Co-culture with TM4 cells enhances the proliferation and migration of rat adipose-derived mesenchymal stem cells with high stemness

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Abstract The proliferation and migration of mesenchymal stem cells (MSCs) are the efficiency determinants in MSCs transplant therapy. Sertoli cells considered as “nurse cell” possesses the ability to enhance the proliferation and migration of umbilical cord mesenchymal stem cells (UCMSCs). However, no reports about TM4 cells’ effect on the proliferation and migration of adipose tissue-derived mesenchymal stem cells (ADSCs) have been found until at present research work. Therefore, this study investigates the effect of TM4 cells on the proliferation and migration of ADSCs. We found that the performance of proliferation and migration of ADSCs were improved significantly while maintaining their stemness and reducing their apoptosis rate. After co-culturing with TM4 cells, the co-cultured ADSCs demonstrated higher proportion of synthetic phase (S) cells and

colony-forming units-fibroblastic (CFU-F) number, lower proportion of sub-G1 phase cells and enhanced osteogenic and adipogenic differentiation ability. Moreover, results confirmed the higher multiple proteins involved in cell proliferation and migration including expression of the phospho-Akt, mdm2, phospho-CDC2, cyclin D1 CXCR4, MMP-2, as well as phospho-p44 MAPK and phospho-p38 MAPK in co-cultured ADSCs. Furthermore, the process of TM4 cells promoting the proliferation of ADSCs was significantly inhibited by the administration of the PI3K/AKT inhibitor LY294002. Obtained results indicated that TM4 cells through MAPK/ERK1/2, MAPK/p-38 and PI3K/Akt pathways influence the proliferation and migration of ADSCs. These findings indicated that TM4 cells were found effective in promoting stemness and migration of ADSCs, that proves adopted co-culturing technique as an efficient approach to obtain ADSCs in transplantation therapy.

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Introduction

Adipose-derived mesenchymal stem cells (ADSCs) are one kind of adult stem cells isolated from adipose

tissues that possess self-renewal and multi-lineage differentiation potential characteristics. Several studies have shown that ADSCs can differentiate into many cell types including osteocytes, adipocytes, cartilage cells, neural cells, vascular endothelial cells, cardio myocytes, pancreatic b cells, hepatocytes, and primordial germ cell-like cells (Majumdar et al. 2000; Zuk et al. 2001; Halvorsen et al. 2000; Halvorsen et al. 2001; Rangappa et al. 2003; Planat-Benard et al. 2004; Konno et al. 2010; Okura et al. 2009; Yin et al. 2015; Wei et al. 2016; Yan et al. 2014). Moreover, over the past few decades, ADSCs have been used for the treatment of numerous diseases, including bone and cartilage tissue repair, cardiovascular disease, digestive system diseases, diabetes, urinary system diseases, nervous system diseases and other diseases (Mesimaki et al. 2009; Cai et al. 2013; Lin et al. 2009; Garcia-Olmo et al. 2009; Yamamoto et al. 2010; Konno et al. 2013; Yanez et al. 2006). Therefore, ADSCs have a good prospect of clinical applications. Most importantly, the proliferation and migration ability are the key factors for affecting cell transplantation efficiency. However, the limitations of proliferation/population doubling and loss of stem cell properties during ex vivo expansion impede ADSCs using in stem cell-based therapies (Cai et al. 2013; Tobita et al. 2015; Baer et al. 2010).

On the other hand, Sertoli cells (SCs) are key cells to maintain testicular function (Huleihel and Lunenfeld 2004) and support primary cells growth as feeder cells; which can secrete multiple trophic factors including glial cell line-derived neurotrophic factor (GDNF), epithelial growth factor (EGF), basic fibroblast growth factor (FGF2), Interleukin-1a (IL-1a) and interleukin-6 (IL-6) (Skinner 2005). Moreover, SCs have been reported to promote proliferation, differentiation, migration and survival of co-cultured cells, e.g. neural stem cells (Yue et al. 2006), umbilical cord mesenchymal stem cells (Zhang et al. 2012), endothelial (Fan et al. 2011), and so on (Tian et al. 2014; Monfared et al. 2016). However, there is a difference in different cell types, co-culturing with SCs can promote the proliferation and migration of ADSCs is still unknown. TM4 cells, which are an established cell line culture derived from immature mouse testis, possess major properties of normal Sertoli cells (Mather 1980). Unlike other cell lines recently established, no external gene was introduced into TM4 cells. In addition, compared with primary cultures of

