

Human adipose-derived mesenchymal stem cells promote breast cancer MCF7 cell epithelial-mesenchymal transition by cross interacting with the TGF- β /Smad and PI3K/AKT signaling pathways

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Abstract. The influence and underlying mechanisms of human adipose-derived stem cells (Hu-ADSCs) on breast cancer cells in the tumor microenvironment remain unclear. Understanding the association between Hu-ADSCs and cancer cells may provide targets for breast cancer treatment and reference for the clinical application of stem cells. Therefore, a Hu-ADSC and breast cancer MCF7 cell coculture system was established to investigate the paracrine effects of Hu-ADSCs on MCF7 cell migration and invasion, in addition to the potential mechanism of action by reverse transcription-quantitative polymerase chain reaction and western blotting. Hu-ADSCs enhanced MCF7 cell migration and invasion by decreasing the expression of epithelial marker E-cadherin, and increasing the expression of interstitial marker N-cadherin and epithelial-mesenchymal transition (EMT) transcription factors *in vitro*. The EMT effect of cocultured MCF7 cells was inhibited with the addition of anti-transforming growth factor (TGF)- β 1 or phosphoinositide 3-kinase (PI3K) inhibitor LY294002, accompanied by a significant decrease in phosphorylated (p)-mothers against decapentaplegic homolog (Smad) and p-protein kinase B (AKT) expression. The data suggested that the paracrine effect of Hu-ADSCs in the tumor

microenvironment promoted the EMT of MCF7 cells by cross interacting with the TGF- β /Smad and PI3K/AKT pathways.

Introduction

Breast cancer is one of the most common malignant tumors in women worldwide. Metastasis and invasion are the predominant causes of mortality in patients (1). A recent study identified that epithelial-mesenchymal transition (EMT) serves an important role in metastasis and invasion in breast cancer (2). EMT is a process by which the epithelial characteristics of a cell are gradually substituted with a mesenchymal phenotype that is associated with increased invasion and metastasis. This process is characterized by the degradation of the extracellular matrix, upregulation of interstitial marker gene expression, downregulation of epithelial marker gene expression, and the loss of cell polarity and invasive ability (3). EMT is a dynamic and complex process that is closely associated with the interaction of various growth factors, protein molecules, transcription factors and pathways. Transcription factors that induce EMT include zinc finger protein SNAI3 (Snail), zinc finger protein SNAI2 (Slug) and twist-related protein 1 (Twist) (4). These transcription factors regulate the generation and development of EMT through various signaling pathways and interactions between these pathways. During EMT, the expression of the epithelial marker gene E-cadherin is inhibited by a number of transcription factors (5).

It is acknowledged that the tumor microenvironment is critically involved in tumor development (3). The tumor microenvironment is complex and necessary for the survival of cancer cells. It is predominantly composed of the extracellular matrix and matrix cells, including inflammatory, immune, endothelial and mesenchymal stem cells (MSCs) (6). In recent decades, MSCs in the tumor microenvironment have been widely researched. MSCs undergo self-renewal, have multiple differentiation potentials and are recruited by tumor cells into their microenvironment (7) to stimulate tumor and/or anti-tumor adjacent cells by releasing endocrine and paracrine signals (8).

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Abbreviations: EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; Hu-ADSC, human adipose-derived stem cell; MSC, mesenchymal stem cell

Key words: co-culture, cross-interacting, Hu-ADSCs, MCF7 cells, paracrine

Transforming growth factor (TGF)- β is a cytokine that mediates complex functions and is widely involved in various pathological and physiological processes. TGF- β is closely associated with the occurrence and development of various conditions, including inflammation, trauma and organ fibrosis (9). However, its association with tumor cells remains unclear. It has been observed that the TGF- β signaling pathway inhibits the proliferation of tumor cells in the early stages; however, promotes tumor migration in the advanced stages of cancer (10).

Notably, TGF- β activates mothers against decapentaplegic homolog (Smad) dependent and Smad independent signaling pathways, which promotes the different effects of TGF- β in tumor cells (11-13). Previous studies have demonstrated that the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway is activated by TGF- β , which has synergistic/antagonistic effects on the Smad signaling pathways (14,15). A previous study additionally demonstrated that adipose-derived stem cells (ADSCs) induce breast cancer cells to secrete TGF- β , which consequently results in EMT in breast cancer cells (16).

In the present study, a coculture model of breast cancer cells and human adipose-derived stem cells (Hu-ADSCs) was established *in vitro* to determine how this microenvironment affected tumor migration and invasion. The underlying signaling pathways were examined in order to clarify the mechanisms involved in these phenomena. The data demonstrated that Hu-ADSCs enhanced the migration and invasion of breast cancer cells, which was accompanied by decreased E-cadherin expression, in addition to increased N-cadherin and EMT transcription factor expression. Notably, it was demonstrated that Hu-ADSCs enhanced EMT in breast cancer cells by cross interacting with the TGF- β /Smad and PI3K/AKT signaling pathways.

Materials and methods

Isolation and culture of ADSCs. The present study was conducted in accordance with the ethical standards in the Declaration of Helsinki (1975) and was approved by the Institutional Ethics Committee at Shengjing Hospital of China Medical University (Shenyang, China). All donors came from the plastic surgery ward between October and December 2017 and were free of major diseases and provided written informed consent. Adult adipose tissues were obtained by facial or abdominal liposuction from 7 female donors (aged 19-52), and Hu-ADSCs were isolated and cultured as previously described (17). Fresh adipose tissues were collected, washed with sterile PBS, minced into small pieces and incubated with 0.1% collagenase (type I; Roche Diagnostics GmbH, Mannheim, Germany) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) for 1 h at 37°C. Subsequently, the tissues were added to an equal volume of DMEM/F12 with 10% fetal bovine serum (FBS; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to neutralize enzyme digestion. This reaction mixture was centrifuged at 1,200 \times g for 10 min at room temperature to remove floating adipose tissues and the supernatant. The deposited cells were seeded into DMEM/F12 medium with 10% MesenCult™ MSC Stimulatory Supplements (Stemcell

Technologies, Inc., Vancouver, BC, Canada) and the cultures were maintained at 37°C in a 5% CO₂ incubator. After 48 h, non-adherent cells were removed. When the adherent cells reached >80% confluency, they were detached using 0.05% trypsin-EDTA (Beijing Solarbio Science & Technology Co., Ltd.) and subcultured at a 1:3 ratio under the same culture conditions. Hu-ADSCs between passages 3-5 were used in all experiments.

