosin, trypsin and fetal bovine serum (FBS) were purchased from Zhengzhou Dingguo Biological Technology Co., Ltd. (Zhengzhou, Henan, China).

Isolation and culture of BMSCs and Sertoli cells

BMSCs were isolated from mouse tibia and femur and cultured as per recently published protocols\textsuperscript{11}. Sertoli cells were isolated from the testes of 2-wk old mice using 0.25% trypsin digestion and cultured in DMEM supplemented with 10% FBS. The third passage of BMSCs and the secondary passage of Sertoli cells were used in the experiments.

Cell counting

The third passage of BMSCs (2×10\textsuperscript{5}/well) was plated in 24-well plates and cultured for 12 h. After that, a group of cells were co-cultured with Sertoli cells in the Transwell co-culture system (Pore size 0.4µm; Corning Costar, Tewksbury, MA, USA) for 24 h. In parallel, another group of cells cultured alone in the common condition were served as controls. Cell numbers were determined using a hemocytometer.

Hematoxylin and eosin (HE) staining

BMSCs and Sertoli cells were fixed with the buffered paraformaldehyde. The HE staining was performed as the standard protocols.

Immunofluorescent and immunochemistry staining

For immunofluorescent staining, BMSCs grown on the coverslips were fixed with 4% buffered paraformaldehyde for 15 min and treated with 0.1% TritonX-100 for 10 min at room temperature (25\degree C). Then, the cells were blocked with 10% goat serum/1% bovine serum albumin (BSA) in PBS for 30 min, and then incubated in dark with PE-conjugated CD44 antibody (1:400) for 1 h followed by another incubation with FITC-conjugated CD90 antibody (1:400) in the dark for 30 min at room temperature. After washing, the coverslips were mounted on slides with ProlongH Gold Antifade Regent with DAPI and viewed under a fluorescence microscope.

For immunochemistry staining, Sertoli cells grown on the coverslips were fixed and blocked as above-mentioned protocols. Then, the cells were incubated with Sox9 primary antibody (1:400) overnight at 4\degree C overnight. The cells were then incubated with Biotinylated goat anti-polyvalent for 10 min at room temperature. After washing 4 times, the cells were incubated Streptavidin peroxidase for 10 min at room temperature followed by one more incubation with DAB Chromogen and DAB Substrate for 5 min at room temperature.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from BMSCs using a RNeasy Mini-Kit and cDNA was synthesized using a SuperScript II 1\textsuperscript{st} Strand DNA Synthesis Kit according to the manufacturer's instructions. PCR reactions were performed as the standard protocols. The primers used for RT-PCR reactions were listed in Table 1.

Western blotting

Proteins were extracted from BMSCs using a RIPA Protein Extraction Kit, and protein concentrations were measured by BCA assay with a BCA protein assay kit. Western blotting assay were performed as per recently published protocols\textsuperscript{12}. In brief, proteins (20 µg/sample) were diluted in 2X loading buffer, heated at 95\degree C for 5 min, loaded into 12% SDS-PAGE gels and separated by electrophoresis. Following electrophoresis, the proteins were transferred onto PVDF membranes. The blots were blocked by 5% non-fat milk in Tris buffered saline with Tween-20 (TBS-T) for 1 h at room temperature, and then incubated with primary antibodies against p53, Mdm2, phosphor-Akt, Cyclin

<table>
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<th>Primer</th>
<th>Sense</th>
<th>Sequence</th>
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<tr>
<td>p53</td>
<td>Forward</td>
<td>5’-GGATAGCAAAGACACAGACAGC-3’</td>
<td>472 bp</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCAGTCTTTCGGACAAGGCGTAC-3’</td>
<td></td>
</tr>
<tr>
<td>Mdm2</td>
<td>Forward</td>
<td>5’-CCAGCATTTTCAGCTTTTTGT-3’</td>
<td>340 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAAAAGCTATCCCTTGCTGCTTCT-3’</td>
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<tr>
<td>( \beta )-actin</td>
<td>Forward</td>
<td>5’-TTTCTTTGAGCTCCTTCTGTGGCG-3’</td>
<td>458 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TGGATGGCTACGTACATGGGCTGGG-3’</td>
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D1 and β-actin (1:1000) in blocking solution at 4°C overnight. After washing with TBS-T for 3 times, the blots were incubated with HRP-conjugated secondary antibodies (1:10000) in blocking solution for 1 h at room temperature. After washing with TBS-T, the blots were incubated with chemilluminescence substrate for 3 min at room temperature, and then exposed to X-ray films. All protein bands were quantified by Image J software.

Statistical analysis
Statistical analysis was performed with SPSS 16.0 software. Data were presented as the mean ± standard deviation (SD). Univariate comparisons of means were evaluated using the Student \( t \) tests. \( P < 0.05 \) was considered as statistically significant difference.

Results
Identification of BMSCs and Sertoli cells
Phase contrast microscope image and HE staining showed that the third passage BMSCs assumed long-spindle and typical fibroblast-like shapes (Fig. 1 A and B). Immunostaining showed that the third passage BMSCs were positive for MSC specific marker CD 44 and CD 90 (Fig. 1 C-F).