SCs, TM4 cells can be grown in the absence of fetal calf serum, which is very important for SCs transplantation (Mather et al. 1982). Most importantly, TM4 cells can be cultured in massive numbers for experiments that require a substantial amount of cellular extracts. In previous studies, co-culturing with TM4 cells may also act to improve proliferation, differentiation, migration and survival of other cells. Our group's previous work (Tian et al. 2014), indicated that the TM4 secreted EGF promotes the proliferation of bone marrow mesenchymal stem cells (BM-MSCs) via PI3K/Akt signaling pathway. Besides, another study exhibited that co-cultured with TM4 cells could promote mouse embryonic stem cells (mESCs) to germ cells differentiation in vitro (Miryounesi et al. 2013). Moreover, FGF2 secreted by TM4 cells regulated the expression of mRNA for ETV5 and GDNF via mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-signaling cascades, which are the essential factors for spermatogonial stem cell maintenance (Simon et al. 2007).

The aim of the present work is to develop a co-culture system with TM4 cells to promote proliferation and migration of ADSC with high cell stemness and to elucidate possible signal transduction pathways that influence the proliferation and migration of ADSCs. In addition, the key components of signaling pathways involved in ADSC's proliferation and migration were explored in this study. In brief, the undertaken study provides a good way to enhance the cell proliferation and stemness of ADSCs.

Materials and methods

TM4 cells

TM4 cells were purchased from American Type Culture Collection (ATCC, Rockefeller, MD, USA). They were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) and supplemented with 5.0% FBS (Gibco), at 37 °C with 5.0% CO₂.

Isolation and culturing of ADSCs

All experiments involving animals were performed according to the guidelines established by the Institutional Animal Care and Use Committee of Chinese Academy of Agriculture Sciences (CAAS).

Adipose tissue from groins was obtained from a 3-week male Sprague–Dawley (SD) rats (Shanghai Jie Esprit experimental animal Co. Ltd, Shanghai, China). The blood vessels and lymph nodes tissues were removed and adipose tissue was minced into small pieces, then digested by collagenase type I (Sigma-Aldrich, St Louis, MO, USA). The cells were plated in 25 cm² culture T flasks (NUNC, Roskilde, Denmark) and cultured in DMEM/F12 medium supplemented with 10.0% FBS, 100 U/mL penicillin and 100 ug/mL streptomycin. Based on preliminary data on cell proliferation, Passage 3 (P3) ADSCs were used to study proliferation and migration of ADSCs alone and co-cultured with TM4 cells.

Flow cytometry (FCM) analysis

Fluorescence Activated Cell Sorter (FACS) (BD Biosciences, San Jose, CA, USA) was used to characterize P3 ADSCs, analyze cell cycle and cell apoptosis as previously described (Tian et al. 2014).

Characterization of P3 ADSCs

Cells were collected and washed twice by PBS. Then allocated the cells into four tubes and the number of cells was adjusted to 5×10^5 cells per tube by PBS. Afterward, cells were incubated on ice for 1 h with anti-rat antibodies (Bioledend, San Diego, CA, USA) which were conjugated with fluorescein thiocyanate (FITC): CD29-FITC, CD90-FITC, and CD45-FITC. FITC-conjugated IgG cultured under the same medium was used as an isotype control. After washing three times, cells were immediately analyzed using FACS. The results were further enumerated with the help of Flow Jo software.

Cell cycle analysis

The ADSCs were harvested, washed and fixed with 70% ethanol. The fixed cells were treated with propidium iodide (PI) (Sigma-Aldrich) at 37 °C for 30 min in the dark, and then FACS was used to analyze the cell cycle phases.

Cell apoptosis analysis

The ADSCs were collected and treated according to the manufacturer's instructions of Annexin V using

the FITC Apoptosis Detection Kit AD10 (DOJINDO, Tokyo, Japan), the Annexin V was labeled with FITC and cells were stained with propidium iodide (PI). Then the treated cells were analyzed by FACS. All results were further enumerated with the help of Flow Jo software.