Hu-ADSC characterization. Hu-ADSCs were analyzed via flow cytometry with respect to cellular membrane marker expression using fluorescein isothiocyanate (FITC)-labeled antibodies (BD Biosciences, Franklin Lakes, NJ, USA) to endoglin (CD105; undiluted; cat. no. 561443), 5'-nucleotidase (CD73; undiluted; cat. no. 561254), Thy-1 (CD90; undiluted; cat. no. 555595), CD34 (undiluted; cat. no. 652802), lymphocyte common antigen (CD45, undiluted; cat. no. 347463) and human leukocyte antigen-antigen D related (HLA-DR, undiluted; cat. no. 347364). The negative control stain was FITC-conjugated mouse immunoglobulin G1-isotype. Hu-ADSCs were suspended in PBS at concentration of 10⁶/ml. Then, 50 μ l cells were incubated with 20 μ l FITC-conjugated CD105, CD73, CD90, CD34, CD45 and HLA-DR for 15 min at 4°C and washed with PBS. Subsequently, the cells were diluted in 500 μ l PBS and analyzed by flow cytometry; 5,000 cell events per sample were acquired on a FACSCalibur flow cytometer (BD Biosciences). Independent experiments were repeated three times. The capacity of Hu-ADSCs to differentiate into adipocytes and osteoblasts was assessed as previously described (18). Hu-ADSCs were treated with an Adipogenesis and Osteogenesis Differentiation kit (CTCC Bioscience, Jiangyin, China). The medium was changed three times per week. After 4 weeks of differentiation, the Hu-ADSCs were fixed with 4% formalin for 15 min and stained with 1% Oil Red O and 0.2% Alizarin Red S, all for 30 min and all at room temperature. Then the stained cells were observed and photographed under an light microscope (magnification, \times 200).

Cell lines and culture conditions. Breast cancer MCF7 cells were obtained from the Cell Biology Department of China Medical University (Shenyang, China). MCF7 cells were cultured in high-glucose DMEM culture medium (Hyclone; GE Healthcare Life Sciences) with 10% FBS (Beijing Solarbio Science & Technology) in a humidified 5% CO₂ incubator at 37°C.

Transwell coculture. A non-contact coculture system of Hu-ADSCs and MCF7 cells was established using a Transwell suspension culture chamber with a polyethylene terephthalate film combined with a 6-pore plate (0.4 μ m pores; Corning, Inc., Corning, NY, USA). Hu-ADSCs were seeded into the upper chamber at 10⁵ cells/well and MCF7 cells were added to the lower chamber at 5 \times 10⁴ cells/well. Hu-ADSC complete medium (DMEM/F12 medium with 10% MesenCult™ MSC Stimulatory Supplements) was used in the coculture system. MCF7 seeded into the 6-pore plate with Hu-ADSC complete medium served as the control group, to eliminate the influence of Hu-ADSC complete medium on MCF7 cells. In another two groups, TGF- β 1 neutralizing antibody anti-TGF- β 1 (cat.

no. ab27969; Abcam, Cambridge, UK) or PI3K inhibitor LY294002 (cat. no. HY-10108; MedChemExpress, LLC, Monmouth Junction, NJ, USA) was added to the upper chamber of the coculture system at a concentration of 8 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{mol}/\text{l}$, respectively. All groups were maintained at 37°C in a 5% CO_2 incubator. After 72 h of culture, MCF7 cells were harvested for the subsequent experiments.

Cell proliferation assay. The effect of Hu-ADSCs on the proliferation of MCF7 cells was evaluated by a Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Following coculture for 72 h, the MCF7 cells (1×10^4 cells/well) were plated in 96-well plates and incubated for 24, 48 and 72 h. MCF7 cells cultured alone were grown for 72 h and subsequently added to 96-well plates as a control. Each group was equipped with four compound pores and 10 μl CCK-8 solution was added to each well. The absorbance was determined at the wavelength of 450 nm after incubation for 1 h.

Transwell migration assay. MCF7 cell migration was evaluated using a 24-well Transwell chamber (8 μm pores; Corning, Inc.). Following coculture for 72 h, the MCF7 cells (1×10^5 cells/well) were plated into the top chamber and high glucose DMEM medium containing 30% FBS was placed in the bottom chamber. MCF7 cells cultured alone were used as the control. Cells were incubated for 24 or 48 h. Cells in the upper chamber were subsequently removed with the aid of a cotton swab and the remaining cells in the lower chamber were fixed with 4% formalin for 15 min at room temperature. Cells that migrated to the lower surface of the membrane were stained with 0.5% crystal violet for 15 min at room temperature and counted with an inverted phase contrast light microscope (magnification, $\times 200$; Motic Incorporation, Ltd., Causeway Bay, Hong Kong). In total, five visual fields were randomly selected for each assay. The mean number of the migrating cells in these five fields was taken as the cell migration number of the group. Independent experiments were repeated three times.

Matrigel invasion assay. MCF7 cell invasion was evaluated in 24-well Transwell chamber (8 μm pores; Corning, Inc.) coated with 40 μl BD Matrigel™ Matrix (1:3 dilution; BD Biosciences). The remaining steps were the same as the Transwell migration assay.

Conditioned medium (CM). When Hu-ADSCs reached ~80% confluency, the culture bottle was washed twice with PBS and completely exchanged with fresh DMEM/F12 serum-free medium. The supernatant was collected after 24, 48 and 72 h. Subsequently, the supernatant was centrifuged at $1,200 \times g$ for 10 min at room temperature to remove cell fragments and filtered with a 0.22- μm filtration membrane to remove bacteria.

