Biochemical and Biophysical Research Communications 427 (2012) 86–90

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Co-culture with Sertoli cells promotes proliferation and migration of umbilical cord mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 27 August 2012
Available online 10 September 2012

Keywords:
Umbilical cord mesenchymal stem cells
Sertoli cells
Co-culture
Proliferation
Migration

ABSTRACT

Human umbilical cord mesenchymal stem cells (hUCMSCs) have been recently used in transplant therapy. The proliferation and migration of MSCs are the determinants of the efficiency of MSC transplant therapy. Sertoli cells are a kind of "nurse" cells that support the development of sperm cells. Recent studies show that Sertoli cells promote proliferation of endothelial cells and neural stem cells in co-culture. We hypothesized that co-culture of UCMSCs with Sertoli cells may also promote proliferation and migration of UCMSCs. To examine this hypothesis, we isolated UCMSCs from human cords and Sertoli cells from mouse testes, and co-cultured them using a Transwell system. We found that UCMSCs exhibited strong proliferation ability and potential to differentiate to other cell lineages such as osteocytes and adipocytes. The presence of Sertoli cells in co-culture significantly enhanced the proliferation and migration potential of UCMSCs ($P < 0.01$). Moreover, these phenotypic changes were accompanied with upregulation of multiple genes involved in cell proliferation and migration including phospho-Akt, Mdm2, phospho-CDC2, Cyclin D1, Cyclin D3 as well as CXCR4, phospho-p44 MAPK and phospho-p38 MAPK. These findings indicate that Sertoli cells boost UCMSC proliferation and migration potential.

1. Introduction

Human umbilical cord mesenchymal stem cells (hUCMSCs) are a novel source of pluripotent progenitors that can differentiate into cells of different lineages, such as osteocytes, adipocytes, chrodrocytes, cardiomyocytes, neurons and steroidogenic cells [1,2]. Compared to bone marrow-derived mesenchymal stem cells (MSCs), UCMSCs can be easily isolated without any trauma to the donors. These cells are able to readily proliferate and migrate in the recipient body after transplant [3]. High proliferation and migration ability ensure that more MSCs reach injured tissues resulting in improved transplant therapeutic efficiency.

Sertoli cells produce essential cytokines and serve as support cells in the process of spermatogenesis [4]. Recent studies have shown that Sertoli cells also promote proliferation of endothelial and neural stem cells in co-culture via excreting cytokines such as epithelial growth factor, nerve growth factor and IL-6 [5,6]. Whether Sertoli cells can also affect the proliferation of UCMSCs has not been examined, and this topic is a focus of the present study.

2. Methods

2.1. Isolation and culture of UCMSCs and Sertoli cells

Umbilical cords from full-term deliveries were obtained from The Affiliated Hospital of Guiyang Medical University and The Third Hospital of Xinxiang Medical University after the written informed consent of mothers. The experiments were performed in accordance with requirements of local ethical committee. The umbilical vessels were removed and the Wharton's jelly was minced into small pieces, plated in 100-mm dishes and cultured in high-glucose DMEM-F12 medium supplemented with 20% FBS, 100 U/mL penicillin and 100 g/L streptomycin. Based on preliminary data on cell proliferation, passage 3 UCMSCs were used to study proliferation and migration of UCMSCs alone or co-cultured with Sertoli cells.

Testes were obtained from 2-week-old mice, minced into small pieces, and Sertoli cells were isolated using 0.25% trypsin digestion. The isolated Sertoli cells were cultured in DMEM-F12 medium supplemented with 10% FBS.