Co-culturing of ADSCs with TM4 cells using Transwell system

To keep the ratio of TM4 to ADSCs 10:1 in co-culture system, ADSCs (4×10^4 cells/well) were co-cultured with TM4 cells (4×10^5 cells/well) in transwell chamber in 6 well plates with 3 µm pore filters (Corning Costar, Kennebunk, ME, USA) for 3 days. ADSCs cultured in DMEM/F-12 medium supplemented with 10.0% FBS were taken as control. The numbers of cells were counted by using Countstar automatic counting cell analyzer IC1000 (Ruiyu, Shanghai, China).

Transwell migration analysis

Transwell plates (Corning Costar) with 8 µm pore filters were used to determine the migration of ADSCs, according to the method previously described by Kim et al. (2010). After incubation, cells on the upper side of Transwell membrane in the chamber were wiped off by cotton swab and stained with crystal violet. Stained cells were observed under a microscope, five fields of view with an equal area were selected to count cells, and their mean values were calculated.

Colony-forming unit-fibroblasts (CFU-F) assay

After co-cultured with TM4 cells, ADSCs were collected and counted the cell number. Then, the cells were diluted to 300 cells per 60 mm petri dish with DMEM/F-12 medium supplemented with 10.0% FBS and inoculated on each plate. The ADSCs cultured alone were taken as control. The medium was changed once after every 3 days. Following 2 weeks, the cells were washed twice with PBS and fixed by 4% poly-formaldehyde (PFA) and then stained by 0.25% crystal violet for 15 min at room temperature. Finally, the cells were rinsed by deionized water and become clearly visible to count colony number.

In vitro osteogenic and adipogenic differentiation

ADSCs were inoculated in 6-well plates at a density of 25,000 cells/cm². When reaching 80–90% confluence, the medium was replaced with osteogenic induction medium and adipogenic induction medium, respectively. The induction media were changed after every 2–3 days. The osteogenic medium consists of L-DMEM, 10.0% FBS, 100 nM dexamethasone, 10 mM sodium β -glycerophosphate, and 50 mg/L L-ascorbic acid 2-phosphate. After 21 days, cells were analyzed by alkaline phosphatase (AP), mineralization and quantitative real-time PCR (qPCR) for osteoblastic biomarkers.

For adipogenic differentiation, the induction medium consists of L-DMEM, 10.0% FBS, 1 μ M dexamethasone, 100 nM L-ascorbic acid 2-phosphate, 10 mg/L insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), and 20 μ M indomethacin. All reagents were purchased from Sigma-Aldrich. After 21 days, cells were analyzed by Oil red staining and qPCR for adipogenic marker.

Alkaline phosphatase (AP) staining

After being cultured in osteogenic induction medium for 21 days, the ADSCs were stained for alkaline phosphatase according to the manufacturer's instructions of BCIP/NBT alkaline phosphatase assay kit C3206 (Beyotime, Shanghai, China).

Alizarin red staining

After being cultured in osteogenic induction medium for 21 days, the ADSCs were rinsed twice with PBS. Then, the cells were fixed in 95% alcohol for 30 min at 4 °C and rinsed with PBS. After that, the cells were stained by 0.1% Alizarin red solution (Sigma-Aldrich) for 30 min at 37 °C.

Oil red staining

After cultured in adipogenic-induction medium for 21 days, the ADSCs were fixed for 10 min in 10% formalin followed by rinsing with 60% isopropanol and PBS. Then, the cells were stained for 15 min at room temperature using 0.5% Oil red O solution (Sigma-Aldrich, St Louis, MO, USA). Then, the cells were visualized under Invitrogen EVOS FL Auto Cell

Imaging System. After that, the lipid droplets were dissolved in isopropanol and the absorbance was measured at 510 nm. The total protein concentration of cells was simultaneously determined as per PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The lipid droplet expression of ADSCs was calculated by dividing OD_{510 nm} with the total protein concentration of cells.

Western blotting (WB) analysis

After the extraction of protein, western blotting was performed by using standard protocols, as previously described by Tian et al. (2014). The following primary antibodies for Western blotting were used against: Mdm2, CXCR4, CDK1 and MMP-2 (Proteintech, Chicago, IL, USA), phospho-Akt, Akt (pan), phospho-CDC2, phospho-p44/42MAPK, p44/42MAPK, phospho-38MAPK, p38 MAPK and Cyclin D1 (Cell signaling Technology, Danvers, MA, USA), collagen type I, Cyclin D3 (BOSTER, Hubei, CHINA), β -actin (Signalway Antibody, College Park, MD, USA). The secondary antibodies were from SAB (Signalway Antibody).