ELISA. TGF- $\beta 1$ expression in the conditioned medium was measured using a double antibody sandwich ELISA according to the manufacturer's protocol (cat. no. DZE10135; Shanghai HoraBio, Inc., Shanghai, China). DMEM/F12 serum-free medium without Hu-ADSCs was placed in the incubator for 24, 48 and 72 h as the control groups. All specimens were tested in duplicate wells.

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction analysis

Target gene	Primer sequences (5'→3')
E-cadherin	F: AGAACGCATTGCCACATACA R: TAAGCGATGGCGGCATTGTA
N-cadherin	F: GGACCATCACTCGGCTTA R: CACTGGCAAACCTTCACG
Snail	F: GCCCCACAGGACTTTGATGA R: AGTGAGTCTGTCTAGCCTTTGTCT
Slug	F: AGCGAACTGGACACACATAC R: GCCCCAAAGATGAGGAGTAT
Twist	F: GGAGTCCGCAGTCTTACGA R: CCAGCTTGAGGGTCTGAATC
β -actin	F: CTTAGTTGCGTTACACCCTTTCTTG R: CTGTCACCTTCACCGTTCCAGTTT

F, forward; R, reverse; Snail, zinc finger protein SNAI3; Slug, zinc finger protein SNAI2; Twist, twist-related protein 1.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene expression in cocultured MCF7 cells and MCF7 cells alone was quantified by RT-qPCR. Total RNA was isolated using an RNAPrep pure cell kit (BioTeke Corporation, Beijing, China) and reverse-transcribed into complementary DNA using a first complementary DNA Synthesis kit with oligo(dT)₁₅ (BioTeke Corporation; 10 min at 25°C, 50 min at 42°C and 5 min at 95°C). The expressions of the genes of interest were measured by PCR (Exicycler™ 96; Bioneer Corporation, Daejeon, Korea) using SYBR Green Master Mix (Beijing Solarbio Science & Technology). The primers were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the primer sequences are listed in Table I. β -actin was used as an endogenous control to normalize gene expression levels. qPCR was performed for 10 min at 94°C followed by 40 amplification cycles (10 sec at 94°C, 20 sec at 60°C and 30 sec at 72°C) to determine the epithelium or interstitial gene expression. The $2^{-\Delta\Delta C_q}$ method (19) was used for the analysis of relative gene expression data. Independent experiments were repeated three times.

Western blot analysis. To assess the expression of phosphorylated (p)-Smad2/3, p-AKT, Smad2/3, AKT, Snail, Slug, Twist, E-cadherin and N-cadherin, total cellular protein was extracted from MCF7 cells from different treatment groups. Protein was isolated from harvested cells using a Total Protein Extraction kit (Wanleibio Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The protein concentration of the samples was determined with a bicinchoninic acid protein assay kit (Wanleibio Co., Ltd.). Total protein (40 $\mu\text{g}/\text{lane}$) was resolved by 5-10% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 h in Tris-buffered saline containing 0.1% Tween-20 and subsequently incubated with diluted primary antibody (1:500; p-smad2/3 antibody cat. no. WL02305, p-AKT antibody cat. no. WLP001, smad2/3 antibody cat. no. WL01520, AKT