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http://dx.doi.org/10.1016/j.bbrc.2012.09.007

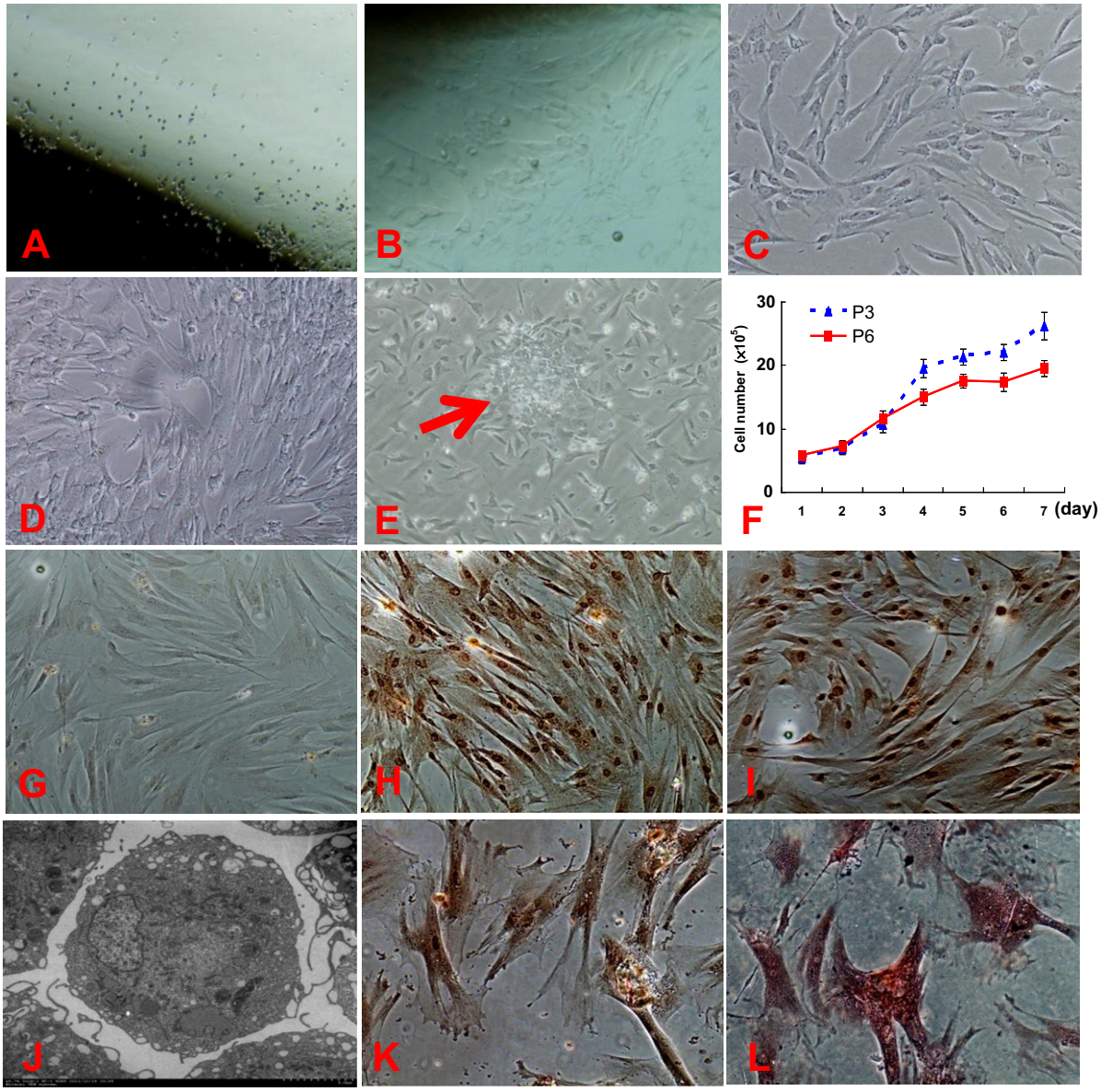

Fig. 1. Identification of UCMSCs. (A, B) The morphology and growth pattern of primary UCMSCs by day 1, 4 and 8 after plating. (C, D) The morphology and growth pattern of passage 3 UCMSCs by day 4 after plating. (E) Growth curve of passage 3 and passage 6 UCMSCs ($n = 5$). (G-I) Immunochemistry shows CD34, cd44 and CD90 expression in passage 3 cells. (J) Ultrastructural image with identifiable mitrochondria, Golgi apparatus and cytoplasmic vesicles in UCMSCs. (K) Immunochemistry shows positive expression of alkaline phosphatase in UCMSCs maintained in osteogenic inductive medium. (L) Oil red staining shows deposition of lipid droplets in UCMSCs maintained in adipogenic inductive medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Co-culture of UCMSCs with Sertoli cells