Total RNA extraction and quantitative real-time PCR

Total RNA extraction was performed by using total RNA isolation kit RP5611 (Bioteke, Beijing, China) according to the manufacturer's instructions. RNA content and purity were detected by NanoDrop 2000 ultra micro spectrophotometer (Thermo Fisher Scientific, Cambridge, MA, USA). After erasing genomic DNA, the first-strand cDNA was obtained by using reverse transcription kit PrimeScript RT reagent Kit RR047A (Takara, Dalian, China) according to the manufacturer's instructions. Relative expression of stemness related genes were detected using CFX96 TouchTM Fluorescence quantitative real-time PCR system (Bio-Rad, Hercules, CA, USA). The specific primers for stemness marker Nanog, Rex1, Oct4 and Sox2, osteoblastic biomarkers Bsp, Coll, Cbfa1, Ap and Ocn, adipocytes biomarkers Pparg, Adipoq and Cfd (See Table 1). PCR reaction procedure is as follows: The samples were denatured at 94 °C for 1 min, followed by 39 amplification cycles of 94 °C for 15 s, 52 °C for 15 s, and 72 °C for 30 s, in a thermal cycler; fluorescence signal intensity was

Table 1 Primer sequences used for qPCR amplification

Target Gene	Sequence of Primers
Nanog	F: 5'-GACTAGCAACGGCCTGACTCA-3' R: 5'-CTGCAATGGATGCTGGGATA-3'
Rex1	F: 5'-AAGACCTTCTTGCCAGGTT-3' R: 5'-CCACCCTTAGCTTCTCTCC-3'
Oct4	F: 5'-GAAGTTGGAGAAGGTGGAAC-3' R: 5'-GGATGGTTGTCTGGCTGAAC-3'
Sox2	F: 5'-CTCGCAGACCTACATGAAC-3' R: 5'-TCGGACTTGACCACAGAG-3'
Bone sialoprotein (Bsp)	F: 5'-GTGCACATGCGTTTTTATCA-3' R: 5'-TGCTCCTTAGACCCACACTC-3'
Collagen type I (Col1)	F: 5'-CGAGTATGGAAGCGAAGG-3' R: 5'-AGTGATAGGTGATGTTCTGG-3'
Core binding factor alpha (Cbfa1)	F: 5'-CTTCGTCAGCGTCTATC-3' R: 5'-CTTCCATCAGCGTCAACA-3'
Alkaline phosphatase (AP)	F: 5'-GAGGAAGGAGGCAGGATT-3' R: 5'-TCAGCAGTAACCAGAGTCA-3'
Osteocalcin (Ocn)	F: 5'-TCTCTGCTCACTCTGCTG-3' R: 5'-GGAGTCTATTACCCACCTTAC-3'
Peroxisome proliferator-activated receptor gamma (Ppar γ)	F: 5'-GTGGAAGCTGTGCAAGAGAT-3' R: 5'-CATGAATCCTTGTCCCTCTG-3'
Adiponectin (Adipoq)	F: 5'-TAATTCAGAGCAGCCCGTAG-3' R: 5'-TGGGGATAACACTCAGAACC-3'
Complement factor D (Cfd)	F: 5'-GGAGTGACCAAGGATGAGG-3' R: 5'-ACCCAGTGAGGCATTGTG-3'
b-actin	F: 5'-TCTGGGTCATCTTTTCACGGTTGGC-3' R: 5'-CTGGATGGCTACGTACATGG-3'

F forward primer sequence; R reverse primer sequence

measured at 72 °C during each cycle. The mRNA relative expression was quantified by $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Statistical analysis was performed using SPSS 19 software (IBM, Armonk, NY, USA). All statistical values are presented as mean $\pm$ standard deviation (SD). Univariate comparisons of means were evaluated by using the Student's test (*t*), $P < 0.05$ was considered statistically significant. The data shown in the figures are representative experiments performed in triplicate.