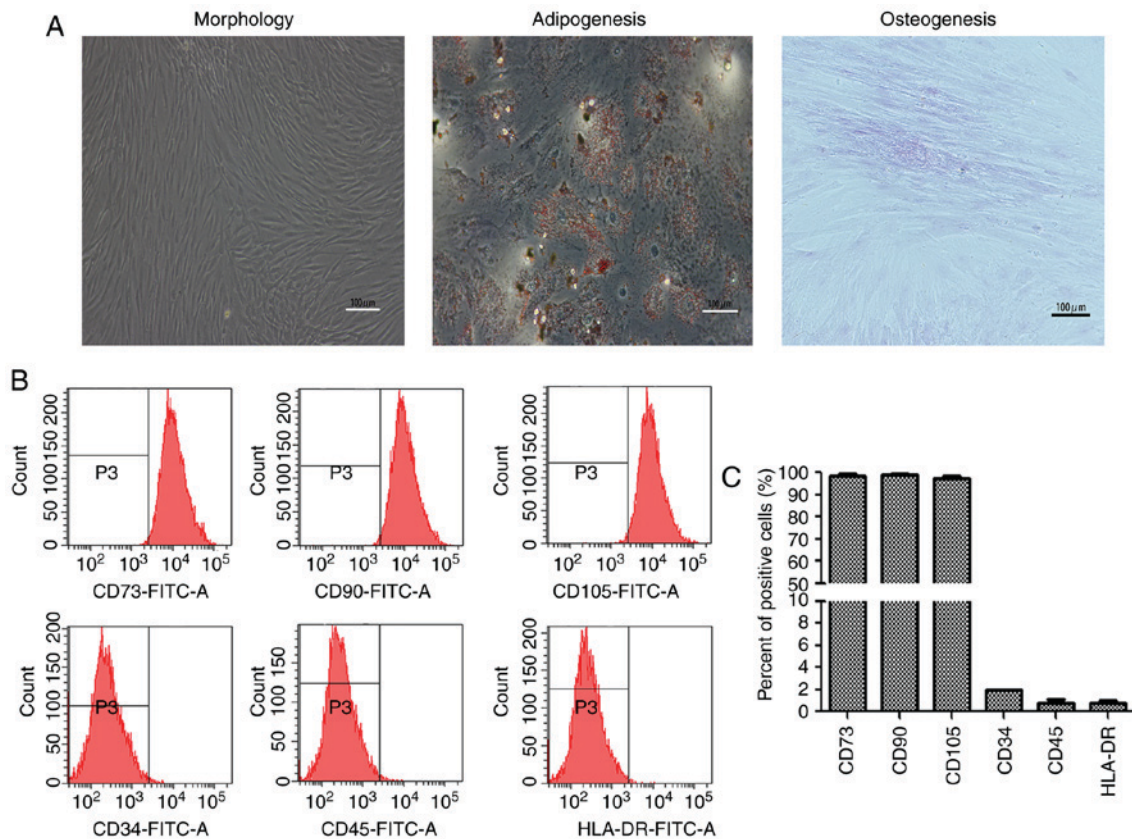


Figure 1. Isolation and identification of Hu-ADSCs. (A) Morphological features of isolated Hu-ADSCs at passage 3. Hu-ADSCs were induced to differentiate into adipocytes and osteoblasts with red indicating lipid droplets and pink indicating calcification. Scale bar, 100 μ m. (B) Hu-ADSCs examined by flow cytometry were positive for CD73, CD90 and CD105 expression, and negative for CD34, CD45 and HLA-DR expression. (C) A total of three independent flow cytometry experiments were quantified. Hu-ADSCs, human adipose-derived stem cells; CD73, 5'-nucleotidase; CD90, Thy-1; CD105, endoglin; CD45, lymphocyte common antigen; HLA-DR, human leukocyte antigen-antigen D related; FITC, fluorescein isothiocyanate.

antibody cat. no. WL0003, Snail antibody cat. no. WL01863, Slug antibody cat. no. WL01508, Twist antibody cat. no. WL00997, E-cadherin antibody cat. no. WL01482, N-cadherin antibody cat. no. WL01047 and β -actin antibody cat. no. WL01845; all from Wanleibio, Co., Ltd.) at 4°C overnight. The membrane was subsequently incubated with a secondary antibody (1:5,000, cat. no. WLA023, Wanleibio Co., Ltd., Shenyang, China) for 45 min at room temperature. Proteins were visualized with an enhanced chemiluminescence reagent (Wanleibio Co., Ltd.). β -actin was used as an internal control to normalize the loading materials. Finally, the protein bands were quantified using Gel-Pro-Analyzer version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All experiments were repeated three times. The results were analyzed by SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean \pm standard deviation. Single comparisons were performed by independent samples t-test and multiple comparisons were performed by one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Isolation and identification of Hu-ADSCs. The Hu-ADSCs were isolated from human adipose tissue and adhered to the

culture plate 24 h after seeding. After 3 or 4 days of culture, the majority of the cells exhibited spindle-shaped, homogeneous and whirlpool-like growth (Fig. 1A). After 4 weeks, Hu-ADSCs were successfully induced to differentiate into adipocytes and osteoblasts (Fig. 1A). To further characterize the cell surface markers of Hu-ADSCs, immunophenotypic analysis was performed by flow cytometry. Hu-ADSCs exhibited positive expression of CD73, CD90 and CD105, and negative expression of CD34, CD45 and HLA-DR (Fig. 1B and C).

Hu-ADSCs increase MCF7 cell migration and invasion.

The migration and invasion of MCF7 cells cocultured with Hu-ADSCs were determined by Transwell and Matrigel assays, respectively, with MCF7 cells cultured alone as the control. In the Transwell migration assay after 24 and 48 h of culture, the numbers of migrating MCF7 cells significantly increased in the cocultured group (78.47 ± 11.37 and 104.93 ± 13.57 , respectively) compared with the control group (37.93 ± 22.00 and 66.53 ± 10.54 , respectively; both $P < 0.01$; Fig. 2A and B). In the Matrigel invasion assay, the same results as the Transwell migration assay were observed after 24 and 48 h: The number of invading MCF7 cells was significantly increased in the cocultured group (50.93 ± 5.71 and 71.53 ± 8.44 , respectively) compared with the control MCF7 cells (24.27 ± 5.35 and 36.80 ± 4.68 , respectively; both $P < 0.01$; Fig. 2C and D). In addition, cell proliferation experiments demonstrated that Hu-ADSCs did not promote MCF7 cell proliferation (Fig. 2E).