UCMSCs were co-cultured with Sertoli cells in Transwell chambers with 3 μm pore filters (Corning Costar, Tewksbury, MA, USA) for 4 days. UCMSCs cultured in high glucose DMEM-F12 medium supplemented with 20% FBS served as control.

2.3. Osteogenic and adipogenic differentiation

To induce osteogenic differentiation, passage 3 UCMSCs were cultured in high glucose DMEM-F12 medium supplemented with 10% FBS, 0.1 μM dexamethasone, 10 μM glycerophophate, and 50 μM ascorbic acid. For adipogenic growth, medium was supplemented with 10% FBS, 1 μM dexamethasone, 10 μg/ml insulin, 0.1 mM 3-isobutyl-1-methyl-xanthine, and 0.2 mM indomethacin [7]. After 30 days, cells were analyzed by immunochemistry using anti-alkaline phosphatase antibody (Santa Cruz Biotechnology, CA, USA) to study calcium deposition or by staining with 0.6% oil red to visualize lipid droplets.

2.4. Cell counting

UCMSCs (5×10^5/well) were seeded in 6-well plates and cell number was determined using a hemocytometer on day 1 through 7. For the co-cultured UCMSCs, cell number was counted after 24 and 96 h of co-culture.

2.5. Immunofluorescence assay

Immunostaining of UCMSCs was performed using standard protocol. All samples were imaged using a Zeiss LSM510 Confocal Microscope.

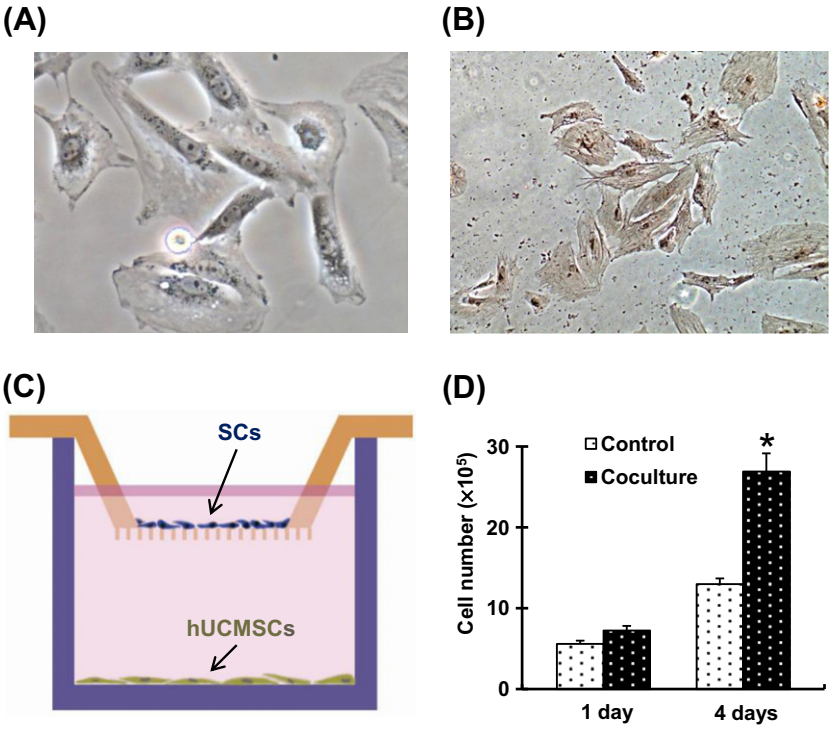

Fig. 2. Co-culture with Sertoli cells promotes UCMSC proliferation. (A) The morphology of primary Sertoli cells. (B) Immunochemistry shows Fas-L, a typical marker of Serteoli cells, in the primary Sertoli cells. (C) The diagram shows the co-culture Sertoli cells and UCMSCs in a Transwell system. (D) Cell number in the control and the co-cultured UCMSCs. Bar graphs represent mean ± SD ($n = 5$ per group). $^{*}P < 0.01$ vs. control.