Results

Proliferation and identification of ADSCs cultured in vitro

A small amount of fusiform adherent cells was observed on the wall of T-flask after 1 day culture of ADSCs isolated from rat tissue (Fig. 1a). After 3 days, many miscellaneous cells were removed by the way of replacing the medium and the cells were assumed as triangular and spindle shaped cells (Fig. 1b). Cell cultures reached 80–90% confluence on day 7 and the cells mainly exhibited spindle and triangular shapes as shown in Fig. 1c. Passage 3 (P3) and passage 6 (P6) cells exhibited spindle shape morphology. Upon reaching complete confluence, cells formed a

Fig. 1 Identification of rADSCs. **a–c** represents the morphology and growth pattern of primary ADSCs on day 1, 2 and 7 after plating. **d** shows the morphology and growth pattern of passage 3 (P3) and passage 6 (P6) ADSCs on day 3 after plating. **e** shows the growth curve of passage 3 (P3) and passage 6 (P6) ADSCs ($n = 3$). **f** represents the diameter of passage 3(P3) and passage 6(P6) ADSCs after digestion with 0.25% trypsin on day three. **g** shows the phenotypic identification of ADSCs by flow cytometry

whirlpool-like pattern, while P6 cells became flatter than P3 cells (Fig. 1d).

To identify ADSCs, specific surface antigen of ADSCs was analyzed by flow cytometry (FCM). The results showed that ADSCs were positive for CD29 and CD90 surface antigens with the percentages above 95%, and no CD45 surface antigen was expressed by ADSCs with a positive rate below 2% (Fig. 1g). Therefore, ADSCs were successfully isolated and cultured in vitro. Besides, on comparing the growth of ADSCs in P3 and P6, results showed that P3 ADSCs had higher proliferative ability than P6, and cultures reached the logarithmic growth phase on days 1 to 3 (Fig. 1e). Accordingly, the mean cell diameter of P6

ADSCs after digestion with trypsin on day 3 was $18.8 \pm 0.19 \mu\text{m}$, which was larger than P3 ADSCs ($17.73 \pm 0.04 \mu\text{m}$) (Fig. 1f).

TM4 cells promote proliferation of ADSCs and the involved signaling pathway

After being co-cultured with TM4 cells in Transwell chamber for 3 days (Fig. 2a), TM4 cells extraordinarily promoted the proliferation of ADSCs in 3 days. Firstly, the cell number of co-cultured ADSCs expanded sextuple, while it was two times more than that of the single cultured ADSCs ($P < 0.01$, Fig. 2b). Additionally, cell cycle analysis further confirmed

Fig. 2 Co-culture with sertoli cells promotes ADSCs proliferation. **a** shows the co-culture TM4 cells and ADSCs in a transwell system. **b** shows cell number in the control and the co-cultured ADSCs. Bar graphs represent mean $\pm$ SD ($n = 3$ per group). * $p < 0.01$ versus control. **c** represents cell cycles analysis. **d** shows expression of Mdm2, phosphor-Akt, phosphor-CDC2, Cyclin D1 and Cyclin D3 in the control and co-cultured ADSCs. **e** shows that the proliferation of ADSCs decreased when the LY294002 added in the medium. **f** shows the proteins involved in the PI3K/AKT signaling pathway by western blotting

these findings and showed that the proportion of S and G2/M phase ADSCs in co-culture mode was higher than that of cells cultured in control (Fig. 2c). Furthermore, the co-cultured ADSCs noticeably increased the expression of cell cycle signals phospho-CDC2, Cyclin D1 and Cyclin D3 as well as cell survival and proliferation signaling biomarkers Mdm2 and phospho-Akt (Fig. 2d).

In addition, cell cycle analysis showed that the proportion of sub-G1 ADSCs in the co-culture group was lower than for individually cultured cells. To explore whether TM4 cells can cause apoptosis of ADSCs upon co-culture, apoptosis of ADSCs was determined using flow cytometry by staining with Annexin V and PI in control and co-cultures for 3 days. Results indicated that the dead cells percentage of ADSCs in co-culture system was 10% lower

than in control group (Fig. 3). Conclusively, such response elucidated that TM4 cells did not lead to apoptosis but affirmed the enhancement in survival promotion of ADSCs.