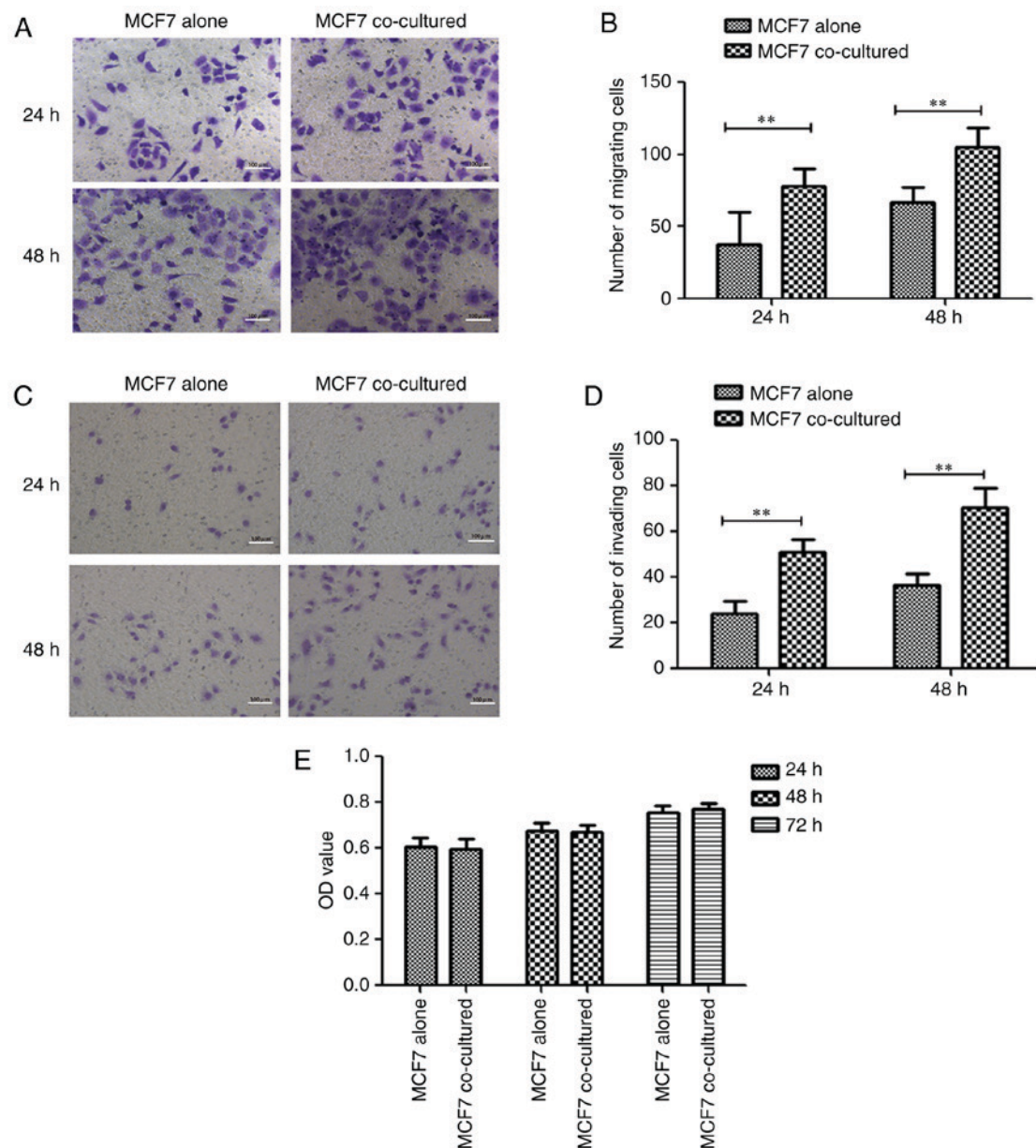


Figure 2. Hu-ADSCs increase MCF7 cell migration and invasion. The migration and invasion capacity of MCF7 cells with Hu-ADSCs was significantly increased at either 24 or 48 h after coculture. (A) Representative images of migrating MCF7 cells with or without Hu-ADSC coculture. (B) Quantification of the number of migrating cells. (C) Representative images of invading MCF7 cells with or without Hu-ADSC coculture. (D) Quantification of the number of invading cells. (E) Hu-ADSCs did not promote the proliferation of MCF7 cells. In the Cell Counting Kit-8 assay, there was no significant difference in the OD value between the cocultured MCF7 cells and MCF7 cells alone ($P>0.05$). Data are presented as the mean \pm standard deviation of three independent experiments. Scale bar, 100 μ m. ** $P<0.01$. Hu-ADSCs, human adipose-derived stem cells; OD, optical density.

This suggested that the ADSC-induced migration and invasion of cocultured MCF7 cells was not caused by a significant increase in the number of MCF7 cells ($P>0.05$); however, rather because the ADSCs specifically promoted cell migration and invasion.

Hu-ADSCs enhance MCF7 cell EMT. E-cadherin is an epithelial marker involved in cell adhesion/polarity and tissue morphogenesis, and normal epithelial cells acquire invasive and migratory properties when they express lower levels of E-cadherin (20). E-cadherin expression was detected in MCF7 cells cocultured with Hu-ADSCs by RT-qPCR and MCF7 cells cultured alone served as a control. Concurrently, alterations in the expression of interstitial marker gene N-cadherin and

EMT-associated transcription factors, including Snail, Slug and Twist, were examined in the cocultured MCF7 cells and the control group. The expression of E-cadherin in the cocultured MCF7 cells was significantly downregulated compared with the control group (Fig. 3A; $P<0.01$), whereas N-cadherin (Fig. 3B), Snail (Fig. 3C), Slug (Fig. 3D) and Twist (Fig. 3E) expression was upregulated compared with the control group (all $P<0.01$).

Hu-ADSCs promote MCF7 cell EMT by cross interacting with the TGF- β /Smad and PI3K/AKT pathways. TGF- β is directly dependent on Smad signaling for tumor inhibition or promotion, and TGF- β may by phosphorylation activate other signaling pathways that have potential involvement in tumor cell survival, growth, migration and invasion. (TGF- β non-Smad

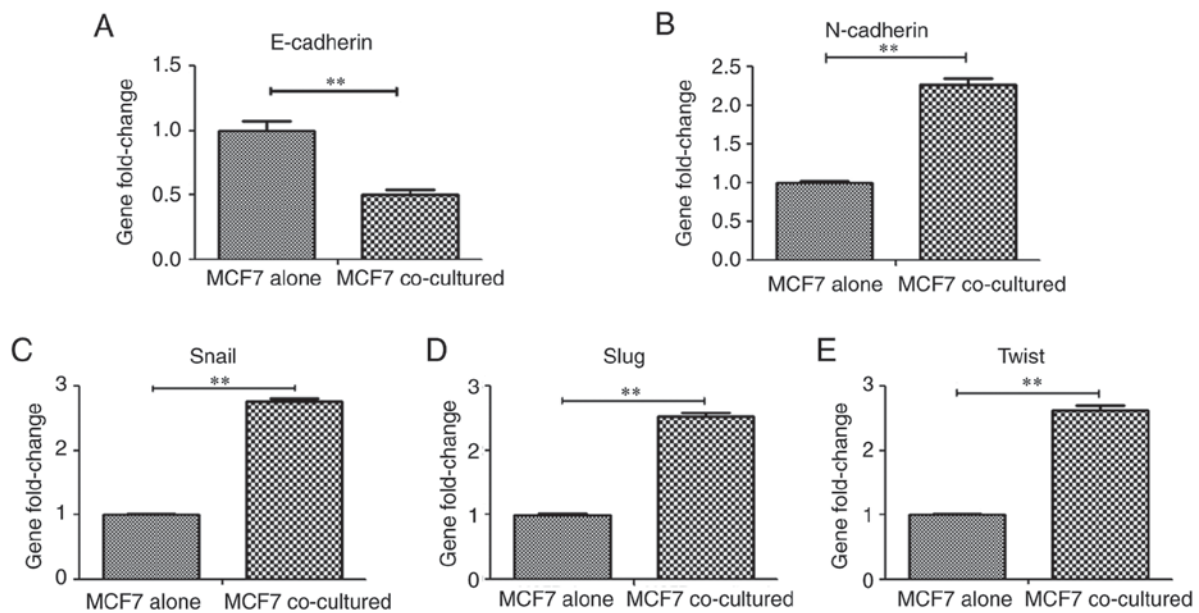


Figure 3. Hu-ADSCs enhance MCF7 cell epithelial-mesenchymal transition as assessed by reverse transcription-quantitative polymerase chain reaction. (A) Expression of E-cadherin was significantly downregulated in MCF7 cells cocultured with Hu-ADSCs. (B) Expressions of N-cadherin, (C) Snail, (D) Slug and (E) Twist were upregulated in MCF7 cells cocultured with Hu-ADSCs. Data are presented as the mean \pm standard deviation of three independent experiments. ** $P < 0.01$. Snail, zinc finger protein SNAI3; Slug, zinc finger protein SNAI2; Twist, twist-related protein 1; Hu-ADSCs, human adipose-derived stem cells.