2.6. Ultrastructural analysis

Passage 3 UCMSCs were washed and centrifuged to prepare the cell blocks, which were fixed with 2% glutaraldehyde for 2 h, and then with 1% osmic acid for 2 h. The cell blocks were embedded in epoxy resin. The ultrathin sections were made, and stained with uranyl acetate and lead citrate, and viewed under H-7650 transmission electron microscope.

2.7. Cell cycle analysis

The UCMSCs were collected, washed and fixed with 70% ethanol. The fixed cells were treated with RNase-A (50 μg/ml) and propidium iodide (25 μg/ml) in PBS containing 0.1% Triton X100 for 30 min in the dark. Analysis of cells in different phases of the cell cycle was conducted by flow cytometer (BD Biosciences, San Jose, CA, USA).

2.8. Transwell migration assay

The migration of UCMSCs was measured using Transwell plates (Corning Costar, Tewksbury, MA, USA) with 8 μm pore filters, as previously described by Kim et al. [8].

2.9. Western blotting

Protein was extraction and Western blotting was performed using standard protocols. Source of primary antibodies for Western blotting: Mdm2, Cyclin D1, Cyclin D3, MMP-2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CXCR4 (ABcam, Cambridage, MA, USA), phospho-Akt, phospho-CDC2, phospho-p44/42MAPK, phospho-38MAPK (Cell signaling Technology, Danvers, MA, USA).

2.10. Statistical analysis

Statistical analysis was performed with SPSS 11.5 software. Data are presented as means ± standard deviation (SD). Univariate comparisons of means were evaluated using the Student t test, $P < 0.05$ was considered statistically significant. The data shown in the figures are representative of 4 or 5 independent experiments.

3. Results

3.1. Identification and proliferation of UCMSCs

One day later after bits of Wharton's jelly tissues were plated, many round cells dissociated from the tissues (Fig. 1A). Three to five days later, the dissociated cells assumed triangular and spindle shapes (Fig. 1B). By day 8, most cells acquired long-spindle shape and reached near-confluence (Fig. 1C). Passage 3 cells exhibited spindle shape and, upon reaching confluence, formed whirlpool-like pattern (Fig. 1D) with occasional clonal expansions (Fig. 1E, arrow).

Cell growth curve showed that passage 3 UCMSCs had higher proliferative ability than passage 6, and cultures reached logarithmic growth phase on day 3– day 5 (Fig. 1F). Immunochemistry showed that UCMSCs were negative for CD34 (Fig. 1G), and expressed MSC markers CD44 (Fig. 1H) and CD90 (Fig. 1I), thus validating purity of isolated UCMSCs. The transmission electron microscopy showed numerous mitrochondria, Golgi apparatus and cytoplasmic vesicles in USMSCs (Fig. 1J). The cytoplasmic vesicles were large and dispersed in the peri-membrane space (Fig. 1J).

3.2. UCMSCs readily to transform into osteoblasts and adipocytes

Mesenchymal stem cells (MSCs) have the potential to differentiate into multilineage cells, such as osteocytes, adipocytes and