Phase contrast microscope image and HE staining showed that Sertoli cells were irregular cells, but their boundaries were regular and smooth, and most of cells assumed a similar morphology (Fig. 2 A and B). This data was consistent with previous reports from other groups. Sox9 is a nuclear transcription factor, which is preferentially expressed in Sertoli cells and viewed as a typical marker of Sertoli cells. Immunochemistry staining showed that Sox9 was positively expressed in Sertoli cells, especially in the nucleus (Fig. 2C). Of note, Sox9 was negative in the nucleus of Sertoli cells when the cells were acquired a negative staining (absence of primary antibody of Sox9) (Fig. 2D).

Effect of Sertoli cells on proliferation of BMSCs
To study the effect of Sertoli cells on proliferation of BMSCs, we co-cultured BMSCs with Sertoli cells...
Fig. 3—Effect of Sertoli cells on BMSC proliferation and p53 and Mdm2 mRNA expression in the co-cultured BMSCs. (A) The diagram shows the co-culture of bone marrow mesenchymal stem cells (BMSCs) with Sertoli cells (SCs) in a Transwell system; (B) Cell number in the control- and co-cultured-BMSCs; (C and D) RT-PCR assay shows p53 and Mdm2 mRNA expression in the control- and co-cultured-BMSCs. [Bar graphs represent mean ± SD (n = 4 per group). *P < 0.05 vs. control]

Effect of Sertoli cells on expression of proliferative signals in BMSCs

Cellular tumor suppressor p53 (p53) is a protein that negatively regulates cell proliferation. Mdm2 can interact with p53 and suppress its expression. RT-PCR assay showed that Sertoli cells in co-culture significantly decreased p53 mRNA expression (Fig. 3C; P < 0.05); however, increased Mdm2 mRNA expression in BMSCs (Fig. 3D; P < 0.01). These data were further confirmed by Western blotting assay, which showed a significant decrease of p53 protein (Fig. 4A; P < 0.05) and a significant increase of Mdm2 protein (Fig. 4B; P < 0.01) in the co-cultured BMSCs. Further, we also observed that marked increases of phospho-Akt and Cyclin D1 in the co-cultured BMSCs (Fig. 4 C and D; P < 0.01).

Discussion

Bone marrow-derived mesenchymal stem cells (BMSCs) are the most promising seed cells for cell-based transplant therapy and have been widely used in clinic to treat many kinds of diseases, such as neurodegeneration, stroke and heart infarction. Proliferation of BMSCs determines the therapeutic efficiency. In this study, we found that Sertoli cells enhanced the growth of BMSCs in co-culture, which was accompanied with an upregulation of pro-proliferative signals such as Mdm2, phosh-Akt and Cyclin D1 and a downregulation of anti-proliferative signal p53.

Sertoli cells are “nurse” cells for formation of sperm cells during spermatogenesis. Their existence supports growth of sperm cells. Previous studies showed that Sertoli cells in co-culture promoted growth of stem cells, including neural stem cells and umbilical cord mesenchymal stem cells. In the present study, we utilized the Transwell with 0.4 µm polyester membrane inserts to co-culture BMSCs with Sertoli cells (Fig. 3A). We found that Sertoli cells promoted proliferation of BMSCs in co-culture. Application of Transwell system to co-culture cells is the most common method to test the paracrine communication of different cell types in vitro. Of note, BMSCs cultured alone in the common culture plates served as the controls in this study, which was the most common way to set controls in the co-culture study and had been also adopted by many other research groups. However, Zhou recently utilized the cells cultured under the empty Transwell
inserts as the controls. This new method sounds more reasonable than culturing cells alone as controls because it can avoid the effects of Transwell inserts themselves on the cells.

We also observed a decrease of p53 and an increase of Mdm2 in the co-cultured BMSCs. The p53 is tumor suppressor gene, which has been demonstrated to inhibit cell proliferation via caspase-mediated cleavage of ERK2/MAPK. Mdm2 is a key regulator of p53. It can interact with p53 and repress p53 transcriptional activity.

The expression of phospho-Akt and Cyclin D1 protein was also markedly increased in the co-cultured BMSCs. Akt is a key molecule for cell survival and proliferation. Its activation mediates a series of cellular processes such as cell proliferation, apoptosis and migration. Cyclin D1 is a cell cycle regulator. It interacts with CDKs to promote progression of cell cycle. Its expression is especially required for cell cycle transition from G1 phase to S phase. Alteration of these proliferation-related signals may be partially responsible for the increased proliferation of BMSCs as the cells were co-cultured with Sertoli cells.

A recent study has provided that Sertoli cells can promote the differentiation of mouse embryonic stem cells into germ cells and spermatogonial stem cells into spermatocytes in a co-culture state via the paracrine communications. So, we conclude that Sertoli cells may also influence the differentiation of BMSCs in a long-time co-culture state as they are able to secret a series of growth factors and cytokines that can stimulate cell differentiation.

Conclusion

In this study, we demonstrated that Sertoli cells promoted the proliferation of BMSCs in co-culture. These findings suggest that co-culture with Sertoli cells may be a viable approach to improve the efficiency of BMSC-based transplant therapy.

Acknowledgment

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References