The expression of phospho-Akt in the co-cultured ADSCs was markedly increased (Fig. 2d), to further clarify whether PI3K/AKT signaling pathways was involved in the process of TM4 cells promoting the proliferation of ADSCs, western blotting were carried out. As shown in Fig. 2e, when LY294002 (at 5 μ M), the PI3K/AKT-specific inhibitor, was added the co-culture group for 1 day, the cells number decreased by approximately 33.3% (Fig. 2e). At the same time, the expression of phospho-Akt and total Akt were also inhibited based on the data by image pro plus analysis (Fig. 2f). This implies that the PI3K/AKT pathway

Fig. 3 TM4 cells increase the survival of ADSCs (evaluated by detecting Annexin V). ADSCs culture alone as control (Control, left), ADSCs co-cultured with TM4 cells (Co-culture, right)

could be involved in the enhanced ADSCs proliferation by co-culture with TM4 cells.

Co-culture with the TM4 cells enhanced the stemness and multipotency of ADSCs

The mRNA expression levels of four key stemness biomarkers including Nanog, Rex-1, Oct4 and Sox2 were detected by qPCR analyses. It showed that the stemness markers tested here were totally up-regulated in the co-culture group of ADSCs (Fig. 4a). This finding was further confirmed by the increased number of colony-forming unit-fibroblasts (CFU-F) in co-culture group in comparison to the control group (Fig. 4b).

The most important characteristic is the osteogenic and adipogenic differentiation ability of ADSCs, which was also enhanced after being co-cultured with TM4 cells. The mRNA expression levels of osteoblastic biomarkers alkaline phosphatase (Ap), Osteocalcin (Ocn), core binding factor alpha (Cbfa1), collagen type I (ColI) and bone sialoprotein (Bsp) as well as adipocytes biomarkers proliferator-activated receptor gamma (Ppar γ), adiponectin (Adipoq) and Complement factor D(cfd) were up-regulated significantly in

the co-culture groups (Fig. 5d, e). These results were further confirmed by the up-regulation of collagen type I secreted by osteoblasts using western blotting analysis (Fig. 5b) and alkaline phosphatase activity was measured by pNPP method (Fig. 5a) and alkaline phosphatase staining [Fig. 5f(a, d)]. Besides, we observed more calcium accumulation in the co-culture group of ADSCs [Fig. 5f(b, e)]. Further, it was observed that more lipids were stained in the co-culture group (Fig. 5c and f(c, f)). All these results aided in obtaining major conclusions for this effect. Hence, it was proven that this approach successfully promoted the stemness and multipotency of ADSCs after co-culture with TM4 cells.

Improvement of migration of ADSCs co-cultured with TM4 cells

As shown in Fig. 6a, crystal violet staining indicated that the number of migrated ADSCs in the co-culture group was significantly higher than that of the control group. This manifested that the migration of ADSCs cultured in Transwell system was significantly enhanced after co-cultured with TM4 cells for 24 h. Furthermore, the regulators CXCR4, MMP-2 and key

Fig. 4 Stemness analysis of ADSCs cultured alone and co-cultured with TM4 cells. **a** represents the mRNA expression levels of stem cell genes Rex, Sox2, Oct4 and Nanog. **b** shows the morphology and number of colonies formed by the cells

under the two culture conditions and the colonies were stained with crystal violet. Bar graphs represent mean $\pm$ SD ($n = 3$ per group). * $p < 0.05$, ** $p < 0.01$ versus control

Fig. 5 Osteogenic and adipogenic differentiation of ADSCs culture alone or in co-culture with TM4 cells. **a** AP activity assay by pNPP method ($n = 5$). **b** shows the analysis of type I collagen of ADSCs by western blotting. **c** represents quantitative analysis of lipid droplets after Oil Red O staining ($n = 5$). **d** shows the mRNA expression levels of osteogenic specific marker ($n = 3$). **e** shows the mRNA expression levels of

adipogenic specific marker ($n = 3$). **f** shows alkaline phosphatase (ALP) staining, Alizarin red staining and Oil red O staining of ADSCs cultured alone and in co-culture with TM4 cells. Controls (top) were cultured alone; experimental groups (bottom) were co-cultured with TM4 cells. Scale bar = 100 μ m (**a–f**). Bar graphs represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ versus control. (Color figure online)

Fig. 6 Migration analysis of MSCs after co-cultured with TM4 mouse Sertoli cells or in individual culture. **a** shows the morphology and number of migrated cells cultured alone or co-cultured with TM4 cells, the cells were stained with crystal violet. Bar graphs represent mean $\pm$ SD ($n = 3$ per group).