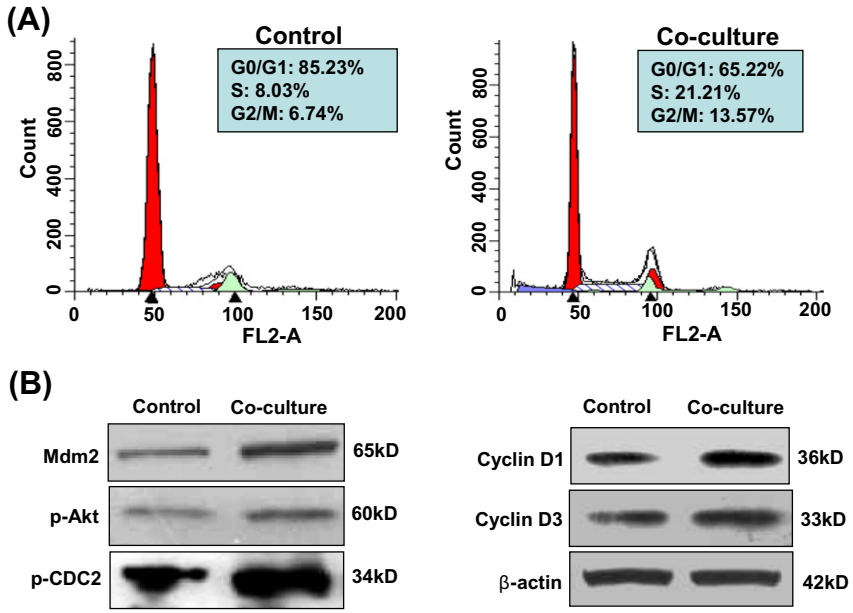

Fig. 3. Analysis of the cell cycle and expression of cell cycle regulatory molecules in UCMSCs plated alone or co-cultured with Sertoli cell. (A) Cell cycle analysis shows higher fractions of S and G2/M phase cell in the co-culture group than in the control. (B) Expression of Mdm2, phospho-Akt, phospho-CDC2, Cyclin D1 and Cyclin D3 in the control and co-cultured UCMSCs.

chrondrocytes. To further confirm identity of the isolated UCMSCs, we examined differentiation of UCMSCs to osteoblasts and adipocytes using appropriate media. Culturing cells in osteogenic inductive medium resulted in appearance of alkaline phosphatase activity (Fig. 1K), whereas cells cultured in adipogenic inductive medium displayed accumulation of lipid droplets in the cytoplasm (Fig. 1L).

3.3. Sertoli cells promote proliferation of UCMSCs

Fig. 2A illustrates the morphological appearance of primary mouse Sertoli cells on day 5 after plating. The cells stained positively for Fas ligand (Fas-L), a typical marker of Sertoli cells (Fig. 2B).

Co-culture of UCMSCs with Sertoli cells in Transwell chamber (Fig. 2C) caused dramatic acceleration of UCMSCs proliferation resulting in quadrupling of cells in 4 days compared to only doubling of cell number in control plates ($P < 0.01$, Fig. 2D). These findings were further confirmed by cell cycle analysis, which showed that the fraction of S and G2/M phase UCMSCs in co-culture was higher than that in control (Fig. 3A). Western blotting data showed that co-cultured UCMSCs exhibit markedly increased expression of cell survival and proliferation signals Mdm2 and phospho-Akt (Fig. 3B), as well as cell cycle signals phospho-CDC2, Cyclin D1 and Cyclin D3 (Fig. 3B).

3.4. Sertoli cells promote migration of UCMSCs

The migration ability of UCMSCs was measured using a Tanswell. After 4 days of co-culture, migration of UCMSCs was significantly enhanced (Fig. 4A). In accordance with previous studies [8–10], we observed significant upregulation of cell migration regulators CXCR4, MMP-2, phosphor-p44MAPK, and phosphor-p38MAPK in the co-cultured UCMSCs (Fig. 4B).