pathways) (9). To clarify the mechanisms of Hu-ADSC action on MCF7 cells, it was determined whether Hu-ADSCs secreted TGF- β 1 by ELISA (Fig. 4A), and the effects of the TGF- β /Smad and PI3K/AKT pathways on tumor EMT were examined by western blotting. The results from ELISA demonstrated that the level of TGF- β 1 in CM greater than that in DMEM/F12 serum-free medium, which was indicated that Hu-ADSCs secreted TGF- β 1. E-cadherin expression in MCF7 cells was significantly decreased following treatment with Hu-ADSCs, and was significantly increased in the coculture of MCF7 cells with anti-TGF- β 1 or PI3K inhibitor LY294002 (Fig. 4B and C; $P < 0.01$). The expressions of N-cadherin (Fig. 4B and D) and EMT-associated transcription factors had the opposite trend (Fig. 4E-H). Concurrently, p-Smad2/3 (data not shown) and p-AKT expression was additionally increased in the cocultured MCF7 cells and this phenomenon was reversed by anti-TGF- β 1 or LY294002 (Fig. 4I-L). These results suggested that the TGF- β /Smad and PI3K/AKT signaling pathways were involved in the promotion of MCF7 cell EMT by Hu-ADSCs. As one of the aims of the present study was to confirm whether TGF- β could activate the PI3K/AKT signaling pathway and what effect the activated PI3K pathway would have on the EMT of the cocultured MCF7 cells, the expression of p-AKT after the addition of anti-TGF- β 1 in the co-culture system was tested. Notably, it was demonstrated that p-AKT was significantly decreased in the cocultured MCF7 cells treated with anti-TGF- β 1 (Fig. 4K and L; $P < 0.01$). This suggested that TGF- β 1 can activated PI3K/AKT signaling pathway and that Hu-ADSCs promoted MCF7 cell EMT via the TGF- β /Smad and PI3K/AKT pathways, and that cross talk between the two pathways existed.

Discussion

MSCs have self-renewal and multiple differentiation functions, and migrate to tumorigenic or inflammatory sites to

exert tumor regulation or immunomodulatory effects by interacting with tumor or inflammatory cells (8,21). Based on these particular biological characteristics, MSCs are considered as candidates for cancer treatment or regenerative medicine applications (22,23). However, accumulating evidence has suggested that the role of MSCs in cancer is controversial. Although they may stimulate the growth, migration and invasion of tumors by direct or indirect actions on tumor cells, they may additionally inhibit tumor growth by inhibiting signal pathways, including Wnt (24,25). Therefore, the present study aimed to investigate the effects of MSCs on tumors and to examine the underlying mechanism involved.

Hu-ADSCs were firstly isolated from adipose tissue. The morphological characteristics of Hu-ADSCs were assessed using an appropriate concentration of collagenase for digestion, and the adherent method was similar to other sources of MSCs, such as bone marrow and human umbilical cords.

Furthermore, the MSCs stably differentiated into adipocytes and osteoblasts, and highly expressed CD90, CD73 and CD105, with low levels of CD34, CD45 and HLA-DR. These results meet the criteria proposed by the Committee of the International Society for Cellular Therapy for the biological characteristics of MSCs (26).

Although it was previously demonstrated that MSCs are recruited by tumor cells into their microenvironment (27), the interaction between them and tumor cells remains unclear. By interacting with the tumor microenvironment, MSCs exert a tumor suppressive effect (28); however, there is accumulating evidence that MSCs have a tumor promoting effect, including tumors of the breast, prostate, liver, ovary and pancreas (29-33). The Hu-ADSC paracrine effect on tumor cells induces an aggressive phenotype, leading to eventual cell migration (34). Park *et al* (35) observed that lung cancer cells promote the differentiation of ADSCs into myofibroblasts and that the direct coculture of these altered ADSCs increased the EMT