** $p < 0.01$ versus control. **b** shows the expression of CXCR4, MMP2, phospho-p44/42MAPK and phospho-p38MAPK in ADSCs and quantitative analysis the result of western blotting by image-pro plus software. Bar graphs represent mean $\pm$ SD ($n = 3$ per group). ** $p < 0.01$ versus control

component factors of the MAPK pathways (phospho-p44MAPK and phospho-rp38MAPK) that related to ADSCs migration were respectively detected by western blotting. As shown in Fig. 6b, all biomarkers tested here presented significant up-regulation at the protein level, indicating the regulatory factors secreted by TM4 cells. Besides, they can trigger the biosynthesis of migration regulators, resulting in the higher migration of ADSCs.

Discussion

In this work, we developed a Transwell insert co-culture system to cultivate MSCs. This demonstrates that Sertoli TM4 cells can significantly enhance the proliferation and migration of rat adipose-derived MSCs with high stemness and elucidates possible signaling pathways in which TM4 cells regulate the migration, and proliferation processes of ADSCs. Our

results suggested that TM4 cells improve the proliferation and migration of ADSCs via up-regulation of regulatory proteins involved in cell cycle progression and key mediator of MSCs migration (Figs. 2a–d, 6). Data in our study are similar to those of previous reports that the co-culturing with SCs promotes the proliferation and migration of UCMSCs (Zhang et al. 2012). Besides, we also observed that co-culturing with TM4 cells enhances the stemness, differentiation potential and survival of ADSCs (Fig. 3, 4, 5), which are obviously the key factors in determining therapeutic efficiency of the stem cells transplantation.

Many studies demonstrated that the proliferation ability and stem cell property of ADSCs were weakened after long-term of culturing in vitro, which are characterized by enlarged and irregular cell shapes along with a decline in cell proliferation, migration and multipotential (Banfi et al. 2000; Cai et al. 2013; Tobita et al. 2015; Lu et al. 2014). In this study, morphology and proliferation of rat ADSCs exhibit

above mentioned characteristics after continuous passages in vitro (Fig. 1d–f), which are similar to the previous report stating that the regeneration ability is decreased in cattle ADSCs (Lu et al. 2014). We infer that the stem cell properties of ADSCs were gradually reduced as a result of prolonged ex vivo culture. Therefore, our finding suggests that the co-culturing with TM4 cells is a good way to improve the therapeutic effect of ADSCs transplantation. However, little is known about the factors and mechanisms involved in TM4 cells enhancing the proliferation, migration of ADSCs progress.

It has been reported that TM4 cells secrete multiple factor as primary SCs (Roberts 2005) and are able to create a proper environment for the proliferation and differentiation of other co-cultured cells via PI3K/AKT and MAPK pathway [e.g., BM-MSCs (Tian et al. 2014), ESCs (Mirzaynesi et al. 2013) and SSCs (Simon et al. 2007)]. However, previous studies showed that some of these factors (such as EGF, TGF β s) could enhance the ADSCs proliferation, stemness, differentiation, survival and migration. Baer's et al. (2009) results showed that there is EGF receptor in ADSCs, which upon incubated with EGF

Fig. 7 Schematic flow diagram of signal transmission from regulatory factors secreted by TM4 cells to ADSCs. TM4 cells secrete multiple trophic factors such as transforming growth factor β (TGF β), epidermal growth factor (EGF) and fibroblast growth factors (FGF). These factors interact with each other and promote the stemness and migration of ADSCs via PI3K/Akt, MAPK/ERK/12 and P38MAPK pathway. Among this, the purple arrows indicate signaling pathways related with apoptosis, the red arrows indicate signaling pathways related with cell proliferation, the blue arrows indicate signaling pathways related with cell proliferation, cell migration and differentiation. The green arrows indicate signaling pathways may be related with cell self-renew and multipotent differentiation potential. (Color figure online)

lead to cell proliferation and induction of cell migration without changing the undifferentiated state of cells by the MEK/ERK1/2 signaling pathway. Exposure of ADSCs to TGF β 1 prior to transplantation enhances their survival in the skeletal tissues following transplantation (Liao et al. 2008). In addition, our group's work showed that FGF2 could promote ADSCs proliferation, inhibit their apoptosis as well as strengthen their stemness and differentiation potential (data not show). Therefore, we suggested that TM4 cells may secrete factors, for instance, EGF, TGF β 1 and FGF2 that act on ADSCs and enhance ADSCs proliferation, survival and migration.