4. Discussion

In this study, we show that Sertoli cells stimulate proliferation and migration of hUCMSCs in vitro. Our results suggest that Sertoli

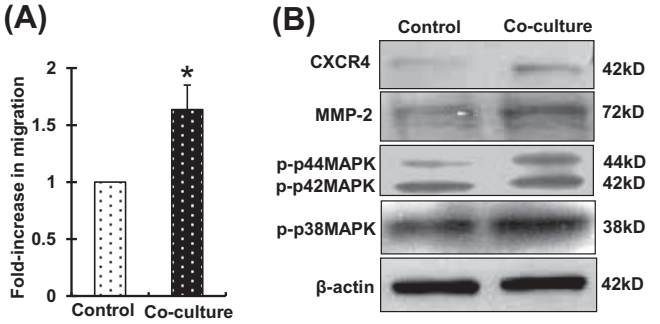

Fig. 4. Analysis of the cell migration and expression of cell migration regulators in UCMSCs plated alone or co-cultured with Sertoli cells. (A) Cell migration rate of co-cultured UCMSCs relative to control UCMSCs. Bar graphs represent mean ± SD ($n = 4$ per group), $^{*}P < 0.01$ vs. control. (B) Expression of CXCR4, MMP2, phospho-p44/42MAPK and phosho-p38MAPK in the control and co-cultured UCMSCs.

cells promote proliferation of UCMSCs via upregulation of genes involved in the regulation of cell cycle progression. Our data are similar to previous observation of enhanced proliferation of endothelial cells by Sertoli cells [5]. Besides the increase in proliferation, we observed that Sertoli cells increased the migration ability of UCMSCs, a key factor in determining therapeutic efficiency of the transplanted material.

It is known that Sertoli cells function to regulate proliferation of other cell lineages such as sperm cells, endothelial cells and neurons [5,6,11]. UCMSCs are very promising as seed cells for stem cell transplant therapy. In the present study, we observed that presence of Sertoli cells in co-culture positively affected UCMSC number. We also observed an increase of Mdm2 and phosphate-Akt content in the co-cultured UCMSCs. Both Akt and Mdm2 are the key molecules for cell survival and proliferation. The activation of Akt mediates a series of cellular processes such as cell glucose metabolism, apoptosis, proliferation and migration [12–15]. The activation of PI3K/Akt pathway is known to modulate cell proliferation via regulation of transcription of multiple genes by phosphorylating FKHRL1, a member of the Forkhead transcription

factor family [12]. Mdm2 is recognized as a potent regulator of cell growth and serves as an important negative regulator of p53tumor suppressor activity. A number of investigators have shown that the activity of Mdm2 is regulated by NF-kB family of transcription factors, particularly RelA (p65) [16,17].

Positive effects of co-culture with Sertoli cells on UCMSC proliferation was further confirmed by cell cycle analysis and the expression of cell cycle regulatory components CDC2, Cyclin D1 and Cyclin D3. In the co-culture group, there were more UCMSCs in S phage and G2/M phages than in the control group. The co-cultured UCMSCs also exhibited markedly increased expression of phospho-CDC2, Cyclin D1 and Cyclin D3. CDC2 (also known as CDK1) is a critical player in cell cycle regulation, which controls the progression of G1 to S, and G2 to M phage [18]. CDKs and their Cyclin partners would form Cyclin-CDK complexes that regulate the progression of cell cycle [19].

Poor migration from the injection sites to the injured regions is one of limitations of MSC transplant therapy. MSCs are able to migrate into the injured tissues after intravenous, intraarterial or intrathecal injection, but the homing rate is very low [20]. Improving the migration ability is one of the most promising strategies to increase the efficiency of MSC transplant therapy. In this study, we observed a significant increase of cell migration after 4 days of co-culture. Several studies have reported CXCR4 and its receptor CXCL12 as well as Akt, ERK, and p38MAPK as key mediators for UCMSC migration [9,10]. In the present study, we found that co-culture with Sertoli cells marked reduced these signals. Recently, Kim et al. reported that matrix metalloproteinase-2 (MMP2) is also a regulator for UCMSC migration, since knockdown of MMP2 markedly decreased oxytocin-induced UCMSC migration [8].

In summary, we provide strong evidence that Sertoli cells can promote the proliferation and migration of UCMSCs in co-culture. Our findings suggest that co-culture with Sertoli cells may be a viable approach to improve the efficiency of UCMSC-based transplant therapy.

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