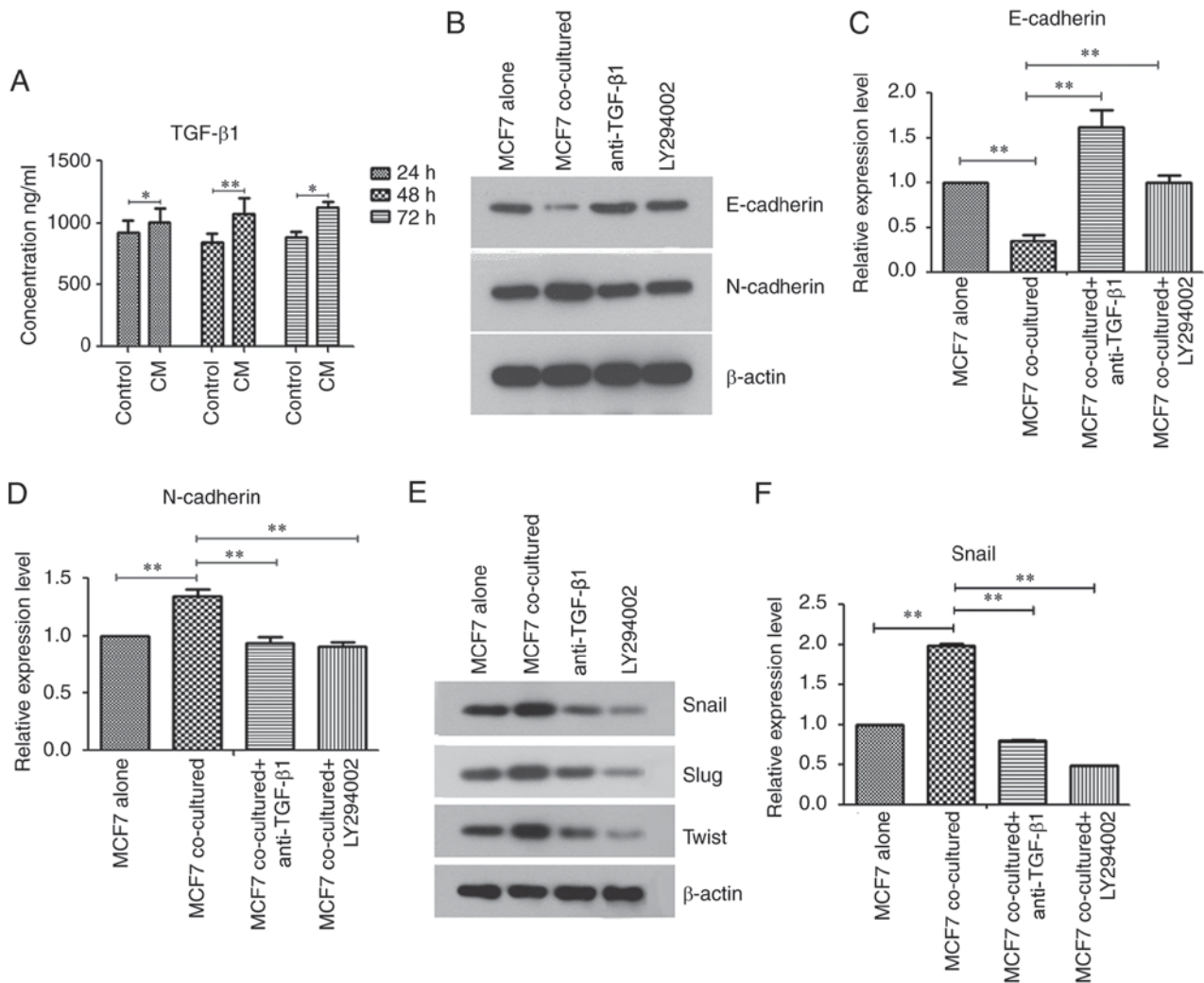


Figure 4. Human adipose-derived stem cells promote MCF7 cell epithelial-mesenchymal transition by cross interacting with the TGF- β Smad and phosphoinositide 3-kinase/AKT pathways. (A) Secretion of TGF- β 1 into CM and DMEM/F12 serum-free medium was assessed by ELISA. (B) Expression of E-cadherin and N-cadherin were determined by western blotting. Relative expression levels of (C) E-cadherin and (D) N-cadherin by densitometric analysis. (E) Expression of Snail, Slug and Twist were determined by western blotting. Relative expression levels of (F) Snail by densitometric analysis. Data are presented as the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01. TGF- β , transforming- β ; Snail, zinc finger protein SNAI3; Slug, zinc finger protein SNAI2; Twist, twist-related protein-1; p, phosphorylated; Smad, mothers against decapentaplegic homolog; AKT, protein kinase B; CM, conditioned medium.

of lung cancer cells. Different from these previous studies, the present study established a Transwell isolated coculture system and determined that the paracrine effect of Hu-ADSCs promoted the EMT of MCF7 cells. A direct coculture method was not adopted, thus allowing Hu-ADSCs to be distinguished from MCF7 cells and allowing a more convenient study of the biological characteristics of MCF7 cells. Although the direct co-culture method may be used to determine the direct contact and paracrine effects of MSCs on tumor cells, it is not easy to separate the two cell types following coculture because MSCs lack specific surface markers, which may additionally be altered following contact with tumor cells (36). Yan *et al* (37) identified that ADSCs promote the proliferation of breast cancer cells. The present study demonstrated that Hu-ADSCs did not increase the number of MCF7 cells; however, promoted tumor cell EMT, characterized by the decreased expression of E-cadherin, and increased expressions of N-cadherin and EMT-associated transcription factors. The transformation of

tumor cell expression from E-cadherin to N-cadherin increases cancer cell migration and invasion (38).

In addition, the expression of Snail, Slug and Twist in cocultured MCF7 cells were increased in the present study, which additionally suggested that Hu-ADSCs promoted MCF7 cell EMT. The expressions of these transcription factors promote tumor cell transition to a mesenchymal state by inhibiting epithelial cell marker expression and inducing mesenchymal cell marker expression (36,39,40).

The results in the present study regarding the signal pathways involved provide insight for the current understanding of the effects of Hu-ADSCs on tumor cell EMT. TGF- β signaling, particularly TGF- β 1, is recognized as a signaling pathway that regulates the EMT process (16,41,42). TGF- β regulates the expression of EMT transcription factors and induces EMT through cross talk with other cell factors, including Wnt, Ras and Notch (43). Therefore, it was hypothesized that the alterations in MCF7 cells were associated with TGF- β 1 released by