Akt and Mdm2 are the two molecules that play an important role in cell survival and proliferation. In this study, we observed an up-regulation of Mdm2 and phosphate-Akt expression by WB in co-cultured ADSCs when compared with ADSCs cultured alone. These results are similar with the previous results of promoting proliferation of hUCMSCs and BM-MSCs by primary cultured Sertoli cells (Fenxi et al. 2012; Zhang et al. 2016). In addition, our results obtained by analyzing the expressions of Annexin V/PI and cell cycle (Figs. 2c, d, 3) indicated that TM4 cells could alleviate apoptosis of ADSCs. However, this finding is slightly different from the previous work that the TM4 cells have no effect on the apoptosis of BM-MSCs (Tian et al. 2014). Moreover, PI3K/Akt signaling pathway plays an important role for ADSCs survival and proliferation. Rosuvastatin is capable to enhance ADSCs viability and decrease their apoptotic rate via PI3K/Akt and MEK/ERK1/2 signaling pathway (Zhang et al. 2013). Moreover, Exendin-4 protects ADSCs from apoptosis induced by hydrogen peroxide through the PI3K/Akt-Sfrp2 pathways (Zhou et al. 2014). The activation of Akt, one of the key downstream molecules of the PI3K pathway, would mediate cellular processes including cells' proliferation, migration, and apoptosis (Brunet et al. 1999; Guo et al. 2014; Choi et al. 2008). Our results further revealed that the addition of LY294002 (a PI3K inhibitor) to the TM4-ADSCs co-culture system could down-regulate phosphate-Akt expression and inhibit ADSCs proliferation (Fig. 2e, f), which means that TM4 cells promote ADSCs proliferation and survival by PI3K/AKT pathway.

Several studies have reported that CXCR4 and its receptors CXCL12, MMP-2, Akt, ERK, and p38MAPK are the key mediators for MSCs migration

(Ryu et al. 2010; Shi et al. 2007; Wynn et al. 2004; Wang et al. 2016). Overexpression of CXCR4 might be considered for increasing the ADSCs migration and proliferation (Cho et al. 2006). Moreover, another study showed that activin B is capable to induce the migration of ADSCs via JNK and ERK signaling (Zhang et al. 2017). In addition, we found that ADSCs co-cultured with TM4 cells increased these signals (Fig. 6b). Hence, it is speculated that TM4 cells regulate the migration process of ADSCs via SDF/CXCR4 and MMP-2 factors by ERK1/2(P42/44 MAPK) and p38 MAPK signaling pathway.

In summary, we provide strong evidences that TM4 cells can promote the proliferation and migration of ADSCs with high stemness. Our studies demonstrated that co-culturing with TM4 cells is a good way to improve the efficiency of ADSCs transplantation therapy. Conclusively, all putative signaling pathways participating in proliferation and migration of ADSCs co-cultured with TM4 cells are depicted in Fig. 7. Yet there exists a gap in exploring the complete pathway and further researches are required to elucidate the challenging mechanism of TM4 cells responsible in affecting the ADSCs fate.

Conclusion

Stem cells with higher stemness and migratory capability are very useful tools for successful transplantation. TM4 cells can enhance the proliferation of ADSCs via PI3K/AKT pathway while maintaining their stemness as well as promoting migration ability. These findings prospect a potential way to improve the efficiency of ADSCs transplant therapy upon passage of comparatively best results in this domain.

Author's contribution This study was designed by MG and HH and experiments were performed by YL, XC and QW. The experimental data was also analyzed by YL. The paper was written by AM, YL and MG. JC and YZ participated in the data discussion. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Availability of data and materials All supporting data are included within the article and its Additional file.

Consent for publication All authors consented to publication of the present manuscript.

Ethics approval and consent to participate The authors declare that this is not a study involving human participants and reporting health related outcomes.

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