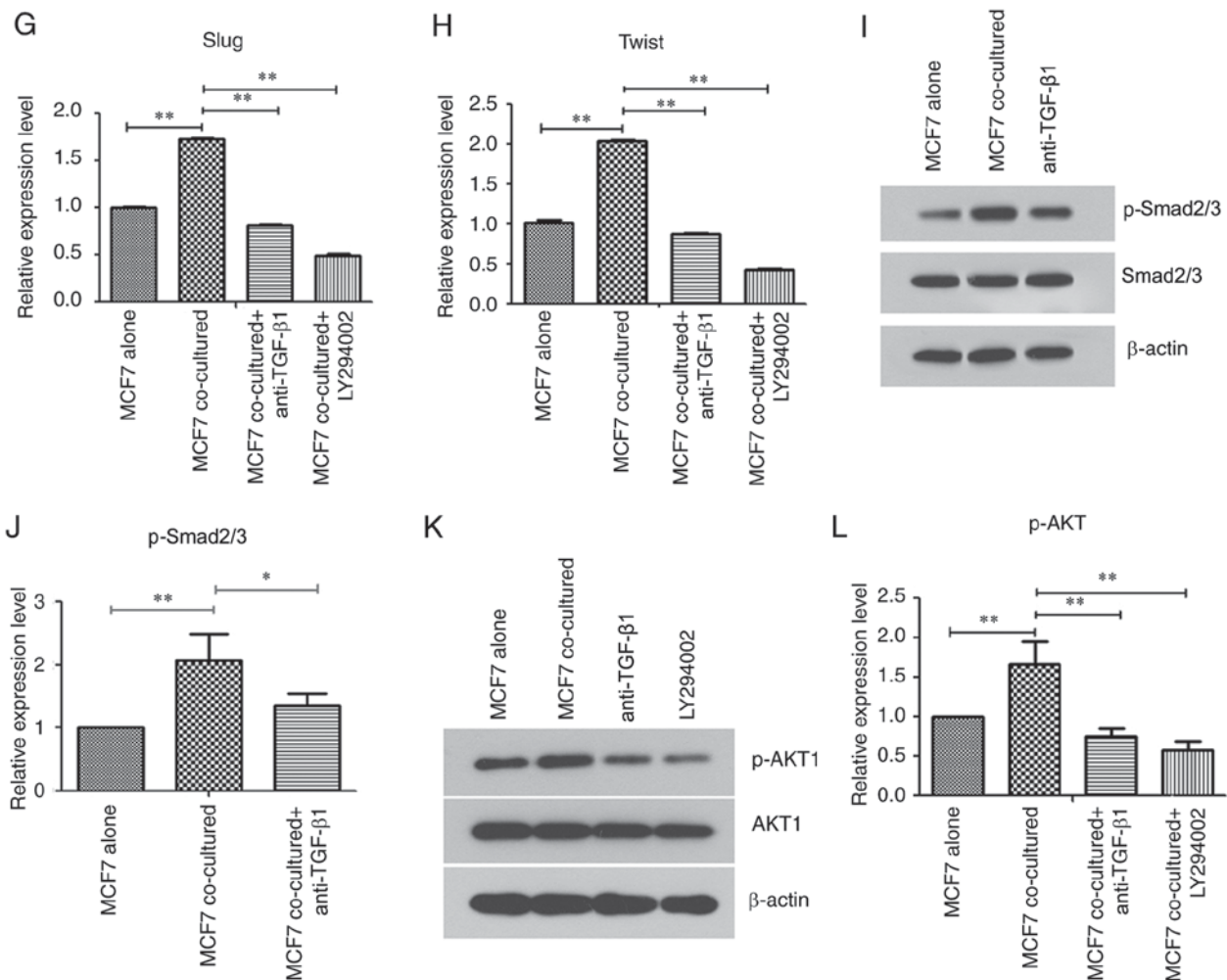


Figure 4. Continued. Relative expression levels of (G) Slug and (H) Twist by densitometric analysis. (I) Expression of p-Smad2/3 was determined by western blotting. Total Smad2/3 was used as a control to confirm equal protein amounts. (J) Relative expression of p-Smad2/3 was determined by densitometric analyses. (K) p-AKT expression was determined by western blotting. Total AKT was used as a control to confirm equal protein amounts. (L) Relative expression of p-AKT was determined by densitometric analyses. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$. Slug, zinc finger protein SNAI2; Twist, twist-related protein-1; p, phosphorylated; Smad, mothers against decapentaplegic homolog; AKT, protein kinase B; CM, conditioned medium.

Hu-ADSCs. To confirm this hypothesis, Hu-ADSC TGF- β 1 secretion was confirmed by ELISA.

Xu *et al.* (16) identified that Hu-ADSCs regulate EMT in MCF7 cells by paracrine and induced autocrine TGF- β signaling. In contrast, the present study identified the signaling pathways associated with TGF- β . Based on accumulating evidence, EMT is closely associated with the activation of the TGF- β /Smad and PI3K/AKT signaling pathways (11,12,14,44). Therefore, the present study aimed to identify the association between the activation of these pathways in Hu-ADSCs, and the effects of Hu-ADSCs on MCF7 cells. The use of anti-TGF- β 1 and PI3K inhibitor LY294002 decreased the EMT of cocultured MCF7 cells. This suggested that Hu-ADSCs promoted the EMT of MCF7 cells, at least in part through the TGF- β Smad and non-Smad dependent PI3K/AKT signaling pathways.

Furthermore, the findings of the present study suggested that the PI3K/AKT signaling pathway may be activated by TGF- β 1 secreted by Hu-ADSCs during the promotion of breast cancer EMT, as p-AKT expression decreased following the addition of anti-TGF- β 1 to the coculture system. This

phenomenon highlighted that suppression of the TGF- β /Smad pathway inhibited the PI3K/AKT pathway, suggesting cross interactions between the two pathways. The external environment tested differed from the internal environment, even if it was close to the internal environment. Further investigations are required to elucidate the underlying mechanisms. Understanding the association between Hu-ADSCs and cancer cells may provide targets for breast cancer treatment.

In conclusion, the present study examined the interaction between Hu-ADSCs and the MCF7 cell line using a Transwell coculture system to examine the paracrine effects of Hu-ADSCs in tumor development. This provided evidence that Hu-ADSCs may stimulate increased MCF7 cell migration and invasion via alterations in E-cadherin, N-cadherin and EMT-associated transcription factor expression, which were at least partially mediated by the activation of the TGF- β Smad and non-Smad dependent PI3K/AKT pathways.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

SMW and XZ were responsible for designing of the study and critical review of manuscript. SMW, YW, ZY, QW and HD performed the experiments. SLW and XL performed statistical analyses. YW and QW wrote the manuscript. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the ethical standards in the Declaration of Helsinki (1975) and was approved by the Institutional Ethics Committee at Shengjing Hospital of China Medical University (Shenyang, China). All donors provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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